Differential Modulation of Transcriptional Activity of Estrogen Receptors by Direct Protein-Protein Interactions with the T Cell Factor Family of Transcription Factors*

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Received for publication, May 3, 2001, and in revised form, August 16, 2001
Published, JBC Papers in Press, August 24, 2001, DOI 10.1074/jbc.M103966200

Two major signaling pathways, those triggered by estrogen (E2) and by the Wnt family, interact in the breast to cause growth and differentiation. The estrogen receptors ERα and ERβ are activated by binding E2 and act as ligand-dependent transcription factors. The effector for the Wnt family is the Tcf family of transcription factors. Both sets of transcription factors recognize discrete but different nucleotide sequences in the promoters of their target genes. By using transient transfections of reporter constructs for the osteopontin and thymidine kinase promoters in rat mammary cells, we show that Tcf-4 antagonizes and Tcf-1 stimulates the effects of activated ER/ERE. For mutants of the promoter, the stimulatory effects of ERα/E2 can be made to depend on Tcf-1, and for the latter promoter the effects of the T cell factors (TCFs) are dependent on ER/E2. Direct interaction between ERs and Tcfs either at the Tcf/ERE binding site on the DNA or in the absence of DNA is established by gel retardation assays or by coinmunoprecipitation/biosensor methods, respectively. These results show that the two sets of transcription factors can interact directly, the interaction between ERs and Tcf-4 being antagonistic and that between ERs and Tcf-1 being synergistic on the activity of the promoters employed. Since Tcf-4 is the major Tcf family member in the breast, it is suggested that the antagonistic interaction is normally dominant in vivo in this tissue.

The mammary gland grows and develops in response both to systemic hormones, which circulate in the body, and to locally produced signaling molecules in the mammary gland itself (1). An example of the former is estrogen (E2), which interacts with the estrogen receptors ERα or ERβ to produce activated transcription factors (2, 3), and an example of the latter is the Wnt family of proteins, the effector of which is the transcription factor family of T cell factors (TCFs) (4, 5). Activated ERα and ERβ receptors recognize specific DNA response elements (ERE), DNA sequences located within the regulatory regions of target genes (6), whereas the Tcf family of transcription factors recognize a different set of consensus sequences typified by the (A/T)(A/T)CAAAG sequence for Tcf-1 in lymphocytes (7). However, it is unknown if these two sets of transcription factors can interact with each other to modify the transcriptional output from target genes.

To investigate if the E2 and Wnt signaling pathways in mammary development can interact at the level of their effector transcription factors, we have chosen the target gene of osteopontin (OPN) and its cognate promoter for such a study. OPN, despite its occurrence in bone, is thought to play a key role in mammary development, since it is specifically overexpressed during pregnancy and lactation (8), and targeted inhibition of its expression causes suppression of lobular alveolar structures and lactational deficiencies in transgenic mice and cultured mammary epithelial cells (9). Moreover, OPN is secreted by epithelial cells of mammary origin in response to interleukin-6 activation of ER (10) and can also be produced by sequestration of Tcf-4 (11) by CAAAG-containing DNA fragments in the cell line rat mammary 37 (Rama 37) (12). The 2.3-kbp promoter for the rat OPN contains two half-ERE or SF1 response elements (SFREs) (13, 14), which are activable as in the mouse (15), and also three Tcf recognition sequences (16), which act to suppress its activity in the Rama 37 cells in culture (17). We now use transient transfections into the Rama 37 cell line of reporter constructs for the OPN promoter to investigate the effects of interaction of the ERs and Tcfs at a promoter that contains sites for both sets of transcription factors.

EXPERIMENTAL PROCEDURES

Cloned DNAs and Oligonucleotides—The full-length human ERα (HEGO) (18) and ERβ (19) within the expression vector pSG5 were obtained from Professor P. Chambon, University of Strasbourg, France, and Dr. S. Ali, Imperial College, London, UK, respectively. Expression vectors containing ERα deletion mutants HEG19 (amino acids 179–595), in pSG5 with activation function-2 (AF-2) at the carboxy-terminal region intact for both the hormone- and DNA-binding domains (18), and HE15 (amino acids 1–282), in pKCR2 with activation function-1 (AF-1) at the amino-terminal region intact for only the DNA-binding domain (21), were gifts from Professor P. Chambon, as described previously (22). The expression vector containing the ERα gene with the transcriptional inactivating point mutation at position 540 (L540Q) (23) was generated with the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) using CGT GGT GCC CCT CTA TGA CCA GCT GCT GGA GAT GCT GGA CGC CC and GGG CGT CCA GCA TCT CCA

* This work was supported by grants from the Cancer and Polio Research Fund and the North West Cancer Research Fund, UK. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: E2, estradiol; AF-1, activation function-1; AP-2, activation function-2; bp, base pair; CAT, chloramphenicol acetyltransferase; ER, estrogen receptor; ERE, estrogen response element; ERα.TKI, thymidine kinase promoter construct; 2ERE.TATA, minimal estrogen response promoter; ICI, ICI, 164384; mAb, monoclonal antibody; OPN, osteopontin; OPNS-M, mutated in first SFRE; OPNS-M, mutated in second SFRE; PAGA, protein AgAl-agarose; pM12-DNA, 20-bp oligonucleotide with Tcf site; Rama 37, rat mammary 37; SFRE, SF1 response element; SFRE-DNA, 29-bp oligonucleotide with SFRE site; Tcf, T cell factor; TK, thymidine kinase; kbp, kilobase pair.
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GCA GCT GTG CAT AGA GGG GCA CCA CG as forward and reverse primers, respectively. 2ERE.TATA is an entirely synthetic enhancer/promoter sequence (24), and ERE.TK (pERE.BCLAT) consists of a consensus ERE linked to the promoter sequence (nucleotides +105 to +115) of the Herpes Simplex Virus thymidine kinase gene (25). All have been cloned into a modified version of the pCI mammalian expression vector, which was used and characterized previously (22). The synthetic double-stranded oligonucleotides pM12-DNA and the CAAAG-deleted pM12-DNA consisted of the following sequences: AAC CAG GCA AAG AGT TGG TTC GC and AAC CAG GGT GTT GGC, respectively (12, 22). Firefly luciferase activity was normalized to β-galactosidase activities; maximum activity was reached by 48 h, and results at 72 h were used. Firefly-luciferase reporter constructs used here. The 2.3-kbp fragment of the 6-kbp rat OPN promoter (16) from Dr. A. Gel Shift Assays—These were performed (7) by incubating 0.5 ng of 32P-pM12-DNA, pM12-D/D-deleted DNA or SFRE-DNA (specific activity ~5 × 106 cpm/μg) with 4 μl of protein extract from a reticulocyte cell-free transcription-translation protein synthesizing lysate pre-treated with 10−M E2 for 1 h at 20°C (12, 22), in 30 μl 0.03 M KCl, 1 mM Na2HPO4, 0.01 M HEPEs (pH 7.9), 0.25 mM dithiothreitol, 10% (w/v) glycerol, 1.4 μg of poly(dI-dC) (Amersham Pharmacia Biotech), 1 μg of single-stranded salmon sperm DNA, 0.01% (w/v) SDS for 40 min at 0°C. Where necessary mouse monoclonal antibody (mAb) to ERα or related mutant promoter firefly-luciferase reporter construct (DLR, Promega) was fixed for 30 min in 10% (v/v) propanol, 10% (v/v) acetic acid, dried for 45 min with equal amounts of protein being loaded per lane. The gel was impregnated with 0.5% (w/v) bromphenol blue, 0.5% (w/v) ponceau S, 0.5% (w/v) Coomassie Brilliant Blue, UK). Biotinylated Tcfs were captured directly onto streptavidin-coated planar aminosilane surfaces (Affinity Sensors, Saxon Hill, Cambridge, UK). Biotinylated Tcfs were generated in a coupled transcription-translation cell-free protein-synthesizing reticulocyte lysate with [35S]methionine and the requisite expression vectors and immunoprecipitated with mAbs to the different transcription factors. For immunoprecipitation in vitro, potential complexes between [35S]methionine-labeled Tcf or control c-Fos products and nonradioactive ERs, usually pre-bound to immobilized synthetic double-stranded SFRE oligonucleotides, were generated with the expression vectors for the ERs, for Tcf-4, for Tcf-1 or with buffer alone in a cell-free protein-synthesizing transcription-translation system to generate the relevant protein as described previously (12, 22). The mAbs to human ERα and ERβ (15) were obtained from Santa Cruz Biotechnology.

Tcf-4 and Tcf-1 cDNAs were obtained from Prof H. Clevers, University of Utrecht, Holland (5), and were separately cloned into the pBK-CMV expression vector (Stratagene) yielding a combination of the above. Tcf-4 and Tcf-1 cDNAs were generated from the Tcf-4 and Tcf-1 expression plasmids using the QuikChange Site-directed Mutagenesis kit (Stratagene) using for the mutated SFRE at position 1551 (OPNS1M) the sequence TGG CTG GTC AGA CAC ACC ACG AGG TTA ATG ATG AGG TTC GTG TCT CTA GAG CTC AGT GGA GGC ACG AGA AT and for the mutated SFRE at position 1579 (OPNS2M) the sequence TGG CTG GTC AGA CAC ACC ACG AGG TTA ATG ATG AGG TTC GTG TCT CTA GAG CTC AGT GGA GGC ACG AGA AT as forward and reverse primers, respectively, and were then ligated into the pBluescript SKII+ (Stratagene) vector of the appropriate, to the expression vectors after a further 24 h (28). Cells were incubated for a further 24 h and harvested in 300 μl of Reporter Lysis Buffer (Promega), and either firefly luciferase and control Renilla luciferase were assayed as described in the Dual Luciferase Reporter Assay System (Promega), or CAT and control β-galactosidase activities were assayed as described previously in 100- and 150-μl aliquots, respectively (12, 22). Firefly luciferase activity was normalized to Renilla luciferase activity, and CAT activity was normalized to β-galactosidase activity; maximum activity was reached by 48 h, and results at 72 h were used. Coimmunoprecipitation and coimmunoprecipitation-CAT reporter constructs (17) gave the same results as the OPN promoter firefly-luciferase reporter constructs used here.

Cell Lines and Transient Transfections—Rama 37 cell line (27) was cultured in Dulbecco’s modified Eagle’s medium, 10% (v/v) fetal calf serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 14.000 μM NaCl, 0.2 mM Na2VO4, 0.5% (w/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 25 μg/ml pepstatin) was added to each sample, mixed, and incubated on ice for 30 min. Twenty μl of mAb A/G-agarose (PAGA, pre-washed three times in immunoprecipitation buffer, were added to each sample and incubated for an additional 4 h at 4°C with rotation to remove any proteins that had interacted nonspecifically with PAGA. PAGA was removed by centrifugation at 14,000 rpm for 3 min in a benchtop centrifuge. The supernatant was incubated with 2.5 μg of the requisite antibody (ERα, ERβ, Tcf-1, or Tcf-4 antibody) overnight at 4°C with rotation. Twenty μl of PAGA were added to each sample and incubated at 4°C for an additional 60 min. PAGA antibody conjugates were washed with immunoprecipitation buffer by centrifugation at 14,000 rpm for 3 min, resuspended in 1 ml of Buffer A (phosphate-buffered saline, 0.2% (w/v) Triton X-100, 350 mM NaCl, and centrifuged. Samples were resuspended in 1 ml of Buffer B (phosphate-buffered saline and 0.2% (w/v) Triton X-100), centrifuged, and resuspended in SDS sample buffer. Samples were resolved by electrophoresis on 10% (w/v) polyacrylamide gels at 200 V for 45 min with equal amounts of protein being loaded per lane. The gel was stained in Coomassie Brilliant Blue, UK). Biotinylated Tcfs were generated in a coupled transcription-translation system as described previously (12, 22). The mAbs to human ERα and ERβ (15) were obtained from Santa Cruz Biotechnology.

Production and Analysis of Biotinylated Products—The biotinylated Tcfs and estrogen receptors were produced by adding 1 μl of Transcend tRNA kit (Promega) containing biotinylated e-amino lysyl-tRNA to 50 μl of the same TNT transcription-translation coupled system as above primed by the expression vectors for Tcf-4 or Tcf-1, incubated at 30°C, and then cooled on ice for 60 min (29). For analysis of biotinylated products, 2-μl samples of the reactants were immunoprecipitated by mAbs to Tcf-4 or Tcf-1, electrophoresed through SDS-polyacrylamide gels as above, and the separated proteins transferred by blotting onto Immobilon membranes (Millipore Corp., Watford, UK) (30). The membranes were incubated with blocking buffer containing 0.02% (w/v) Tris-HCl (pH 7.0), 0.02% (w/v) NaCl, 0.5% (w/v) Tween 20 (w/v) Triton X-100, 350 mM NaCl, and centrifuged. Samples were resuspended in 1 ml of Buffer B (phosphate-buffered saline and 0.2% (w/v) Triton X-100), centrifuged, and resuspended in SDS sample buffer. Samples were resolved by electrophoresis on 10% (w/v) polyacrylamide gels at 200 V for 45 min with equal amounts of protein being loaded per lane. The gel was stained in Coomassie Brilliant Blue, UK). Biotinylated Tcfs were generated in a coupled transcription-translation system as described previously (12, 22). The mAbs to human ERα and ERβ (15) were obtained from Santa Cruz Biotechnology.

Complex Detection by Direct Binding—Binding reactions were carried out in an Iasys two channel resonant mirror biosensor at 20°C on planar aminosilane surfaces (Affinity Sensors, Saxon Hill, Cambridge, UK). Biotinylated Tcfs were captured directly onto streptavidin-derivatized aminosilane surfaces from 20 μl of the above protein-synthesizing reticulocyte lysates. Alternatively, recombinant human ERα was captured on the aminosilane surface by cross-linking with
Rama 37 cells were transiently cotransfected with the luciferase promoter reporter gene coupled downstream from the OPN promoter or with the CAT reporter gene coupled downstream from the ERE.TK or downstream of the 2ERE.TATA together with optimal concentrations of expression vectors for ER<sub>E2</sub>, ER<sub>E2</sub>, Tcf-4, Tcf-1, and/or pM12-DNA. Transfections were also conducted without or with estradiol (E<sub>2</sub>) and without or with ICI in the culture medium. The mean ± S.D. of the promoter activity of the promoter-reporter constructs with various additions relative to the promoter-reporter constructs alone for three separate experiments is shown for the optimum inputs of the different additives.

### Table I

| Additions | Relative promoter activity ± S.D. |
|-----------|----------------------------------|
|           | OPN | ERE.TK | 2ERE.TATA |
| ER<sub>E2</sub> | 1.0 | 1.0 | 1.0 |
| ER<sub>E2</sub> | 1.2 ± 0.2 | 1.2 ± 0.3 | 1.2 ± 0.3 |
| E<sub>2</sub> | 6.5 ± 0.8 | 9.9 ± 0.1 | 5.9 ± 0.2 |
| ICI | 0.8 ± 0.3 | 1.1 ± 0.2 | 0.6 ± 0.3 |
| Tcf-4 | 0.5 ± 0.2 | 1.3 ± 0.2 | 1.0 ± 0.2 |
| pM12 | 0.6 ± 0.1 | 1.0 ± 0.2 | 1.2 ± 0.3 |
| Tcf-1 | 2.7 ± 0.2 | 1.2 ± 0.3 | 1.1 ± 0.1 |
| pM12 | 2.6 ± 0.2 | 0.9 ± 0.4 | 0.5 ± 0.2 |
| Tcf-1 | 9.6 ± 0.4 | 12.6 ± 0.2 | 8.6 ± 0.3 |
| Tcf-4 | 2.7 ± 0.1 | 1.2 ± 0.3 | 0.6 ± 0.2 |
| pM12 | 4.4 ± 0.3 | 0.9 ± 0.2 | 0.8 ± 0.4 |
| Tcf-4 | 4.6 ± 0.4 | 1.0 ± 0.2 | 0.9 ± 0.3 |
| Tcf-1 | 13.5 ± 1.0 | 13.1 ± 0.5 | 8.4 ± 0.5 |
| Tcf-1 | 4.2 ± 0.5 | 0.8 ± 0.4 | 0.7 ± 0.2 |
| E<sub>2</sub> | 1.1 ± 0.2 | 1.1 ± 0.2 | 1.1 ± 0.3 |
| ER<sub>E2</sub> | 1.4 ± 0.3 | 5.4 ± 0.1 | 0.9 ± 0.2 |
| ER<sub>E2</sub> | 0.6 ± 0.2 | 1.1 ± 0.3 | 1.1 ± 0.2 |
| pM12 | 2.8 ± 0.4 | 8.9 ± 0.2 | 1.2 ± 0.3 |
| ER<sub>E2</sub> | 4.8 ± 0.6 | 9.8 ± 0.6 | 1.4 ± 0.5 |

The stimulation achieved by the expression vector for ER<sub>E2</sub>, either alone or in combination with other additves, was abolished completely when the anti-estrogen ICI 164384 (ICI) was present in the culture medium, but ICI was without effect on the stimulation achieved by vectors containing pM12-DNA or Tcf-1 cDNA (Table I). The lack of effect on the OPN promoter-reporter construct of ICI in the presence of pM12-DNA indicates that the increase produced by pM12-DNA is not due to ER activity. In contrast the expression vector for ER<sub>E2</sub> failed to stimulate significantly the OPN promoter-reporter construct alone or enhanced its activity with vectors containing pM12-DNA or Tcf-1 cDNA (p ≥ 0.3). These results suggest that Tcf-4 and Tcf-1 alter the activity of the OPN promoter-reporter construct at a site different to that for ER<sub>E2</sub>, which in turn is not recognized by ER<sub>E2</sub>.

**Effect of ERs/Tcfs on the ERE Promoter-Reporter Constructs**

Transient transfection of reporter CAT constructs containing only sequences recognized by ER<sub>E2</sub> and not the Tcf CAAAG core recognition sequences gave different results. Thus the activity of the TK promoter-reporter construct, which contained a consensus estrogen responsive element (ERE.TK), and the minimal estrogen-responsive promoter-reporter construct (2ERE.TATA) were not altered significantly in Rama 37 cells by cotransfection of optimal amounts of Tcf-1, Tcf-4 expression vectors, nor by pM12-DNA (p ≥ 0.3). However, cotransfection of Rama 37 cells with optimal amounts of Tcf-1, Tcf-4 expression vectors, or of pM12-DNA in the presence of ER<sub>E2</sub> significantly altered the activities of these two promoter-reporter constructs over those achieved by expression vectors for ER<sub>E2</sub> alone (p ≥ 0.01). Vectors for Tcf-1 and pM12-DNA increased these promoter-reporter activities by 1.3–1.5-fold and for Tcf-4 suppressed their activities by 5–10-fold (Table I). The stimulated activities of the two ERE promoter-reporter constructs produced by cotransfections of expression vectors for ER<sub>E2</sub> and for Tcf-1, ER<sub>E2</sub> and pM12-DNA were this time completely abolished by ICI in the culture medium (p ≥ 0.7). Once again the stimulations achieved by cotransfection of Rama 37 cells with pM12-DNA were lost when it was replaced by the CAAAG-deleted pM12/D-DNA (not shown). The results for the activities of the ERE.TK promoter-reporter construct were similar for cotransfection of Rama 37 cells with pM12-DNA.
TABLE II

Specificity of ERα/Tcf-1 in activation of the OPN promoter

Rama 37 cells were transiently cotransfected with the luciferase reporter gene coupled downstream from the OPN promoter or one of its mutated forms with one (OPNS,M or OPNS,M) or both (OPNS,M or its SFREs missing and optimal concentrations of expression vectors for ERα, L540Q mutant of ERα (ERα,M), HE15 an ERα mutant containing only AF-1, HEG19 an ERα mutant containing only AF-2, c-Fos, and/or Tcf-1. Transfections were also conducted without or with E2 in the culture medium.

The mean ± S.D. of the activity of the OPN promoter-reporter constructs with various additions relative to that for the OPN promoter-reporter construct alone for 2 or 4° separate experiments is shown for the optimum inputs for the different additives.

| Promoter | ER  | E2 | c-Fos | Tcf-1 | Relative promoter activity ± S.D. |
|----------|-----|----|-------|------|----------------------------------|
| OPN      | −   | −  | −     | −    | 1.0                              |
| OPN      | ERα | −  | −     | −    | 1.2 ± 0.3                        |
| OPN      | ERα | −  | −     | −    | 6.9 ± 0.5                        |
| OPN      | AF-2| +  | −     | −    | 7.5 ± 0.4                        |
| OPN      | ERα,M| + | −     | −    | 1.7 ± 0.2                        |
| OPN      | AF-1| +  | −     | −    | 1.5 ± 0.3                        |
| OPN      | ERα | −  | −     | −    | 1.3 ± 0.1                        |
| OPN      | ERα | −  | −     | +    | 1.7 ± 0.4                        |
| OPN      | ERα | +  | −     | −    | 7.7 ± 0.5                        |
| OPN      | −   | −  | −     | +    | 5.0 ± 0.4a                       |
| OPN      | −   | −  | +     | +    | 4.7 ± 0.5                        |
| OPN      | −   | −  | +     | −    | 4.7 ± 0.2                        |
| OPN      | ERα | −  | +     | −    | 18.9 ± 0.2                       |
| OPNS,M   | ERα | +  | −     | −    | 2.7 ± 0.3                        |
| OPNS,M   | ERα | +  | −     | +    | 5.1 ± 0.4                        |
| OPNS,M   | ERα | +  | −     | +    | 1.4 ± 0.2                        |
| OPNS,M   | ERα | +  | −     | +    | 4.9 ± 0.3                        |
| OPNS,M   | ERα | +  | −     | +    | 6.0 ± 0.5a                       |
| OPNS,M   | ERα | +  | −     | +    | 12.4 ± 0.5a                      |
| OPNS,M   | AF-2| −  | −     | −    | 5.9 ± 0.3                        |
| OPNS,M   | AF-2| −  | −     | +    | 11.4 ± 0.6                       |
| OPNS,M   | ERα,M| − | −     | +    | 5.3 ± 0.4                        |
| OPNS,M   | AF-1| +  | −     | +    | 5.7 ± 0.3                        |

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cells with the ERα expression vector, and the stimulation achieved by ERα/E2 was altered in the same way by cotransfection with Tcf-1, Tcf-4 expression vectors or with pM12-DNA (stimulation 1.6–1.8-fold, inhibition 5-fold), and the stimulated activities were abolished completely by ICI (not shown). The expression vector for ERα/E2, however, was unable to stimulate the activity of the 2ERE.TATA promoter-reporter construct under any conditions (Table I). These results show that the effects with Tcf-1, Tcf-4, and pM12-DNA on promoters containing only ERs are totally dependent on the presence of the ER and E2 and suggest that the relevant Tcfs may bind to the ERs themselves for transactivation.

Specificity of the Effect of ERα/Tcf-1 on the OPN Promoter-Reporter Construct—The estrogen receptor consists of several functional domains including the ligand-binding site and the DNA-binding site (18, 21). However, only the expression vector HEG19 with both functional binding domains, AF-2 with E2, increased the activity of the OPN promoter-luciferase reporter construct by about the same 6–7-fold value as the expression vector for the complete ERα, with E2 (Student’s t test, p = 0.32). Expression vectors for the transactivation inactive L540Q mutant and for HE15 with a functional DNA binding domain without an active hormone-binding site AF-1 were without significant effect (p = 0.24). The expression vector for a transcription factor without a recognition site in the OPN promoter (16), c-Fos (26), was also included in the transient transfection assays. By itself the expression vector for c-Fos was without effect, and it did not modify the stimulatory activity of the OPN promoter-reporter construct produced separately by the expression vector for ERα/E2 nor by the expression vector for Tcf-1 (Student’s t test, p ≥ 0.25) (Table II). The enhanced 2.5–3.5-fold stimulation of the OPN promoter-reporter construct produced by simultaneous addition of expression vectors for ERα/E2 and Tcf-1 over those obtained by each expression vector alone was lost completely without E2 (p = 0.25), showing the dependence for this stimulatory effect on the presence of the ligand E2.

The rat OPN promoter (16) contains two possible estrogen control sites in the form of two potential SF1 response elements (SF1REs) (13, 14). When transfected with the expression vector for ERα/E2 in Rama 37 cells, the OPN promoter-reporter constructs mutated in the first (OPNS,M or second (OPNS,M) SFRE produced significant 61% (p = 0.010) or near significant 26% (p = 0.058) decreases in activity, respectively, compared with the nonmutated promoter-reporter construct. The activities of these two promoter-reporter constructs with the expression vector for ERα/E2 were also significantly different from one another (p = 0.026), and that for the double mutant with no active SF1REs (OPNS,M) was not significantly different from control (p = 0.51) (Table II). As anticipated, this doubly mutated OPN operator-reporter construct was still stimulable to the same degree as the wild-type construct by the expression vector for Tcf-1 (p = 0.57). When transfected simultaneously with the expression vectors for ERα/E2 and for Tcf-1, the activity of the doubly mutated OPN promoter-reporter construct was still increased by 2.5-fold over that with the Tcf-1 expression vector alone (Table II). This increase in activity was not significant without the E2 ligand (p = 0.12). Moreover, when expression vectors for the different domains of ERα and for Tcf-1 were transfected with the doubly mutated OPN promoter-reporter construct, only the expression vector the AF-2 domain produced a similar 2.3-fold stimulation over that with the expression vector for Tcf-1 alone (p = 0.21), the expression vectors for the point mutated ERα and for AF-1 were without significant effect over the control (p ≥ 0.12) (Table II). This stimulation of the doubly mutated OPN promoter-reporter construct by the expression vector for AF-2 in the presence of Tcf-1 was also dependent on E2 (p = 0.007) and was also not significant without the E2 ligand (p = 0.13) (Table II). These results suggest that ERα can interact with Tcf-1 through its AF-2 domain in an E2-dependent manner to activate the OPN promoter-reporter construct at its Tcf recognition sites.

Effect of ERα/Tcfs on Gel Mobilities of CAAAG- and SFRE-containing DNA—To test whether ERs could interact with the Tcfs when the latter were bound to the promoter region through their CAAAG core recognition sequence, the 32P-labeled 20-bp double-stranded oligonucleotides 32P-pM12-DNA was incubated with reticulocyte lysates in which ERα/E2 and/or Tcf-4 was expressed from the appropriate vectors in a coupled transcription-translation system. Without the expression vectors the reticulocyte lysate failed to retard the electrophoretic mobility of pM12-DNA on polyacrylamide gels (Fig. 1A). When the Tcf-4 expression vector was included in the protein-synthesizing lysate, a single retarded band for pM12-DNA was produced (Fig. 1A, lanes 1 and 8). When both Tcf-4 and ERα/E2 expression vectors were included in two separate lysates, a further reduction in the mobility of pM12-DNA was observed, and this component now split into two bands (Fig. 1A, lanes 2 and 9). When the 32P-pM12-DNA was replaced by the CAAAG-deleted pM12/D-DNA and incubated with ERα, and Tcf-4-containing lysates, no retarded bands were observed (Fig. 1A, lanes 4 and 12), and no retarded bands were observed when pM12-DNA was incubated with lysates containing only ERα (Fig. 1A, lane 5). Antibodies to ERα, but not to ERβ, when added to incubations containing 32P-pM12-DNA and combined ERα/E2 and Tcf-4 lysates produced a similar-sized single band as with Tcf-4 lysates alone (Fig. 1A, lanes 10 and 11). The same results were obtained when lysates contained ERα instead of...
Functional Interactions of Tcfs and Estrogen Receptors

To test the reciprocal interaction between Tcf-4 and ERα when the latter was bound to the promoter region through its SFRE recognition sequence, the 32P-labeled 29-bp double-stranded oligonucleotide 32P-SFRE-DNA was incubated with reticulocyte lysates expressing ERα alone (lanes 1 and 2), for ERα (lanes 3 and 4), for Tcf-4 (lanes 5 and 6), and for Tcf-1 (lanes 7 and 8). The resultant proteins were immunoimmunoprecipitated with a monoclonal antibody to ERα (mERα) (lanes 1 and 4), to ERβ (mERβ) (lanes 2), and for Tcf-4 (lanes 5 and 8), and to Tcf-1 (mTcf-1) (lanes 6 and 7) analyzed on polyacrylamide gels. The autoradiogram shows the position of the 32P-labeled proteins. The positions of authentic ERα, Tcf-4, ERβ, and Tcf-1 are shown by arrows on the left-hand side and of standard marker proteins on the right-hand side of the autoradiogram. B, coimmunoprecipitation of Tcfs and ERs. 35S-Labeled proteins (*) produced in reticulocyte lysates by expression vectors for Tcf-4 (lanes 1, 3, and 5–8) or for Tcf-1 (lanes 2 and 4) were incubated with nonradioactive proteins produced in reticulocyte lysates by expression vectors for ERα (lanes 1, 2, and 5) or for ERβ (lanes 3, 4, 5, and 6) treated for 1 h with 10−6 M E2. Any resultant complexes were immunoprecipitated with a monoclonal antibody to ERα (mERα) (lanes 1, 2, 5, and 7) and to ERβ (mERβ) (lanes 3, 4, 6, and 8) and were analyzed on polyacrylamide gels. The positions of authentic Tcf-4 (++) and Tcf-1 (−−) are indicated by arrows.

To investigate whether the ERs and Tcfs could interact directly without the necessity to bind to DNA, coimmunoprecipitation of the relevant molecules produced by suitable expression vectors in coupled transcription-translation reticulocyte lysates was attempted. That the correct molecules were produced in these lysates was confirmed by separately using the expression vectors for ERα, ERβ, Tcf-4, and Tcf-1 to direct protein synthesis with 35S-methionine and obtaining the correct sized 35S-labeled proteins of 65, 62, 65, and 33 kDa, respectively, on polyacrylamide gels after immunoprecipitation with only the cognate (Fig. 2A, lanes 1, 3, 5, and 7) and not the noncognate antibody (Fig. 2A, lanes 2, 4, 6, and 8). 35S-Tcf-4 or 35S-Tcf-1-containing lysates were then incubated with nonradioactive ERα- and ERβ-containing lysates with E2, immunoprecipitated with the relevant anti-ER, and analyzed on polyacrylamide gels (Fig. 2B). In the presence of ERα/E2 an antibody to ERα coimmunoprecipitated a 35S-labeled protein of 65 kDa from Tcf-4-producing lysates (Fig. 2B, lane 1) and a 35S-labeled protein of 33 kDa with Tcf-1-producing lysates (Fig. 2B, lane 2). These proteins corresponded to the major radioactive proteins in their respective lysates and possessed the same molecular weights as those reported for human Tcf-4 and Tcf-1, respectively (7, 32). The same results were obtained when ERβ replaced ERα-containing lysates, and the resultant complexes were immunoimmunoprecipitated with an antibody to ERβ (Fig. 2B, lanes 3 and 4). No such 35S-labeled proteins were detected in the resultant immunoprecipitates when lysates containing ERα/E2 or ERβ/E2 were omitted from the incubations (Fig. 2B, lanes 5–8). Sometimes smaller, considerably less abundant 35S-labeled protein bands were also observed on the gels (e.g. Fig. 2B); these minor products...
FIG. 3. Specificity of immunoprecipitated complexes. A, control immunoprecipitations. 35S-Labeled proteins (*) produced in reticulocyte lysates by expression vectors for Tcf-4 (lanes 1, 3, and 4) or for c-Fos (lanes 2, 5, and 6) were applied directly to the gel (lanes 1 and 2) or were incubated with nonradioactive proteins produced in reticulocyte lysates by the expression vector for ERE pretreated with 10−6 M E2 for 1 h (lanes 4 and 6). Any resultant complexes were immunoprecipitated with mAbs to ERα (lanes 3–6) and analyzed on polyacrylamide gels. The positions of the products of HE15 (AF-1) and HEG19 (AF-2) are indicated by the arrow. B, specificity of ERα/Tcf-4 interaction. 35S-Labeled proteins (+) produced in reticulocyte lysates by expression vectors HE15 for AF-1 (lanes 1–3), HEG19 for AF-2 (lanes 4–8), or for the point mutated ER_L540Q (ERαM) (lanes 9 and 10) were applied either directly to the gel (lanes 1 and 4) or were incubated with nonradioactive proteins produced in reticulocyte lysates by the expression vector for Tcf-4 (lanes 3 and 7–10). The products of HEG19 lysates (AF-2) and of L540Q lysates were also preincubated with 10−6 M estradiol (E2) before inclusion (lanes 6, 8, and 10). Any resultant complexes were immunoprecipitated with mAb to Tcf-4 (mTcf-4) (lanes 2, 3, 5, and 10) and analyzed on a polyacrylamide gel. The positions of the products of HE15 (AF-1) and HEG19 (AF-2) are indicated by the arrow.

might have arisen from proteolytic cleavage of the major proteins during incubation.

The specificity of the immunoprecipitated complexes formed between ERs and Tcf-4 was tested with an unrelated transcription factor, c-Fos, and with mutants in the activating functions of ERα. When 35S-labeled c-Fos or 35S-Tcf-4-containing lysates were incubated with nonradioactive ERα/ERαM-containing lysates and then immunoprecipitated with anti-ERα, no radioactive c-Fos protein of 55 kDa (26) was observed on the resultant polyacrylamide gels (Fig. 3A, lanes 2, 5, and 6), despite the presence of radioactive Tcf-4 (Fig. 3A, lanes 1, 3 and 4). When the expression vectors for the ERα deletion mutants HE15 (AF-1) and HEG19 (AF-2) were transcribed/translated in the reticulocyte lysate, they produced proteins of about 45 kDa consistent with their sizes (Fig. 3B, lanes 1 and 4); whereas that for the point mutated L540Q was similar to the parental ERα (not shown). However, only the expression vector HEG19 with both ERα functional domains (AF-2) produced a product that in the presence of Tcf-4-containing lysates was precipitable with mAb to Tcf-4; the expression vectors HE15 with only the DNA-binding domain (AF-1) and the transactivation-inactive L540Q failed to do so (Fig. 3B, lanes 2, 3, 5–7, 9, and 10). Moreover, the amount of 35S-HEG19 precipitable product depended on the presence of Tcf-4 lysates and was enhanced by the inclusion of E2 (Fig. 3B, lanes 6–8). These results suggest that ERα can interact specifically with Tcf-4 through its AF-2 domain in an E2-dependent manner.

Binding of ERs to Immobilized Tcfs—Binding of ERs to immobilized Tcfs was also explored in an optical biosensor. Biotinylated e-aminocaproic acid was used to synthesize biotinylated proteins in a reticulocyte lysate directed by an expression vector for Tcf-4 or Tcf-1. Immunoprecipitation of 35S-labeled protein aliquots directed separately by Tcf-4 and Tcf-1 and their analysis on polyacrylamide gels confirmed that they produced the same molecular mass proteins of 65 and 33 kDa, respectively, with the cognate but not with the noncognate antibodies as authentic Tcf-4 and Tcf-1, and these proteins could react with streptavidin peroxidase (not shown). Three different surfaces were prepared by immobilizing the in vitro synthesized biotinylated proteins on streptavidin-derivatized biosensor surfaces for endogenously synthesized proteins and for approximately equal quantities of Tcf-4 or Tcf-1 proteins (8–9 fmol/mm2) produced by expression vectors in the reticulocyte lysate. Addition of lysates directed by expression vectors for ERα or ERαM to the control surface of endogenously synthesized proteins failed to increase the response over that obtained with endogenously synthesized proteins alone (Fig. 4A). In contrast, addition of ERα- or ERαM-containing lysates to the Tcf-4 surface elicited a greater than 2-fold increase in response (Fig. 4B). Addition of ERαM-containing lysates to the Tcf-1 surface produced a 2–5-fold increase in binding, respectively (Fig. 4C). When a second aliquot of ERαM- or ERαM-containing lysate was added, binding only increased by a further 20–50%, indicating that each interaction ERα, with Tcf-4 and Tcf-1, and ERαM with Tcf-4 and Tcf-1 was saturable (not shown). Within the limits of the loss of binding observed after regeneration (10–40%) (33), inclusion of 9 nM estradiol had little effect on the interaction between ERs and Tcfs. In controls for testing the interaction the other way around, nonbiotinylated pure recombinant ERα was cross-linked to the biosensor surface. Addition of lysates directed by an expression vector for Tcf-1 elicited a 2.4-fold increase in response over that with a nonprogrammed lysate, and lysates containing c-Fos were without any effect (Fig. 4D). The 5- or more fold differences in the amount of ERα, coating the cuvette compared with the biotinylated Tcfs reflected the different methods of attachment. These results suggest that whichever partner ER or Tcf was attached to the surface of the biosensor cuvette, they can still interact in a specific manner.

DISCUSSION

Both ERα, like other nuclear receptors (34) and the Tcf family of transcription factors (32), interact separately with accessory proteins to modulate transcription of presumptive target genes. ERs can also bind to other transcription factors and modulate their activity at non-ERE sites in promoters (35, 36). Here we show that the ERα/ERαM and the Tcfs that recognize entirely different sequences in the promoter can interact and form a complex using two independent methods, coimmunoprecipitation and refractive index changes in a biosensor. We also show that this complex can assemble at a Tcf recognition sequence on a small 20-bp oligonucleotide and at an SFRE recognition sequence on another separate 29-bp oligonucleotide, as judged by gel mobility shift experiments. Although the ERs and Tcfs used in the experiments reported herein have been synthesized from expression vectors in cell-free coupled transcription-translation systems, control experiments demonstrate that they are the major products synthesized and that there are no complexes formed with material from unprimed cell-free systems alone. Moreover, reticulocyte protein-synthesizing lysates that contain a transcription factor c-Fos, with no
recognition sites on the rat OPN promoter-reporter construct (37, 38), fail to increase its activity with or without ER/\textit{Tcf-1} and also fail to bind to ER/\textit{Tcf} in either the coimmunoprecipitation or biosensor assays. This result eliminates the possibility of nonspecific interaction with any transcription factor. However, the identification of protein complexes \textit{in vivo} does not necessarily prove that such complexes occur \textit{in vitro} and direct recourse to other types of experiments that assay for complex formation \textit{in vitro}, such as the yeast one-hybrid system (39, 40), will be necessary to establish this fact unequivocally.

The functional significance of the interaction between ERs and Tcfs, either off or on the DNA, is supported by the fact that effects of \textit{Tcf-1}, \textit{Tcf-4}, and pM12-DNA can occur on promoters containing only ER\textit{E}s and not any Tcf recognition sites and that these Tcf-related effects are totally dependent on an activated ER. Moreover, when the two SFRE recognition sites are removed by standard inactivating mutations from the OPN promoter-reporter construct leaving only the Tcf recognition sites, expression vectors for ER/E\textsubscript{2} are still capable of stimulating this doubly mutated construct in an E\textsubscript{2}-dependent manner in the presence of the expression vector for Tcf-1. This result confirms that only Tcf-1-occupied Tcf sites are required for further stimulation of the OPN promoter-reporter construct by ER/E\textsubscript{2}. The functional results have been obtained by transient transfection of promoter-reporter constructs together with cotransfection of expression vectors for ERs and Tcfs in the Rama 37 cell system. These results are likely to reflect the activity of the endogenous promoters in the Rama 37 cells, at least for OPN, since the activity of the same promoter-reporter construct in the Rama 37 cells accurately reflects the levels of endogenous OPN mRNA produced (12, 17). Moreover, control experiments have demonstrated that all the effects on the activities of the promoter-reporter constructs produced by transient cotransfection of the expression vectors for the ERs and Tcfs are dose-dependent, up to a maximum input of a particular expression vector and reflect also the levels of the appropriate ER/Tcf synthesized (not shown). Since the Rama 37 cells can mimic some of the differentiation processes observed in the normal mammary gland (41), it is possible that the results obtained by transient transfections of this cell line in culture may reflect control of transcription of endogenous genes like OPN \textit{in vivo}.

At first sight it may be surprising that a vector with one Tcf-binding site, when transfected into the Rama 37 cell line, can produce a discernible titration effect on the level of available Tcf molecules in the nucleus of the resultant transfectants. However, transient transfection of the same optimum concentration of vector containing pM12-DNA results in a dramatic fall in the level of nuclear Tcf-4 protein (12, 17), similar to that observed in cells permanently transfected with about 100 copies of the same DNA (17, 29). Both methods of transfection presumably yield enough copies of pM12-DNA to sequester a sufficient number of Tcf molecules in the nucleus so that the majority of those bound to the OPN promoter are removed, thereby stimulating its transcription (12, 17). Moreover, the stimulatory effect on the OPN promoter-reporter construct of the vector containing the Tcf-binding site can be gradually reversed by increasing concentrations of the expression vector for Tcf-4 (12, 17), consistent with this idea. The stimulatory effect with pM12-DNA on the ER/E\textsubscript{2}-activated ER promoters is entirely dependent on the Tcf recognition sequence contained within the pM12-DNA, since there is no effect when this Tcf site is deleted. This result suggests that, in the Rama 37 cells, it is Tcf-4 rather than Tcf-1 that is normally complexed with the ERs, thus inhibiting the activity of the two ERE promoter-reporter constructs used here. Sequestration of Tcf-4 by pM12-DNA in the Rama 37 cells can then stimulate the activity of the two ERE promoter constructs still further. The fact that Tcf-4 can inhibit and Tcf-1 can stimulate the promoter-reporter activity of ER/E\textsubscript{2} in transient transfection assays in the Rama 37 cell system may be due to subtle differences in binding, as reported for ER\textsubscript{2} and antiestrogens (42).

The rat OPN promoter (16) contains no classical ERE sequences but two potential SF1 response elements (SFRE\textsubscript{s}), a single half-site preceded by a consensus trinucleotide (13, 14). That ER\textsubscript{a}/E\textsubscript{2} but not ER\textsubscript{y}/E\textsubscript{2} can transactivate the rat OPN promoter-reporter construct is consistent with the report that ER\textsubscript{y}/E\textsubscript{2} fails to transactivate the mouse OPN promoter, which also contains only SFRE\textsubscript{s} (17), and with our results and the results of others (43) for the minimal ERE.TATA. In contrast, the more complex ERE.TK is capable of transactivation by both ER\textsubscript{a}/E\textsubscript{2} and ER\textsubscript{y}/E\textsubscript{2}, as reported by others (43). In addition, the
The estrogen receptor α consists of several functional domains. The amino-terminal region exhibits a hormone-independent transactivation function (AF-1), the central region is principally involved in interactions with the DNA, and the carboxyl-terminal region is involved with hormone-dependent transactivation functions (AF-2) (18, 21). The results with the doubly mutated OPN promoter-reporter construct with both SREs inactivated suggests that ERα can interact with Tcf-1 through its AF-2 domain to transactivate this promoter via Tcf-binding sites on its DNA. Similar conclusions have been obtained from direct ERα and Tcf-4 interactions observed by immunoprecipitation techniques. In both cases the interactions require E2 for full effect. However, there is a very limited transactivation of the doubly mutated OPN promoter-reporter construct via Tcf-1 with lysates containing complete ERα or the AF-2 product without E2. This limited interaction in the absence of E2 between ERα and Tcf-4 is also observed to a more marked extent when measured by immunoprecipitation techniques and is most marked when measured in the biosensor where little dependence on E2 is preserved. However, transactivation of the OPN promoter-reporter construct is conducted inside the rat mammary cell line Rama 37, whereas immunoprecipitation is performed in the presence of reticulocyte lysates that are even more diluted in the biosensor due to the immobilization and washing processes. Thus the different conditions of assay may reflect decreasing concentrations of an inhibitor for ERα whose inhibition is abrogated by binding E2; one such example is heat shock protein 90 (44).

Transgenic mouse studies have separately implicated the E2/ER (45) and Wnt/Tcf signaling systems (46) in various developmental functions; different family members have more or less defined functions. Now we have shown that some of the different family members of the two sets of downstream transcription factors ER/ERα and Tcf-1/Tcf-4 can interact directly and thereby modulate the activity of promoters for potential target genes. The precise modulation, however, depends on the identity of the family member in a particular cellular environment. In the case of the mammary gland, the major Wnt family member Wnt-4 is expressed in early pregnancy from a low level in virgin mice (47, 48) and in reconstituted glands causes secondary branching of ducts that terminate in very incomplete and lactation-deficient alveolar-like structures (49). Similar secondary-branching structures are also seen in transgenic mice in which OPN expression is specifically inactivated in the mammary glands of pregnant/lactating animals (9), and this early developmental period in normal pregnant mice corresponds to the period of absolute minimum expression of OPN mRNA (20). Thus it is possible that Wnt and E2 can interact to control this switch from secondary to lobular alveolar development through OPN in vivo, as we have shown their effecter transcription factors Tcf-4 and ERα, can interact to control OPN promoter activity in cultured mammalian cells in vitro.

Acknowledgments—We thank Dr. A. Ridall, University of Texas, for the original 6-kbp osteopontin promoter; Professor H. Clevers, University of Utrecht, the Netherlands, for the cDNAs and antibodies to human Tcf-1 and Tcf-4; Professor P. Chambon, University of Strasbourg, France, and Dr. S. Ali, Imperial College, London, UK, for expression vectors for ERα deletion mutants and ERα, respectively.

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Differential Modulation of Transcriptional Activity of Estrogen Receptors by Direct Protein-Protein Interactions with the T Cell Factor Family of Transcription Factors
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J. Biol. Chem. 2001, 276:41675-41682.
doi: 10.1074/jbc.M103966200 originally published online August 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103966200

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