Prostate cancer is the second most prevalent cancer among men in the United States and ranks second to lung cancer in terms of annual mortality (Weir et al., 2003). Prostate cancer begins as an androgen-dependent tumour that undergoes clinical regression in response to pharmacological and surgical strategies that reduce testosterone concentration. Despite this treatment, the cancer eventually regrows as an androgen- or hormone-independent tumour (Feldman and Feldman, 2001). Microarray analysis of both androgen dependent and independent tumour xenografts (Amler et al., 2000; Mousses et al., 2001) and human prostate cancer samples (LaTulippe et al., 2002) during disease progression has identified several candidate targets, including the AR itself, for prostate cancer therapy and diagnosis associated with the androgen independent phenotype. Thus, aberrant changes in AR signalling are likely to play a role in the progression to androgen independence (Grossmann et al., 2001). Chen et al. (2004) showed, using microarray-based profiling of isogenic prostate cancer xenografts, that increases in AR mRNA were the only changes in vitro, and in animal models (Craft et al., 1999; Yeh et al., 1999). ErbB2 is also required for IL-6 activation of AR (Qiu et al., 1998). However, studies of the role of ErbB2 in clinical prostate cancer remain inconclusive (Grossmann et al., 2001), and initial clinical trials indicate that the anti-ErbB2 antibody Herceptin (trastuzumab) does not show significant clinical activity as a single agent (Ziada et al., 2004). ErbB3 has not been as extensively studied, but analysis of clinical prostate cancer specimens indicates that overexpression of ErbB3 has been linked to a less favourable prognosis (Leung et al., 1997).

EGF-like ligands have been shown in vitro and in vivo to stimulate growth of prostate cancer cells. For example, in vitro AR is activated in a ligand independent manner by EGF (Culig et al., 1999).
1994). Torring et al (2003) recently showed that LNCaP cells constitutively express EGF ligands and that ErbB1 activity is necessary for androgen-induced proliferation. Endogenous stromal derived factors such as Heparin binding-EGF attenuate the response of AR to its ligands, resulting in androgen independent growth of LNCaP cells (Adam et al, 2002). By contrast, the ErbB3/4 ligand HRG is highly expressed in basal, luminal and stromal cells of the normal prostate, but not in prostate tumours (Lyne et al, 1997). Further, HRG protein is not detected in the prostate cancer cell lines LNCaP, DU-145 or PC-3 (Grasso et al, 1997a; Lyne et al, 1997). HRG mRNA could be detected in these cell lines only by RT–PCR in one study (Lyne et al, 1997), but was not detected in another (Grasso et al, 1997b). HRG inhibits growth and induces differentiation of AR positive, ErbB-3 positive LNCaP cells, but has little effect on proliferation of ErbB1–3 positive, AR negative DU 145 and PC-3 cells (Grasso et al, 1997b; Lyne et al, 1997). In addition, HRG induces the expression of the tumour suppressor p53 and the CDK inhibitor p21 in LNCaP cells (Bacus et al, 1996). Most recently, Tal-Or et al (2003) have demonstrated that HRG activates ErbB2/3 heterodimers and induces apoptosis of LNCaP cells. These combined findings suggest that HRG signals may contribute to growth restriction or differentiation of prostate epithelia.

Our laboratory has recently demonstrated that a protein Ebp1, isolated by its binding to HRG’s cognate receptor ErbB3 (Yoo et al, 2000), binds AR in vitro and in vivo (Zhang et al, 2002). Ebp1 is expressed in both normal prostate epithelial cells and in the prostate cancer cell lines LNCaP, DU-145 and PC-3 (Xia et al, 2001b). Ectopic expression of ebp1 inhibits ligand-mediated transcriptional activation of both artificial and natural AR regulated promoters in COS cells transfected with wild-type AR and in LNCaP cells that express a mutant AR. The transcription of the endogenous PSA gene is also decreased in LNCaP cells stably transfected with Ebp1 (Zhang et al, 2002). However, the effect of Ebp1 on transactivation of wild-type AR in prostate cancer cells was not established. The purpose of the present study was to extend our finding that Ebp1 represses AR transactivation and to determine if the ErbB3 ligand, HRG, affects Ebp1’s interactions with AR.

MATERIALS AND METHODS

Cell culture

All cell lines except PC-3 AR were obtained from the American Type Culture Collection (Manassas, VA, USA). PC-3 AR cells (Long et al, 2000) were a gift of Dr Angela Brodie. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Cell lines were routinely cultured in RPMI 1640 media supplemented with 10% foetal bovine serum (FBS) (Sigma, St Louis, MO, USA).

Plasmids

The PSA reporter luciferase construct was a gift from Dr Martin Gleave and contains −630/+12 of the 5’ PSA flanking region. The Probasin (−285/+32) luciferase reporter and the pS55-hAR expression construct were gifts of Dr O Janne. The MMTV-luciferase plasmid was obtained from Dr Joseph Fondell (Wang and Fondell, 2001). The ebp1 expression construct has been previously described (Xia et al, 2001a).

Immunoprecipitation, GST-pulldowns and Western blot analysis

To measure ErbB3–Ebp1 interactions, LNCaP cells were incubated overnight in serum-free RPMI-1640 media. Where indicated, cells were treated with 20 ng ml⁻¹ of HRG β1 (R&D Systems, Mpls, MN, USA) for the indicated times. Cell lysates were prepared and immunoprecipitated as described previously (Fernandes et al, 1999). Briefly, cells were lysed with buffer containing 50 mM HEPES (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100 and Complete™ protease inhibitor. Protein concentrations were measured using a detergent compatible kit (BioRad, Hercules, CA, USA). Cell lysates were precleared with Protein A/Protein G agarose and immunoprecipitated for 4 h at 4°C with 2 μg of a monoclonal antibody directed against ErbB3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 20 μl packed Protein A/G agarose beads. The immunoprecipitates were washed and resuspended in Laemmli sample buffer. Proteins were resolved by SDS–PAGE. After electrophoresis, the proteins were transferred onto Immobilon-P membranes, and immunoblotted as described (Xia et al, 2001b) using a monoclonal antibody to ErbB3 (Santa Cruz) or a rabbit polyclonal antibody that detects both phosphorylated and unphosphorylated forms of Ebp1 (Xia et al, 2001b) (Upstate, Lake Placid, NY, USA). To measure the association of endogenous AR and endogenous Ebp1, LNCaP cells, growing in complete media, were switched to phenol-red free RPMI 1640 containing 1% charcoal stripped calf serum (CSS) (Sigma) and 10⁻⁸ M R1881 (NEN, Boston, MA, USA) for 24 h. Cells were then stimulated with or without HRG β1 (R&D Systems, Mpls, MN, USA) for 1 h. Cell lysates were immunoprecipitated as described above using the polyclonal antibody to Ebp1. Western blot analysis was performed using a monoclonal antibody to AR (Santa Cruz) or the Ebp1 antibody.

Luciferase reporter assays

Cells (5 × 10⁴) were plated in 12-well plates in complete media. When cells reached 50–60% confluence, they were transfected using the Fugene-6 Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer’s instructions. Cells were transfected with 0.5 μg of the indicated reporter plasmids, 0.5 μg of pS55-hAR (where specified), and 0.5 μg of pcDNA3 or wild-type ebp1 expression plasmids and 5 ng of the TK-Remilla plasmid (Promega, Madison, WI, USA) as an internal control. Complete medium was replaced 24 h after transfection with phenol red free RPMI 1640 with CSS with or without R1881 (10⁻⁸ M) (Sadar and Gleave, 2000). Luciferase activity was determined using the Promega Dual luciferase assay kit as described by the manufacturer. The levels of luciferase activity were normalised using the renilla luciferase as an internal control. The ratio of luciferase activity to the renilla control derived from cells that were transfected with vector alone and not treated was given a Relative Luciferase Activity value of 1. All values presented in the individual figures were derived by comparison to this ratio observed in control cells. Transfection efficiency was approximately 30% as judged by parallel experiments using the EGFP-N1 plasmid (Clontech, Palo Alto, CA, USA). All transfection experiments were carried out in triplicate wells.

Gene silencing with small interfering RNAs

The siRNA oligonucleotides were purchased from Dharmacon Research Inc (Lafayette, CO, USA). COS-7 cells were cultured in 12-well plates until 60% confluent. Cells in 1 ml of antibiotic-free culture media were transfected with 60 nM final concentration of annealed oligonucleotides using Lipfectamine 2000 according to the manufacturer’s instructions. The Ebp1 siRNA sequences corresponded to the coding regions beginning at nucleotides 476 and 995 (Genbank accession number U87954). The target sequences were AACGGCAGGAGUUAUACUCU and AAGU-GAGGGUAGGGCGUUU respectively. These sequences do not match any other human genomic sequences as determined by BLAST analysis using the NCBI Website. Scrambled oligonucleotides of these sequences were used as negative controls. The
next day, cells were transfected with an expression construct for wild-type AR and the MMTV-luciferase and TK plasmids using Fugene-6.

**Statistical analysis**

Results were analysed using a two-tailed Students’-test. Significance was established at $P \leq 0.05$.

**RESULTS**

**Ebp1 inhibits transactivation of wild-type AR in prostate adenocarcinoma cell lines**

We previously demonstrated that Ebp1 inhibited transactivation of the artificial ARE$_{DS}$ promoter in COS cells transfected with wild-type AR, and the PSA promoter in LNCaP cells that harbor a mutant AR (Zhang et al., 2002). We were interested in determining if Ebp1 represses wild-type AR in prostate cancer cell lines. We therefore tested the ability of Ebp1 to repress AR-mediated transcription of the MMTV-luciferase reporter plasmid in androgen-independent DU145 cells transiently transfected with a wild-type AR and PC-3 AR cells stably transfected with wild-type AR (Long et al., 2000). Cells were transfected with the MMTV luciferase reporter construct, ebp1 or the pcDNA vector control, and in the case of DU145 cells, the expression construct for AR. After 24 h, the cells were stimulated with either R1881 or vehicle for 16 h. Cells were harvested and monitored for dual luciferase activity. Transfection of ebp1 at 0.5 μg per individual well routinely results in a two- to three-fold increase in Ebp1 expression levels as determined by Western blot analysis (data not shown). As expected, R1881 stimulated luciferase activity four- to five-fold in DU145 and PC-3 AR cells (Figure 1). Ectopic expression of ebp1 reduced AR transactivation to basal levels in both cell types.

Corepressors may have dissimilar effects on the activity of natural AR regulated promoters due to differential binding of the AR to androgen response elements within those promoters (Claessens et al., 2001). We had previously demonstrated that Ebp1 represses both exogenous and endogenous PSA promoter activity in LNCaP cells (Zhang et al., 2002). We therefore tested the ability of Ebp1 to repress the MMTV-luc and probasin native androgen responsive promoters. LNCaP cells were stimulated with R1881 and the induction of luciferase activity in the absence of exogenous ebp1 was measured. Probasin and MMTV promoters were strongly activated by R1881. AR activation of both promoters was reduced significantly ($P \leq 0.05$) with the ectopic expression of ebp1 (Figure 2). However, a small but significant increase in AR activity after R1881 treatment was noted even in the presence of ebp1.

We next wished to determine if endogenous Ebp1 was important in AR signalling. COS-7 cells were first transfected with siRNA targeted to two regions in the Ebp1 cDNA as described in the Materials and Methods. Cells were then transfected with the AR expression plasmid, and the MMTV-luciferase reporter construct the next day. Cells were stimulated with R1881 on day 3 and lysates collected on day 4. The results of Western blotting experiments showed that transfection of siRNA directed against Ebp1 reduced proteins levels about 80% at Day 4 (Figure 3A). This decrease was not observed in cells transfected with scrambled oligos. Decreased expression of Ebp1 resulted in a significant ($P \leq 0.05$) 3.5-fold increase in the luciferase activity of the MMTV promoter in the absence of androgen. No such stimulation was observed in cells lacking AR. R1881 stimulation of the reporter plasmid was decreased by inhibition of ebp1 expression, but this change was not significant at the $P \leq 0.05$ level (Figure 3B). These results suggest that Ebp1 may be important in repression of AR in the absence of androgen.

**HRG regulates the binding of Ebp1 to ErbB3 and AR**

We next determined if ErbB3 could bind Ebp1 in human prostate cell lines as it does in breast carcinoma cells (Yoo et al., 2000) and if HRG could affect this binding. Lysates of serum starved LNCaP cells were incubated with either a mouse monoclonal antibody to ErbB3 or control IgG. Proteins were resolved by SDS–PAGE and immunoblotted with antibody to Ebp1. Ebp1 was found in ErbB3, but not control, immunoprecipitates (Figure 4A). Next, we determined if the binding of Ebp1 to ErbB3 could be regulated by HRG. LNCaP cells were serum starved and treated with HRG (20 ng ml$^{-1}$) for 0, 15, 60 and 120 min and 24 h. Cell lysates were immunoprecipitated with antibody to ErbB3 and Ebp1 was found in ErbB3 immunoprecipitates of untreated cells (Figure 4B). There was a decrease in the level of Ebp1 associated with ErbB3 starting at 15 min. No Ebp1 was found in the Erb3 immunoprecipitates 60 min after treatment. Binding was increased 2 h after treatment and by 24 h after HRG treatment, Ebp1 binding to ErbB3 was restored.
As HRG treatment resulted in the release of Ebp1 from ErbB3, we next determined whether HRG could regulate the association of endogenous Ebp1 with endogenous AR. LNCaP cells, growing in complete media, were switched into phenol-red free RPMI 1640 media with 1% CSS and R1881 (10\(^{-8}\)M) overnight. Cells were then treated with 20 ng ml\(^{-1}\) of HRG /\(\beta\)1 for 1 h, a time when we could not detect Ebp1 in ErbB3 immunoprecipitates. Cell lysates were immunoprecipitated with the Ebp1 antibody. Western blot analysis of the immunoprecipitates indicated that HRG treatment enhanced the interaction of Ebp1 with AR (Figure 5A). Examination of cell lysates revealed that HRG treatment did not increase the level of AR protein at this 1 h time point (Figure 5B).

HRG enhances Ebp1 transcriptional repression. We reasoned that if HRG could change the association of Ebp1 with AR, it could affect Ebp1 induced repression of AR transactivation. LNCaP cells were transiently transfected with the MMTV luciferase reporter plasmid and limiting amounts of an ebp1 expression construct. Ebp1 at low concentrations (0.1 \(\mu\)g) reduced AR luciferase activity 55%. Maximal inhibition of 90% was observed at 0.5 \(\mu\)g of the ebp1 plasmid. This was more than the 80% inhibition previously observed (Figure 1) and probably due to changes in transfection efficiencies as different batches of cells and plasmids were used in these different experiments. Concentrations of HRG (20 ng ml\(^{-1}\)), previously demonstrated to increase association of Ebp1 and AR, significantly (\(P \leq 0.05\)) enhanced Ebp1-mediated repression at low (0.1 and 0.2 \(\mu\)g) amounts of the Ebp1 plasmid (Figure 6).

**DISCUSSION**

We have previously established that Ebp1, a protein cloned in our laboratory via its interactions with the ErbB3 receptor, inhibits AR-mediated transcription and growth of the AR positive LNCaP cell line (Zhang et al, 2002). In this report, we confirm and extend our findings by demonstrating that Ebp1 is capable of inhibiting receptor transactivation independent of cell type or AR target promoter. In addition, we demonstrate that HRG, the ErbB3 ligand, stimulated the association of Ebp1 with AR and increased Ebp1 mediated repression of AR activity, providing further evidence for a link between ErbB ligands and AR function.

This study first demonstrated that Ebp1 inhibition of AR transactivation was neither promoter nor cell type specific. We had previously demonstrated that Ebp1 inhibits AR transactivation of...
the artificial ARE2 luciferase reporter in COS cells and the PSA luciferase reporter in LNCaP cells (Zhang et al., 2002). However, recent studies have demonstrated that AR mediated gene transcription is influenced by the cell type examined (Kotaja et al., 1999; Claessens et al., 2001; Holter et al., 2002). For example, Holter et al. (2002) have shown that DAX1 inhibition of PSA and the ARE reporter was more potent in COS-7 than HeLa cells. This variability has been attributed to the complement of transcription factors and coregulators in different cell types. Therefore, we examined AR transactivation of MMTV-luc in two androgen-independent prostate cancer cell lines, PC3 and DU145, that had been transfected with wild-type AR. Ebp1 inhibited AR-regulated transcription in these androgen-independent cells. Similarly, Cyclin D1 inhibits AR transactivation across a wide variety of both prostate and nonprostate derived cell lines (Petre-Draviam et al., 2003). In addition, a number of AR coregulators demonstrate promoter specificity. For example, ARIP3 enhances transcription from minimal AREs, yet represses the probasin promoter (Kotaja et al., 2000). Activation of both the PSA and probasin promoters requires interactions of the N and C terminal domains of AR, but this association is not required for activation of MMTV (He et al., 2000). Thus, it was important to examine the effects of Ebp1 on different native promoters. Here we show that Ebp1 also inhibited the probasin and MMTV reporters in LNCaP cells as well as PSA as previously demonstrated (Zhang et al., 2002). Our previous studies had also shown that inhibition of AR transactivation is specific, as Ebp1 did not affect the estrogen induced responsiveness of an ERE luciferase reporter or the thyroid hormone mediated activity of a TRE luciferase reporter plasmid (Zhang et al., 2002). It is of interest to note that while ebp1 overexpression completely inhibited AR activity in DU145 and PC-3 AR transfected cells, ebp1 was unable to completely suppress the response to R1881 in LNCaP cells. This discrepancy may have been due to different expression levels of AR in the DU145 and PC-3 cells lines as compared to LNCaP cells, the
fact the AR receptor is mutated in LNCaP cells, or to different transfection efficiencies of the ebp1 plasmid among the three cell lines.

Studies using siRNA demonstrated that inhibition of endogenous Ebp1 expression resulted in increased activity of an androgen regulated reporter construct in the absence of androgens. No such effect was observed in cells not expressing the AR. Thus, the increase in promoter activity in the absence of androgens was mediated via the AR. This finding suggests that Ebp1 may play a role in inhibition of AR signalling in the presence of no or extremely low levels of androgens. Ebp1 has the ability to bind DNA (Zhang and Hamburger, 2004) and histone deacetylases (Zhang et al, 2003), and we postulate that Ebp1 might reside on AR regulated promoters in the absence of androgens to inhibit transcription. ChIP assays to demonstrate Ebp1 occupancy on AR promoters are underway in the laboratory. The fact that abolition of Ebp1 protein enhanced basal, but not AR stimulated transcription, is somewhat puzzling in light of the fact that overexpression of Ebp1 inhibits R1881 stimulated, but not basal, transcription of AR regulated genes. It is possible that basal transcription by AR is so low in unstimulated LNCaP cells that Ebp1-mediated repression in the absence of androgens may have gone undetected. Conversely, overexpression of Ebp1 might drive Ebp1 to AR promoters in the presence of androgens. Ebp1 might then recruit HDACs, important in AR transcriptional repression (List et al, 1999), to the promoter to inhibit gene transcription. The fact that inhibition of expression of Ebp1 leads to increased transcriptional activation of AR suggests that endogenous Ebp1 may function to regulate AR signalling in prostate cancer cells.

Although Ebp1 was shown to be associated with ErbB3 in breast cancer cells (Yoo et al, 2000), the interaction of Ebp1 with ErbB3 in prostate cells had not yet been demonstrated. Here, we determined that Ebp1 could also bind ErbB3 in LNCaP cells. To the best of our knowledge, this is the first demonstration of a direct interaction between ErbB3 and the AR. It is of interest that Bonaccurso et al (2004) recently reported the physical association and subcellular colocalisation of the EGFR with AR in PC3 cells transfected with the AR. These studies further support the concept that ErbB receptors and AR interact in vivo.

We then determined the effects of HRG on Ebp1 function. HRG treatment resulted in dissociation of Ebp1 from the ErbB3 receptor in LNCaP cells. The present studies also demonstrate that HRG mediated the interaction of Ebp1 with the AR receptor. First, we demonstrated that endogenous Ebp1 associated with endogenous AR in vivo. However, the efficiency of the Ebp1:AR interaction was relatively low in the absence of HRG. HRG treatment of LNCaP cells for 1h enhanced the association of Ebp1 with AR in LNCaP cells. The basis of the increased association of Ebp1 with AR after HRG treatment is not known. We have found that HRG increases phosphorylation of Ebp1 in breast cancer cells (Lessor et al, 2000) and studies are underway in the laboratory to examine if enhanced phosphorylation of Ebp1 increases its binding to AR. The intracellular compartment in which AR and Ebp1 interact in either the presence or absence of HRG is not clear at this time. Immunofluorescence analysis in our hands indicates that both Ebp1 and AR are located in the nucleus and the cytoplasm of LNCaP cells in the absence of HRG and/or R1881 (data not shown).

The fact that HRG enhances the binding of Ebp1 to AR suggests that in the absence of HRG, Ebp1 may not optimally affect AR function. Indeed, HRG potentiated the ability of limiting amounts of Ebp1 to inhibit AR promoter activity. HRG has been previously shown to inhibit growth of AR + but not AR – prostate cancer cell lines (Grasso et al, 1997b; Lyne et al, 1997). Similarly, Abreu-Martin et al (1999) found that MEKK-1, a downstream mediator of HRG signalling, induced apoptosis of AR +, but not AR –, prostate cancer cell lines. We hypothesise that Ebp1 may be one mediator of the effect of HRG on AR function. In the presence of low concentrations of HRG, such as has been observed in prostate cancer tissues (Lyne et al, 1997), the activity of Ebp1 may be suboptimal, resulting in increased AR signalling. Thus, although Ebp1 may be present in prostate cancer cells (Xia et al, 2001b), it may not be fully functional.

In summary, Ebp1 is a potent corepressor of AR with broad specificity. Ebp1 maintains its corepressor activity independent of cell type and promoter examined. Thus, Ebp1 joins a small but growing group of AR corepressors (Culig et al, 2003). The fact that interactions of Ebp1 and AR were regulated by HRG suggests a link between the HRG-generated growth inhibitory signals transduced through the ErbB3 receptor and the AR receptor. Further studies are needed to characterise the interactions of ErbB3, Ebp1 and AR in the progression of prostate cancer.

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