Evidence that the mouse insulin receptor substrate-1 belongs to the gene family on which the promoter is activated by estrogen receptor α through its interaction with Sp1

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Abstract

In the present study, the molecular mechanism underlying the up-regulatory effect of estradiol (E2) on mouse insulin receptor substrate-1 (IRS-1) promoter was investigated in CHO cells on which the same promoter had first been functionally characterized. The mouse IRS-1 promoter bears four consensus half Estrogen Responsive Elements (ERE) sequences and thirteen AP-1- and ten Sp1-binding elements. We performed molecular dissection of this promoter gene providing 38 different deleted constructs, containing the same AP-1 rich region with a progressively increased number of ERE half sites located downstream. None of these constructs was responsive to E2, while a downstream region (nt –1420 to –160) rich in GC elements was induced by E2. However, the latter region lost its intrinsic E2 responsiveness when the whole IRS-1 promoter was mutated for deletion in all four ERE half sites. Deletion analysis of the ERE half sites demonstrated that only ERE located at the position –1500 to –1495, close to the GC-rich region, was able to maintain the induced activatory effect of E2 on the IRS-1 gene. Deletion analysis of the ERE half sites demonstrated that only ERE located at the position –1500 to –1495, close to the GC-rich region, was able to maintain the induced activatory effect of E2 on the IRS-1 gene. Electrophoretic mobility shift and chromatin immunoprecipitation assays identified the region containing the half ERE/Sp1 (nt –1500 to –1477) as the one conferring E2 responsiveness to the whole promoter. This effect occurs through the functional interaction between E2/ERα and Sp1.

Journal of Molecular Endocrinology (2006) 36, 91–105

Introduction

Estrogen and insulin-like growth factor (IGF) regulate breast cancer cell growth and survival through the activation of distinct transductional pathways (Dickson & Lippman 1987, 1995, Surmacz 2000). However, evidence of a cross-talk between estrogen and growth factors (such as IGFs) has been documented, addressing an additive effect of these two mitogenic systems on breast cancer cell growth and survival (Molloy et al. 2000, Yee & Lee 2000).

The previous findings of ourselves and others have disclosed novel arguments to sustain the cross-talk between the two signals, demonstrating how exposure of breast cancer cells to estradiol (E2) enhances the expression of insulin receptor substrate-1 (IRS-1), a key molecule linked to phosphatidylinositol-3 kinase (PI-3K)/Akt and ERK1/ERK2 pathways, crucial for cell proliferative response and survival (Surmacz 2000, Molloy et al. 2000, Yee & Lee 2000, Mauro et al. 2001). This deduction fits well with previous evidence reporting on how the mitogenic effects of insulin or IGF-I were amplified by exposure to estrogen (Ando et al. 1998, Lee et al. 1999). In addition, breast cancer cells overexpressing IRS-1 show a marked growth advantage and reduced or abrogated estrogen growth requirements (Guvakova & Surmacz 1997). Our recent data have demonstrated that E2 is able to increase IRS-1 mRNA level through activation of the regulatory region of the IRS-1 gene (Mauro et al. 2001). Mouse IRS-1 promoter, characterized for the first time by Araki et al. (1995) in CHO cells, has furthermore been analyzed and our results show four consensus half Estrogen Responsive Elements (ERE) sequences and thirteen AP-1- and ten Sp1-binding elements. These might be important regulatory sites for the actions of estrogen. The up-regulatory effect induced by E2 on this promoter activity in both MCF-7 and CHO cells, expressing estrogen receptor α (ERα), seems to underscore a general mechanism which is not strictly related to the cell type (Mauro et al. 2001).

In the present study, we have demonstrated, through a molecular dissection of the IRS-1 promoter, how the region bearing the ERE half site, separated by 12
nucleotides from the Sp1 site (5'-AGGTCA[N]12CCG CCC-3') within nt –1500 to –1477, is responsible for the E2-induced activation of the whole IRS-1 promoter. Both electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay confirmed that the above-mentioned sequence is functionally involved in mediating the up-regulatory effect induced by E2 on IRS-1 expression. The effect, as documented for other E2-responsive genes, occurs through the interaction between ERα and Sp1 proteins, bound separately to the ERE half sequence and Sp1 responsive element respectively and present in the ERE/Sp1 region of the IRS-1 promoter (Dubik & Shiu 1992, Wu-Peng et al. 1992, Krishnan et al. 1994, Rishi et al. 1995, Porter et al. 1996, 1997, Scholz et al. 1998, Petz & Nardulli 2000, Saville et al. 2000, Khan et al. 2003).

Materials and methods

Materials

Dulbecco’s modified essential medium (DMEM)/Ham’s F-12, t-glutamine, penicillin/streptomycin, calf serum (CS), bovine serum albumin (BSA), aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, 4-OH-tamoxifen and E2 were purchased from Sigma (Milan, Italy). FuGENE 6 and poly (di-l-c) were from Roche Applied Science (Milan, Italy). Taq DNA polymerase, T4 poly nucleotide kinase, 1 kb DNA ladder, dual lucerase kit, pGL2 basic vector and dual luciferase reporter vector (pRL-TK) were obtained from Promega (Madison, WI, USA). [γ32P]ATP, Sephadex G50 spin columns and the enhanced chemiluminescence (ECL) system were from Amersham Biosciences. Sp1 (1C6), ERα F10 and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IRS-1 antibody was from UpState Biotechnology (New York, NY, USA). Human recombinant ERα and Sp1 proteins were obtained from Invitrogen (Carlsbad, CA, USA) and Alexis (Lausen, Switzerland) respectively.

The plasmid pBluescript SKII containing mouse IRS-1 promoter (3·3 kb) and a codifying region of the IRS-1 gene (3·4 kb) was kindly given by Dr Kaku Tsuruzoe (Research Division, Joslin Diabetes, Boston, MA, USA). pHEGO plasmid, containing the full length IRS-1 promoter (3·3 kb) and a codifying region of the IRS-1 gene (3·4 kb) was kindly given by Dr Kaku Tsuruzoe (Research Division, Joslin Diabetes, Boston, MA, USA). pHEGO plasmid contains the full-length ERα cDNA. The Renilla luciferase reporter vector pRL-TK (Promega) was used as a transfection standard to normalize transfection efficiency.

PCR mutagenesis

The plasmids pEREmut-luc, pERE1,2,3,mut-luc and pERE4 mut-luc were generated by PCR mutagenesis (Clackson et al. 1991).

pEREmut-luc contained mutation of all ERE half sites, located at sites nt –2218 to –2213, –2128 to –2123, –2050 to –2045 and –1500 to –1495. To generate this plasmid, PCRs were performed using the primers 1s (3‘-GGCACCTCAAGAGATGTTG-5‘) and 2a (5‘-TTGGGGGCGCTGGGGCAGGGGAGCAGCACCAAGGTAAGGCCGCCCCCG-3‘) to obtain the amplified product A (nt –3350 to –2240); the non-mutagenic external primer 3s (3‘-CGTCTAATGC TCGTGCAAAAC-5‘) and the mutagenic internal primer 6a (5‘-TTGGGGGCGCTGGGGCAGGGGAGCAGCACCAACAGGTAAAGGCGACCCCCC-3‘) to obtain the amplified product B (nt –2026 to –1466); the mutagenic internal primer 5s (5‘-CGGGGTCCGCTCTTTACCTGTGTGGTCTGTCCCCCTCCGCCGCCACGC GCCCCCA-3‘) and the non-mutagenic external primer 4a (3‘-AGGAAGATAGCCCTGATCCGA-5‘) to obtain the amplified product C (nt –1536 to –170). Products B and C were used as templates in a second PCR using the primers 3s and 4a (product D, nt –1536 to –170). Products A and D were restricted by BamHI (specific sites were present in primers 2a and 3s) and ligated to obtain product E, lacking all ERE half sites, which was further

et al. (1995). The MatInspector V2-2 software is available at the web url http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl; it allows the identification of consensus sequences of transcriptional factors through the TRANSFAC data base (http://transfact.gbf.de/TRANSFAC/) (Quandt et al. 1995).

Plasmid pIRS-1-luciferase (luc) was generated by inserting the 3·3 kb fragment of the mouse IRS-1 gene promoter into the pGL2 expression vector containing a luciferase gene. The sequence was confirmed by automated sequencing analysis with a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA).

IRS-1 promoter fragments (pIRS-1A-luc, pIRS-1B-luc, pIRS-1C-luc) were synthesized by unidirectional deletion using the exonuclease III reaction (Ausubel et al. 1988). pIRS-1D-luc was obtained by PCR using 5‘-GCC TCCCTCACTCCTTGGT-3‘ and 5‘-GGAAGATAGCCTGATCCCGAG-3‘ as the sense and antisense primers respectively.

Alligation products were transformed into competent Escherichia coli cells (Invitrogen). Plasmids were isolated, and clones were confirmed by DNA sequencing.

Materials and methods

Journal of Molecular Endocrinology (2006) 36, 91–105
digested by SacI and HindIII (specific sites were present in primers 1s and 4a) and inserted in the pGL2 plasmid.

pERE1,2,3,mut-luc contained mutations of the three ERE half sites, located at the sites nt -2218 to -2213, -2128 to -2123 and -2050 to -2045, and was generated using product A, as described above, and product F (nt -2026 to -170) obtained by PCR using primers 3s and 4a. Products A and F were restricted by BamHI, ligated to obtain product G, lacking the three ERE half sites, which was further digested by SacI and HindIII and inserted in the pGL2 plasmid.

pERE4 mut-luc was mutated in the ERE half sites corresponding to nt -1500 to -1495, and was constructed using product C, as described above, and product H (nt -3350 to -1466) obtained by PCR using the primers 1s and 6a. Products C and H were used as templates in a second PCR using the primers 1s and 4a to obtain product L. The latter product was digested by SacI and HindIII and inserted in the pGL2 plasmid.

Point mutations were done by PCR-mediated site-specific mutagenesis using degenerate primers, replacing one G with T in the ERE sequences. Namely, the ERE half site was located at position nt -2218 to -2213: AGtTCA; the ERE half site was located at position nt -2128 to -2123: TtACCC; the ERE half site was located at position nt -2050 to -2045: TCAaG; the ERE half site was located at position nt -1500 to -1495: AGtTCA (mutations are shown as lower case letters).

Cell lines and culture conditions

CHO cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Wild-type human breast cancer (MCF-7) cells were a gift from Dr E. Surrancz (Kimmel Cancer Institute, Philadelphia, PA, USA). The cell lines were cultured in DMEM/Ham’s F12 (1:1) medium supplemented with 5% CS, 1% l-glutamine and 1% penicillin/streptomycin. The cells were cultured in phenol red-free, serum free medium, DMEM (PRF-SFM-DMEM) containing 0·5% BSA, 1% -glutamine and 1% penicillin/streptomycin, 24 h before each experiment.

Transfections and luciferase assay

CHO and MCF-7 cells were seeded (1 × 10⁵ cells/well) in DMEM/F-12 supplemented with 5% CS in 24-well plates. CHO cells were cotransfected with pIRES-luc promoter construct and pHEGO. Cells were transfected in SFM using FuGENE6 according to the manufacturer’s instructions with a mixture containing 1 µg/well of each specific plasmid and 25 ng/well of TK Renilla luciferase plasmid. An empty pGL2 vector was used as the control vector to measure basal activity. Twenty-four hours after the transfection the medium was changed and the cells were treated in PRV-SFM-DMEM in the presence of 10 pM and 1, 10 and 100 nM E₂. The firefly and Renilla luciferase activities were measured by using a dual luciferase kit. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

Western blotting

CHO and MCF-7 cells were grown in 100 mm dishes to 70–80% confluence, shifted to SFM for 24 h and lysed. Protein lysates were obtained with a buffer containing 50 mM HEPES, pH 7·5, 150 mM NaCl, 1·5 mM MgCl₂, 10 mM EGTA, pH 7·5, 10% glycerol, 1% Triton X-100 and protease inhibitors (2 µM Na₃Vo₄, 1% PMSF and 20 µg/ml aprotinin).

The expression of IRS-1 was tested by Western blotting in 50 µg protein lysates using an anti-IRS-1 antibody. Proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane, probed with primary antibody and then stripped and reprobed with β-actin antibody. The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with a peroxidase-coupled anti-IgG antibody and revealed using the ECL system. Blots were then exposed to film and bands were quantified by densitometer. The results obtained are expressed in terms of arbitrary densitometric units.

Gel mobility shift assay

Nuclear extracts were prepared from CHO cells as previously described (Andrews & Faller 1991). The probe was generated by annealing single stranded oligonucleotides and labeled with [γ³²P]ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors were as follows (the nucleotide motifs of interest are underlined, mutations are shown as lower case letters): Sp1 5'-GGTGAGCTTTGGCAGGt CGCCCTCCCCCCCTGGCCAAG-3'; ERE/Sp1 5'-GAAGCAGTGACCtCCGCCCCGGTTCCCCCTGtGCCCA GCGCCCCCACCCCTG-3'. The DNA sequence used as ERE cold competitor was as follows: 5'-TCCCCC TGCCAAAGTACGCTGGGCACCCGTG-3'. Oligonucleotides were synthesized by MWG Biotech (Ebersberg, Germany). The protein binding reactions were carried out in 20 µl buffer (20 mM HEPES, pH 8, 1 mM EDTA, 50 mM KCl, 10 mM dithiothreitol (DTT), 10% glycerol, 1 mg/ml BSA, 50 µg/ml poly (dI-dC)) with 50 000 c.p.m. labeled probe, 20 µg CHO nuclear protein or an appropriate amount of ERα or Sp1 human recombinant proteins, and 5 µg poly (dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled.
competitor oligonucleotides. The specificity of the binding was tested by adding specific antibodies (anti-ERα and anti-Sp1) to the reaction mixture. The entire reaction mixture was electrophoresed through a 4% polyacrylamide gel in 0·25 × Tris borate-EDTA for 3 h at 150 V. The gel was dried and subjected to autoradiography at −70 °C.

**ChIP and Reverse (Re)-ChIP assays**

We followed the ChIP methodology described by Morelli *et al.* (2004). CHO cells were transiently transfected with pHEGO plasmid and treated with 100 nM E2 for 1 h, or left untreated in SFM. The cells were then cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with anti-ERα antibody (Ab) F-10 (Santa Cruz Biotechnology). Pellets were washed as reported in Morelli *et al.* (2004), eluted with elution buffer (1% SDS and 0·1 M NaHCO3 and digested with proteinase K (Morelli *et al.* 2004). DNA was obtained by phenol/chloroform extractions and precipitated with EtOH. PCR was carried out with the primers amplifying the IRS-1 promoter region containing the three upstream CACCCAGAGC-3′ and downstream CGACATC-3′ ERE/Sp1 primers: upstream 5′-CTGAGGCAGTCTAGTGGATTGA-3′ and downstream 5′-TGTGTATATGTTAGCAGATGTTTG-3′.

In Re-ChIP experiments, complexes from ERα immunoprecipitations (IPs) were eluted in RE-ChIP buffer (0·5 mM DTT, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris–HCl, pH 8·1) and subjected again to the ChIP procedure by using anti-Sp1 antibody PEP2 (Santa Cruz Biotechnology). Inputs were used as loading control and were obtained by eluting DNA from 5 µl cell lysates prior to the IP step. Negative control was performed using normal IgGs in place of the primary antibody.

**Statistical analysis**

Each data point represents the means ± s.d. of at least three experiments. The data were analyzed by ANOVA using the STATPAC computer program (Statpac Inc., Bloomington, MN, USA).

**Results**

**Analysis of the IRS-1 mouse promoter sequence**

Sequencing of the IRS-1 mouse promoter by MatInspector V2-2 software (see Materials and methods) led us to the identification of new potential transcriptional regulatory sites, in addition to those characterized by Araki *et al.* (1995), namely thirteen AP-1, ten Sp1 and four ERE half sites (Fig. 1). All these sites could be potential targets of E2 action resulting in the activation of the IRS-1 promoter.

**Identification of an E2-activated IRS-1 promoter region**

In CHO cells transiently co-transfected with pHEGO and pIRS-1-luc, encoding the full length of IRS-1 gene promoter linked to the firefly luciferase reporter gene, E2 (10 pM and 1, 10 and 100 nM) was able to induce luciferase activity (Fig. 2A). The same results were obtained in ER-positive human breast cancer MCF-7 cells transfected with pIRS-1-luc (Fig. 2B). E2 up-regulated IRS-1 protein content in both CHO and MCF-7 cells in a dose-dependent manner (Fig. 3).

Previous findings have demonstrated that ER may indirectly modulate transcription by interaction with the AP-1 sites (Umayahara *et al.* 1994, Paech *et al.* 1997, Webb *et al.* 1999); thus, we checked the potential role of the AP-1-rich region in mediating the activatory effect induced by E2 on the IRS-1 promoter. In the same vein, we checked how the four ERE half sites may work, together with AP-1, in mediating the up-regulatory effect of E2 on the activity of the whole IRS-1 promoter. With this aim, we transiently co-transfected CHO cells with pHEGO and three different 3′-deleted constructs conjugated with a luciferase reporter gene containing the same AP-1-rich region with a progressively increased number of ERE half sites, namely: (i) pIRS-1A-luc, containing one ERE half site (nt −3370 to −2180), (ii) pIRS-1B-luc, containing three ERE half sites (nt −3370 to −1650) and (iii) pIRS-1C-luc, containing four ERE half sites (nt −3370 to −1308). None of these constructs was stimulated by E2 (Fig. 4).

On the contrary, the plasmid pIRS-1D-luc, containing the residual downstream region (nt −1420 to −160), rich in GC elements, was responsive to E2 action (Fig. 4). The potential positive influence of AP-1 sites in the construct rich in Sp1 sequences has been ruled out since in previous experiments the deletion of the AP-1 site in the construct pIRS-1D-luc did not elicit substantial effects in the E2responsiveness (data not shown). Earlier reports (Saville *et al.* 2000) have shown how Sp1 cis-elements located in the promoter region of some genes are more responsive to E2; we therefore ascertained if the IRS-1 GC-rich region, present in the full length of IRS-1 promoter, was able to yield estrogen responsiveness independently on the four ERE half sites. Consequently, we performed site-specific mutagenesis at the ERE half sites of the IRS-1 mouse promoter located upstream to the GC-rich region. The effects of the site-specific mutagenesis on basal activity and E2...
Figure 1 Regulatory sites of the IRS-1 mouse promoter. The analysis of the IRS-1 mouse promoter sequence by MatInspector V2.2 software allowed identification of 13 AP-1 (underlined nucleotides), ten Sp1 (nucleotides in the boxes) and four half ERE (nucleotides in grey boxes) potential regulatory sites.

ER/Sp1 complex activates IRS-1 gene promoter: M L PANO, L MAURO and others

-370 ggtcctatac caggggttgg gcaacctcagta gcaaggtggtc taaccttctct taatcagaa
-330 taacttattcat taaactactac ctgccaccaag ccctgctgccg taaactactac ccctgctgccg
-320 tcaataattat attttttttta tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-310 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-300 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-290 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-280 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-270 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-260 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-250 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-240 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-230 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-220 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-210 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc
-200 ggtacctcttt tttttttttt tttgtttttc tttgtttttc tttgtttttc tttgtttttc tttgtttttc

ER/Sp1 complex activates IRS-1 gene promoter: M L PANO, L MAURO and others

-2050 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-2030 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-2010 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-2000 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1980 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1960 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1940 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1920 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1900 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1880 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1860 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1840 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1820 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1800 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1780 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1760 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1740 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1720 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac
-1700 tcaagtctaa aagttgctgtc ctgtcgtgca aacatcgaac aacatcgaac aacatcgaac aacatcgaac

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-1450 gcaccgctag tcgaaccacca ctctcaactc gctctcgtgtg ttctcctcag tttcctcctcag
-1390 tccccccctc aacaccaacc ctgctgtgact ggttgggcttc gttggcctgtc ttttcctcctcag
-1330 tccccccctc aacaccaacc ctgctgtgact ggttgggcttc gttggcctgtc ttttcctcctcag
-1270 gacacacagg ccggccgctt gggcctcag tggccgcttc cggcctcag tggccgcttc cggcctcag
-1210 agggcggact ctcgctgccg cggccagcag cgcgcggttg cgcgcggttg cgcgcggttg cgcgcggttg
-1150 gcgcactctg gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-1090 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-1030 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-970 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-910 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-850 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-790 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-730 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-670 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-610 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-550 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-490 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-430 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-370 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-310 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-250 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-190 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-130 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc
-70 gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc gcgcgcggc

Figure 1 Regulatory sites of the IRS-1 mouse promoter. The analysis of the IRS-1 mouse promoter sequence by MatInspector V2.2 software allowed identification of 13 AP-1 (underlined nucleotides), ten Sp1 (nucleotides in the boxes) and four half ERE (nucleotides in grey boxes) potential regulatory sites.
responsiveness were determined in transient transfection studies using the plasmids pEREmut-luc, pERE4 mut-luc and pERE1,2,3 mut-luc (see Materials and methods) (Fig. 5A).

Using the IRS-1 promoter mutated for deletion of all four ERE half sites, the CG-rich region, present in the full length of the IRS-1, was unable per se to yield E2 responsiveness. On the contrary, only the ERE half site localized at the position nt −1500 to −1495, close to the Sp1 elements, was crucial in maintaining the E2

![Graph A](image)

**Figure 2** E2 enhances IRS-1 promoter activity. (A) CHO cells were co-transfected with pIRS-1-luc and pHEGO together with pRL-TK. (B) MCF-7 cells were transiently transfected with DNA mixture containing pIRS-1-luc and pRL-TK. The transfectants were treated in the absence (C, control) or in the presence of 10 pM and 1, 10 and 100 nM E2 for 24 h. These results represent the means±S.D. of five different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. pGL2 basal activity was measured in cells transfected with pGL2 basal vector. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

![Graph B](image)

![Graph C](image)

**Figure 3** E2 up-regulates IRS-1 protein expression. Protein expression of IRS-1 in (A) CHO and (C) MCF-7 cells treated in the presence or absence of 10 and 100 pM and 1, 10 and 100 nM E2 for 24 h. β-actin served as loading control. Representative results are shown. The histograms represent the means±S.D. of three separate experiments performed in (B) CHO and (D) MCF-7 cells, in which the band intensities were evaluated in terms of arbitrary densitometric units and expressed as the percentage of the control assumed as 100%. *P<0.01 vs control.
transactivation of the IRS-1 promoter. Similar results were obtained by performing point mutations in ERE half sequences (data not shown). The important role of the fourth ERE half site together with the Sp1 region in conferring E2 responsiveness was confirmed by a functional study performed with the following construct bearing: (i) the whole IRS-1 promoter, (ii) the fragment containing the first three ERE half sites and (iii) the fragment containing the fourth ERE half site close to the Sp1 elements (Fig. 5B). The results showed that pERE4/Sp1-luc, transfected in CHO cells, reproduced the E2 responsiveness pattern similar to the full length of IRS-1 promoter (Fig. 5B).

On the basis of these findings, our attention was focused on the sequence ERE/Sp1 assumed to be a putative regulatory region target of E2 action.

**EMSA study**

Nuclear extracts of CHO cells were incubated in the presence of ERE/Sp1-labeled oligonucleotide to prove if this region was able to bind ERα and/or Sp1 proteins. Nuclear proteins from CHO cells revealed the presence of a single band (Fig. 6, lane 1), which was inhibited by a 100-fold molar excess of the homologous ERE/Sp1 cold competitor (Fig. 6, lane 2), but remained substantially unchanged in the presence of the cold mutated competitor (Fig. 6, lane 3). The results showed an enhanced binding of the nuclear extracts, obtained from CHO cells over-expressing ERα, to the ERE/Sp1-labeled oligonucleotide (Fig. 6, lane 5) and particularly evident upon E2 treatment (Fig. 6, lane 8). Both bands were abrogated by a 100-fold molar excess of the cold competitor.

![Diagram](https://example.com/diagram.png)
competitor (Fig. 6, lanes 6 and 9). The specificity of the binding was demonstrated by immunodepletion induced by ERα and Sp1 antibodies (Fig. 6, lanes 10 and 11).

Using a labeled Sp1 oligonucleotide containing a GC box motif present in the Sp1-rich region of the IRS-1 mouse promoter (from nt −490 to nt −455), in a cell-free system, ERα protein per se was unable to bind the Sp1 sequence (Fig. 7, lanes 1 and 2). However, the combined presence of ERα at a lower concentration and Sp1 proteins resulted in a clearly enhanced Sp1–DNA binding (Fig. 7, lanes 8 and 9) with respect to the one observed with Sp1 protein alone (Fig. 7, lanes 6 and 7). Sp1 protein was able to up-regulate its binding to the ERE/Sp1 sequence in a dose-related manner (Fig. 7, lanes 6 and 7). Such an up-regulatory effect produced by ERα was drastically attenuated in the presence of cold ERE oligonucleotide (Fig. 7, lanes 10 and 11). On performing a new set of experiments using the same ERE/Sp1-labeled oligonucleotide, as mentioned, it emerged that both Sp1 and ERα were able to bind this sequence (Fig. 8A, lanes 1 and 4). Both bindings were abrogated by a 100-fold molar excess of cold competitor (Fig. 8A, lanes 2 and 5) and restored in the presence of a mutated cold competitor (Fig. 8A, lanes 3 and 6). The specificity of the separate binding of the two proteins to the ERE/Sp1 sequence was demonstrated by the immunodepletion obtained in the presence of the two specific antibodies (Fig. 8A, lanes 8 and 9).

An increased amount of ERα enhanced Sp1 binding to the ERE/Sp1 sequence (Fig. 8B, lanes 6 and 7). In contrast, a progressive increase of Sp1 did not elicit any apparent influence on ERα binding (Fig. 8B, lanes 4 and 5). This up-regulatory effect was reversed by an excess of cold ERE oligonucleotide (Fig. 8B, lanes 8 and 9).

No cross-reaction was observed between the two specific antibodies (data not shown).

**ERα and Sp1 are recruited to the ERE/Sp1 sequences of the IRS-1 promoter in CHO cells**

The binding of ERα and Sp1 to the ERE/Sp1-containing sequence of the IRS-1 gene promoter was confirmed by ChIP assays. CHO cells were transiently transfected with pHEGO and treated or not treated with E2 for 1 h. The chromatin was immunoprecipitated with anti-ERα anti-
Figure 6 Binding of nuclear extracts from CHO cells and CHO cells transfected with pHEGO (CHO/HEGO) to ERE/Sp1 oligonucleotide. Nuclear extracts from CHO (lanes 1–4) and CHO/HEGO cells (lanes 5–11) were incubated with a double stranded ERE/Sp1 sequence probe labeled with [γ-32P]ATP and subject to electrophoresis in a 4% polyacrylamide gel. CHO and CHO/HEGO nuclear extracts treated with 100 nM E2 for 24 h incubated with probe are shown in lanes 4 and 8 respectively. Competition experiments were performed by adding as competitor a 100-fold molar excess of unlabeled ERE/Sp1 probe (lanes 2, 6 and 9) or a cold mutated (mut) competitor (lanes 3 and 7). The specificity of the binding was tested by adding to the reaction mixture an ERα antibody (ω) (lane 10) or an Sp1 antibody (ω) (lane 11). Lane 12 contains probe alone. The location of the ERα/Sp1/DNA (ERα/Sp1) complex is indicated by the arrow.
body. Eluates from ERα IPs were re-immunoprecipitated with anti-Sp1 antibody (Re-ChIP) to confirm the co-existence of ERα/Sp1 complex on the promoter (Fig. 9). The recovered DNA, opportunely amplified by using specific primers mapping the ERE/Sp1-containing sequence, showed an increased occupancy of this region by the two proteins under E2 treatment (Fig. 9). No effect was observed when the amplified DNA region contained the other three non-functional ERE half sequences (data not shown).

Figure 7 Binding of Sp1 to the Sp1 oligonucleotide in the presence of ERα. 32P-labeled oligonucleotide containing the Sp1-binding site was incubated with: (i) 360 fmol (lane 1) or 540 fmol (lane 2) of ERα and (ii) 10 ng (lanes 3 and 6) or 15 ng (lane 7) of Sp1 and subjected to electrophoresis in a 4% polyacrylamide gel. ERα (360 fmol) was incubated in the presence of 10 ng (lane 8) or 15 ng (lane 9) Sp1 protein. A competition experiment was performed by adding as competitor a 100-fold molar excess of unlabeled probe (lane 4) or a cold mutated competitor (lane 5) or a cold ERE (lanes 10 and 11). Lane 12 contains probe alone. The location of the Sp1/DNA (Sp1) complex is indicated by the arrow.
Discussion

Our previous findings have shown how treatment of CHO cells and estrogen-responsive positive breast cancer cell lines with E2, for up to 24 h, revealed an up-regulatory effect of E2 on the regulator region of the mouse IRS-1 gene (Mauro et al. 2001). We have here demonstrated that E2 at concentrations ranging from 10 pM to 100 nM produces the same up-regulatory effect on IRS-1 protein content in both CHO and human breast cancer MCF-7 cells. This suggests that there is a common regulatory mechanism controlling IRS-1 expression in the human and the mouse.

In order to investigate the molecular mechanism underlying the up-regulatory effect of E2 on the mouse IRS-1 promoter we used the same cell type where it had been first functionally characterized. With this aim, we transiently co-transfected CHO cells with the mouse IRS-1 promoter and pHEGO and tested the response to different doses of E2 ranging from 10 pM to 100 nM. The mouse IRS-1 gene, like other housekeeping genes, lacks the typical TATA and CAAT boxes (Araki et al.

Figure 8 ER-enhanced binding of Sp1 to the half ERE/Sp1-binding site. (A) 10 ng Sp1 or 360 fmol ERα proteins were incubated with a double stranded ERE/Sp1 sequence probe labeled with [γ-32P]ATP and subject to electrophoresis in a 4% polyacrylamide gel (lanes 1 and 4 respectively). The specificity of the binding was proved by incubating the reaction mixture containing Sp1 and ERα proteins with an anti-Sp1 or anti-ERα antibodies (lanes 8 and 9 respectively). Competition experiments were performed by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2 and 5) or a cold mutated (mut) competitor (lanes 3 and 6). Lane 10 contains probe alone. The location of the Sp1/DNA (Sp1) and ERα/DNA (ERα) complexes are indicated by the arrows. (B) 32P-labeled oligo containing the half ERE/Sp1-binding site was incubated with 10 ng Sp1 and 360 fmol ERα (lane 1) and subjected to electrophoresis in a 4% polyacrylamide gel. Competition experiments were performed by adding as competitor a 100-fold molar excess of unlabeled probe (lanes 2) or a cold mutated (mut) competitor (lanes 3). Binding reaction contained 10 ng (lane 4) or 15 ng (lane 5) Sp1 protein in combination with 360 fmol ERα protein. Sp1 (10 ng) was incubated with 360 fmol (lane 6) or 540 fmol (lane 7) ERα, in the absence (lanes 6 and 7) or presence (lanes 8 and 9) of cold ERE. Lane 10 contains probe alone. The location of the Sp1/DNA (Sp1) and ERα/DNA (ERα) complexes are indicated by the arrows.
and contains thirteen AP-1- and ten Sp1-binding sites and four ERE half sites. It appears that in addition to binding to a classic ERE element, the ER may also modulate transcription indirectly by interaction with other DNA-binding proteins. Actually, ER interaction with AP-1-bound fos and jun proteins confers E\textsubscript{2} responsiveness to the ovoalbumin (Gaub et al. 1990), e-fos (Weisz & Rosales 1990), collagenase (Webb et al. 1992) and IGF-I (Umayahara et al. 1994) genes. However, previous studies have demonstrated ligand- and cell-context specific differences in ER\textalpha/\textgamma and ER\beta/\textgamma action. For example, in HeLa cells both estrogens and anti-estrogens activated ER\textalpha/\textgamma but only anti-estrogens activated ER\beta/\textgamma (Paech et al. 1997). Thus, the latter observation led us to investigate whether the region of the IRS-1 promoter rich in AP-1-binding sites was responsive to E\textsubscript{2}. Our results have shown that the AP-1-rich region failed to be up-regulated by E\textsubscript{2}.

When we extended the molecular dissection downstream to implement the AP-1-rich region with the ERE half sites, the unresponsiveness to E\textsubscript{2} was still persistent. In contrast, the remaining downstream region of the IRS-1 promoter rich in Sp1-binding sites appears per se to be responsive to E\textsubscript{2} stimulation.

EMSA studies, performed in a cell-free system, using an oligonucleotide reproducing the Sp1 sequence present in the mouse IRS-1 promoter, revealed how ER\textalpha did not bind the Sp1 sequence but was able to enhance Sp1 binding to its own responsive element.

Sp1 was originally described as a trans-acting factor that bound to the GC box and activated transcription of the SV40 promoter (Dyan & Tjian 1983, Gidoni et al. 1984). However, it has been subsequently identified as a higher affinity consensus Sp1 site 5'-GGGGCGG GGC-3' and it has been also discovered that the sequences that varied from this consensus sequence displayed decreased affinities for Sp1 (Briggs et al. 1986). It is worth noting how the GC-rich region, when present in the full length of the IRS-1 mouse promoter mutated for deletion in all four ERE half sites, loses its intrinsic E\textsubscript{2} responsiveness. This led us to investigate the potential role of ERE half site as involved in IRS-1 E\textsubscript{2} responsiveness, taking into account the ability of ER\textalpha to bind as a monomer to the consensus ERE half site (Wood et al. 1998). Among the different ERE half sites tested, only the ERE at the position nt -1500 to -1495, appears to be crucial in conferring E\textsubscript{2} responsiveness to the whole promoter. The latter ERE half site was the closest one to the Sp1 sequence nt -1482 to -1477. Indeed, in CHO cells, only the construct bearing the ERE/Sp1 sequence has reproduced the same pattern of E\textsubscript{2} responsiveness as that given by the full length IRS-1 promoter. Because of this we reasonably postulated a functional interaction between ERE half sites and the Sp1-rich region downstream.

Results from the EMSA showed that the binding of the untreated nuclear extract to the labeled ERE/Sp1 oligonucleotide, bearing both ERE half site and Sp1 sequence (5'-GGTC\textalpha\textgamma\textalpha\textgamma\textalpha\textgammaCCGCCC-3'), resulted in a single band which was enhanced in the presence of ectopic ER\textalpha and drastically increased upon prolonged E\textsubscript{2} exposure. In the latter condition, a clear immunodepletion occurred in the presence of either anti-Sp1 or anti-ER\textalpha antibodies.
The ability of Sp1 and ERα to bind separately was demonstrated by two distinct bands which were abrogated in the presence of an excess of cold oligonucleotide and immunodepleted in the presence of an anti-ERα antibody in a cell-free system.

Progressively increased amounts of purified ERα protein enhanced Sp1 binding to the half ERE/Sp1-binding site in a dose-dependent manner, while increased amounts of Sp1 were unable to do so.

All these data have demonstrated that ERα enhances Sp1 binding and that both ERα and Sp1 can bind directly to the half ERE/Sp1-binding site. On the other hand, the binding of ERα and Sp1 to the ERE/Sp1-containing sequence of the IRS-1 gene promoter was confirmed by ChIP assay which showed an increased occupancy of this region by the two proteins under E2 treatment. In contrast, this was not observed when, in the ChIP assay, we used the sequence containing the first three ERE half sites. On the basis of these findings, it emerges that the ERE/Sp1-binding site is crucially involved in mediating E2 responsiveness to the IRS-1 gene. In contrast, the three non-functional ERE half sequences upstream of the ERE/Sp1 site are not involved in the process. This finding acquired relevance when we became aware of how the functional synergism between Sp1 and ERα, through the formation of the ERα/Sp1 complex, was responsible of the activation of other E2-responsive genes. For instance, in the promoter region of such genes the half palindromic ERE sequence and Sp1 were separated by a number of nucleotides ranging from 10 to 23 nt, such as cyclin D1, bcl2, retinoic acid receptor α1, IGF-binding protein 4, adenosine deaminase, DNA polymerase α, c-fos, cathepsin D, transcription factor-E2F1, creatine kinase B, human progesterone receptor A promoter and, recently, cad gene (Dubik & Shiu 1992, Wu-Peng et al. 1992, Krishnan et al. 1994, Rishi et al. 1995, Porter et al. 1996, 1997, Scholz et al. 1998, Wang et al. 1998, Petz & Nardulli 2000, Salvato et al. 2000, Saville et al. 2000, Tanaka et al. 2000, Vyhildal et al. 2000, Li et al. 2001, Khan et al. 2003).

While models of DNA are typically drawn in a linear array, the packaging of DNA and proteins into the nucleus of a cell requires tremendous compaction. This compaction could facilitate interaction between trans-acting factors bound to more distant cis elements. For instance, both ERα and SP1 are known to directly associate with the Transcription Factor (TFII) component. In particular, Sp1 has been reported to recruit TFII/TFII-binding protein and mediate formation of the transcription preinitiation complex on the TATA-less promoter (Pugh & Tjian 1991). On the other hand, ERα as is known, interacts with the TATA-binding protein (TBP) transcription factor IIb (TFIIb) and TBP-associated factor (TAF)430 (Ing et al. 1992, Jacq et al. 1994, Sabbah et al. 1998)). Thus, we can reasonably assume that the interaction of ERα and Sp1, by recruiting TFII/TFII-binding protein, could foster the formation of a protein–protein network that helps to establish an active transcriptional complex. Furthermore, the E2-dependent recruitment of coactivators such as CREB binding protein (CBP)/p300, which can function as a histone acetyltransferase (Ogryzko et al. 1996), could help remodel chromatin in different promoters and enhance formation of an interconnected protein–protein and protein–DNA network involved in activation of the IRS-1 gene.

Thus, the active complex ERα/E2-Sp1 could trigger the interaction between trans-acting factors bound to more distant cis elements, like the GC downstream elements, potentiating the transcriptional machinery at the level of the whole GC-rich region of the IRS-1 promoter, which is a region reported to have positive active elements on IRS-1 promoter activity (Araki et al. 1995).

Thus, with the present findings, we have demonstrated the molecular mechanism through which E2/ERα up-regulates mouse IRS-1 expression, thereby amplifying IGF-I/insulin signaling.

Since IRS-1 is sufficient to increase rRNA synthesis and cell size (Sun et al. 2003), its enhanced expression, upon prolonged E2 exposure, may establish another intriguing link between E2, cell growth and its mitogenic potentiality.

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