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

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 InI1 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 corepressors.

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), 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 phoshatidylinositol 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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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 μg/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 Alu-72 protein, NIH 3T3 cells were transfected with the HA-tagged version of ets-2 Alu-72 by the calcium phosphate method as described (3, 19), and glycolymin-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 [35]methionine, cells were placed in DMEM that lacked both serum and methionine for 45 min, then incubated with DMEM containing 90 μCi/ml [35]methionine (1200 Ci/mmol; ICN, Irvine, CA) for 2 h. Cells were harvested and 15,000 × g pellets of ets-2 MCF-7 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 μg of BRCA1-luciferase plasmid and 1 μg of a neomycin-resistance vector using LipofectAmine Plus (Invitrogen). 48 h after transfection, cells were selected with G418 (400 μg/mL). The neomycin-resistant colonies were picked every 3 days. 14 days after selection, there were about 100 colonies on the dish. The cells were pooled and cultured with 200 μg/mL G418 and used for subsequent experiments.

Plasmids, DNA Transfections, and Northern Blots—The vector used for expression of the HA-tagged version of ets-2 and ets-2 Alu-72 proteins were prepared as 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 CgGAAAGAG2 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-p-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 μg/DNA precipitate). Relative luciferase activity is equal to (raw luciferase activity/raw activity of the internal control × 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 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 μg of a puromycin-resistant expression vector, pBabe-puro-mycin (6). 36 h after transfection, cells were selected with 4 μg/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 an Amer sham Biosciences Phosphorlmager.

Immune Reagents and Analysis—The antibodies specific for ets-2 and phosphorylated ets-2 (ETS21128–ETS21303) 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 11n1 from Anthony Imbalzano (University of Massachusetts).

For standard immunoprecipitations, 2.5–3 × 106 cells were placed in 500 μl 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 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 11n1 from Anthony Imbalzano (University of Massachusetts).

In the present report evidence is presented indicating that ets-2 and BRCA1 co-repressor (20). Therefore, SWI/SNF appears to act as both co-activator and co-repressor (20).

For direct protein interaction assays, a portion of Brg-1 corresponding to the pointed homology region (amino acids 60–187) 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 antibody was phosphorylated in a reaction that contained 10 μg of ets-2 affinity column, 30 μl of kinase buffer (30 mM Hepes, pH 7.2, 20 mM MgCl2, 2 mM dithiothreitol), 100 μM ATP, 10 μM of (γ-32P)ATP (500 Ci/mmole, PerkinElmer Life Sciences), and 5 μl of activated recombinant MAPK p42 (Upstate Biotechnology, Lake Placid, NY). The GAGA domain of the ets-2 protein corresponding to the ets-2 pointed domain that was phosphorylated or the phosphorylated ets-2 affinity column were incubated with cell extracts prepared in lysis buffer as above for 16 h at 4 °C. Washing procedures and analysis were performed as for the immunoprecipitations above.

For standard immunoprecipitations, 2.5–3 × 106 cells were placed in 500 μl of lysis buffer consisting of 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 3 mM MgCl2, and 1% Nonidet P-40. 36 h after transfection, cells were selected with 4 μg/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 an Amer sham Biosciences Phosphorlmager.

Affinity Chromatography and Protein Interaction Assays—Recombinant ets-2 protein corresponding to the pointed homology region (amino acids 60–187) 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 μg of ets-2 affinity column, 30 μl of kinase buffer (30 mM Hepes, pH 7.2, 20 mM MgCl2, 2 mM dithiothreitol), 100 μM ATP, 10 μCi of (γ-32P)ATP (500 Ci/mmole, PerkinElmer Life Sciences), and 5 μl of activated recombinant MAPK p42 (Upstate Biotechnology, Lake Placid, NY). The recombinant ets-2 protein was incubated in lysis buffer for 16 h at 4 °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 phosphorimagining.

Western analysis was performed as previously described (5, 6). A Loading Dye Reagent (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 of ets-2 (amino acids 334–466) was produced using the pGEX expression vector system and purified by gluta thione-Sepharose chromatography (Amer sham Biosciences) as previously described (28). Purified protein was used in the electrophoretic mobility shift assays using standard conditions as previously
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’-GTTAAGATTCTCCCTCTTCGCGCCTCTTTGG; BRCA1 M1, 5’-GTTAAGATTCTCCCTCTTCGCGCCTCTTTGG; BRCA1 M2, 5’-GTTAAGATTCTCCCTCTTCGCGCCTCTTTGG; and BRCA1 M1 + M2, 5’-GTTAAGATTCTCCCTCTTCGCGCCTCTTTGG. 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 est-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 (3, 28), 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 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 (ACGGAGAGGT, 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 (ACCGAGAGATG, M2). Mutation of the G residue in the ets core motif (ACGTAGAGGT, M1) or a mutation of both G residues (ACGTAGAGGT, 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...
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. 2B). 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. 2B). 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 (ACG-1TAAGAGT2, 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. 2C).

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. 2D). 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 Thr-72A 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. 2D). 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
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 (3) following 24 h of induction (Fig. 3). Induction of HA-ets-2 repressed expression of endogenous ets-2, remaining low in the cells following induction of the oncogene, and estrogen treatment persistently stimulates MAPK kinases activity and ets-2 phosphorylation at Thr-72, as shown). Analysis of proteins from NIH 3T3 cells that bound to the unphosphorylated ets-2 column (Fig. 4A, lane 6) did not bind to the phospho-ets-2 column (Fig. 4A, lane 6). Notably, 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-32P]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. 4A, lane 6). Notably, p200 and p50 did not bind to the phospho-ets-2 column (Fig. 4A, 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.

These immunoprecipitation experiments demonstrated that the HA-tagged T72A version of ets-2 was expressed at the same level as endogenous protein (Fig. 4A, 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. 4A, lanes 3 and 4). These proteins were also detected in ets-2 immunoprecipitates obtained serum-starved NIH 3T3 cells (Fig. 4A, lane 1).

In contrast, these bands were not detected in HA immunoprecipitates prepared from NIH 3T3 cells (Fig. 4A, lane 1). Furthermore, when the immunoprecipitaiton 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. 4B). 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.
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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 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.

Fig. 5. Ets-2 forms a complex with components of mammalian SWI/SNF. Panel A, Western analysis of proteins that co-immunoprecipitated 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-
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 50% 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-32P]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 32P-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 32P-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 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 reported, 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
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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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