Transcriptional Activation of the Porcine P450 11A Insulin-like Growth Factor Response Element in MCF-7 Breast Cancer Cells*

Randal J. Urban§§ and Yvonne Bodenburg††

From the §Division of Endocrinology, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas 77555-1060

Insulin-like growth factor-I (IGF-I) stimulates the growth of MCF-7 breast cancer cells. This study determined the transcriptional activity of an IGF-I-responsive region (IGFRE) of porcine P450 11A (P450scct) after transfection into MCF-7 cells. IGF-I induced transcriptional activity of a porcine P450scct core promoter luciferase construct containing the IGFRE transfected in MCF-7 cells. Electrophoretic mobility shift assay with nuclear protein extract from MCF-7 cells showed two transcription factors binding to the IGFRE. Supershift assay determined that one transcription factor was Sp1.

Studies in human breast cancer cells show that insulin-like growth factors (IGFIs) act through cell membrane receptors (1, 2) to stimulate cell growth (3, 4). In MCF-7 cells, estrogen increases insulin-like growth factor-I (IGF-I)-stimulated cell growth by increasing IGF-I receptor concentration (5). However, IGF-I also stimulates breast cancer cell growth in estrogen-unresponsive MDA M231 cells (5). A luteinizing hormone-releasing hormone antagonist inhibits IGF-I-stimulated cell growth in cultures of MCF-7 cells by direct effects on IGF autocrine/paracrine loops (6). The stable expression of IGF-I receptor antisense RNA in MCF-7 cells will inhibit both IGF-I and serum-induced cellular proliferation (7).

While much is known regarding the regulation of IGF-I and its binding proteins (8), the molecular mechanisms through which IGF-I stimulates gene transcription are unknown. IGF-I-responsive regions (IGFREs) have been identified in the chicken β1-crystallin gene (9, 10) and rat elastin gene (11–13). These regions are GC-rich domains that bind the ubiquitous transcription factor, Sp1 (10, 13). We identified a GC-rich, 30-base pair IGFRE in the porcine P450 cholesterol side-chain cleavage gene, P450 11A (P450scct) (14).

In this study, we determined that the porcine P450scct IGFRE could induce IGF-I-stimulated gene expression in MCF-7 cells. Moreover, we showed that Sp1 and another unknown transcription factor, P2, bound to the IGFRE and mediated IGF-I-induced gene expression.

EXPERIMENTAL PROCEDURES

Materials—All restriction enzymes were obtained from Life Technologies, Inc. Radioactive nucleotides were obtained from Amersham. Pure human recombinant IGF-I was obtained from Bachem Inc. (Torrance, CA).

Reporters Gene Constructs—The luciferase reporter gene constructs containing the porcine P450scct core promoter and IGFRE have been previously described (14). Briefly, the –2320 construct contains the entire sequence upstream from the IGFRE (see Table I). The –100 construct contains the porcine core promoter without the IGFRE (14).

The oligonucleotides mWT and mMI-mM7 represent the porcine IGFRE and mutations to the GC box of the IGFRE (see Table I). Sense and antisense oligonucleotides were synthesized with BovHII and SuI sites for directional cloning into pSVPLUC. The plasmid pSVPLUC is a modified pGEM3 plasmid containing the luciferase gene and the enhancerless SV40 early region promoter (15). The plasmid was obtained from Dr. Allan Brasier, University of Texas Medical Branch, Galveston, TX. Annealed oligonucleotides were used in electrophoretic mobility shift assays (EMSA) and selected mutants (mWT, mM1, mM2, and mM6) were cloned into pSVPLUC for transfection experiments in MCF-7 cells. All constructs were verified by sequencing.

Transient Transfection in MCF-7 Cells—MCF-7 cells were cultured in Earle’s minimum essential medium + 10% fetal bovine serum with 1 ml of penicillin/streptomycin/100 ml of medium. Transient transfection was done by lipofection (Tfx-50 Reagent, Promega, Madison, WI). Transfection experiments were done on 60-mm plates as per the Promega protocol for Tfx-50 Reagent. A 3 to 1 ratio (1 μg of DNA/2.5 μl of Tfx-50) was used for each transfection. The control plasmid pSV2Apap containing the SV40 early promoter, enhancer region, and the human placental alkaline phosphatase gene; Ref. 16) was cotransfected with the chimeric construct of interest. After transfection, cells were maintained in 2.5% fetal bovine serum. Cells were harvested and measured for luminescence as described previously (14). The remaining lysate was measured for alkaline phosphatase activity using p-nitrophenyl phosphate (Sigma) and measuring absorbance at 405 nm.

EMSA and Supershift Assays—Oligonucleotides were 32P-labeled by polynucleotide kinase and [γ-32P]ATP (4500 Ci/mmol). Nuclear extract protein from MCF-7 cells (10 μg), 5 × gel shift binding solution (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5), 0.5 μg of poly(dl-dC-poly(dl-dC)-dl) and 32P-labeled oligonucleotide (50,000 cpm) were incubated at room temperature for 15 min for shift assays. For supershift assays, 2 μl of Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated at room temperature for 15 min with the nuclear extract protein, 5 × gel shift binding solution, and labeled oligonucleotide. The resulting complex was added for an additional 15 min at room temperature. The protein-DNA complexes were separated on a 4% polyacrylamide gel by electrophoresis in 0.5 × TBE buffer (1 mM Tris-HCl, 1 mM boric acid, 20 mM EDTA) and visualized by autoradiography.
**IGF-I Transcriptional Activity in MCF-7 Cells**

**RESULTS**

**IGF-I-stimulated Gene Expression in MCF-7 Cells**—The –2320 porcine P450sec construct, containing the porcine IGFRE, showed a significant increase in luciferase activity when transfected into MCF-7 cells with a control plasmid (pSV2Apap) and treated with IGF-I (Fig. 1). The porcine P450sec core promoter alone (–100) was not stimulated with IGF-I treatment (Fig. 1), nor was the pOLUC (promoterless luciferase) plasmid alone (data not shown). The activity of the porcine P450scc core promoter was minimal, but detectable in MCF-7 cells.

**MCF-7 Transcription Factors Binding to the Porcine IGFRE**—EMSA with MCF-7 nuclear extract protein and an oligonucleotide of the porcine IGFRE identified two transcription factors binding to the IGFRE (Fig. 2). The binding of both factors was competitively inhibited by 100 × unlabeled IGFRE oligonucleotide, but 100 × unlabeled consensus Sp1 oligonucleotide (Santa Cruz Biotechnology) only inhibited binding of the top band (Fig. 2). To further show that Sp1 was binding to the IGFRE, supershift assays were done with an Sp1 antibody that recognizes both isoforms of Sp1 (Santa Cruz Biotechnology). The top band was supershifted with Sp1 antibody, although not completely (Fig. 3). The lower, unknown band was designated as P2.

**Effects of IGF-I on Transcription Factor Binding Activity to the IGFRE**—To determine whether IGF-I treatment affected the binding activity of Sp1 and P2 to the IGFRE, MCF-7 nuclear extract protein was collected on cells treated for 48 h with IGF-I (20 nM). EMSA found the binding activity of Sp1 and P2 are summarized in Table I.

**Determination of the Transcription Factors Mediating IGF-I-stimulated Gene Expression**—A series of mutations were made to the porcine IGFRE in the form of synthetic oligonucleotides to create mutants that bound one transcription factor and not the other. These mutations were made only to the GC box of the IGFRE and were more selective than mutations reported previously (14). In all, seven different mutants were tested in EMSA. The mutants and their binding activity for Sp1 and P2 are summarized in Table I.

**Because of the low activity of the porcine P450sec core promoter in MCF-7 cells, constructs of selected mutants of the IGFRE were made in pSVPLUC. The constructs were mM1 (no binding of either transcription factor), mM2 (binds Sp1 only), and mM6 (binds P2 only). The EMSA results for the selected mutants are shown in Fig. 5 (mWT and mM2) and Fig. 6 (mWT, mM1, and mM6). These mutant constructs were transfected
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**TABLE I**

| Mutation | Sequence | Binding activity |
|----------|----------|------------------|
| mWT     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | +Sp1, +P2 |
| mM1     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | −Sp1, −P2 |
| mM2     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | +Sp1, −P2 |
| mM3     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | −Sp1, −P2 |
| mM4     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | +Sp1, | P2 |
| mM5     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | +Sp1, +P2 |
| mM6     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | +Sp1, +P2 |
| mM7     | ATCCTGAGTCGAGAGGCTTTGTTGCCC | +Sp1, +P2 |

The GC box is underlined for each oligonucleotide. + indicates normal binding, − indicates loss of binding, \( \downarrow \) indicates reduced binding.

**FIG. 5.** EMSA of a porcine IGFRE mutant that binds Sp1 alone. Autoradiogram of nuclear extract protein from MCF-7 cells incubated with \(^{32}\)P-labeled mWT (porcine IGFRE, lanes A–C) and mM2 (mutant that binds only Sp1, lanes D–F). Lanes A and D are probe alone, lanes B and E are the respective oligonucleotide and protein, and lanes C and F are oligonucleotide, protein, and Sp1 antibody.

**FIG. 6.** EMSA of a porcine IGFRE mutant that binds P2 alone. Autoradiogram of nuclear extract protein from MCF-7 cells incubated with \(^{32}\)P-labeled mWT (porcine IGFRE, lane A), mM6 (mutant that binds only P2, lanes B and C), and mM1 (mutant that binds neither protein, lanes D and E). Lanes B and D are free probe, while lanes C and F are oligonucleotide and protein.

**FIG. 7.** Transfection of mutants in MCF-7 cells and their response to IGF-I. Constructs containing selective mutations to the IGFRE were transfected in MCF-7 cells and treated for 48 h with IGF-I (20 nm). The mutations are described in Fig. 5 and 6 and Table I. Transient transfection experiments were done as described in Fig. 1. The data represent six replicates. The asterisks indicate statistical significance as determined by paired t test.

**DISCUSSION**

With this study, we have shown that Sp1 and another unknown transcription factor, P2, bind to the porcine IGFRE in MCF-7 cells. The binding of both transcription factors is necessary for basal and IGF-I-stimulated transcriptional activity of the porcine IGFRE in MCF-7 cells.

IGF-I-stimulated gene expression is mediated by transcription factors that bind to GC-rich domains of genes. The IGFREs identified contain only one GC box and are located approximately 100–150 base pairs from a TATA box (9, 13, 14). This promoter arrangement differs from other promoters that contain multiple GC boxes and often lack the TATA box sequence (19). As expected, the GC boxes found in the IGFREs bind Sp1 (9, 13). Studies in the promoter of chicken \( \beta \)-crystallin gene identified two transcription factors binding to a GC-rich domain approximately 100–150 base pairs from a TATA box (9, 13, 14). This GC box is underlined for each oligonucleotide.

The GC box identified contains two transcription factors binding to a GC-rich domain of genes. The IGFREs found in breast cancer cell growth. Breast cancer cell initial development, growth, and later metastases involve the IGF-I system (4). The expression of IGF-I in breast cancers is of independent prognostic significance and relates to the malignant histopathological features of the breast cancer cells (20). The beneficial effect of tamoxifen on the treatment of breast cancer is linked to its lowering of plasma concentrations of IGF-I (21). Stable expression of IGF-I receptor antisense RNA in MCF-7 breast cancer cells will reduce IGF-I and serum-stimulated cellular prolifer-
ication (7). Any therapy that reduces the expression of Sp1 in breast cancer cells would likely have adverse effects on normal cells and tissues because the transcription factor is ubiquitous and involved in the regulation of many genes. However, there is the potential for more cell-specific actions on breast cancer cell growth with the other transcription factor, P2. The extent of this factor’s expression and its importance in normal cell function can only be assessed once the factor has been identified. Therefore, isolation and identification of P2 could result in a therapy that prevents the growth and metastasis of certain breast cancers.

In summary, we determined that MCF-7 cells contain transcription factors (Sp1, P2) that mediate IGF-I-stimulated gene expression of a known IGFRE (porcine 450scc). Further characterization of the P2 transcription factor may result in a therapy that prevents IGF-I-mediated breast cancer cell growth.

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