

Ets-2 and Components of Mammalian SWI/SNF Form a Repressor Complex That Negatively Regulates the *BRCA1* Promoter*

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Ets-2 is a transcriptional activator that can be modulated by *ras*-dependent phosphorylation. Evidence is presented indicating that *ets-2* can also act as a transcriptional repressor. In the breast cancer cell line MCF-7, exogenous *ets-2* repressed the activity of a *BRCA1* promoter-luciferase reporter dependent on a conserved *ets-2*-binding site in this promoter. Conditional overproduction of *ets-2* in MCF-7 cells resulted in repression of endogenous *BRCA1* mRNA expression. To address the mechanism by which *ets-2* could act as a repressor, a biochemical approach was used to identify proteins that interacted with the *ets-2* pointed domain. From this analysis, components of the mammalian SWI/SNF chromatin remodeling complex were found to interact with *ets-2*. Brg-1, the ATP-hydrolyzing component of the SWI/SNF complex, along with the BAF57/p50 and In1 subunits could be co-immunoprecipitated from cells with *ets-2*. The pointed domain of *ets-2* directly interacted *in vitro* with the C-terminal region of Brg-1 in a phosphorylation-dependent manner. The combination of Brg-1 and *ets-2* could repress the *BRCA1* promoter reporter in transfection assays. These results support a role for *ets-2* as a repressor and indicate that components of the mammalian SNF/SWI complex are required as co-repressors.

The ETS family, encompassing approximately 30 vertebrate members, encodes for sequence-specific DNA-binding proteins that are transcriptional activators and repressors (1). The family is defined by a highly conserved DNA binding domain referred to as the ETS domain. However, the DNA-binding properties of these factors are similar and cannot entirely account for the specificity required for the precise activation of target genes that occurs during the diverse biological processes mediated by individual family members. Modification of discrete family members by signal transduction pathways provides an additional mechanism to determine specificity (1).

For example, members of the ETS family of transcription factors are important for mediating both transient and persistent changes in gene expression patterns in response to *ras*-

signaling pathways (2–4). The ETS family member *elk-1* and related factors are directly phosphorylated by mitogen-activated protein kinases (MAPK),¹ a modification required for the activation of immediate early target genes like *c-fos* (2). Similarly, phosphorylation of the ETS family members *ets-1* and *ets-2* by *ras*-dependent pathways leads to persistent expression of target genes including extracellular proteases such as urokinase plasminogen activator (uPA) and stromelysin/MMP-3 (3, 4). *Ets-1* and *ets-2* are phosphorylated at a conserved residue (threonine 38 and threonine 72, respectively) by the well characterized *ras*-effector pathway, the Raf/MAPK pathway (4–7). Additionally, the same residue in *ets-2* can also be phosphorylated by another major *ras*-effector pathway, the phosphatidylinositol 3-kinase/Akt pathway (8).

Understanding at the molecular level how phosphorylation modifies the activity of *ets-1* and *ets-2* will be critical for defining how these factors selectively regulate target genes. The key phosphorylation event occurs within a region of *ets-1* and *ets-2* that is conserved through evolution with the *Drosophila* pointed P2 protein, and has been termed the pointed domain (9, 10). Phosphorylation of the conserved threonine residue within this region leads to an increased ability of *ets-1* and *ets-2* to activate target promoters (3–7). Previous work indicated that phosphorylation of *ets-1* or *ets-2* did not affect protein turnover, nuclear localization, or intrinsic DNA binding activity of the factors (3, 4, 7). In addition, the N-terminal region of *ets-2* fused to the heterologous gal4 DNA binding domain is still regulated by the *ras*/Erk pathway (11). The pointed domain appears similar to domains in other transcription factors, for example in the cAMP responsive enhancer-binding protein, that are regulated by phosphorylation-dependent protein-protein interactions with transcription co-activators (12). *Ets* factors can also act as repressors of gene expression, and the pointed homology domain has been implicated in this activity in some family members (1). Thus, whereas *ets-1* and *ets-2* have been considered to be activators of gene expression, it is possible that they also repress target gene expression.

Genetic and biochemical evidence demonstrate that the products of the SWI/SNF genes, first defined in *Saccharomyces cerevisiae* as co-activators of gene expression, form a complex with the ability to remodel chromatin (13). The SWI/SNF complex is conserved in mammals and can act as a co-activator for steroid hormone receptors (13–16). The complex has ATP-dependent chromatin remodeling activity and can alter the conformation of the nucleosome core in a reversible fashion (13, 14). The mammalian ATPase hydrolyzing subunit Brg-1 interacts with the retinoblastoma tumor suppressor protein (Rb)

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¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; uPA, urokinase plasminogen activator; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; GR, glucocorticoid receptor; MMP, matrix metalloproteinase.

(17). The Brg-1 remodeling complex is required for E2F/Rb-mediated repression of gene expression (18, 19). Recently, experiments performed in *S. cerevisiae* have definitively established the ability of the ATPase-hydrolyzing subunit of the complex (SNF2) to act as a co-repressor of gene expression (20). Therefore, SWI/SNF appears to act as both co-activator and co-repressor (20).

In the present report evidence is presented indicating that ets-2 can repress the *BRCA1* promoter, a promoter previously reported to contain functional ets-binding sites (21–24), in the breast cancer cell line MCF-7. Ets-2 can bind to the *BRCA1* proximal promoter, and ets-2 co-expression results in repression of a *BRCA1* promoter-luciferase in transient transfection assays. Conditional overexpression of ets-2 in MCF-7 cells resulted in repression of the endogenous *BRCA1* promoter, whereas expression of other targets, uPA and MMP3, were stimulated by ets-2 overexpression. Biochemical evidence is presented indicating that ets-2 physically interacted with discrete sets of cellular proteins dependent on the phosphorylation status of the factor. We show that components of the mammalian SWI/SNF (mSWI/SNF) chromatin remodeling complex comprised one set of proteins that interacted with unphosphorylated ets-2. The pointed homology region of ets-2 directly interacted with the C-terminal region of Brg-1, the ATP-hydrolyzing component of the mammalian complex, in a phosphorylation-dependent manner *in vitro*. Co-expression of Brg-1 with ets-2 resulted in repression of a stably integrated *BRCA1* reporter gene in SW13 cells, a *Brg-1* null cell line. These results demonstrate that ets-2 can act as a repressor and that a chromatin remodeling complex is necessary for this activity.

EXPERIMENTAL PROCEDURES

Cell Lines—To generate the tetracycline-controlled inducible ets-2 MCF-7 cells, an influenza virus hemagglutinin (HA)-epitope tagged version of wild-type ets-2 (3) was cloned into the tetracycline operator-expression vector, pUHD10-3 (25), to generate pUHD10-3/HA-ets-2. The MCF-7/Tet-off cell line (Clontech) was transfected with pUHD10-3/HA-ets-2 to establish stable expression cell lines that can be induced to express HA-ets-2. Transfections were done using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) as described previously (26). The tetracycline-controlled inducible ets-2 MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) without phenol red with 10% charcoal-treated fetal bovine serum and 1 μ g/ml tetracycline (Sigma). To induce ets-2 expression, adherent cells were washed 3 times with phosphate-buffered saline and then re-fed with 10% charcoal-treated fetal bovine serum/DMEM without tetracycline.

For generating stable cell lines expressing ets-2 Ala-72 protein, NIH 3T3 cells were transfected with the HA-tagged version of ets-2 Ala-72 by the calcium phosphate method as described (3), and hygromycin-resistant clones were selected in 200 units/ml hygromycin (Calbiochem, Inc.) and analyzed by Western blotting. Cells were grown in DMEM with 10% calf serum containing 100 units/ml hygromycin. For metabolic labeling of cellular proteins with [³⁵S]methionine, cells were placed in DMEM that lacked both serum and methionine for 45 min, then incubated with DMEM containing 90 μ Ci/ml [³⁵S]methionine (1200 Ci/mmol; ICN, Irvine, CA) for 2 h.

Human carcinoma SW13 cells were obtained from American Type Culture Collection (Bethesda, MD) and cultured in DMEM plus 5% bovine calf serum. SW13 cells that contained stably integrated *BRCA1* promoter-luciferase reporter were generated by the co-transfection of 3 μ g of *BRCA1*-luciferase plasmid and 1 μ g of a neomycin-resistance vector using LipofectAMINE Plus (Invitrogen). 48 h after transfection, cells were selected with G418 (400 μ g/ml, Invitrogen) and media was changed every 3 days. 14 days after selection, there were about 100 colonies on the dish. The cells were pooled and cultured with 200 μ g/ml G418 and used for subsequent experiments.

Plasmids, DNA Transfections, and Northern Blots—The vector used for expression of the HA-tagged versions of ets-2 and ets-2 Ala-72 proteins were previously described (3, 5). The expression vectors for Brg-1 and Brg-1 K798R were previously described (16). The luciferase reporters for the human *BRCA1* (21), and murine MMP14/MT1-MMP (27) promoters were kindly provided by Ellen Soloman (Guy's Hospital, London) and Joseph Madri (Yale University), respectively. The *BRCA1*

promoter with the ets-site double-point mutation CGTAAGAGT was constructed by site-directed PCR mutagenesis as previously described (3). The mutation was verified by sequencing.

Transient transfections in MCF7 or SW13 cells were performed by the calcium phosphate method as described (3, 16). For the transient assays either a Rous sarcoma virus- β -galactosidase (MCF-7 cells) or an expression vector for *Renilla* luciferase (pRL-CMV, Promega, SW13 cells) was included as an internal control for transfection efficiency (0.1 μ g/DNA precipitate). Relative luciferase activity is equal to (raw luciferase activity)/(raw activity of the internal control \times the protein concentration of the extract) (3). Fold-repression is the ratio of relative luciferase activity for the *BRCA1*-luciferase reporter alone (with empty expression vectors) to the activity in the presence of ets-2, Brg-1, or the combination of both ets-2 and Brg-1, as indicated in the figure legends.

The SW13 cells containing the stably integrated *BRCA1* promoter reporter were transfected using LipofectAMINE Plus (Invitrogen). *Brg-1* and ets-2 expression plasmids (see Fig. 7B) were cotransfected with 0.5 μ g of a puromycin-resistant expression vector, pBABE-puro-myc (6). 36 h after transfection, cells were selected with 4 μ g/ml puromycin and subsequently harvested 36 h after the initial selection. Luciferase activity was adjusted by cell lysate protein concentration.

RNA was isolated and analyzed by Northern blotting as previously described (5). Levels of RNA expression were quantified using a Amersham Biosciences PhosphorImager.

Immune Reagents and Analysis—The antibodies specific for ets-2 and phosphorylated ets-2 (pT72-ets-2) have been previously described (6). The anti-HA antibody was purchased from Babco, Inc. (Richmond, CA). Polyclonal anti-rabbit antibodies specific for Brg-1 and Brm-1 were gifts from Said Sif (Ohio State University), BAF57/p50 from Gerry Crabtree (Stanford University), and In11 from Anthony Imbalzano (University of Massachusetts).

For standard immunoprecipitations, 2.5–3 \times 10⁶ cells were placed in 500 μ l of lysis buffer consisting of 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 3 mM MgCl₂, and 1% Nonidet P-40. For more stringent analysis, the same buffer was used except that it contained 500 mM NaCl and 0.5% deoxycholate in addition to 1% Nonidet P-40. The buffer contained a mixture of protease and phosphatase inhibitors that was previously described (5, 6). The pre-cleared cell lysates were incubated with specific antibody (2–4 μ g) and 20 μ l of a 50% slurry of protein-G beads (Amersham Biosciences) overnight at 4 $^{\circ}$ C. The immunoprecipitates were washed 4 times in lysis buffer and analyzed by SDS-PAGE.

Affinity Chromatography and Protein Interaction Assays—Recombinant ets-2 protein corresponding to the pointed homology region (amino acids 60–167) was prepared as described (5, 6) and covalently linked to Affi-Gel 10 beads (10 mg of ets-2/ml of beads; Bio-Rad). The ets-2 column was phosphorylated in a reaction that contained 10 μ l of ets-2 affinity column, 30 μ l of kinase buffer (30 mM Hepes, pH 7.2, 20 mM MgCl₂, 2 mM dithiothreitol), 100 μ M ATP, 10 μ Ci of [γ -³²P]ATP (500 Ci/mmol, PerkinElmer Life Sciences), and 5 μ l of activated recombinant MAPK p42 (Upstate Biotechnology, Lake Placid, NY). Either the unphosphorylated or the phosphorylated ets-2 affinity columns were incubated with cell extracts prepared in lysis buffer as above for 16 h at 4 $^{\circ}$ C. Washing procedures and analysis were performed as for the immunoprecipitations above.

For direct protein interaction assays, a portion of Brg-1 corresponding to amino acids 1108–1686 at the C-terminal of the protein (17) were expressed as a GST fusion protein in *Escherichia coli*, and 1 μ g of fusion protein was immobilized using glutathione beads (Amersham Biosciences). The beads were incubated with 1 μ g of the recombinant ets-2 pointed domain that was unphosphorylated, or phosphorylated by recombinant MAPK p42 in a reaction that contained 10 μ Ci of [γ -³²P]ATP but no cold ATP (see above for details of the kinase reaction). Beads and ets-2 protein were incubated in lysis buffer for 16 h at 4 $^{\circ}$ C. Beads were washed and the material bound analyzed by Western blotting using the ets-2 specific antibody, or for ³²P-labeled protein by autoradiography and phosphorimaging.

Western analysis was performed as previously described (5, 6). A Lumi-Imager (Roche Diagnostics) was used for quantification of chemiluminescent signals. For estimation of ets-2 concentration, standard curves for both ets-2 antibodies were prepared using recombinant protein corresponding to the ets-2 pointed domain that was phosphorylated with MAPK p42 as described above.

Electrophoretic Mobility Shift Assays—Recombinant ets-2 corresponding to the DNA binding domain (amino acids 334–466) was produced using the pGEX expression vector system and purified by glutathione-Sepharose chromatography (Amersham Biosciences) as previously described (28). Purified protein was used in the electrophoretic mobility shift assays using standard conditions as previously

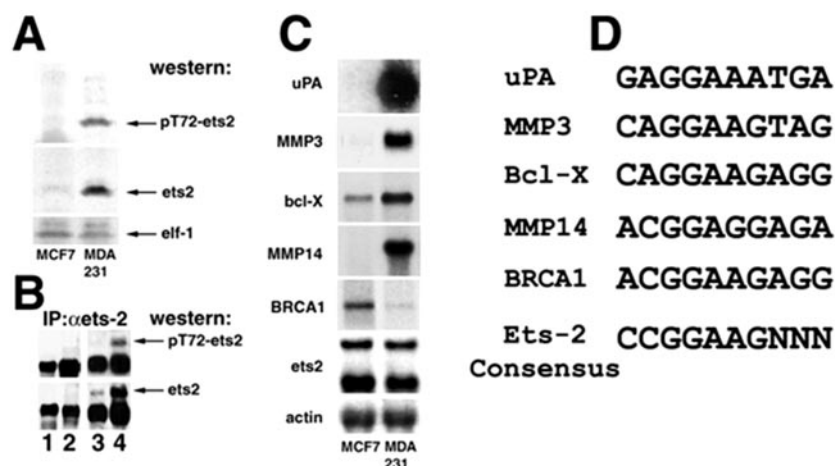


FIG. 1. Expression of phosphorylated ets-2 correlates with expression of target genes in the breast cancer cell lines MCF-7 and MDA231. A, nuclear extracts from MCF-7 and MDA231 breast cancer cells were analyzed by SDS-PAGE, followed by Western blotting with the anti-pT72 ets-2 (top panel). The same membrane as in the top panel was reprobed with a pan-ets-2 antibody (middle panel). Blot was reprobed with antibody specific for the ets factor elf-1 as a loading control (bottom panel). The arrows indicate the position of ets-2. B, ets-2 was immunoprecipitated from MCF-7 (lane 3) and MDA231 (lane 4) cells using the non-phosphodiscriminating anti-ets-2 antibody. The immunoprecipitate was analyzed by Western blotting using the same two antibodies used in panel A, as indicated in the figure. Lanes 1 and 2, preimmune ets-2 antiserum was used for control immunoprecipitations from MCF-7 and MDA231, respectively. Arrows indicate the position of ets-2. The unlabeled band present in each lane is immunoglobulin heavy chain. C, mRNA expression of ets-2 target genes in MCF-7 (lane 1) and MDA231 (lane 2). Total RNA was prepared and analyzed by Northern blotting (10 μ g per lane) with uPA (first panel), MMP3/stromelysin (second panel), bcl-x (third panel), MMP14 (fourth panel), BRCA1 (fifth panel), and ets-2 (sixth panel) probes utilized as indicated. Note that there are two ets-2 mRNA isoforms of 2.2 and 4.5 kb that differ in the 3' non-coding region. The blot was reprobed with a γ -actin probe (seventh panel) as a control for sample loading. D, comparison of the target gene promoter sequences encompassing the ets-2-binding site. uPA (GenBankTM number X02419), MMP3 (GenBankTM number U43511), bcl-x (GenBankTM number D30746), MMP14 (GenBankTM number AF158733), and BRCA1 (GenBankTM number U37574) sequences are displayed. Also shown is the consensus sequence for an ets-1/ets-2-binding site (1).

described (28). Double stranded oligonucleotides were end-labeled with polynucleotide kinase. The sequences of the oligonucleotides used were (sense strand) (BRCA1 oligos correspond to -208 to -182 relative to the ATG): BRCA1 wild-type, 5'-GGTAGAATTCCTCTTCCGCTCTC-TTGG; BRCA1 M1, 5'-GGTAGAATTCCTACTCTTCCGCTCTTGG; BRCA1 M2, 5'-GGTAGAATTCCTCTCTTACGCTCTCTTGG; and BRCA1 M1 + M2, 5'-GGTAGAATTCCTACTCTTACGCTCTCTTGG. A Amersham Biosciences PhosphorImager was used to quantify the amount of protein-DNA complex formed.

RESULTS

Expression of ets-2 Correlates with Expression of ets-2 Target Genes in Breast Cancer Cells—Previous work demonstrated that expression of phosphorylated ets-2 correlates with a more invasive, mesenchymal phenotype in both ovarian and prostate cancer tumor cell lines (29, 30). To determine whether a similar correlation was detected in breast cancer cell lines, we studied expression of ets-2 and ets-2 target genes in MCF-7 cells, an estrogen receptor-positive non-invasive cell line, and in MDA231 cells, representative of invasive breast tumor cell lines (Fig. 1, A and B).

Western analysis of these two cell lines indicated that ets-2 was expressed at ~10-fold higher levels in MDA231 cells than in MCF-7 (Fig. 1A). Furthermore, the pT72 phosphorylated, activated form of ets-2 could be detected in MDA231 cells but not MCF-7 cells, as assayed using a pT72 ets-2 phospho-specific antibody (5). Similar results were observed if ets-2 were first immunoprecipitated from these two cell lines using the non-phosphodiscriminating antibody, then analyzed by Western analysis using the same antibody or the pT72 ets-2 antibody (Fig. 1B, lanes 3 and 4, respectively). From these Western blots, we estimated that ~40–50% of ets-2 was phosphorylated at position Thr-72 in MDA231 (see “Experimental Procedures”).

Correlating with phospho-ets-2 expression, the mRNA expression levels for three known ets-2 target genes, uPA (3, 28), MMP3/stromelysin (31), and bcl-X (8, 32), were all higher levels in MDA231 cells versus MCF-7 cells (Fig. 1C). Additionally, the expression of another metalloprotease implicated in mammary tumorigenesis MMP14/MMP-MT1 (27, 33, 34) was higher in

MDA231 cells than in MCF-7. MMP14 expression is reported to be increased by ras signaling pathways (35), and the proximal promoter for the mouse and human genes contain a consensus ets-like site that is related to sites found in other ets-regulated genes (Fig. 1D). Interestingly, the expression of ets-2 mRNA is identical in MCF-7 and MDA231 cells, indicating that differences in ets protein expression between the two cell lines (see Fig. 1, A and B) are because of a post-transcriptional regulatory mechanism.

In contrast to the known ets-2 target genes, the expression of tumor suppressor BRCA1 was ~5-fold lower in MDA231 in comparison to MCF-7 (Fig. 1C). The BRCA1 α promoter, the predominant promoter active in breast cells (36), also contains a consensus ets-binding site conserved in both mouse and human promoters (21–24) (Fig. 1D). Thus, the expression of ets-2 protein and BRCA1 mRNA were inversely correlated in MCF-7 and MDA231 cells.

Ets-2 Binds to the BRCA1 Promoter and Represses BRCA1 Promoter Activity in MCF-7 Cells—To test the hypothesis that ets-2 might repress BRCA1 expression, we first performed electrophoretic mobility shift assay analysis to determine whether ets-2 could bind to the ets-consensus site in the BRCA1 promoter (Fig. 2A). Recombinant ets-2 bound to the BRCA1 oligonucleotides containing the wild-type ets-binding site (ACGGAAGAGG, wild-type). However, 10-fold lower levels of ets-2-DNA complex was formed when the consensus site was mutated by one base outside of the GGA core (ACGGAAGAGT, M2). Mutation of the G residue in the ets core motif (ACGTAAGAGG, M1), or a mutation of both G residues (ACGTAAGAGT, M1 + M2) ablated ets-2 recognition of the BRCA1 sequence (Fig. 2A). These mutated oligonucleotides also failed to compete effectively for binding to wild-type sequence in competition assays (data not shown).

To determine whether ets-2 binding to the BRCA1 promoter had functional significance, transient transfections were performed in MCF-7 using BRCA1 and MMP14 promoter-luciferase reporters. The BRCA1 promoter was highly active in

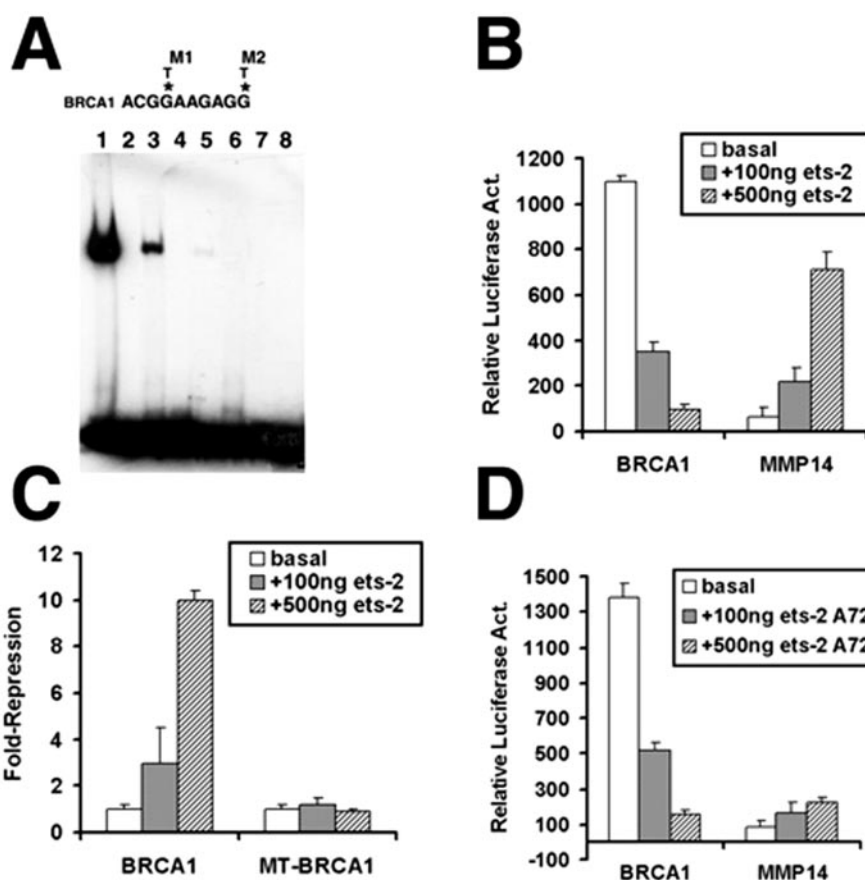


FIG. 2. Ets-2 binds to the *BRCA1* promoters and represses *BRCA1* promoter activity in MCF-7 cells. *A*, electrophoretic mobility shift analysis with recombinant ets-2. The wild-type *BRCA1* sequence (–208 to –182 relative to the ATG) along with nucleotides mutated (shown by asterisk) are represented above the gel. Wild-type *BRCA1* oligonucleotides M2 (lane 3), M1 (lane 5), and M1 + M2 (lane 7) were end-labeled with ^{32}P and incubated with 1 μg of recombinant ets-2 protein, as indicated. Probes incubated without recombinant protein were included in even numbered lanes (WT, M2, M1, and M1 + M2 in lanes 2–8, respectively). *B*, MCF-7 cells were transfected with 5 μg of human *BRCA1* or mouse *MMP14* luciferase reporters and 100 ng of Rous sarcoma virus- β -galactosidase reporter (an internal control). Either empty expression vector or vector for ets-2 (either 100 or 500 ng) as indicated, were co-transfected. Relative luciferase activity, adjusted for the internal control and for protein concentration of the cell-free extracts, is presented. *C*, MCF-7 cells were transfected with either 5 μg of *BRCA1* promoter luciferase reporter containing the wild-type ets-binding site (*BRCA1*) or the mutated ets-binding site M1 + M2 (*MT-BRCA1*, see panel *A*), with internal control as above, and with or without the addition of ets-2 expression vector (either 100 or 500 ng) as indicated. The activity is expressed as fold-repression, which is, the ratio of relative luciferase activity for the *BRCA1* or *MT-BRCA1* reporter alone (with empty expression vector) to the relative luciferase activity in the presence of ets-2. *D*, MCF-7 cells were transfected with 5 μg of human *BRCA1* or mouse *MMP14* luciferase reporters, internal control, and with or without mutated ets-2 T72A expression vector (either 100 or 500 ng) as indicated. Relative luciferase activity, as above, is presented. For panels *B–D*, the average of three independent experiments performed in duplicate are presented. Error bars indicate the standard deviation obtained from the three independent experiments.

MCF-7 cells, but co-transfection of an ets-2 expression vector resulted in a concentration-dependent repression of *BRCA1* promoter activity, with a maximum repression of ~10-fold observed (Fig. 2*B*). In contrast, *MMP14* promoter activity was low in MCF-7 cells, and ets-2 co-expression stimulated *MMP14* promoter activity by 12-fold in MCF-7 cells (Fig. 2*B*). When a uPA promoter reporter was tested, results were similar to those observed for the *MMP14* promoter (data not shown).

To demonstrate whether ets-2 repression of *BRCA1* promoter activity required a functional ets-binding site, a *BRCA1* promoter luciferase reporter with either a wild-type ets-binding site (ACGGAAGAGG) or a mutated ets-binding site (ACGTAAGAGT, M1 + M2) were studied by transient transfection in MCF-7 cells. Titration of ets-2 expression vector resulted in a concentration-dependent repression of the wild-type *BRCA1* promoter with a maximum repression of 10-fold observed, as above. However, ets-2 was not able to repress activity of the mutated *BRCA1* promoter, indicating that the ets-binding site is necessary for ets-2-mediated repression (Fig. 2*C*).

Previous work has demonstrated that the ets-2 transactivation potential is enhanced by phosphorylation at position

Thr-72 (3, 4, 7). To determine the role of residue Thr-72 in ets-2 activity in the transient assays in MCF-7 cells, we studied the effect of co-transfection of a vector encoding the ets-2 T72A mutation with both *MMP14* and *BRCA1* reporters (Fig. 2*D*). In this analysis, *MMP14* promoter activity was activated ~2-fold in the assays, and this activation was not dose dependent, results that are consistent with what was observed with ets-2 T72A in NIH 3T3 fibroblasts (3). In contrast, ets-2 T72A repressed the activity of the *BRCA1* promoter in the same way as ets-2 Thr-72 (Fig. 2*D*). These results indicate that an intact Thr-72 site is required for transactivation of *MMP14* and similar targets, but not for repression of *BRCA1*.

Ets-2 Overexpression in MCF-7 Cells Inhibits *BRCA1* Expression and Increases uPA and *MMP3* Expression—To test whether expression of exogenous ets-2 would affect the expression of the endogenous *BRCA1* gene in MCF-7 cells, we constructed MCF-7 cell lines that conditionally express an HA epitope-tagged ets-2. Conditional expression of ets-2 was accomplished by putting the gene under the control of the tetracycline operator and tetracycline-VP16 activator, such that ets-2 was expressed when tetracycline was removed from the

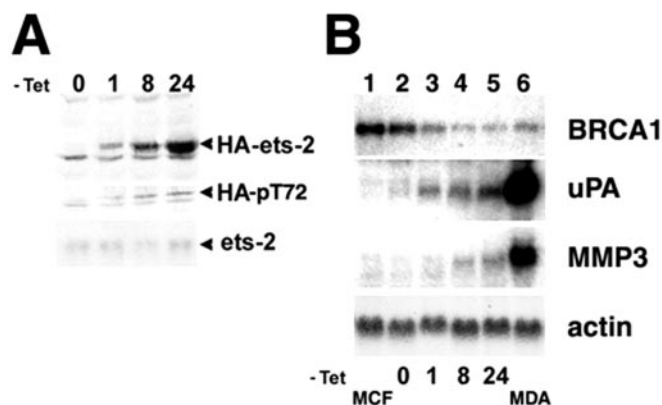


FIG. 3. Conditional expression of Ets-2 alters target gene expression in MCF-7 cells. *A*, after growing MCF-7/tetracycline activator cells in the absence of tetracycline ($-Tet$) for 0, 1, 8, and 24 h, as indicated, equal amounts of cell lysates were analyzed by SDS-PAGE, followed by Western blot with the anti-HA antibody (top panel), the anti-pT72 ets-2 (middle panel), or with an ets-2 non-discriminating antibody (lower panel). Arrows indicate the position of ets-2. *B*, total RNA was prepared from cells treated as in panel *A*, and analyzed by Northern blotting (10 μ g/lane) with probes specific for *BRCA1*, *uPA*, *MMP3*, and γ -actin, as indicated.

cell culture media (26). The results with one cloned cell line, MCF-7 clone 21B, are presented in Fig. 3.

After growing cells in the absence of tetracycline for 0, 1, 8, and 24 h, cell lysates were subjected to Western analysis using the anti-HA epitope tag antibody. HA-ets-2 could be detected 1 h after stimulation, and expression levels were maximal following 24 h of induction (Fig. 3*A*, first lane). The phosphorylated pT72 form of HA-ets-2 was present (Fig. 3*A*, second lane) but the levels of the pT72 form were estimated to be 5–10% of the total ets-2 pool, significantly lower than in MDA231 cells (see Fig. 1). The level of expression of endogenous ets-2 remained low in the cells following induction of the HA-ets-2 protein (Fig. 3*A*, third lane).

The expression of *BRCA1*, *uPA*, and *MMP3* mRNA was studied by Northern analysis in the tetracycline-regulated cell line (Fig. 3*B*). Induction of HA-ets-2 repressed *BRCA1* mRNA levels 5-fold (Fig. 3*B*, lane 1), to a level similar to the level of expression observed in MDA231 cells (compare lanes 5 and 6). In contrast, the expression of *uPA* mRNA and *MMP3* mRNA levels were increased ~4–5-fold following induction of HA-ets-2 expression (Fig. 3*B*, lanes 2 and 3, respectively). However, the levels of *uPA* and *MMP3* RNA induced demonstrated that levels of expression were approximately still 5-fold lower than in MDA231 cells (compare lanes 5 and 6), consistent with the lower levels of phosphorylated ets-2 expressed in the tetracycline-regulated MCF-7 cell line. Similar results were obtained for all 4 genes studied with an independent cell clone that expressed 3-fold lower levels of ets-2 than clone 21B (data not shown).

The ets-2 Pointed Domain Interacts with Distinct Sets of Nuclear Proteins in a Phosphorylation-dependent Manner—The results presented above indicated that ets-2 could act as both a repressor and activator of gene expression. We postulated that ets-2 might interact with both co-repressors and co-activators via the conserved pointed domain.

To begin testing this hypothesis, NIH 3T3 cells were constructed that expressed the HA-tagged ets-2 T72A mutation. As shown in Fig. 2*D*, this mutated form of ets-2 could act as a repressor but was only a weak activator, and thus we might expect ets-2 T72A to interact preferentially with co-repressors. Cells that expressed ets-2 T72A (Fig. 4*A*, lanes 3 and 4) were compared with serum-starved NIH 3T3 cells that also have a low level of the phosphorylated form of ets-2 (5, 6) (Fig. 4*A*,

lanes 1 and 2). Extracts from cells labeled with [35 S]methionine were lysed and immunoprecipitated under native conditions with antibody specific for ets-2 (Fig. 4*A*, lanes 1 and 3) or for the HA tag (Fig. 4*A*, lanes 2 and 4). In preliminary experiments the positions of ets-2 and HA-ets-2 proteins, indicated by arrowheads in the figure, were determined in the denaturing gels by Western blotting; the positions of authentic proteins were subsequently confirmed by comparing their mobility with the mobility of ets-2 and HA-ets-2 proteins overexpressed in COS cells and run on the same gels (data not shown).

These immunoprecipitation experiments demonstrated that the HA-tagged T72A version of ets-2 was expressed at the same level as endogenous protein (Fig. 4*A*, arrowheads). Additionally, several other proteins were reproducibly present in both immunoprecipitates, in particular species of apparent molecular mass 200 and 50 kDa (*p200* and *p50*, indicated by arrows in Fig. 4*A*, lanes 3 and 4). These proteins were also detected in ets-2 immunoprecipitates obtained serum-starved NIH 3T3 cells (Fig. 4*A*, lane 1).

In contrast, these bands were not detected in HA immunoprecipitates prepared from NIH 3T3 cells (Fig. 4*A*, lane 1). Furthermore, when the immunoprecipitation was performed with higher salt concentration and inclusion of an ionic detergent, these bands are no longer contained in the ets-2 or HA-ets-2 immunoprecipitates (Fig. 4*B*). These control experiments indicate that the *p200* and *p50* bands are present in a complex with either endogenous or HA forms of ets-2, and are not likely to be nonspecific or cross-reacting proteins found in the immunoprecipitates.

In a complementary biochemical approach a recombinant ets-2 protein, corresponding to the pointed domain (amino acids 67–170) that includes residue threonine 72 (5, 6), was overexpressed in *E. coli* and covalently linked to an Affi-Gel 10 matrix. Whole cell lysates prepared from [35 S]methionine-labeled NIH 3T3 cells were incubated with the ets-2 affinity column. After washing, the proteins that bound to the column were eluted and analyzed by SDS-PAGE along with the immunoprecipitated proteins (Fig. 4*A*, lane 5). The experiment demonstrated that proteins with the same mobility as those detected in the HA immunoprecipitate, in particular *p200* and *p50*, also bound to the ets-2 pointed domain affinity column. The results support the conclusion that these proteins form a specific complex with ets-2.

In a parallel experiment, the ets-2 affinity column was first incubated with recombinant, active MAPK p42 to phosphorylate threonine 72. Previous studies demonstrated that threonine 72 is the only site in the recombinant protein phosphorylated by MAPKs (5, 6). By using a trace amount of [γ - 32 P]ATP in the kinase reaction, we were able to calculate that >90% of the covalently linked ets-2 protein was phosphorylated (data not shown). Analysis of proteins from NIH 3T3 cells that bound to the phospho-ets-2 affinity column indicated that a discrete set of proteins distinct from those bound to the unphosphorylated column were detected (Fig. 4*A*, lane 6). Notably, *p200* and *p50* did not bind to the phospho-ets-2 column (Fig. 4*A*, lane 5 versus lane 6).

To determine whether the phosphorylation of ets-2 *in vivo* could affect interaction with the *p200* and *p50* proteins, immunoprecipitations were performed in ER-Raf/3T3 cells (6). These cells contain an estrogen responsive form of the activated Raf oncogene, and estrogen treatment persistently stimulates MAPK kinases activity and ets-2 phosphorylation at Thr-72, as well as ets-target gene activation (6). In these experiments, the *p200* and *p50* proteins could again be co-immunoprecipitated with endogenous ets-2, but stimulation of Raf signaling had no significant effect on the relative amount of these proteins found

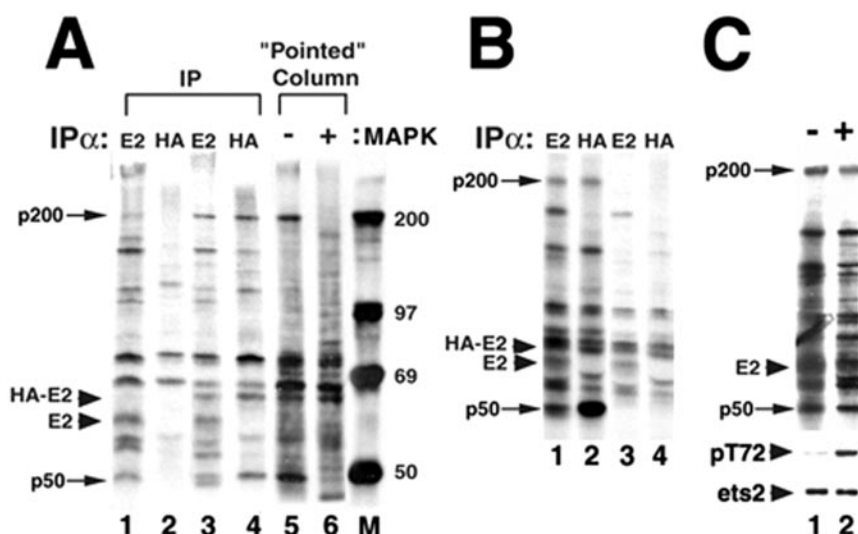


FIG. 4. Ets-2 interacts with nuclear proteins in a phosphorylation-dependent manner. *A*, serum-starved NIH 3T3 cells (lanes 1 and 2), or NIH 3T3 cells that expressed HA-tagged Ala-72 ets-2 (lanes 3 and 4) were labeled with [³⁵S]methionine and protein extracts were prepared. The labeled extracts were incubated with either an ets-2 specific antibody (E2, lanes 1 and 3) or with the anti-HA antibody (HA, lanes 2 and 4). In parallel experiments, labeled NIH 3T3 extracts were incubated with an ets-2 pointed domain affinity column that was either unphosphorylated (lane 5) or phosphorylated by MAPK p42 (lane 6). Proteins present in the immunoprecipitates or bound to the affinity column were separated by SDS-PAGE and visualized by autoradiography. The position of ets-2 proteins (endogenous and HA-tagged, determined by Western blotting) are indicated by arrowheads, and co-immunoprecipitated proteins of relative mass 200 and 50 kDa are indicated by arrows. Note that the band with similar mobility to HA-ets-2 in lanes 5 and 6 does not cross-react with the HA or ets-2 antibodies. The position of molecular weight markers are also indicated in the figure. *B*, immunoprecipitation as in panel A, using ets-2 and HA antibodies as indicated. NIH 3T3 extracts were in lanes 1 and 3, whereas extracts from cells expressing HA-ets T72A were in lanes 2 and 4. Lanes 1 and 2 were performed under standard conditions (150 mM NaCl and 1% Nonidet P-40), whereas lanes 3 and 4 were performed with more stringent conditions (500 mM NaCl and 0.5% deoxycholate). *C*, immunoprecipitation as in panel A, using ets-2 antibody and labeled extracts prepared from ER-Raf/NIH 3T3 cells (6) grown in the absence (lane 1) or presence of 10^{-6} M β -estradiol (lane 2). Position of endogenous ets-2 is indicated by the arrowhead, whereas the p200 and p50 bands are shown by arrows. The lower panels show the results of Western blotting with these cells, using pT72 or non-discriminating ets-2 antibody, as indicated.

in the immunoprecipitates (Fig. 4C, lane 1 compared with lane 2). Western analysis of a portion of these immunoprecipitates demonstrated that phosphorylated ets-2 could be detected following activation of Raf (Fig. 4C, lower panels). We estimated that about 50–60% of ets-2 was phosphorylated at position Thr-72 in the experiment shown (see “Experimental Procedures”). Thus, a significant pool of unphosphorylated ets-2 was present in the cells and immunoprecipitates analyzed in this experiment.

Ets-2 Co-immunoprecipitates with Brg-1 and Components of the Mammalian SWI/SNF Complex—As a first attempt at identifying the proteins that co-immunoprecipitated with ets-2, we took a candidate protein approach and obtained antibodies for known co-activators and co-repressors in the 200-kDa size range. Fortuitously, one of the first antibodies tested was directed against Brg-1, the ATPase hydrolyzing subunit of the mSWI/SNF complex (13, 14), and recognized the p200 protein (Fig. 5A). In addition, antibody against the BAF-57/p50 subunit of the mSWI/SNF complex (13) recognized the p50 protein present in anti-ets-2 immunoprecipitates (Fig. 5A). The Ini1 subunit of mSWI/SNF (12–14) could also be detected in a complex with ets-2 in NIH 3T3 cells (Fig. 5A). In contrast, the Brm-1 protein, a protein highly related to Brg-1 that can be found in a distinct mSWI/SNF complex (13, 14), was not immunoprecipitated with ets-2 (Fig. 5A).

The MCF-7 cells engineered to conditionally express ets-2 were used to determine whether the ets-2/mSWI/SNF interaction could be detected in human cells (Fig. 5B). For these experiments, cells were grown for 8 h after removing tetracycline from the culture media followed by isolation of ets-2 complexes by immunoprecipitation. These experiments showed that Brg-1 and Ini1 could be detected in complex with ets-2 after tetracycline removal, but not in cells maintained in tetracycline (Fig. 5B). The BAF157/p50 antibody did not cross-react with proteins present in MCF-7 cells in our hands (data not shown).

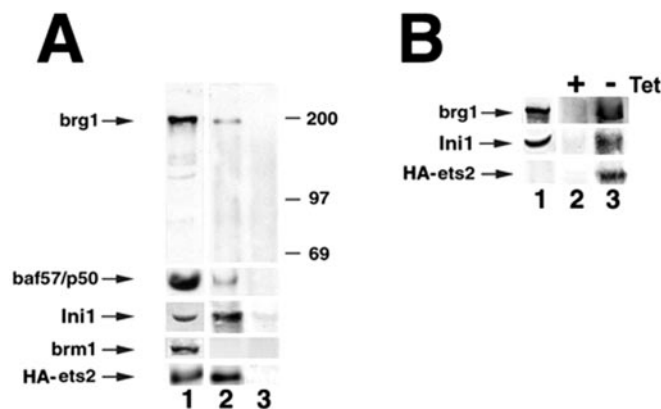


FIG. 5. Ets-2 forms a complex with components of mammalian SWI/SNF. *Panel A*, Western analysis of proteins that co-immunoprecipitate with HA-ets-2 Ala-72 in NIH 3T3 cells that express this exogenous form of ets-2 (lane 2). Components of the mSWI/SNF complex, Brg-1, BAF57/p50, and Ini1, all were found in complex with the HA-ets-2 T72A (panels 1–3), but Brm-1 is not found in the complex (lane 2, panel 4). Lane 1 is a Western blot performed on total nuclear extracts prepared from these cells. As a control, immunoprecipitations with HA antibody were performed on normal NIH 3T3 cells that do not express the HA-tagged protein (lane 3). *Panel B*, Western analysis of proteins that co-immunoprecipitated with HA-ets-2 in the tetracycline-off MCF-7 cells (see Fig. 3). Tetracycline was removed from cells for 8 h, HA-ets-2 was immunoprecipitated with HA antibody, and the immunoprecipitates were analyzed by Western blots with antibodies as indicated (lane 3). Lane 1 represents Western analysis of crude nuclear extracts with the same antibody, whereas lane 2 is a Western blot performed on material in the HA immunoprecipitate prepared from cells grown in the presence of tetracycline (non-inducing conditions).

The ets-2 Pointed Domain Forms a Phosphorylation-dependent Complex with Brg-1 in Vitro—To determine whether the interaction of Brg-1 with ets-2 was direct and dependent on ets-2 phosphorylation, GST pull-down experiments were per-

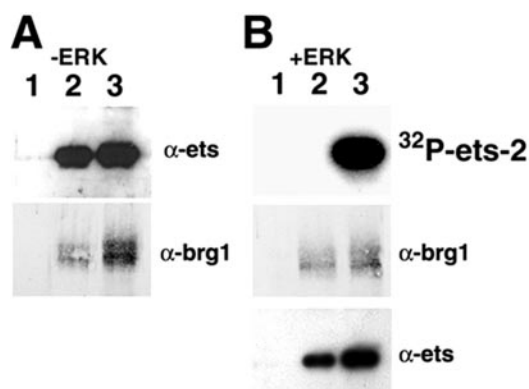


FIG. 6. Phosphorylation-dependent direct interaction between Brg-1 and Ets-2 *in vitro*. *A*, recombinant GST-Brg-1 (1 μ g) was immobilized on GST beads and then incubated with the recombinant, unphosphorylated pointed domain of ets-2 (1 μ g). Protein bound to beads was analyzed by SDS-PAGE and Western blotting, using ets-2 or Brg-1 specific antibodies as indicated. *Lane 1*, GST only + ets-2 pointed; *lane 2*, GST-Brg-1 + unphosphorylated ets-2; *lane 3*, 50% of input of ets-2 and Brg-1. *B*, pull-down assay with GST-Brg-1 and ets-2 pointed phosphorylated by Erk using [γ - 32 P]ATP. *Upper panel* is an autoradiograph to detect 32 P-labeled ets-2 pointed. Radioactivity was quantified using an Amersham Biosciences PhosphorImager. *Two lower panels* are Western blots probed with Brg-1 and ets-2 antibodies, respectively. *Lane 1*, GST only + ets-2 pointed; *lane 2*, GST-Brg-1 + 32 P-labeled ets-2; *lane 3*, 50% of input of ets-2 and Brg-1.

formed. A recombinant GST-Brg-1 fusion protein containing the entire C-terminal portion of the protein from amino acids 1108 to 1686 was used in "pull-down" assays with the recombinant ets-2 pointed region (Fig. 6A). In these experiments, GST-Brg-1 could form a complex with the ets-2 pointed protein, whereas GST alone did not (Fig. 6A, top panel, lane 2 versus lane 1). Approximately 30% of the ets-2 input was present in the complex (lane 3 represents 50% of ets-2 input).

In a parallel experiment, the ets-2 pointed region was phosphorylated *in vitro* using recombinant MAPK p42 and [γ - 32 P]ATP. Cold ATP was not added to the kinase reaction, so that only a trace amount of the ets-2 pointed protein would be phosphorylated. The 32 P-labeled ets-2 pointed protein was used in the pull-down assay with GST-Brg-1 as above (Fig. 6B). In this case, less than 5% of the 32 P-labeled ets-2 protein was found in a complex with the GST-Brg-1 protein (Fig. 6B, top panel, lane 2 versus lane 3). In contrast, Western analysis demonstrated that ~30% of the unlabeled ets-2 pointed domain could still be detected in the pull-down fraction.

Brg-1 and ets-2 Repress the BRCA1 Promoter in SW13 Cells—To test the functional significance of the Brg-1/ets-2 interaction, the effects of these nuclear factors on the activity of the BRCA1-luciferase reporter were studied (Fig. 7). For these experiments, the tumor cell line SW13, which lacks detectable Brg-1 and Brm-1 proteins (15, 16, 37), was used. First, the BRCA1-luciferase reporter was introduced into cells with the combination of expression vectors for ets-2 and Brg-1 in transient transfection assays (Fig. 7A, left panel). The results of the experiments, expressed as fold-repression, indicated that neither expression vectors for ets-2 nor Brg-1 alone repressed the BRCA1 reporter. However, the combination of the two resulted in an approximate 3-fold repression of reporter activity (Fig. 7A, left panel). If an expression vector for a Brg-1 gene encoding a protein with a mutation in the ATP-binding domain, Brg-1 (K798R), was used in the assay, repression of the BRCA1 reporter was not observed in either the presence or absence of ets-2 (Fig. 7A, left panel). As a control, we also studied the ability of Brg-1 to act as a co-activator for the glucocorticoid receptor (GR) using an artificial glucocorticoid responsive reporter that contained 8 GR-binding sites. As previously re-

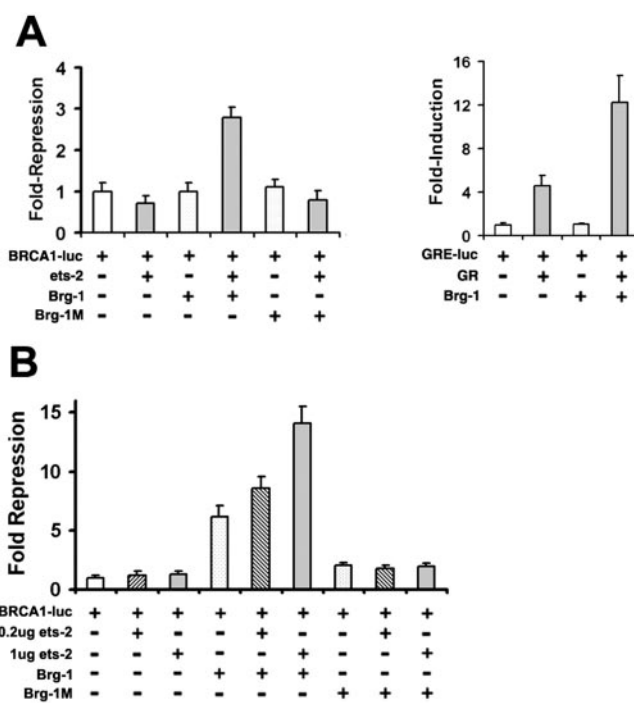


FIG. 7. Brg-1 and ets-2 repress BRCA1 promoter reporter in SW13 cells. *A*, transient transfections performed in SW13 cells. 5 μ g of human BRCA1-luciferase reporter was co-transfected with 100 ng of expression vector for ets-2 and 250 ng of expression vector for Brg-1, or Brg-1 K798R, or with a combination of 100 ng of ets-2 vector and 250 ng of Brg-1, or Brg-1 K798R vectors, as indicated (left bar graph). In parallel experiments shown in the bar graph to the right, 5 μ g of an 8 \times GRE reporter was co-transfected with 100 ng of rat GR expression vector (47), 250 ng of expression vector for Brg-1, or with a combination of 100 ng of GR vector and 250 ng of Brg-1 vector. All experiments were performed in the presence of 10^{-7} M dexamethasone in this analysis. *Fold-induction* indicates the ratio of 8 \times GRE relative activity alone compared with activity seen with GR or Brg-1 alone, or a combination of the two. *B*, transfection with stably integrated BRCA1-luciferase reporter. For these experiments, 1 μ g of Brg-1 or Brg-1 K798R alone or in combination with ets-2 (0.2 or 1 μ g, as indicated) were transfected into SW13 cells that contained stably integrated copies of the BRCA1-luciferase reporter. For both panels A and B, fold-repression is the ratio of relative luciferase activity (see "Experimental Procedures") for the BRCA1-luciferase reporter alone (with empty expression vectors) to the activity in the presence of ets-2, Brg-1 (Brg-1 K798R), or a combination of both ets-2 and Brg-1 (Brg-1 K798R). Results of three independent experiments performed in duplicate are presented. *Error bars* indicate standard deviation of the measurements.

ported, the combination of Brg-1 and GR expression vectors could stimulate a target reporter more efficiently than either gene alone (16) (Fig. 7A, right panel).

Because transient DNA templates may not always be organized as chromatin (16, 38), we also performed assays with SW13 cells that contained stably integrated BRCA1-luciferase reporter genes (Fig. 7B). In these experiments, ets-2 expression by itself had little effect on the reporter activity. Brg-1 expression alone could repress the BRCA1 reporter ~7-fold, whereas the combination of ets-2 and Brg-1 resulted in a further dose-dependent repression of BRCA1 promoter activity (Fig. 7B). A 14-fold reduction was observed with the higher concentration of ets-2 expression vector. As in the transient assays, Brg-1 (K798R) did not repress BRCA1 reporter activity alone or in combination with ets-2.

DISCUSSION

Ets-2 has previously been characterized as an activator of gene expression, and the results presented here indicate that this factor can also act as a repressor. Results from transient transfection assays and following conditional expression of

ets-2 demonstrate that ets-2 could both repress and activate target genes in MCF-7 cells. Furthermore, ets-2 directly interacted with Brg-1, the ATPase component of the mSWI/SNF complex, and Brg-1 behaved as a transcriptional co-repressor along with ets-2. Specificity for target gene regulation within the ets family of transcription factors can be achieved in part through the ability of signaling pathways to selectively activate ets family members (1). For example, the ras pathway selectively activates only a subset of ets family members, including ets-2. The results presented here may begin to provide insight into how signaling pathways modulate the activity of ets-2.

At least *in vitro*, the pointed domain of ets-2 interacted with distinct sets of cellular proteins, including Brg-1, dependent on phosphorylation of residue threonine 72, a target of ras/MAPK signaling. These results suggest the hypothesis that ras-dependent phosphorylation of ets-2 switches the activity of this factor from repressor to activator by modulating interactions with co-repressors like Brg-1. However, the fact that pools of both phosphorylated and non-phosphorylated ets-2 are present even in cells that have high activation of the ras/raf/MAPK signaling pathways as can be achieved with the ER-Raf system (6), makes it difficult to determine *in vivo* whether phosphorylation of ets-2 strictly modulates its activity as repressor, or even its interaction with Brg-1. Whereas the model that signaling pathways can modulate the pools of ets-2 that act as either repressor or activator within cells is attractive, additional work is required to validate this model. It is possible that signaling pathways never lower the level of non-phosphorylated ets-2 sufficiently to affect repression of target genes. A significant portion of ets-2 may always be present in a repressor complex within cells, and phosphorylation may only increase the pool of ets-2 that is able to activate target genes.

The results presented here provide further evidence that the mammalian SWI/SNF complex can also repress gene expression, in addition to the well characterized role as a co-activator in mammalian cells (13, 15, 16). At least two models can be proposed to account for the activity of the ets-2-Brg-1 complex as a repressor instead of as an activator of gene expression. One possibility is that direct interaction with ets-2 may cause a conformational change in the mSNF/SWI complex resulting in an increased ability to form closed nucleosome structures over the open, altered nucleosome structure (14).

A second possibility that may account for our results is that distinct subcomplexes may mediate activation and repression functions of mSWI/SNF (20, 39). In mammalian cells, discrete Brg-1 and Brm-1 subcomplexes containing mSin3 co-repressors have been characterized (39), whereas recent work in yeast suggests that the co-repressor function may be mediated by the ATPase-hydrolyzing SNF2 subunit alone, and not require additional complex subunits (20). Our analysis indicates that at least two known components of mSWI/SNF (Ini1 and BAF57/p50) in addition to Brg-1 are present in the putative ets-2 co-repressor complex. The closely related gene product Brm-1 could not be detected in a complex with ets-2, consistent with evidence suggesting that Brg-1 and Brm-1 complexes are biochemically and functionally distinct (39, 40). However, additional antibodies that are available for mSWI/SNF complex subunits, for example, p155 and p170, were not sufficiently specific, at least in our hands, to allow us to determine with certainty whether other components were associated with ets-2. Defining the exact composition of the complex, and demonstrating that the ets-2 containing complex has chromatin remodeling activity, are important questions that need to be addressed by future work.

New mechanisms by which the tumor suppressor *BRCA1* might be down-regulated in mammary tumor cells are biologically significant. Germline mutations in *BRCA1* account for

approximately one-half of inherited breast cancers, but mutations of *BRCA1* are infrequent in sporadic breast cancer (41). Several studies indicate that *BRCA1* expression is down-regulated in primary breast tumors *versus* normal breast tissue (42–45). Aberrant methylation of CpG islands in the *BRCA1* promoter may be one mechanism that leads to decreased gene expression in sporadic breast cancer (42–44). However, hypermethylation of the *BRCA1* promoter region is only found in ~13% of sporadic breast cancer cases (46), suggesting that additional mechanisms may be involved in *BRCA1* silencing. Ets-2 as both a repressor of *BRCA1* and an activator of extracellular proteases like uPA, MMP3, and MMP14 in a subset of breast cancer cases provides an attractive model.

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