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Transcriptional Activation of Insulin-Like Growth Factor-Binding Protein-4 by 17β-Estradiol in MCF-7 Cells: Role of Estrogen Receptor-Sp1 Complexes*

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ABSTRACT

Insulin-like growth factor-binding protein-4 (IGFBP-4) is expressed in MCF-7 human breast cancer cells, and treatment of these cells with 17β-estradiol (E_2) resulted in induction of IGFBP-4 gene expression (>3-fold) and protein secretion (>6-fold). To identify genomic sequences associated with E_2 responsiveness, the 5′-promoter region (−1214 to +18) of the IGFBP-4 gene was cloned into a vector upstream from the firefly luciferase reporter gene, and E_2 induced a 10-fold increase in luciferase activity in MCF-7 cells transiently transfected with this construct. Deletion analysis of this region of the IGFBP-4 gene promoter identified two GC-rich sequences at −559 to −553 and −72 to −64 that were important for E_2-induced *trans*-activation. Gel mobility shift assays using ^{32}P-labeled −569 to −540 and −83 to −54 oligonucleotides from the IGFBP-4 gene promoter showed that Sp1 protein bound these oligonucleotides to form a retarded band, and the intensity of the band was competitively decreased after coincubation with unlabeled IGFBP-4-derived and consensus Sp1 oligonucleotides. Mutation of the GC-rich sites within these sequences resulted in loss of the retarded band formation. Wild-type human estrogen receptor did not bind directly to the IGFBP-4 oligonucleotides; however, human estrogen receptor enhanced Sp1-DNA binding in a concentration-dependent manner. The results of this study demonstrate that at least two GC-rich sequences at −559 to −553 and −72 to −64 are required for induction of IGFBP-4 gene expression by E_2 in MCF-7 cells. (*Endocrinology* **140:** 2501–2508, 1999)

INSULIN-LIKE growth factor (IGF)-binding proteins (IGFBPs) are widely expressed in mammalian cells, and these proteins play an important role in transport and tissue availability of IGFs. Six IGFBPs have been identified, and these are characterized by cysteine-rich N- and C-terminal domains and their high binding affinities for IGFs (1–7). In addition, new proteins have been proposed as members of the IGFBP family (7–11). The distribution, regulation, and function of IGFBPs have been extensively investigated in human breast cancer cells (12–23). For example, IGFBP-3 is widely expressed in human mammary tumors and breast cancer cells. IGFBP-3 alone inhibits the growth of both estrogen receptor (ER)-positive and ER-negative human breast cancer cells and induces apoptosis, and several antimitogenic compounds and polypeptides, such as retinoids, the antiestrogen ICI 182,780, and transforming growth factor-β (TGFβ), also induce IGFBP-3 (24–32).

IGFBP-4 has also been widely detected in breast tumors and cells in culture, and it has been reported that IGFBP-4 expression positively correlated with ER status in mammary tumors (12, 33). E_2 induces IGFBP-4 messenger RNA (mRNA) and protein levels in ER-positive breast cancer cells, and antiestrogens inhibited these responses (34–37); however, the molecular mechanisms of ER action have not been determined. Structural and functional analyses of the IGFBP-4 gene promoter have recently been reported (38, 39), and the 5′-promoter region contains multiple *cis*-elements including cAMP response elements, activated protein-1 (AP-1)/AP-2 sites, Egr-1 sites, and GC-rich sequences. This study shows that E_2 induces IGFBP-4 expression, and analysis of the 5′-promoter region has identified GC-rich sites at −559 to −553 and −72 to −64 that bind Sp1 protein and are important for E_2 responsiveness. ER/Sp1 action at GC-rich sites has previously been reported for cathepsin D, retinoic acid receptor α1, and c-*fos* genes and does not require direct interaction of the ER with genomic DNA (40–44). The results obtained in this study for IGFBP-4 further extend the number of E_2-responsive genes regulated by this transcription factor complex.

Materials and Methods

Chemicals, cells, and oligonucleotides

All cells used in this study were obtained from American Type Culture Collection (Manassas, VA). MCF-7 cells were maintained in MEM with phenol red and supplemented with 0.22% sodium bicarbonate, 10% FBS, 0.011% sodium pyruvate, 0.1% glucose, 0.24% HEPES, 10^{-6}% insulin, and 10 ml/liter antibiotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown in 100-cm^2 culture plates in an air-carbon dioxide (95:5) atmosphere at 37 C and were passaged every 5 days. Cells for various experiments were seeded in phenol red-free DMEM/Ham's F-12 medium with 5% charcoal-stripped FBS. DMEM/Ham's F-12 medium without phenol red, PBS, acetyl coenzyme A, E_2, and antibiotic solution were purchased from Sigma Chemical Co. FBS was obtained from Intergen (Purchase, NY). The STAT-60 RNA Extract Kit was purchased from Tel-Test (Friendswood, TX). [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]CTP (3000 Ci/mmol) were purchased from NEN Research Products (Boston, MA). Horseradish peroxidase substrate for Western

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blot analysis was purchased from DuPont NEN (Boston, MA). Hybond-N nylon membrane for Northern blot analysis and Hybond enhanced chemiluminescence nitrocellulose membrane for Western blot analysis were purchased from Amersham International (Aylesbury, UK). IGFBP-4 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polydeoxy-(inosinic-cytidylic) acid [polyd(I-C)], restriction enzymes (*Hin*dIII and *Bam*HI), and T4 polynucleotide kinase were purchased from Boehringer Mannheim (Indianapolis, IN). The human estrogen receptor (hER) expression plasmid was provided by Dr. Ming-jer Tsai (Baylor College of Medicine, Houston, TX). Recombinant human Sp1 protein, reporter lysis buffer, and luciferase reagent for luciferase studies were purchased from Promega Corp. (Madison, WI), and baculovirus-expressed hER proteins were obtained from Panvera (Madison, WI). β-Galactosidase (β-Gal) reagent was purchased from Tropix (Bedford, MA). The plasmid preparation kit was purchased from Qiagen (Chatsworth, CA). All other chemicals and biochemicals were the highest quality available from commercial sources. All primers and oligonucleotides used in this study were synthesized and/or sequenced by Texas Agricultural Experiment Station, Department of Veterinary Pathobiology, Texas A&M University (College Station, TX). InstantImager and LumiCount were purchased from Packard (Meriden, CT).

Northern blot analysis

MCF-7 cells were seeded in 5% charcoal-stripped FBS/DMEM/Ham's F-12 medium for 24 h and in serum-free DMEM/Ham's F-12 medium for another 24 h. Fresh serum-free medium was then used, and cells were treated with E_2, antiestrogens, or dimethylsulfoxide (DMSO) for different times before harvesting. Total RNA was isolated using the STAT-60 Kit (Tel-Test). Twenty micrograms of total RNA were diluted in 2 × FPF [20% formaldehyde, 1.65% Na_2HPO_4 (pH 6.8), 63.5% formamide, and 1 × loading buffer] and separated on 1.2% agarose gel with 1 M formaldehyde in 1 × SPC buffer. After transfer onto Hybond-N nylon membrane (Amersham), the blots were prehybridized and hybridized in NENhybe solution (5 × SSPE, 10% dextran sulfate, 0.1% polyvinylpyrolidine, 0.1% Ficoll, 0.1% BSA, and 1% SDS) at 65 C without or with [^{32}P]CTP-labeled IGFBP-4 complementary DNA probe for 16 h. Blots were visualized by autoradiography and quantitated on InstantImage (Packard). The membrane was then stripped and rehybridized with β-tubulin probe as a control. The IGFBP-4 c-DNA probe was determined by RT-PCR using the following primers: IGFBP-4 forward, 5′-TGC AGA AGC ACT TCGCCA AA-3′ (+702/+721); and IGFBP-4 reverse, 5′-ACA GGA CTC AGA CTC AGA CT-3′ (+1141/+1160).

Western blot analysis

MCF-7 cells were seeded in 5% charcoal-stripped FBS/DMEM/Ham's F-12 medium in six-well plates. At 85% cell confluence, cells were washed twice with sterile PBS buffer and incubated in serum-free DMEM/Ham's F-12 medium for 24 h. Cells were then changed to fresh serum-free DMEM/Ham's F-12 medium and treated with E_2 or DMSO (solvent control). After incubation for 24 h, cells were changed to 0.75 ml fresh serum-free medium and treated again. After 16–24 h, conditioned medium was collected, and cells in each well were counted to normalize results. The conditioned medium was concentrated using microconcentrators (12,000 × *g*, 60 min) to less than 100 μl and transferred to fresh 1.5-ml tubes. Samples were boiled for 2 min, separated on 12% SDS-PAGE at 180 V for 4 h, and transferred to Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham) at 14 V overnight at 4 C. Nitrocellulose membranes were then soaked in 5% milk/Tris-buffered saline with gentle shaking for 15 min and incubated in fresh 5% milk-TBS with 1:500–1000 primary antibody (Santa Cruz Biotechnology, Inc.) for 1–2 h with gentle shaking. After washing with TBS for 15 min (once) and 5 min (twice), the membrane was incubated in 5% milk-TBS with 1:1000–2000 secondary antibody for 1–2 h with gentle shaking. The membrane was washed with TBS for 15 min (once) and 5 min (twice); 10 ml horseradish peroxidase substrate (DuPont NEN) were then added and incubated for 1.0 min. The membrane was exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, NY), visualized by autoradiography, and quantitated by densitometry using the Molecular Dynamics, Inc. Zero-D software package (Sharp Corp., Mahwah, NJ).

Cloning

pXP2 luciferase reporter plasmid (American Type Culture Collection) was modified with the insertion of TATA sequence into its polylinker site immediately upstream of the luciferase expression gene. IGFBP-4 promoter fragments (−1214 to −379, −575 to −379, −569/−569m to −540, and −83/−83m to −54) were amplified/enzyme cut or synthesized as double stranded DNA with 5′-overhangs and inserted into the vector between *Hin*dIII and *Bam*HI polylinker sites; fragments (−373 to +18 and −125 to +18) that contain TATA sequences were inserted into pGL2 luciferase reporter plasmid (Promega Corp.) at *Kpn*I and *Xho*I sites. All plasmids are designated with a p, followed by the size of the promoter insert. All ligation products were transformed into competent *Escherichia coli* cells. Plasmids were isolated and clones were confirmed by restriction enzyme mapping and DNA sequencing. High quality plasmids for transfection were prepared using QIAGEN Plasmid Mega Kit. The sequences of primers and oligonucleotides are listed below. GC-rich elements are capitalized, and mutations in the oligonucleotides are *underlined*; (s)* means sense; (m)** means mutant: −1214 forward primer, cca agc ttc tcg tga tct gcc; −575 forward primer, cca agc ttc cct ggg gag a; −354 reverse primer, aga aag gga ctt cct a; −373 forward primer, gcg gta ccc aga gcc ggg agt cc; −125 forward primer, gcg gta ccg cga ctc agg aca gc; +18 reverse primer, cga gct cgg cag ggg gct gag; −569 oligonucleotide(s)*(−569 to −540), agc ttg gga gat tgc gGG GGC GGG aga ggt tgc aag; −569 oligonucleotide(s)*(m)**, agc ttg gga gat tgc gGA ATC TTG aga ggt tgc aag; −83 oligonucleotide(s)*(−83 to −54), agc ttt ctc ccc ctc gCC CGC CCC ggc tcc ccc acg; −83 oligonucleotide (s)*(m)**,

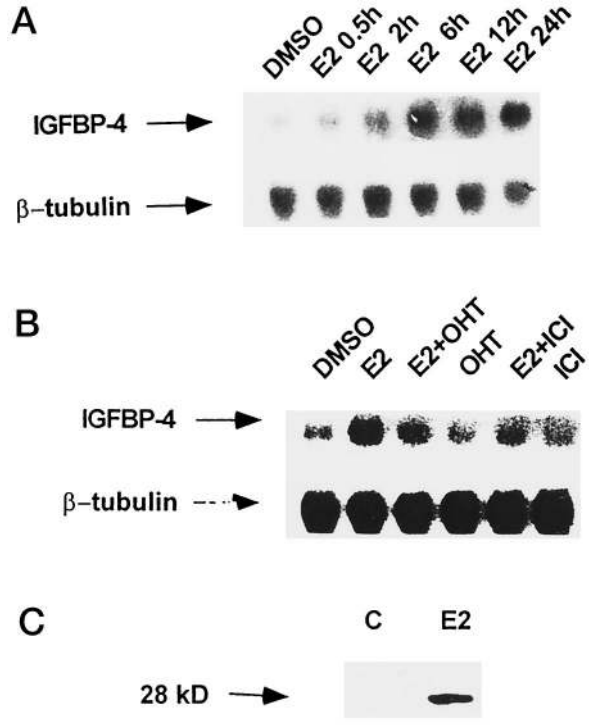

FIG. 1. Effects of E_2 and antiestrogens on IGFBP-4 in MCF-7 cells. A, E_2-mediated *trans*-activation. MCF-7 cells were treated with 10^{-8} M E_2 for different times, and IGFBP-4 mRNA levels were determined by Northern analysis as described in *Materials and Methods*. Results are expressed as the mean ± SD for three separate determinations (**, $P < 0.05$, significantly higher than control mRNA levels). B, Northern analysis. MCF-7 cells were treated with 10^{-8} M E_2, 10^{-6} M hydroxytamoxifen (OHT), or 10^{-6} M ICI 182,780 (ICI), alone or in combination, for 6 h, and IGFBP-4 mRNA levels were determined by Northern blot analysis as described in *Materials and Methods*. C, Western blot analysis of IGFBP-4. MCF-7 cells were treated with 10^{-8} M E_2 for 6 h, and the glycosylated 28-kDa IGFBP-4 protein was determined as described in *Materials and Methods*. Levels of the immunoreactive protein were more than 6-fold higher after treatment with E_2.

agc ttt ctc ccc ctc gCA AGA TCC ggc tcc ccc acg; and consensus Sp1 oligonucleotide(s)*, agc tta ttc gat cgg ggc ggg gcg agc g.

Transient transfection and luciferase activity assay

Cultured MCF-7 cells were seeded in charcoal-stripped FBS/DMEM/Ham's F-12 medium in 60-mm plates 1 day before transfection. Five micrograms of test plasmid, 2.5 μg wild-type hER, and β-Gal-*lacZ* plasmid (1.0 μg) obtained from Invitrogen (Carlsbad, CA) were cotransfected into MCF-7 cells using the calcium phosphate-DNA coprecipitation method. After incubation for 16–20 h, cells were washed with PBS and treated with 10 nM E_2 or DMSO (as control) in fresh medium for 40 h. Cells were then washed with PBS and lysed with 400 μl 1 × reporter lysis buffer (Promega Corp.). Cell lysate was frozen in liquid nitrogen and thawed at room temperature; 20 μl cell extract were assayed with luciferase (Promega Corp.) and β-Gal reagents (Tropix). LumiCount (Packard) was used to quantitate luciferase and β-Gal activities. The luciferase/β-Gal ratio was used to represent normalized luciferase activity for each treatment group.

Gel electrophoretic mobility shift assay (GEMSA)

Oligonucleotides were synthesized, purified, and annealed, and 10 pmol of specific oligonucleotides were ^{32}P-labeled at the 5′-end using T4 polynucleotide kinase and [γ-^{32}P]ATP. GEMSAs were performed by incubating varying amounts of recombinant human Sp1 protein (Promega Corp.) in 25 μl 1 × binding buffer (5% glycerol, 0.477% HEPES, 0.546% KCl, 1 mM EDTA, and 0.4 mM dithiothreitol, pH 8.0) and 0.16 mg/ml BSA. After incubation for 10 min at 4 C, ^{32}P-labeled oligonucleotides (100,000 cpm) were added to the reaction mixture in the presence of 1 μl polyd(I-C) and 0.33% Ficoll and incubated for an additional 15 min at 25 C. Competition studies were carried out with excess unlabeled DNA before the addition of ^{32}P-labeled oligonucleotides. The following procedures were used for ER-enhanced Sp1 binding studies: 1) 0–400 fmol pure hER protein (Panvera) in 1 × binding buffer containing 40 mM E_2 and BSA were incubated for 10 min at 4 C; 2) different amounts of Sp1 protein were added to the mixture and incubated on ice for 5 min; 3) ^{32}P-labeled oligonucleotides (100,000 cpm) were added to the reaction mixture in the presence of 1 μl polyd(I-C), and the mixture was incubated for another 15 min at 25 C. Five percent polyacrylamide gel (acrylamide-bisacrylamide, 30:0.8) was used to separate the reaction mixture. Electrophoresis was carried out at 110 V in 1 × TBE (0.9 M Tris-borate and 2 mM EDTA, pH 8.3). Gels were dried, and protein-DNA interactions were determined by scanning on an InstantImage (Packard) and visualized by autoradiography.

Statistical analysis

Statistical differences between treatment groups were determined by ANOVA and Scheffe's test for significance. The data are presented as the mean ± SD, and at least three determinations were carried out for each treatment group.

Results

The results of preliminary studies using [^{125}I]IGF-I and Western ligand blot analysis showed that E_2 caused a 2-fold induction of secreted IGFBP-4 (37), and this was confirmed by Western and Northern blot analyses of extracts from MCF-7 cells treated with 10 nM E_2 (Fig. 1). Northern analysis showed that E_2 significantly induced IGFBP-4 mRNA levels within 2 h after treatment, and a more than 3-fold increase was observed after 6 h; moreover, 10^{-6} M 4′-hydroxytamoxifen and 10^{-6} M ICI 182,780 inhibited E_2-induced mRNA levels. A more than 6-fold increase in the 28-kDa glycosylated form of IGFBP-4 was detected in conditioned medium by IGFBP-4 antibodies 6 h after treatment with E_2.

Transient transfection of various plasmids containing IGFBP-4 gene promoter inserts linked to a bacterial luciferase were determined in MCF-7 cells. Basal promoter activity for several constructs demonstrated that maximal responses were observed for the full-length plasmid (−1214 to +18) and p−373/+18 that contained the −373 to +18 region of the IGFBP-4 gene promoter (Fig. 2A). The loss of activity for p−125/+18 indicated that elements within the −373 to −125 region of the IGFBP-4 gene promoter were essential for maximal basal activity in MCF-7 cells. E_2 caused an approximately 3-fold increase in luciferase activity in MCF-7 cells

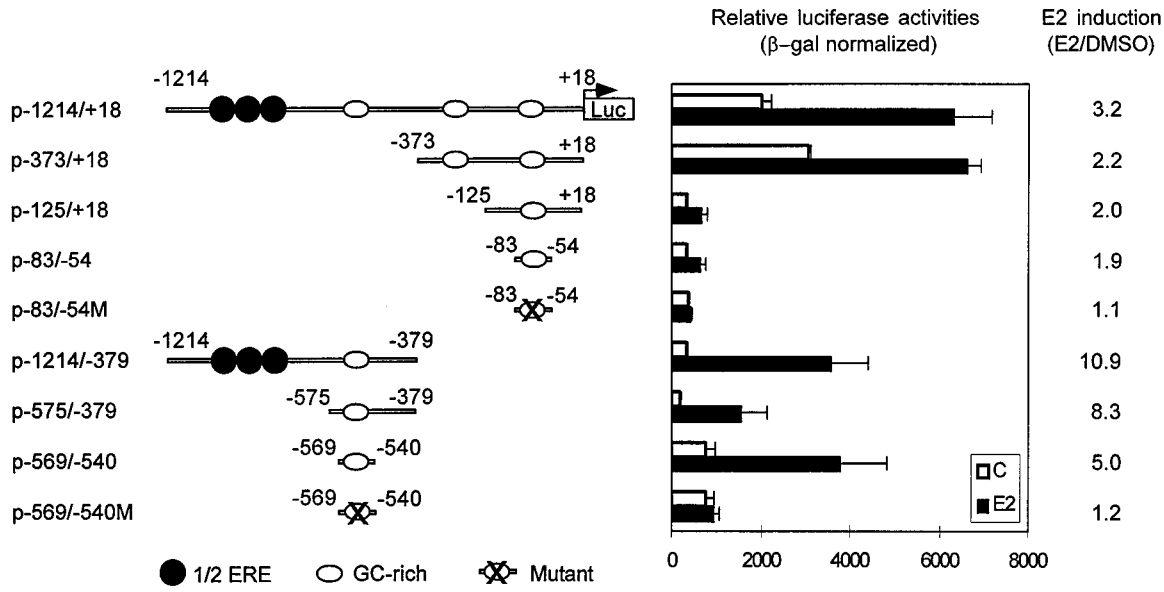

FIG. 2. Basal and E_2-induced *trans*-activation of IGFBP-4 gene promoter constructs. MCF-7 cells were transiently transfected with the appropriate plasmid treated with DMSO (solvent control) or in 10^{-8} M E_2, and luciferase activity was determined as described in *Materials and Methods*. Significant induction ($P < 0.05$) was observed for all wild-type constructs, whereas no induction was observed for mutant p−569/−540m or p−84/−54m. All results are the mean ± SD for three separate determinations and β-gal determinations were used to correct for transfection efficiency.

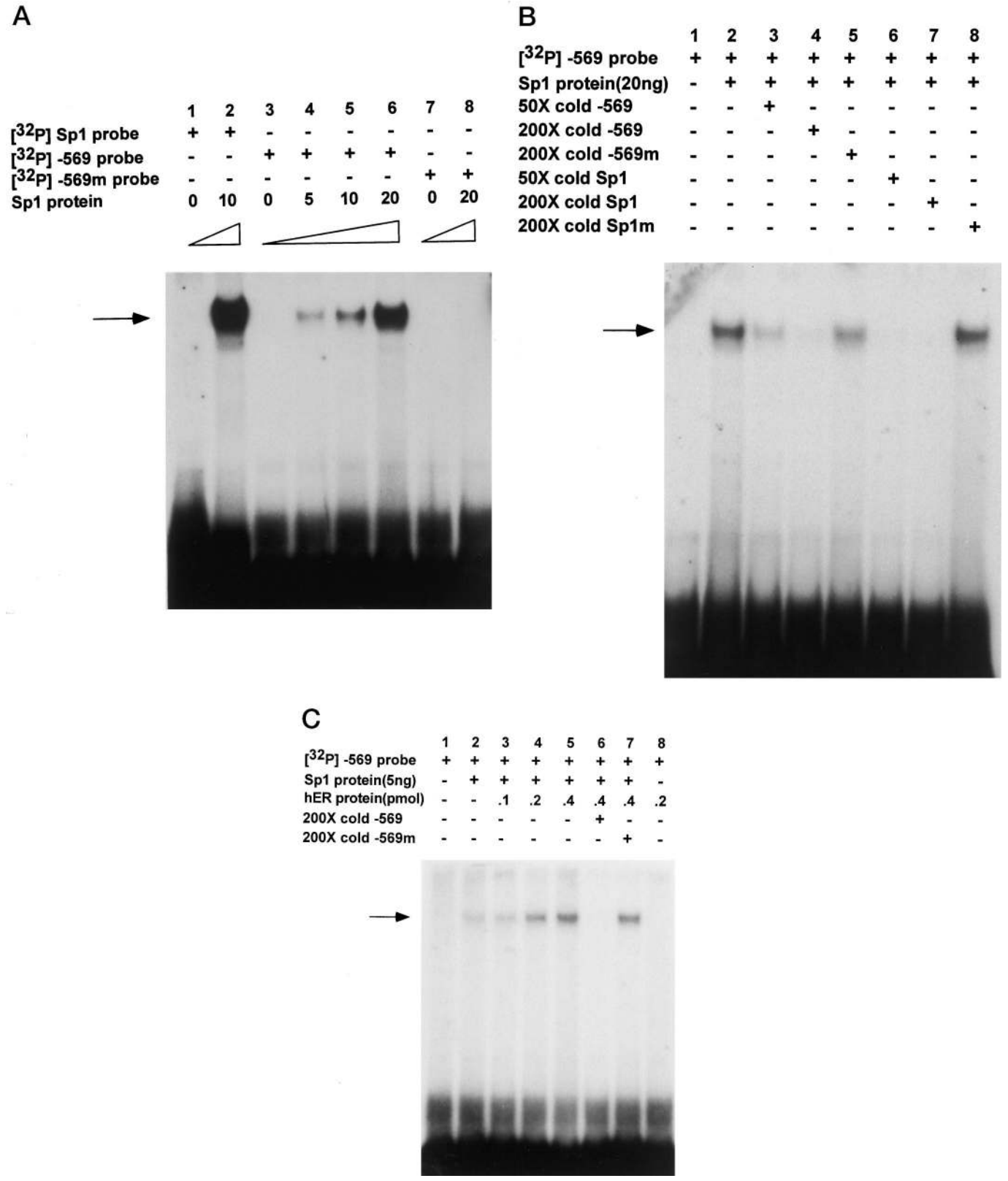

FIG. 3. GEMSAs with −569 oligonucleotide. A, Direct binding of [^{32}P]−569 with recombinant Sp1 protein. [^{32}P]Sp1, [^{32}P]−569, and mutant [^{32}P]−569m were incubated in the presence or absence of 20 ng Sp1 protein as described in *Materials and Methods* section. The specifically bound Sp1-DNA retarded band is indicated with an *arrow*. B, Competition for [^{32}P]−569-Sp1 retarded band. Sp1 protein was incubated with [^{32}P]−569 in the presence or absence of unlabeled wild-type −569, mutant −569m, consensus Sp1, or mutant Sp1m oligonucleotides as described in *Materials and Methods*. Unlabeled −569 and consensus Sp1 oligonucleotides decreased band intensities (lanes 3, 4, 6, and 7); a 200-fold excess

transfected with the full-length construct (−1214 to +18) and induction was more than 10-fold using the 3′-deletion construct p−1214/−379 (Fig. 2B). The E_2 responsiveness of this upstream region of the IGFBP-4 gene promoter was further investigated by 5′- and 3′-deletion analysis (p−575/−379, p−569/−540, and p−569/−540m), and the GC-rich site at −559 to −553 was identified as the important upstream *cis*-element required for ER action. The downstream −373 to +18 (p−373/+18) region of the promoter was also E_2 responsive and contained two GC-rich sites at −313 to −305 and −72 to −64. Transient transfection of MCF-7 cells with p−373/+18, p−125/+18, or p−83/−54 showed that only the −72 to −64 GC-rich site was required for hormone responsiveness in MCF-7 cells (Fig. 2B). Mutation of the −72 to −64 GC-rich site (p−83/−54m) resulted in the loss of E_2 responsiveness.

The results summarized in Fig. 3 compare the binding of human recombinant Sp1 protein to consensus [^{32}P]Sp1 oligonucleotide (lane 2), [^{32}P]−569 (lanes 3–6), and mutant [^{32}P]−569m probes (lanes 7 and 8). A retarded band with similar mobility (see *arrow*) was observed only for the consensus [^{32}P]Sp1 oligonucleotide and [^{32}P]−569 probe. The specificity for [^{32}P]−569-Sp1 binding was confirmed in competitive binding studies (Fig. 3B), showing that the intensity of the retarded band was decreased after competition with excess unlabeled −569 probe (lanes 3 and 4) and consensus Sp1 oligonucleotide (lanes 6 and 7), but not mutant −569m probe (lane 5) or mutant Sp1m oligonucleotide (lane 8). The effects of recombinant hER protein on formation of the [^{32}P]−569-Sp1 retarded band were also investigated by GEMSA (Fig. 3C). The intensity of the retarded band (lane 2) was incrementally increased after the addition of 0.1, 0.2, and 0.4 pmol recombinant human ER protein (lanes 3–5); the retarded band intensity was decreased after incubation with excess unlabeled −569 probe (lane 6), but was unaffected by coincubation with mutant −569m probe (lane 7). ER protein alone did not form a retarded band with [^{32}P]−569 (lane 8). Recombinant human Sp1 protein also formed a retarded band with [^{32}P]Sp1 oligonucleotide (Fig. 4A, lanes 1 and 2) and [^{32}P]−83 probe (lanes 3 and 4), but not with mutant [^{32}P]−83m (lanes 5 and 6). The specifically bound [^{32}P]−83-Sp1 band (Fig. 4B, lane 2) was competitively decreased by competition with excess unlabeled −83 probe (lanes 3 and 4) and Sp1 oligonucleotide (lanes 6 and 7), but not by mutant −83 probe (lane 5) or Sp1m (lane 8). Recombinant human ER protein alone did not bind [^{32}P]−83 probe (Fig. 4C, lane 8); however, ER enhanced retarded band intensity after coincubation with Sp1 protein/[^{32}P]−83 (lanes 3–5) compared with the band formed with Sp1 protein alone (lane 2). The intensity of the specifically bound retarded band was decreased after competition with unlabeled wild-type −83 probe (lane 6), and band intensity only slightly decreased after competition with −83m probe. The latter result could be attributed to upstream (CCCCCTCG) and downstream (CCCCCACG) motifs that could also bind Sp1 protein. These results are consistent with previous studies using ^{32}P-labeled consensus Sp1 oligonucleotide and GC-rich motifs in the cathepsin D, c-*fos*, and retinoic acid receptor α1 gene promoters (40–44).

Discussion

IGFBPs are widely expressed in mammalian tissues, and their biological actions are highly variable and dependent on cell/tissue type. This family of proteins is characterized by their binding to IGFs; however, there is growing evidence that some IGFBPs modulate IGF-dependent and independent responses. Several studies show that IGFBP-4 functions in many cell types as an inhibitor of IGF action. For example, in osteoblast and colon cancer cells, IGFBP-4 inhibits IGF-mediated proliferation; in CaCo2 colon cancer cells, IGFBP-4 also modulates growth (45) and differentiation (45–49). In contrast, IGFBP-4 mRNA and immunoreactive protein are induced by E_2 in ER-positive MCF-7 cells, and the results of this study (Fig. 1) confirm that IGFBP-4 is E_2 responsive. Qin and co-workers (39) previously analyzed the promoter activity of the IGFBP-4 gene 5′-flanking region in COS-7 monkey kidney cells and three human osteoblast-like osteosarcoma (SaOS-2, TE-85, and MG-63) cell lines. Deletion analysis showed that maximal basal activity in all four cell lines required the −836 to −54 regions of the IGFBP-4 gene promoter, and important elements that may contribute to this activity include putative Sp1, AP-2, and EGR-1 sites (39). Results obtained in MCF-7 cells (Fig. 2) demonstrate that high basal activity is primarily associated with the −373 to −125 region of the IGFBP-4 gene promoter containing a GC-rich Sp1-binding site at −313 to −305 and an AP-2 site centered at −247.

Multiple *cis*-elements have been identified within the 5′-promoter region of the IGFBP-4 gene; these include a TATAA element (−18 to −14), binding sites for ATF/CREB (−1169 to −1161), AP-1 (−855 to −859), early growth response factor-1 (−562 to −554), an Alu repetitive element (−1150 to −848), and numerous GC-rich Sp1-binding sites (−1204 to −1199, −904 to −899, −559 to −553, −313 to −305, and −72 too −64) (38, 39). Classical palindromic estrogen-responsive elements were not detected in the IGFBP-4 gene promoter; however, recent studies show that E_2-induced *trans*-activation can be mediated through ER interactions with DNA-bound AP-1 or Sp1 proteins (40–44, 50). Deletion analysis of the IGFBP-4 gene promoter showed that constructs containing both upstream (p−1214/−379) and downstream (p−373/+18) sequences were E_2 responsive in transient transfection studies in MCF-7 cells (Fig. 2B). Subsequent deletion analysis showed that E_2 responsiveness was primarily associated with the upstream GC-rich sequence at −559 to −553 (p−569/−540). In addition, E_2 induced luciferase activity (2-fold) in MCF-7 cells transfected with p−83/−54, but not −85/−54m (GC mutations), suggesting that the

of mutant −569 oligonucleotide slightly decreased the intensity of the retarded band (lane 5), whereas mutant Sp1m did not affect retarded band intensities. C, ER/Sp1 interactions. [^{32}P]−569 was incubated with human recombinant Sp1 protein (5 ng), alone (lane 2) or in combination with 0.1–0.4 pmol ER (lanes 3–8) as described in *Materials and Methods*. ER alone did not bind to [^{32}P]−569 (lane 8), but enhanced formation of the specifically bound Sp1-DNA retarded band (lanes 3 − 5). Band intensities in lanes 3–5 compared with lane 2 (arbitrarily set at 1.0) were 1.58 ± 0.43, 2.38 ± 0.76, and 3.03 ± 1.0, respectively (for three separate determinations).

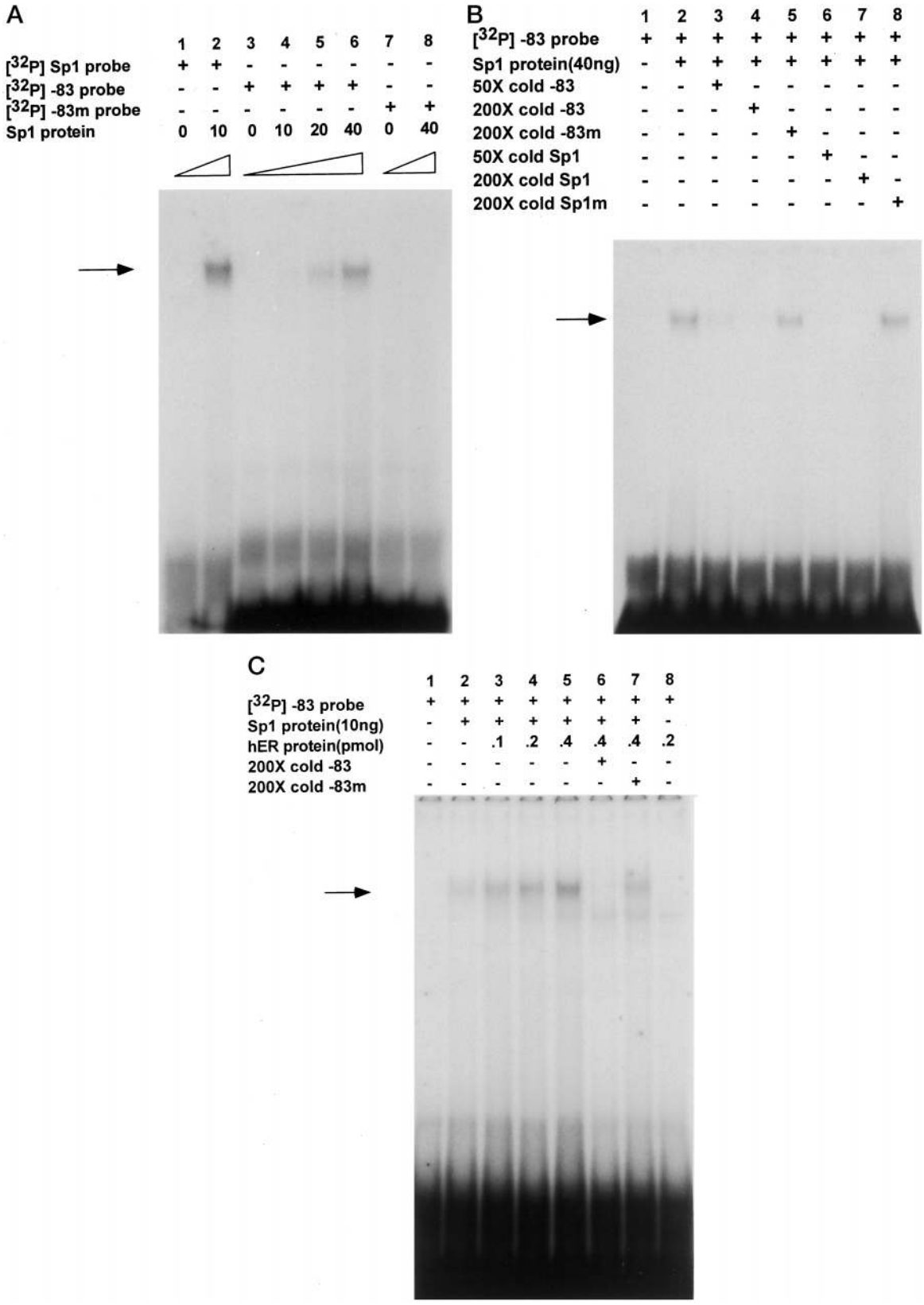

FIG. 4. GEMSAs with −83 oligonucleotide. A, Direct binding of [^{32}P]−83 with recombinant human Sp1 protein. ^{32}P-Labeled consensus Sp1, −83, and mutant −83m oligonucleotides were incubated with pure Sp1 protein (0–40 ng) as described in *Materials and Methods*. A specifically bound retarded band (see *arrow*) was formed with Sp1 (lane 2) and −83 (lanes 5 and 6), but not −83m (lane 8), oligonucleotides. B, Competition for the [^{32}P]−83-Sp1 retarded band. Competition studies were carried out as described in *Materials and Methods*. Incubation of [^{32}P]−53 formed

downstream Sp1-binding site at −72 to −64 was also weakly E_2 responsive.

Results of GEMSAs with [^{32}P]−569 and [^{32}P]−83 (Figs. 3 and 4) demonstrated that both oligonucleotides formed specifically bound Sp1-DNA complexes. Coincubation with ER did not result in a supershifted ER/Sp1-DNA ternary complex, but caused a 2-fold increased intensity of the binary Sp1-DNA retarded band. Previous gel mobility shift studies using ^{32}P-labeled consensus Sp1 oligonucleotide or similar GC-rich sequences from the c-*fos*, retinoic acid receptor α1, adenosine deaminase, and cathepsin D gene promoters also showed that wild-type ER enhanced the rate of Sp1-DNA complex formation (40–44) and increased (2- to 3-fold) the binding capacity for the retarded band; this was comparable to results obtained with [^{32}P]−569 and [^{32}P]−83 (Figs. 3 and 4). Coimmunoprecipitation and pulldown assays using chimeric glutathione-*S*-transferase Sp1 and ER proteins confirmed that ER and Sp1 physically interact, and ER preferentially bound to the C-terminal regions of Sp1 protein (40). The failure of ER to form a supershifted ternary ER/Sp1-DNA complex is not unprecedented, as it has also been reported that other nuclear proteins, including human T cell leukemia virus, type I Tax, sterol regulatory element-binding protein, and cyclin D1, enhanced binding of bZIP, Sp1, and ER to their cognate enhancer elements (51–53).

In summary, the results of this study have identified GC-rich regions in the IGFBP-4 gene promoter that are important for E_2 responsiveness. ER/Sp1-mediated *trans*-activation through GC-rich sites plays an important role in the induction of several genes, including c-*fos*, cathepsin D, and retinoic acid receptor α1 in MCF-7 cells, and this complements ER/Sp1 action through Sp1(N)$_x$ERE half-sites identified in c-*myc*, creatinine kinase B, cathepsin D, and heat shock protein 27 genes (54–57). The importance of the Sp1 protein for transcriptional activation by members of the nuclear receptor superfamily is not confined to the ER, as progesterone receptor-Sp1 complex interactions with GC-rich sites in the p21 gene promoter are required for progesterone-mediated *trans*-activation (58). Current studies in this laboratory are further investigating the integrating role of Sp1 in the cell-specific regulation of several E_2-responsive genes and genes regulated via other nuclear transcription factors.

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a retarded band with Sp1 protein (lane 2). The intensity of the band was decreased after coincubation with excess unlabeled −53 and consensus Sp1 oligonucleotides (lanes 3, 4, 6, and 7), but only minimal change was observed after coincubation with their corresponding mutant oligonucleotides (lanes 5 and 8). C, ER/Sp1 interactions. [^{32}P]−83 was incubated with 10 ng human recombinant Sp1 protein alone (lane 2) or in the presence of 0.1–0.4 pmol ER (lanes 3–8) as described in *Materials and Methods*. ER significantly ($P < 0.05$) enhanced Sp1-DNA complex formation (lanes 3–5), but did not bind [^{32}P]−83 alone. Band intensities in lanes 3–5 compared with that in lane 2 (arbitrarily set at 1.0) were 3.47 ± 0.32, 4.14 ± 0.05, and 4.02 ± 0.22, respectively (for three separate determinations).

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