Identification of a Novel Estrogen Response Element in the Breast Cancer Resistance Protein (ABCG2) Gene

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Abstract

The breast cancer resistance protein (BCRP) is an ATP-binding cassette half transporter that confers resistance to anticancer drugs such as mitoxantrone, anthracyclines, topotecan, and SN-38. Initial characterization of the BCRP promoter revealed that it is TATA-less with 5 putative Sp1 sites downstream from a putative CpG island and several AP1 sites. The promoter showed that the region between Sp1 sites downstream from a putative CpG island and several AP1 sites is TATA-less with 5 putative estrogen response element (ERE). We showed that estrogen enhanced the expression of BCRP mRNA in the estrogen receptor (ER)-positive T47D/A18 cells and PA-1 cells stably expressing ERα. In BCRP promoter-luciferase assays, sequential deletions of the BCRP promoter showed that the region between −243 and −115 is essential for the ER effect. Mutation of the ERE found within this region attenuated the estrogen response, whereas deletion of the site completely abrogated the estrogen effect. Furthermore, electrophoretic mobility shift assays revealed specific binding of ERα to the BCRP promoter through the identified ERE. Taken together, we provide evidence herein for a novel ERE in the BCRP promoter.

Introduction

The breast cancer resistance protein (BCRP) belongs to the ATP-binding cassette G family and is also known as ABCG, MXR, or ABCG2 (1–3). BCRP-overexpressing cancer cells have been isolated from breast, colon, gastric, lung, or ovarian carcinomas (3–6) where BCRP acts as an efflux pump, resulting in decreased intracellular concentrations of anticancer drugs. It was initially found to be associated with mitoxantrone resistance (2, 3, 5, 7) and later found to confer resistance to other drugs such as topotecan, SN-38, anthracyclines, and flavopiridol (4, 7–9). More recently, methotrexate and its polyglutamates have also been named as BCRP substrates (10–12).

Cells that overexpress a mutated BCRP (R482T and R482G) retained sensitivity to methotrexate (10–12). Cells that overexpress a mutated BCRP (R482T and R482G) retained sensitivity to methotrexate (10–12).

Among normal tissues, BCRP is expressed in the syncytiotrophoblasts of the placenta, in the epithelium of the small intestines and colon, in the liver canalicular membrane, in hematopoietic stem cells, and in the ducts and lobules of the breast (13, 14). Whereas the precise physiological role of BCRP is still unknown, its localization in these cells (13, 14) suggests that it may play a protective role against toxic substances and metabolites by extruding them across the apical membrane. A number of endogenous substrates of BCRP have been named lately, and these include 17β-estradiol-17-β-D-glucuronide), sulfated steroidal compounds such as dehydroepiandrosterone sulfate, and folic acid (10, 15, 16). The identification of more physiological substrates may eventually provide insights into the functions of BCRP in the body.

To date, little is known about the molecular mechanisms controlling BCRP expression. The BCRP gene comprises 16 exons and 15 introns spanning 66 kb in length and is located on chromosome 4q22 (17). Initial characterization of the BCRP promoter revealed that it is TATA-less with 5 putative Sp1 sites downstream from a putative CpG island and several AP1 sites (17). The present study was undertaken with the goal of identifying factors responsible for regulating BCRP expression. Here we examined the sequence of the 5′-flanking region of the BCRP gene and found a novel estrogen response element (ERE) in this promoter. We showed that BCRP mRNA expression was induced by 17β-estradiol (E2) in estrogen receptor (ER)-positive cell lines and that this effect can be reversed by the antiestrogen ICI 182,780. We investigated the transcriptional activities of the 5′-flanking region of the BCRP gene and confirmed that the BCRP gene is a direct target of the E2/ER complex through the identified ERE.

Materials and Methods

Hormones and Chemicals. E2 was purchased from Calbiochem (San Diego, CA) and ICI 182,780 was a gift from AstraZeneca Pharmaceuticals (Macclesfield, United Kingdom).

Cell Culture. All of the cells were grown at 37°C in a humidified atmosphere containing 5% CO2. Cell culture reagents were purchased from BioWhittaker (Walkersville, MD) unless otherwise stated. The ER-positive human breast cancer cell line, T47D/A18, has been described before (18) and was maintained in RPMI 1640 containing fetal bovine serum (10%; Sigma, St. Louis, MO), penicillin (100 units/ml), streptomycin (100 μg/ml), and t-glutatmine (2 mM). The ER-negative human ovarian cancer cell line, PA-1, was obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM containing fetal bovine serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and t-glutatmine (2 mM). The PA-1+ER cell line was established by transfecting PA-1 cells with an expression vector for human ERα (pSG5-HEG0; a gift from Dr. Pierre Chambon, CNRS/INSERM/ULP, Illkirch Cedex, France) and pcDNA3.1 in the ratio of 3:1 using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Positive clones were maintained in G418 (2 mg/ml; Meditech, Inc., Herndon, VA).

Plasmids and Cloning. The human BCRP promoter and deletion fragments (−1285/+362, −243/+362, and −115/+362) were subcloned into a pGL3-basic reporter plasmid (Promega, Madison, WI) and were described previously (17). The vectors −243mut1, −243mut2, −243mut3, and −243del were generated from −243/+362 using sequential PCR steps (19) and subcloned into the pGL3-basic reporter vector. All of the constructs were verified by sequencing. The vector, Triplite ERE-luciferase, containing three copies of the Xenopus vitellogenin A2 ERE upstream of the luciferase gene, was described before (20).
ESTROGEN RESPONSE ELEMENT IN BCRP

RNA Isolation and Semiquantitative Reverse Transcription-PCR Analysis. Total RNA was extracted from T47D:A18 and PA-1+ER cells using Trizol (Invitrogen). The first-strand cDNA was synthesized with 3 μg of the total RNA using the ThermoScript Reverse Transcript-PCR system (Invitrogen). Using one twentieth of the cDNAs as a template, the PCR was carried out for each transcript under these conditions, 20–35 cycles of 94°C 30 s, 60°C 60 s, and 72°C 80 s to determine the exponential phase of amplification. As an internal control, amplification of β-actin mRNA was carried out with similar cycle optimization. The following primer sets were used, sense, 5'-TGG AGC AGA GAG -3' for BCRP, sense, 5'-TGG AGC AGA GAG GAG GCA A-3' and antisense, 5'-GCC GAG CTC TGG GAG GAC TAA TCA-3' for pS2, and sense, GAG AAG ATG ACC CAG ATC ATG T-3'; and antisense, 5'-TCG TCA TAC TCC TGC TTG CAG-3' for β-actin.

Luciferase Assays. To examine the promoter activities in response to estrogenic stimulation, PA-1 cells were cultured in phenol red-free MEM (Invitrogen) supplemented with 10% charcoal/dextran-stripped fetal bovine serum (Gemini, Woodland, CA) for at least 3 days. PA-1 cells were grown at a density of 2 × 10^6 cells/well in six-well plates and cotransfected with 1.5 μg of the luciferase constructs, 0.25 μg hERα expression vector (pSG5-HEG0) and 0.5 μg β-galactosidase reporter plasmids by Lipofectamine Plus, according to the manufacturer’s protocol. After 16–18 h, fresh medium containing 10 nm E2 dissolved in ethanol or ethanol alone (0.1%) was added to the transfected cells. Twenty-four h later, the cells were harvested and analyzed for both the luciferase and β-galactosidase activities using assay kits from Promega. Relative luciferase activities were normalized with β-galactosidase activities. Each experiment was performed in triplicate and repeated at least twice.

Electrophoretic Mobility Shift Analysis. Radiolabeled probes harboring the ERE of the BCRP gene were amplified using 243/362 as a template in a standard PCR reaction to incorporate [α-32P]dCTP (Amersham Biosciences, Piscataway, NJ) into the probe. The binding reactions were carried out in the presence of 10 nm E2 with 5 pmol recombinant ERα protein (Affinity Bioreagents, Golden, CO) and 32P-labeled 110-μb probes (5 × 10^6 cpm/reaction) in 20 μl of buffer [5 mM HEPES (pH 7.9) containing 100 mM NaCl, 0.25 mM EDTA, 0.25 mM EGTA, and 0.25 mM DTT]. Fifty μg/ml poly(deoxyinosinic-deoxycytidylic acid; Amersham Biosciences) was used as a nonspecific carrier DNA in the reaction. The mixture was placed on ice for 30 min. Protein-DNA complexes were separated from the free probe by nondenaturing 5% PAGE at 200 V with running buffer consisting of 25 mM Tris/25 mM borate/1 mM EDTA. Gels were dried, and the radioactive bands were visualized by autoradiography. For competition EMSA, a 100-fold excess of the unlabeled probe was added during the preincubation period. A 100-fold excess of unlabeled oligonucleotides with ERE deleted (BCRP-EREme) was also added in a separate reaction. Nonspecific unlabeled oligonucleotides that do not contain any known binding sequences were used as a negative control.

**Results**

Identification of a Putative ERE in the BCRP Gene. We examined the sequence of the 5'-flanking region of the BCRP gene and found a putative ERE in the promoter (Fig. 1A) using our own computer software, PAGen@UIC,4 to search for known DNA-binding protein recognition sites. Previous analyses of the 5'-flanking region of the human BCRP gene had not revealed any indication of an ERE (17). This sequence is highly similar to the ERE found in genes for human coagulation Factor XII (21, 22) and human c-fos (23), as shown in Fig. 1B. Although not perfectly palindromic as with most known elements identified to date, at least 10 bp of the putative ERE of the BCRP gene are identical to the consensus ERE, in particular, to that found in human coagulation Factor XII (21, 22) and human c-fos (23). Furthermore, the imperfect palindrome is separated by exactly 3 bp, thereby retaining the length of the ERE critical for high affinity ER-ERE binding (24).

**Fig. 1.** The estrogen response element (ERE) in the promoter region of the BCRP gene. A, the human BCRP promoter region that contains the ERE. Nucleotide numbers are denoted with the transcription start site assigned as +1. The ERE sequence is underlined. B, comparison of the ERE sequence of the BCRP gene with the consensus sequence and that of the human coagulation Factor XII and c-fos genes. The invariant sequence is underlined. Pn, purine; Py, pyrimidine; N, any nucleotide.

Estrogen Treatment Increases BCRP mRNA Levels. To determine whether BCRP mRNA levels are regulated by E2, we placed ER-positive T47D:A18 breast cancer cells in a medium containing charcoal/dextran-stripped serum for 3 days before treating them with 10 nm E2 for 24 h. Semiquantitative reverse transcription-PCR was performed and β-actin was used as a loading control. As shown in Fig. 2A, BCRP mRNA levels were increased significantly with E2 treatment. The estrogen-inducible gene, pS2, was used as a positive control and showed a similar increase. This effect was effectively abolished with concomitant treatment with a pure steroidal antiestrogen, ICI 182,780.

Next we asked if a similar trend would be observed in an ER-negative ovarian cancer cell line PA-1, stably transfected with ERα, PA-1+ER. As shown in Fig. 2B, BCRP mRNA levels were also increased in PA-1+ER cells with E2 treatment. As expected, no change was observed in the parental PA-1 cells after E2 treatment (data not shown).

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**BCRP Gene Contains a Functional ERE.** These results encouraged us to analyze whether BCRP promoter activity is E₂-regulated. We transfected PA-1 cells with the full-length promoter-luciferase construct −1285/+362, together with the human ERα expression plasmid, and treated them with 10 nM E₂ only, or with a combination of 10 nM E₂ and ICI 182,780 at concentrations ranging from 0.01 to 1 μM. As shown in Fig. 3A, the promoter activity was increased up to 6.2-fold in the presence of E₂ alone and decreased in a dose-dependent manner in the presence of ICI 182,780, reflecting a blockade of ER activity. As a positive control, we transfected the cells with a Triplet ERE-luciferase plasmid and found a dramatic increase in promoter activity with E₂ treatment of 22-fold (Fig. 3B).

The ERE in the BCRP Promoter Binds ERα. To determine whether the ERE in the BCRP gene can be recognized by ERα like a known ERE, electrophoretic mobility shift analyses were performed using purified recombinant ERα protein incubated with 10 nM E₂ and radiolabeled oligonucleotides harboring the putative ERE of the BCRP gene. Fig. 4 (Lane 2) shows efficient binding to the radiolabeled oligonucleotides. The binding was competed by adding a 100-fold excess of unlabeled ERE probe (Fig. 4, Lane 3). When a 10–100-fold excess of unlabeled oligonucleotides with the ERE deleted was added, no competition of binding occurred (Fig. 4, Lanes 4 and 5), indicating that binding only occurred with specific recognition of the ERE by the recombinant ERα protein. A nonspecific unlabeled competitor used also did not result in any changes in binding (Fig. 4, Lane 6). Collectively, these data show that the ERE of the BCRP gene behaves similarly as the consensus ERE and that it can bind ERα.

**Discussion**

In this study, we found that BCRP is induced by nanomolar (physiological) concentrations of E₂ at the transcriptional level, and we revealed the underlying molecular mechanism by identifying a functional ERE in the BCRP promoter. The consensus ERE contains a palindrome of PuGGTCA motifs separated by 3 bp (24, 25). These elements are bound by ER dimers, with one receptor interacting with each PuGGTCA motif. This ER dimer-motif interaction is highly specific, as consensus EREs are expected to be found only in every 4 million bp in random DNA sequences (25). Stimulation of BCRP gene expression in response to E₂ is likely to be mediated by the “classical” mechanism.
mechanism, whereby E2-ligated ER binds directly to the identified ERE, and interacts with coactivator proteins and components of the RNA polymerase II transcription machinery, resulting in enhanced transcription (26).

BCRP is highly expressed in the placenta, or more specifically, in the placental syncytiotrophoblast, at the apical surface of the chorionic villus (14), where steroid hormones such as estrogens are produced and could reach a maximum concentration of 150 nm (27). The localization of BCRP in the placenta suggests that BCRP plays a protective role for the fetus by effluxing drugs and toxic metabolites that enter the placenta back into the maternal circulation. In fact, Jonker et al. (28) showed that the fetal penetration of topotecan is increased at least 2-fold by the BCRP inhibitor GF120918 in pregnant mdr1a/1b knockout mice. Our findings suggest for the first time that estrogens may augment this protective function, by inducing the endogenous expression of BCRP in the presence of ER.

Our findings also raise the possibility that estrogens can directly stimulate the production of this multidrug resistance transporter in hormone-responsive tumors clinically. We have shown the E2 induction of the BCRP gene expression in ER-positive breast and ovarian tumor cells and the reversal of this induction with a specific antiestrogen ICI 182,780. Intratumoral concentrations of estrogens are much higher than that in the serum and adjacent tissues (27), and this reservoir may cause induction of the BCRP gene and potentiate multidrug resistance in these tumors. Conversely, clinical treatment of hormone-responsive tumors with antiestrogens may help to reduce the incidence of drug resistance. It will be of interest, therefore, to determine whether ERα analysis in tumor biopsies correlates with BCRP expression and drug resistance.

Most studies of estrogen action have focused on ERα because ERβ was discovered more recently (29). Studies have shown that ERα and ERβ may respond differently to ER ligands in a cell-dependent and target gene context-dependent manner (30, 31). The above consideration raises the concern that in tumor cells expressing both ERα and ERβ, the latter may counteract ERα induction of BCRP. Studies examining the effect of ERβ on BCRP gene expression are ongoing.

The regulation of BCRP by estrogen is a complex issue. We have shown the influence of estrogen on BCRP gene expression. To date, several studies on the effect of estrogen on BCRP transport functions have been reported (15, 32). Imai et al. (15) found that free estrogens are not transported by BCRP, although they are able to block intravesicular estrone-3-sulfate uptake. Estrogen agonists and antagonists were found to increase cellular accumulation of topotecan in BCRP-overexpressing K562 cells (32). It is important to note, however, that in the latter study the BCRP gene was driven by a constitutive (cytomegalovirus) promoter (33) and not its endogenous promoter. Hence, no conclusions can be made regarding the effect of estrogen on BCRP gene expression.

In conclusion, a novel imperfect palindromic ERE was found in the BCRP gene promoter, and E2 was shown to activate the promoter through the classical pathway that involves binding of E2/ER complexes on the ERE. Given the abundance of BCRP in the placenta, our findings could shed light on its physiological regulation. At the same time, our results may have a clinical impact, because repression of BCRP expression might be an important result of antiestrogen therapy.

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References


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