Selective Interactions of Krüppel-like Factor 9/Basic Transcription Element-binding Protein with Progesterone Receptor Isoforms A and B Determine Transcriptional Activity of Progesterone-responsive Genes in Endometrial Epithelial Cells*

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The Sp/KLF transcription factor basic transcription element-binding protein (BTEB1) regulates gene transcription by binding to GC-rich sequence motifs present in the promoters of numerous tissue-specific as well as housekeeping genes. Similar to other members of this family, BTEB1 can act as a transactivator or transrepressor depending on cell and promoter context, although the molecular mechanism underlying these distinct activities remains unclear. Here we report that BTEB1 can mediate signaling pathways involving the nuclear receptor for the steroid hormone progesterone in endometrial epithelial cells by its selective interaction with the progesterone receptor (PR) isoforms, PR-A and PR-B. Functional interaction with ligand-activated PR-B resulted in superactivation of PR-B transactivity, facilitated the recruitment of the transcriptional integrator CREB-binding protein within the PR-dimer, and was dependent on the structure of the ligand bound by PR-B. By contrast, BTEB1 did not influence agonist-bound PR-A transactivity, although it augmented PR-A inhibition of PR-B-mediated transactivation as well as potentiated ligand-independent PR-A transcriptional activity in the presence of CREB-binding protein. We also demonstrate similar positive modulatory actions of BTEB1-related family members Krüppel-like family (KLF) 13/FKLF2/BTEB3 and Sp1 on PR-B transactivity. Further, we provide support for the potential significance of the selective functional interactions of PR isoforms with BTEB1 in the peri-implantation uterus using mouse and pig models and in the breast cancer cell lines MCF-7 and T47D. Our results suggest a novel mechanism for the divergent physiological consequences of PR-A and PR-B on progesterone-dependent gene transcription in the uterus involving select KLF members.

Progesterone (P)† plays a predominant role in the control of uterine endometrial growth and differentiation (1). In the absence of P, the unopposed actions of estrogen can lead to uncontrolled cellular proliferation at the expense of cellular differentiation, an event highly correlated with the development of endometrial carcinoma (2–3). The intracellular actions of P are mediated by the progesterone receptor (PR), a member of the nuclear receptor superfamily of ligand-activated transcription factors (4–5). PR exists as two isoforms, PR-A and PR-B, which are transcribed from a single gene and display similar hormone and DNA binding specificities (6, 7). PR-B differs from PR-A by the presence of an additional 164 residues in the amino-terminal region of PR-B, and the two proteins exhibit distinct transcriptional activities as a function of promoter and cellular contexts. In general, PR-B is a stronger transcriptional activator than PR-A (8, 9). Moreover, PR-A exerts an inhibitory effect on PR-B transactivity as well as that of other steroid hormone receptors (10–12), and endometrial differentiation and secretory phenotype are positively associated with PR-B rather than PR-A expression (8, 13). Concomitant with a general decrease in the number of endometrial cells expressing PR, a shift in the ratio of PR-A to PR-B, favoring the predominance of PR-A-expressing cells, was noted during the progression of the endometrium from normal to hyperplastic to tumorigenic states (14).

The mechanism underlying the distinct transcriptional activities of the two PR isoforms is not well understood, although this has been attributed to discrete protein-protein interactions that are mediated by the B-upstream sequence unique to PR-B (15). In particular, B-upstream sequence has been shown to repress the inhibitory activity of a segment within the N-terminal region of PR-A, which interferes with the activation functions of the AF-1 and AF-2 domains of the PR (16). PR-A has also been shown to interact preferentially with co-repressors and less efficiently with co-activators, compared with PR-B (17–18). In a recent study (18), however, PR-A was demonstrated to positively modulate the transcriptional activity of PR-B on a gene promoter that is induced synergistically by P and cAMP by binding to liver activating protein (LAP), an isoform of CCAAT/enhancer-binding protein β (C/EBPβ) in human endometrial stromal cells. Thus, promoter as well as cellular context can influence the transcriptional activity of each

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‡ The abbreviations used are: P, progesterone; KLF, Krüppel-like factor; PR, progesterone receptor; BTEB, basic transcription element-binding protein; DMEM, Dulbecco’s minimal essential medium; ECL, enhanced chemiluminescence; UF, uteroferrin; Luc, luciferase; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; FBS, fetal bovine serum; TFBS, Tet system approved fatty bovine serum; LSM, least-square means; ANOVA, analysis of variance; SRC-1, steroid receptor co-activator-1; Dox, doxycycline.
isofrm, and binding to consensus PREs may not be necessarily required for P-responsiveness of target genes (19, 20).

In a previous study, we have defined basic transcription element-binding protein (BTEB1), a member of the Sp/Krüppel-like family (KLF) of transcription factors (21–23) as a PR-B interacting protein (24). We also showed that BTEB1 transactivates genes containing GC-rich recognition motifs (25, 26), but in the presence of ligand-activated PR-B, the functional complex formed between the PR-B dimers and BTEB1 depends on the induction of PRE-containing promoters (24). Because the relative levels of PR-A, PR-B and BTEB1 are tightly regulated in normal uterine endometrium, and deviations in PR isoform ratios can affect tissue responsiveness to P, we examined whether BTEB1 alters the transactvity of PR-A for different promoters. Here we show that BTEB1 has no effect on PR-A transactivation but enhances PR-A-mediated repression of PR-B transcriptional activity. We also demonstrate that BTEB1 cooperates with the transcriptional integrator CREB-binding protein (CBP) (27) in enhancing the transcriptional activities of ligand-bound PR-B and unliganded PR-A, respectively. Further, we identify FKF2/BTEB3 (28) and Sp1 as functional PR-B transcriptional partners, with BTEB3 closely mimicking the activity of BTEB1. Finally, we provide evidence to suggest that the functional interactions of BTEB1 and PR-B noted from transient transfection studies may be biologically relevant in the context of early pregnancy in the mouse and pig uteruses and in the breast carcinoma cell lines MCF-7 and T47D. These results suggest that the selective utilization of BTEB1 and related KLF members by PR isoforms may underlie, in part, the distinct repertoire of progestin-regulated genes mediated by homodimers of PR-A and PR-B as well as by the PR-APR-B heterodimer, respectively, in target cells (29).

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were obtained from the following sources: Taq DNA polymerase and restriction endonucleases from Roche Molecular Biochemicals (Indianapolis, IN); PCR optimizer, complementary DNA (cDNA) cycle, and TA cloning systems from Invitrogen; cell culture media from Invitrogen; and [3H]deoxycytidine triphosphate (3000 Ci/mmol) from ICN Radiochemicals (Irvine, CA). Rabbit antibodies raised against human Sp1 and human Pr were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Type I (ZSK9289, nonapristone) and Type II (R1486, mifepristone) PR antagonists were kindly provided by Dr. Linda Tseng (State University of New York, Stony Brook, NY). All molecular biology-grade chemicals and solvents were purchased from Fisher Scientific (Pittsburgh, PA).

**Plasmid DNAs and Expression Constructs**—The DNA constructs used for transient transfection studies were obtained from the following sources: rat BTEB1 in pcDNA3 (Dr. Horiako Imataka, McGill University, Quebec); rat PR-B in pcMV5 (Dr. Benita Katzenellenbogen, University of Illinois, Urbana-Champaign, IL); human Sp1 in pCMV (Dr. Robert Tjian, University of California, Berkeley, CA); human BTEB3 in pcDNA3.1 and pcDNA3.1 empty vector (Dr. An Song, Stanford University, Palo Alto, CA); human PR-A in pSV and pVP16, respectively, and pSG5 (Dr. Dean Edwards, University of Colorado, Denver, CO); human PR-A and -B isoforms in pSG5 vector and mouse mammary tumor virus (MMTV)-chlamyaphenol acetyltransferase (CAT) reporter construct (Dr. Pierre Chamoun, University of Strasbourg, Strasbourg, France); and CBP in pSG5 (Dr. James Bierek, Mount Sinai School of Medicine, New York, NY). A luciferase reporter construct (UF-Luc) containing 1143 bp of the 5′ promoter and regulatory region of the P-regulated porcine uterotelin (UF) gene was previously described (24). All plasmid DNAs were prepared using the Maxiprep system (Qiagen, Valencia, CA).

**Cell Lines and Culture**—The human endometrial carcinoma cell line HeC-1-A, a model of well differentiated carcinoma, was a gift of the late Dr. John Hog (Hershey Medical Center). HeC-1-A was routinely cultured in McCoy’s 5A in the presence of 10% (v/v) fetal bovine serum (FBS). Monkey kidney COS-1 cells (American Type Culture Collection (ATCC), Manassas, VA) were propagated in DMEM containing 10% FBS. The human mammary carcinoma cell line T47D was maintained in RPMI 1640 containing bovine insulin (0.2 IU/ml) and 10% FBS. The human mammary carcinoma MCF-7 Tet-On cell line (Clontech, Palo Alto, CA) and its clonal derivatives B5 and C3 that were generated in our laboratory (see “Results”) were cultured in DMEM containing 10% Tet system approved fetal bovine serum (TFBS) and 4G18 (0.1 μg/ml), following the manufacturer’s instructions. Cells were incubated at 37 °C in an atmosphere of 5% CO₂ in air.

**Transient Transfection and Reporter Gene Assays**—Transfections were carried out using polyethylene (Hexadimethrine Bromide, Sigma) for HeC-1-A cells or LipofectAMINE (Invitrogen) for COS-1 cells, as previously described (24). Approximately 6 × 10⁵ cells were plated in 6-well plates 24 h before transfection. Four h after transfection, HeC-1-A cells were treated with 25% MeSO in Hank’s Balanced Salt Solution (HBSS, pH 7.4) for 4 min, washed twice with HBSS, and then incubated for an additional 48 h in fresh HBSS supplemented with charcoal-stripped FBS (10%) in the presence or absence of the synthetic progesterin R5020 (100 nm; PerkinElmer Life Sciences). COS-1 cells transiently transfected with the chloramphenicol acetyltransferase reporter gene construct pSG5CAT (24) and specific combinations of p-PR-A chimeras, pVP16-PR-A chimeras, (30), and pCGDNA-BTEB1 expression vectors (each added at 0.5 μg/well), as well as other constructs (described under “Results”) were incubated for 6 h at 37 °C and then fed fresh medium containing 20% FBS. Eighteen h later, the cells were transferred to charcoal-stripped FBS (10%)-containing medium with or without 100 nM R5020 (24). Treated cells were further incubated for 24 h, and Luc or CAT activity was measured on whole cell extracts as previously described (24). Results were normalized to total protein content for each sample, as determined by the Bradford method (31), and are presented as least-square means (LSM) ± S.E. Individual transfections were done in triplicate and performed three or four times using cells of comparable passage numbers.

**RT-PCR**—Total cellular RNA from cells and uterine endometrial tissues was prepared by the TriZol method. Reverse transcription of RNA into cDNA and PCR amplifications were performed using commercially available cDNA synthesis and PCR kits (Invitrogen). For PCR analysis, the following primers were used: pig/human progesterone receptor PR (A/B) (sense) 5′-AGTCTTAAATGCTACCCCATC-3′ and (antisense) 5′-CTGAGAAAATCCACACATTAGT-3′ (290 bp); pig/human PR-A (sense) 5′-ACCTTCTACGTCGGT-3′ and (antisense) 5′-TCCAAAGACTCTGACACG-3′ (220 bp); pig β₂-microglobulin (sense) 5′-CTGCTCTCAGTGCCTG-3′ and (antisense) 5′-ATCGAGAGTCACCGCTG-3′ (288 bp); pig/human CBP (sense) 5′-AAGCAGAACCACACAC-3′ and (antisense) 5′-GGTGTGGCGACCCCTTCTTCTC-3′ (489 bp); human cyclin D1 (sense) 5′-GCGTCACTGAACTCTG-3′ and (antisense) 5′-GCTCACCTGATCACCT-3′ (482 bp); and human β-actin (sense) 5′-TCAACCAAGCCGGAGCATG-3′ and (antisense) 5′-TACCCCGGATCCATAGC-3′ (243 bp). The PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. The PR-B and CBP PCR products were transferred to a nylon membrane, which was subsequently hybridized with 32P-labeled 18S rRNA probes. Quantification of the intensities of the ethidium bromide staining (for PR (A/B), β₂-microglobulin, cyclin D1, and β-actin) and hybridization signals (for PR-B and CBP) were quantified using microdensitometry.

**Western Immunoblot and Co-immunoprecipitation Assays**—Nuclear extracts from tissues or cells, prepared following previously described protocols (32), were fractionated on 10% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes. Standard Western blot techniques were used to detect the levels of nuclear proteins (PR-A and -B isoforms, BTEB1, and Sp1) using the appropriate primary (1:1000 final dilution for each) and secondary antibodies as previously described (24, 26), and the enhanced chemiluminescence (ECL) detection system (Amersham Life Sciences). Co-immunoprecipitation with anti-rat BTEB1 antibody followed previously described protocols (24).

**Animals and Tissue Isolation**—Mice (Harlan, Indianapolis, IN) were housed in the animal care facility at the University of Florida in accordance with National Institutes of Health standards for the care and use of experimental animals. Adult female C57BL/6J mice were assigned randomly into groups of 6–8 animals per group. Animals were divided into two treatment groups, a control group, which consisted of mice fed chow ad libitum but were not mated and a group of mice mated with males of the same strain to induce pregnancy. Day 0.5 of pregnancy was designated as the morning of observing the vaginal plug. Whole uteri were taken at the indicated pregnancy days and frozen for analysis after flushing with PBS. Pig uterine tissues were isolated as described previously (25).

**Statistical Analysis**—All numerical data were compared with appropriate controls and analyzed using ANOVA following the general linear models procedure of the Statistical Analysis System (SAS). Comparisons between groups were assessed using predicted differences of the LSM. The statistical model included treatment and experiment, and only

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experiment, in addition to pG5CAT reporter, are indicated by performed in triplicate. The components present in each transfection and the pG5CAT reporter construct (5 μg) in the presence or absence of pCDNA3-BTEB1 expression vector or empty (pCDNA3 alone) expression vector (0.5 μg). Twenty-four h post-transfection, cells were treated with vehicle (ethanol) or R5020 (100 nM) for 24 h. CAT activity in transfected cells was analyzed as described previously (24) and was normalized to protein content of cellular lysates. Data are presented as LSM ± S.E. from three independent experiments, each performed in triplicate. The components present in each transfection experiment, in addition to pG5CAT reporter, are indicated by +, * designates significant difference (p ≤ 0.05) from the CAT activity of pm-PR-A/pVP16-PR-A group in the absence of added R5020.

preplanned comparisons were made. Treatment means were considered significantly different at p ≤ 0.05.

RESULTS

Lack of Functional Interaction of the PR-A Isoform with BTEB1—To investigate whether the PR-A isoform interacts with BTEB1, as was previously demonstrated for PR-B (24), two functional assays involving the quantification of promoter-reporter activities in cells transiently transfected with expression constructs for PR-A and BTEB1 were utilized. In the mammalian two-hybrid assay, the full-length PR-A, fused to the Gal4-DNA-binding domain in the pG vector or the activation domain in the pVP16 vector were co-transfected with pG5CAT in the presence or absence of full-length BTEB1 expression construct. Transfected COS-1 cells were then incubated for 24 h in medium containing R5020 (100 nM) in ethanol (vehicle) or vehicle alone. In the absence of BTEB1, the combination of the two PR single hybrids increased basal reporter activity with added R5020, suggesting the formation of a functional PR-A dimer (Fig. 1). BTEB1, however, had no effect on the basal or R5020-stimulated reporter activity in the presence of both PR-A fusion proteins.

To determine whether PR-A functionally interacts with BTEB1 within the context of a natural promoter with recognition sequences for both BTEB1 and PR, the UF promoter, which contains functionally responsive sequences for BTEB1 (25) and PR (34), was linked to the Luc reporter gene and used in transient transfections of HeLa cells. As expected, BTEB1 alone increased UP promoter activity (Fig. 2). In the absence of its ligand, PR-A or PR-B decreased basal transcription from the UF promoter-Luc reporter, which was further attenuated with the co-expression of the two isoforms. Ligand-activated PR-B increased transcriptional activity from the reporter, and this was enhanced in the presence of BTEB1 consistent with our previous findings (24). By contrast, ligand-bound PR-A had no effect on this promoter’s activity in the presence or absence of BTEB1. Moreover, PR-A inhibited P-dependent PR-B transactivity, and this repression was augmented by BTEB1 (Fig. 2). Results indicate that although BTEB1 preferentially interacts with PR-B homodimer to enhance PR-B transactivation, it acts as a negative modulator of P-dependent gene transcription mediated by PR-A/PR-B heterodimer.

PR-B Interaction with BTEB1 Is Dependent on Ligand Type—Because the type of ligand bound to a steroid receptor influences its subsequent interaction with other nuclear co-factors, the effects of type I (ZK98299) and type II (RU486) PR antagonists on the functional relationship of PR-B dimer and BTEB1 were compared with that of R5020 (PR agonist). RU486 and ZK98299 have higher and lower affinities, respectively, for PR than PR agonists, although both are known to elicit the formation of the PR-B dimer (30), resulting in inhibition of P-dependent promoter activity (see Ref. 35 for review). The transactivity of the PR-B dimer was observed only when bound to R5020, and this was augmented by BTEB1 co-expression (Fig. 3). By contrast, the respective complex formed with either ZK98299 or RU486, when used at the same concentration as R5020, had no effect on basal promoter activity but, as expected, inhibited the induction of this promoter’s activity by R5020-bound PR-B. Interestingly, the negative effect of either compound on PR-B transactivity was not reversed by the presence of BTEB1. Taken together, results suggest that the initial formation of PR-B dimer of the correct conformation that is able not only to bind to its cognate response element but also permits the binding of other nuclear proteins is requisite for subsequent interaction with BTEB1.

CBP Modulates Ligand-dependent PR-B Interaction with BTEB1—To determine whether the transcriptional integrator CBP, which has been shown recently to act synergistically with the steroid receptor co-activator-1 (SRC-1) in PR transactivation (36), modulates the transcriptional activity of either PR-A or PR-B in the presence of BTEB1, co-transfection experiments with the P-responsive MMTV-CAT reporter (37) and various
combinations of expression constructs were carried out in COS-1 cells. As shown in Fig. 4A, the MMTV-promoter activity was unaffected by BTEB1, alone or in combination with either CBP or PR-A. On the other hand, CBP and unliganded PR-A individually increased basal promoter activity, albeit their combined effects were not additive. The activities of PR-A and CBP when added together were enhanced by BTEB1, independent of the presence of ligand. The P-dependent PR-B transactivity was not augmented by CBP (Fig. 4B). However, the combination of BTEB1, PR-B and CBP maximally increased promoter activity, but only in a ligand-dependent manner (Fig. 4B). These findings indicate that though BTEB1/PR-B dimer association facilitates the recruitment of CBP into the PR-B complex, the functional interaction of PR-A with BTEB1 and CBP occurs through distinct mechanism(s). The latter finding may underlie, in part, the differing effects of each isoform on P-dependent gene transcription.

Effects of KLF Members Sp1 and FKLF2/BTEB3 on PR-B Activity—Sp1, BTEB3, and BTEB1 exhibit significant homologies in their DNA-binding domains but differ in their transactivation domains, which are located primarily in the amino-terminal regions of these proteins (21–23, 28). To assess the modulatory roles of Sp1 and BTEB3 relative to BTEB1 on PR-B transactivation activity, transient transfection experiments were performed using expression constructs for each, singly and in combination with PR-B. Sp1 had minimal, albeit significant, effects on P-dependent PR-B transcriptional activity of the MMTV-CAT promoter relative to BTEB1 in COS-1 cells, which express both BTEB1 and Sp1 endogenously (Fig. 5A). By contrast, BTEB3 mimicked the effect of BTEB1. In particular, both BTEB1 and BTEB3 showed no activity on the MMTV-CAT promoter but enhanced the P-dependent PR-B transactivity to a similar extent (Fig. 5B). Interestingly, within the context of the UF gene promoter, which contains recognition motifs for KLF members and PR-B, the transcriptional activities of BTEB1 and BTEB3 differed (Fig. 5C). Although both stimulated P-dependent PR-B transactivation of the UF promoter, only BTEB1 had a positive effect on basal transcription. Moreover, BTEB3 had no effect on BTEB1-mediated transcriptional activity, although both are known to bind GC-rich sequences within gene regulatory regions (21–23, 28). These findings suggest that the DNA-binding domain, which is conserved among KLF members, is sufficient for interaction of Sp1, BTEB1, and BTEB3, respectively, with the PR-B dimer, although other domains within these molecules may contribute to the strength of their inductive effects on PR-B transactivation.
Expression Levels of PR-A, PR-B, and CBP in Pregnancy Endometrium

To begin to assess the biological significance of BTEB1 interaction with specific PR isoforms in the presence of CBP, the expression levels of PR-A, PR-B, and CBP were evaluated in pig pregnancy endometrium by semi-quantitative RT-PCR and/or Western blot analysis. In previous studies (24, 38), we have shown that the levels of uterine endometrial BTEB1 protein did not change with stage of pregnancy. By contrast, the levels of total PR(A/B) mRNAs shown here were higher at early than at late pregnancy, with peak levels observed at day 12, the period of maternal recognition of pregnancy for this species (39) (Fig. 6A). The pattern of PR-B mRNA levels followed that of PR(A/B), with levels highest at early pregnancy (days 12–14) and dropping precipitously beginning at day 30 until late pregnancy days (Fig. 6B). Similar to PR-B, CBP

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

**Fig. 6. Gene expression of PR isoforms A and B and CBP in pregnancy endometrium.** Total RNA was isolated from pregnant pig endometrium (n = 3 animals/pregnancy (Px) day) and analyzed for expression of the indicated mRNAs (A, PR A/B; B, PR-B; C, CBP) by RT-PCR, as described under “Experimental Procedures.” Each point represents the mean (LSM ± S.E.) of the ethidium bromide or hybridization intensities of the generated PCR products after normalizing to that of β2-microglobulin, as a function of pregnancy day. * indicates significant difference (p < 0.05) from the values obtained at pregnancy day 12. D, nuclear extracts (100 μg protein per lane) from pig endometrial tissues isolated at the indicated pregnancy days were analyzed for the presence of immunoreactive PR-A and PR-B proteins by Western blot as described previously (24). Each lane represents a different animal as source of nuclear extract. Results are representative of two experiments, with each experiment using different pairs of animals at the indicated pregnancy days. The migration position of the 100 kDa molecular mass marker is shown.

**Expression Levels of PR-A, PR-B, and CBP in Pregnancy Endometrium**—To begin to assess the biological significance of BTEB1 interaction with specific PR isoforms in the presence of CBP, the expression levels of PR-A, PR-B, and CBP were evaluated in pig pregnancy endometrium by semi-quantitative RT-PCR and/or Western blot analysis. In previous studies (24, 38), we have shown that the levels of uterine endometrial BTEB1 protein did not change with stage of pregnancy. By contrast, the levels of total PR(A/B) mRNAs shown here were higher at early than at late pregnancy, with peak levels observed at day 12, the period of maternal recognition of pregnancy for this species (39) (Fig. 6A). The pattern of PR-B mRNA levels followed that of PR(A/B), with levels highest at early pregnancy (days 12–14) and dropping precipitously beginning at day 30 until late pregnancy days (Fig. 6B). Similar to PR-B, CBP

Selected Modulation of PR Isoform Transactivity by KLF9/BTEB1
mRNA levels were highest at early pregnancy and declined by day 30 (Fig. 6C). Western blot analysis of nuclear extracts prepared from uterine endometrial tissues indicated that although PR-B protein was easily detected at pregnancy days 10 and 12, a loss of this protein was observed at days 30, 60, and 90. On the other hand, PR-A protein remained constitutively expressed across most of pregnancy, except at day 90 when a modest increase in its levels was noted (Fig. 6D).

Interaction of PR and BTEB1 in Other Tissue and Cell Contexts—PR is a key regulator of diverse events in reproduction and in breast cell proliferation and differentiation (1, 40). The interactions of PR and BTEB1 within the context of these physiological events were evaluated using mouse uterus at peri-implantation and two human mammary carcinoma cell lines, MCF-7 and T47D. The endogenous BTEB1 expression in these tissues or cell lines was initially determined by Western blot analysis. BTEB1 protein was expressed in the mouse uterus on days 1–8 of pregnancy (Fig. 7A), although the levels did not vary considerably during this period, consistent with the previous report for the pig uterus (24, 38). Similarly, T47D cells had relatively abundant expression of BTEB1 protein, which was higher than that for MCF-7 cells (Fig. 7A). Nuclear extracts were prepared from early pregnancy mouse uterus and from T47D cells treated with R5020 (100 nM) for 48 h, immunoprecipitated with anti-BTEB1 antibody (2 μg), and then immunoblotted with a specific anti-PR antiserum that recognized both PR-A and PR-B isoforms. Results indicate that although T47D cells (Fig. 7A) and peri-implantation mouse uterus (41, 42) express both PR isoforms, only PR-B (~110 kDa) co-immunoprecipitated with endogenous BTEB1 in these systems, consistent with the results obtained for the pig pregnancy uterus (Fig. 7B) (24). Parallel immunoprecipitations carried out with normal rabbit serum IgG did not detect any immunoreactive band corresponding to PR-A or -B in Western blots with anti-PR (data not shown). These data indicate that in P-responsive tissues or cells that endogenously express BTEB1 and both PR isoforms, direct physical association of BTEB1 with ligand-bound PR-B occurs preferentially, over that with PR-A.

To demonstrate the functional relevance of BTEB1 and PR interactions within the context of an endogenous gene that responds to both PR- and BTEB1-mediated transactivation, the expression of cyclin D1 was examined in the MCF-7 Tet-On clonal derivative line B5, whose endogenous expression of BTEB1 was increased in response to the tetracycline homolog doxycycline (Dox).2 The parental MCF-7 cell line from which B5 was derived is P-responsive and expresses low levels of BTEB1 (Fig. 7A), hence, it is ideal for evaluating PR-mediated transcriptional activity as a function of cellular BTEB1 content. The B5 clonal line was grown in the absence or presence of Dox (2 μg/ml) for 24 or 48 h, and Dox-treated and control vehicle (alone) cells were then evaluated for cyclin D1 and β-actin gene expression levels by semi-quantitative RT-PCR. Induction by Dox of BTEB1 protein levels (approximately by 2-fold as confirmed by Western blots; data not shown) increased cyclin D1 gene expression within 24 h, and this increase was maintained in cells treated for 48 h (Fig. 8A). By contrast, a similar induction in β-actin gene expression was not observed in these cells (Fig. 8A), consistent with previous findings in a human endometrial carcinoma clonal line that overexpressed BTEB1 (43). In a control line C3, a clonal line derived from MCF-7 Tet-On cells that were stably transfected only with the empty expression vector (pTRE-6XH1N), treatment with Dox under the same conditions did not result in induction of cyclin D1 gene expression (data not shown).

To examine P effects on cyclin D1 gene expression in Dox-treated cells, B5 cells treated with ethanol (vehicle), Dox (2 μg/ml), R5020 (100 nM), or a combination of Dox and R5020 for 24 h were evaluated by semi-quantitative RT-PCR. Cyclin D1 gene expression was induced and repressed, respectively, in Dox- and R5020-treated cells, relative to those of control cells (Fig. 8B). In cells treated with both R5020 and Dox, the expression of cyclin D1 was further diminished from that of cells treated only with R5020. Thus, BTEB1 enhanced the repressive effects of R5020 on cyclin D1 gene expression, suggesting functional relevance of PR/BTEB1 interaction in the regulation of breast cell proliferation.

DISCUSSION

Here we show that the KLF family member KLF9/BTEB1 acts as a positive or negative modulator of PR-B transactivity, depending on whether PR-B exists as a homodimer or a het-

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2 D. Zhang, X.-L. Zhang, R. R. Eason, F. A. Simmen, and R. C. M. Simmen, unpublished data.
Eroderod with PR-A. In addition, we found that BTEB1, while having no effect on the transcriptional activity of the PR-A homodimer, functions as an activator of unliganded PR-A transactivity in the presence of the transcriptional integrator CBP. Moreover, BTEB3 and to a much lesser extent Sp1, mimicked the modulatory role of BTEB1 on ligand-dependent PR-B transcriptional activity, irrespective of gene promoter context. Further, we identified CBP as a transcriptional activator of ligand-bound PR-B, but only in the presence of BTEB1. Finally, we determined that the functional interactions of BTEB1 and PR-B noted here by transient transfection assays may be biologically relevant in the pregnancy endometrium of two species (mouse and pig) and to events that regulate breast cell proliferation, physiological conditions characterized by high P levels. Taken together, these results define BTEB1 as an important determinant of the cellular response to P.

The PR in humans and rodents, and as reported here also in pigs, has two well characterized isoforms, which are produced from a single gene either by transcription from two distinct promoters or by translation initiation from two alternative AUG sites (6–7). Studies in mice null for both or either isoform demonstrated distinct as well as overlapping biological functions for each in a target tissue-specific manner (40, 41, 44), consistent with results obtained from analyses of promoter transactivation in transiently transfected cells (45, 46) as well as of cell phenotypes upon over-expression of either isoform (47–49). In general, PR-A is a weaker transcriptional activator than is PR-B (8–9) and indeed can suppress PR-B as well as ER- and androgen receptor-mediated transactivation (10–12; 16); exhibits ligand-independent transcriptional activity in some contexts (50, 51); and demonstrates altered expression levels relative to those of PR-B in normal versus transformed cells (14, 52). In the present study, we provide evidence to indicate that the relative ability to interact with BTEB1 may serve to distinguish the transcriptional activities of PR-B homodimer from those of PR-A homodimer or PR-A/PR-B heterodimer. First, we demonstrated the lack of functional as well as physical interactions of PR-A homodimer with BTEB1, both of which were demonstrably present with PR-B. Second, we showed that the transactivation function of the PR-A/PR-B heterodimer, which is significantly reduced from that of PR-B homodimer, is further inhibited when BTEB1 is present. Third, we found that the functional interaction of unliganded PR-A or ligand-bound PR-B homodimer with CBP occurred only in the presence of BTEB1. Taken together, these results are consistent with the notion that BTEB1 interaction (or lack thereof) with each PR isoform defines in part the subsequent pathway by which each interacts with other transcriptional components, likely resulting in the modulation of a distinct repertoire of P-regulated genes (29).

One of the questions raised by our study is the exact mechanism by which BTEB1 mediates the selective transcriptional signaling of the two PR isoforms. Although this remains to be clarified, the differential recruitment of CBP/p300 or related transcriptional co-activators, co-activators, or co-repressors to the agonist-bound PR may be involved. Based on data presented indicating the lack of a functional interaction between antagonist-bound PR-B and BTEB1 (Fig. 3), consistent with recent studies that focused on the proper conformation of the PR dimer as requisite for subsequent interactions with co-activators (27, 35), the effect of BTEB1 in enhancing PR-A inhibition of PR-B transactivity may be a function of the conformation of PR-A/PR-B heterodimer distinct from that of PR-B homodimer, likely resulting in different contact points with BTEB1. Prior studies have reported that BTEB1 can contribute to the formation of a higher order "repressome" complex by its ability to bind the co-repressor mSin3b (53) and its ability to physically interact with the related Sin3a (54). Thus, the conformation of the PR-A/PR-B heterodimer might permit the subsequent recruitment of a co-repressor by BTEB1, in lieu of transcriptional co-activators or integrators such as CBP. However, we could not detect PR-A isoform when endogenous protein complexes associated with BTEB1 in intact cells (P-treated T47D) and tissues (pregnancy uterus) were immunoprecipitated with anti-BTEB1 antibody that recognized both PR isoforms, suggesting that BTEB1 does not physically associate with PR-A in these contexts. Alternatively, sequestration of co-activators that normally bind to the heterodimer might be responsible for the negative effect of BTEB1, independent of its physical interaction with the complex.

It was recently reported (36) that the synergistic effects of the SRC-1 and p300 in enhancing PR-dependent transactivation require the ordered recruitment by PR of SRC-1, followed by p300. Our present findings that document the selective physical (and functional) interactions between PR-B and BTEB1 (but not between PR-B and CBP) in a number of physiological contexts, coupled with the recent report (55) that the
KLF family member KLF13/BTEB3 with the greatest homology to KLF9/BTEB1, interacts with p300/CBP are consistent with the notion of a similar sequential order of recruitment of BTEB1 and then CBP by PR-B, which does not necessarily occur with PR-A homodimer. We cannot extrapolate the present data to infer the hierarchy in the recruitment by PR-B of BTEB1 relative to SRC-1, or the extent of BTEB1 contribution to the formation of a functional transcriptional preinitiation complex in the presence of SRC-1. Moreover, it is possible that BTEB1 might function independent of SRC-1, in a manner similar to that recently delineated for a novel PR-interacting protein, jun dimerization protein 2, which induces P-dependent PR-mediated transactivation by interacting with the AF-1 rather than the SRC-1 interacting AF-2 region of the PR molecule (56).

Recently, BTEB1 was identified as a P-induced gene in a screen of T47D mammary epithelial cells expressing both PR-A and PR-B isoforms (29). In uterine epithelial cells, we have shown that BTEB1 up-regulates the expression of cyclin-dependent kinase 2 (43), which others have demonstrated to phosphorylate seven sites within the PR-B receptor in vitro, contributing to its ligand-dependent activation (57). Taken together with our present findings that selective BTEB1/PR-B interactions result in enhanced PR-mediated transactivation whereas that of BTEB1 with PR-A/PR-B heterodimer results in transrepression, it is likely that the possible existence of this autoregulatory loop may serve to amplify the effects of P to ensure optimal target gene expression in different contexts. In both mouse and pig uterus, BTEB1 appeared to be constitutively expressed at high levels across pregnancy (Refs. 24 and 38 and this study) in contrast to PR-B, suggesting the existence of both PR-B-dependent and -independent functions for this nuclear protein. Preliminary findings from our laboratory utilizing a recently generated BTEB1 null mouse (58) indicated that litter size is compromised in homozygous females relative to PR-A/PR-B heterodimer results in transrepression, it is likely that the possible existence of this autoregulatory loop may serve to amplify the effects of P to ensure optimal target gene expression in different contexts. In both mouse and pig uterus, BTEB1 appeared to be constitutively expressed at high levels across pregnancy (Refs. 24 and 38 and this study) in contrast to PR-B, suggesting the existence of both PR-B-dependent and -independent functions for this nuclear protein. Preliminary findings from our laboratory utilizing a recently generated BTEB1 null mouse (58) indicated that litter size is compromised in homozygous females relative to control and heterozygous counterparts. Further analysis of this mouse model should delineate important PR-related signaling pathways of BTEB1 in the uterus.

To begin to identify other possible partners of PR-B in P-regulated gene transcription, two other members of the KLF family were evaluated for their ability to modulate ligand-dependent PR-B-mediated transactivity. Sp1 exhibits significant homology with BTEB1 only in the DNA-binding region (21, 23), whereas KLF13/BTEB3, also known as FKL2, has extensive homologies with BTEB1 in primary sequence, including the amino-terminal region as well as in size (28). Results demonstrating a modest effect of Sp1 on PR-B-mediated transactivation in contrast to the very robust effect of BTEB3 analogous to that of BTEB1 suggest that although the activity is common to KLF proteins, the distinct contributions of the amino-terminal region are critical to these proteins’ interactions. Surprisingly, however, BTEB3 does not transactivate the UF gene promoter that contains G/C rich sequences, although these motifs were previously shown to bind both Sp1 and BTEB1 with comparable affinity, resulting in an induction of basal UF gene transcription (25, 26). BTEB3 has been reported to function as a transactivator or transrepressor, depending on whether it interacts with members of the histone acetyltransferase co-activators such as p300/CBP (55) or the co-repressors Histone deacetylase-1 and mSin3A (59). The absence of either type of activity for BTEB3 in the present study suggests that Hec-1-A cells used for the transient transfection assays may be lacking these nuclear factors or that the promoters tested here do not utilize these complexes. Analysis of the functional domains in BTEB1 that are required for mediating induction of PR-B transactivity will provide the essential amino acid sequences shared by KLF members in this novel mechanism of gene activation by PR.

Pregnancy is a P-dominated physiological state, and the appropriate expression of P-regulated genes, resulting in structural modifications as well as changes in specialized cellular functions of the uterine endometrium, is requisite for both pregnancy initiation and maintenance. Similarly, P plays a pivotal role in mammary gland morphogenesis and development (60), and abnormal mammary gland proliferation leading to carcinogenesis is highly dependent on PR function (61). In the present study, we assessed the potential physiological relevance of the functional protein complex formed by BTEB1 and PR-B/P in the context of the peri-implantation uterus and mammary epithelial cells by: 1) evaluating the preferential ability of endogenous BTEB1 to physically interact with PR-B, relative to PR-A under conditions of high P; 2) establishing a positive relationship between the expression profiles of PR-B, BTEB1, and CBP across pregnancy; and 3) demonstrating the functional consequence of PR and BTEB1 interaction on the endogenous expression of a P- and BTEB1-regulated gene, cyclin D1 (62, 63). The collective results from these studies are congruent with the preferential interaction of BTEB1 with PR-B, support a model where CBP participates in the formation of a multimeric complex with PR-B and BTEB1, and define a role for BTEB1 as a P-B interacting partner in the control of cell proliferation. In this regard, a previous report (48) demonstrated that in endometrial cells transfected with either PR isofrom, PR-B caused a much more dramatic decrease in cell growth than did PR-A. In the context of early pregnancy, the consequence would likely be an endometrium with a differentiated secretory, rather than a proliferative phenotype, consistent with the requirement for production and secretion of essential proteins or other bio-molecules for embryo development and implantation during this time (64). We speculate that the stability of the higher order complex formed by PR-B-CBP-BTEB1 in cooperation with other well established PR co-activators (27), may ultimately guide the biological outcome of PR-mediated signaling. Clearly, direct physical evidence that this complex exists in vivo at defined physiological states that provide additional support for defined mechanism. Chromatin immunoprecipitation using specific antibodies to each component will be useful in this regard (65).

The present study was aimed at dissecting the components of the PR signaling mechanism involving KLF family members. The differential effects of BTEB1 on the assembly of a functional complex involving PR isofroms provide a molecular basis in support of the hypothesis that selective utilization by PR of distinct partners may have evolved to provide a highly fine-tuned regulatory mechanism to ensure appropriate progesterin action in the uterus, the biological consequence of which is requisite for normal growth and differentiation of this tissue.

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