Activation of adenosine deaminase in MCF-7 cells through IGF–estrogen receptor α crosstalk

W Xie, R Duan and S Safe

Department of Veterinary Physiology & Pharmacology, Texas A&M University, 4466 TAMU, College Station, Texas 77843–4466, USA

(Requests for offprints should be addressed to S H Safe; Email: ssafe@cvm.tamu.edu)

ABSTRACT

Adenosine deaminase (ADA) regulates cellular levels of adenosine and deoxyadenosine, and 17β -estradiol (E_2) induces ADA mRNA in MCF-7 human breast cancer cells. IGF-I also induces ADA gene expression in these cells, and induction of this response through IGF activation of estrogen receptor α (ER α) was further investigated. IGF and other polypeptide growth factors induce reporter gene expression in MCF-7 cells cotransfected with ER α expression plasmid and pADA211, a construct containing the –211 to +11 region of the ADA gene promoter which is required for high basal and E_2 -inducible activity. Deletion analysis of this

promoter demonstrates that IGF activates ER α /Sp1 interactions with multiple GC-rich sites in the promoter and this response is abrogated in cells transfected with ER α containing mutations at Ser¹¹⁸ or Ser¹⁶³. IGF induces both MAPK (mitogen-activated protein kinase) and PI3-K (phosphatidylinositol-3-kinase) phosphorylation cascades in MCF-7 cells; however, using a series of inhibitors and dominant negative constructs, our results show that induction of ADA by IGF activation of ER α /Sp1 is dependent on the MAPK signaling pathway.

Journal of Molecular Endocrinology (2001) **26**, 217–228

INTRODUCTION

17β -Estradiol (E_2), insulin and growth factor polypeptides, such as insulin-like growth factor-I (IGF-I), transforming growth factor- α (TGF α) and epidermal growth factor (EGF), induce proliferation of estrogen receptor (ER)-positive breast cancer cell lines (Furlanetto & DiCarlo 1984, Bates *et al.* 1988, Dickson & Lippman 1988, 1991, 1995, Karey & Sirbasku 1988, Wilding *et al.* 1988, Fernandez *et al.* 1994, Westley & May 1994, Kato *et al.* 1998, Smith 1998, Weigel & Zhang 1998). Growth factor and E_2 -induced growth of breast cancer cells is accompanied by an increase in the number of cells in S phase of the cell cycle and enhanced expression of some of the same genes including immediate-early genes such as *c-fos*, *myc* and early growth response-1 (*egr-1*), several genes involved in cell cycle progression, and other genes/gene promoter constructs that are E_2 -responsive (Furlanetto & DiCarlo 1984, Ignar-Trowbridge *et al.* 1992, 1993, 1996, Aronica & Katzenellenbogen 1993, Musgrove *et al.* 1993, Foster & Wimalasena 1996, Hafner *et al.* 1996,

El-Tanani & Green 1997, Lee *et al.* 1997, Planas-Silva & Weinberg 1997, Prall *et al.* 1997, Pratt *et al.* 1998, W. Wang *et al.* 1998, Wilding *et al.* 1988). Although insulin/IGF growth factor polypeptides and E_2 induce cell proliferation and gene expression through distinct receptors and their downstream signaling pathways, growth factor–ER crosstalk has been observed in ER-positive breast cancer cells and other E_2 -responsive tissues such as the rodent uterus. For example, both E_2 , IGF and EGF induce a similar pattern of uterotrophic responses in female mouse uterus (Gannon *et al.* 1976, Das *et al.* 1994); however, in ER α knockout mice, EGF-induced DNA synthesis and progesterone receptor (PR) gene expression were absent (Curtis *et al.* 1996). Moreover, the estrogen-like activity of EGF in the uterus was inhibited by antiestrogen ICI 164 384.

Growth factors such as IGF-I induce proliferation and/or DNA synthesis of ER-positive breast cancer cells, and these responses are inhibited by phosphatidylinositol-3-kinase (PI3-K) inhibitors such as wortmanin and LY294002, the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate, and antiestrogens (Vignon *et al.* 1987, Wakeling *et al.*

1989, Freiss *et al.* 1990, Ignar-Trowbridge *et al.* 1992, 1993, 1996, Aronica & Katzenellenbogen 1993, de Cupis *et al.* 1995, Hafner *et al.* 1996, Dufourny *et al.* 1997, El-Tanani & Green 1997, Lee *et al.* 1997, Kato *et al.* 1998, Weigel & Zhang 1998, Ahmad *et al.* 1999, Zimmermann & Moelling 1999, Wang *et al.* 2000). Inhibition of growth factor-induced proliferation by antiestrogens demonstrates that some of the mitogenic activity of IGF-I is linked to regulation of estrogen-responsive genes. Growth factors mediate transcriptional activation of constructs containing estrogen-responsive element (ERE) promoter inserts and activation of estrogen-responsive genes through kinase-dependent phosphorylation of Ser¹¹⁸ or Ser¹⁶⁷ of ER α in the absence of estrogen (Kato *et al.* 1995, 1998, Joel *et al.* 1998). Thus, growth factor induction of kinase pathways causes ligand-independent activation of ER and some ER-dependent genes.

Research in this laboratory has identified a number of E₂-responsive genes which are ligand (E₂)-activated through interaction of ER α /Sp1 with specific GC-rich promoter elements. This induction process involves ER α -protein (Sp1) but not ER α -DNA interactions and gene promoters that are induced by this pathway include cathepsin D, E2F1, IGF binding protein 4, retinoic acid receptor α 1, thymidylate synthase, adenosine deaminase (ADA), *c-fos* and *bcl-2* (Porter *et al.* 1996, Duan *et al.* 1998, Sun *et al.* 1998, Wang *et al.* 1998, W. Wang *et al.* 1999, Dong *et al.* 1999, Qin *et al.* 1999, Xie *et al.* 1999, 2000). Recent reports also suggest that the ER α /Sp1 pathway may also be important for induction of the human PR, EGF receptor, and receptor for advanced glycation end products (Petz & Nardulli 2000, Salvatori *et al.* 2000, Tanaka *et al.* 2000). The ADA gene is involved in nucleotide metabolism (Aronow *et al.* 1992, Dusing & Wiginton 1994), and this study uses ADA as a model to investigate mechanisms of growth factor activation of ER α /Sp1-dependent genes in breast cancer cells (Xie *et al.* 1999). The results show that IGF-I-induced expression of ADA in MCF-7 cells is associated with ligand-independent ER α activation and interactions of ER α /Sp1 with multiple GC-rich sites in the proximal promoter region of the ADA gene promoter.

MATERIALS AND METHODS

Chemicals, MCF-7 cells and oligonucleotides

MCF-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were routinely maintained in minimal

essential medium with phenol red and supplemented with 10% fetal bovine serum plus a 1% antibiotic-antimycotic solution (Sigma Chemical Co., St Louis, MO, USA) in an air:carbon dioxide (95:5) atmosphere at 37 °C. For transient transfection studies, cells were grown for 1 day in DME/F12 medium without phenol red and 5% fetal bovine serum treated with dextran-coated charcoal. The constructs pADA211, pADA84 and pADA56 contained ADA gene promoter inserts linked to a bacterial chloramphenicol acetyltransferase (CAT) reporter gene and were provided by Dr Dan A Wiginton (University of Cincinnati College of Medicine and Children's Hospital Research Foundation, Cincinnati, OH, USA) or previously prepared in this laboratory (Dusing & Wiginton 1994, Xie *et al.* 1999). The wild-type human ER α (hER) expression plasmid was provided by Dr Ming-Jer Tsai (Baylor College of Medicine, Houston, TX, USA). The dominant negative Sp1 plasmid pEBG-Sp1 was provided by Dr G Thiel (University of Cologne, Cologne, Germany). Serine ER α mutants, HEGO-S¹¹⁸, HEGO-S¹⁶⁷, and HEGO-S^{118/167}, were kindly provided by Dr D Lannigan (Center for Cell Signaling, University of Virginia, Charlottesville, VA, USA) (Joel *et al.* 1998). pC3-luc containing the -1806 to +58 from the human complement C3 gene promoter was kindly provided by Dr Donald McDonnell, Department of Pharmacology, Duke University (Durham, NC, USA) (Norris *et al.* 1996). PD98059 and LY294002 were purchased from Calbiochem Co. (La Jolla, CA, USA). The inhibitory MAPK (mitogen-activated protein kinase) construct was kindly provided by Dr A Gutierrez-Hartmann (University of Colorado, Denver, CO, USA) (Conrad *et al.* 1994) and the dominant negative Ras was kindly provided by Dr J Baldassare (St Louis University, St Louis, MO, USA) (Weber *et al.* 1997). β -Galactosidase activity in cotransfection studies was determined using an assay kit purchased from InVitrogen (Carlsbad, CA, USA). Acetic acid (0.1 M) was used as solvent for growth factors, EGF, IGF and TGF α which were purchased from Sigma Chemical Co. All other chemicals and biochemicals were the highest quality available from commercial sources.

Oligonucleotides derived from the ADA gene promoter and a consensus Sp1 oligonucleotide were synthesized by the Gene Technologies Laboratory, Texas A&M University (College Station, TX, USA). Structures of these oligonucleotides (sense strands) are summarized below and the putative GC-rich sites are underlined. Mutations incorporated in the mutant oligonucleotides are denoted by an asterisk.

ADASp1·4 (–86/–65)
 5'-AGC TTG GCG AGA GGG CGG GCC CCG GGA G-3'
 ADASp1·4 m (–86/–65)
 5'-AGC TTG GCG AGA GT*T* A*A*A* GCC CCG GGA G-3'
 Sp1 (consensus)
 5'-AGC TTA TTC GAT CGG GGC GGG GCG AGC G-3'

Cloning

The pBLTATA-CAT plasmid was made by digesting the pBLCAT2 vector with BamHI and XhoI to remove the thymidine kinase promoter; the double-stranded E1B oligonucleotide containing complementary 5' overhangs was then inserted into the corresponding sites. ADASp1·4 and ADASp1·4 m oligonucleotides were cloned into the pBLTATA-CAT vector at the HindIII and BamHI sites to give the pADASp1·4 and pADASp1·4 m constructs respectively, as described (Xie *et al.* 1999).

Northern blot analysis

ADA and β -tubulin gene cDNAs were purchased from ATCC. ADA mRNA levels were measured by using a 1·1 kb HindIII fragment of the human ADA cDNA. β -Tubulin mRNA levels were measured by using a 1·1 kb EcoRI fragment of human β -tubulin cDNA. RNA was extracted from MCF-7 cells after treatment with vehicle (control) or 10 nM IGF using the RNazol B method (Tel-Test Inc., Friendswood, TX, USA) followed by electrophoretic separation on a 1·2% agarose gel, then transferred to a nylon membrane. The membrane was then exposed to u.v. light for 5 min to crosslink RNA to the membrane and baked at 80 °C for 2 h. The membrane was prehybridized in a solution containing 0·1% BSA, 0·1% Ficoll, 0·1% polyvinylpyrrolidone, 10% dextran sulfate, 1% SDS and 5 $\times$ SSPE (0·75 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA) for 18–24 h at 65 °C and hybridized in the same buffer for 24 h with the ³²P-labeled DNA probe (10⁶ c.p.m./ml). DNA probes were labeled with α -³²P-dCTP using the random primed DNA labeling kit (Boehringer-Mannheim, Indianapolis, IN, USA). The resulting blots were quantitated using an Instant Imager System (Packard Instruments, Downers Grove, IL, USA) and visualized by autoradiography using X-Omat film (Eastman Kodak, Co., Rochester, NY, USA). ADA mRNA levels were standardized against β -tubulin mRNA.

Transient transfection assay

MCF-7 cells were transfected utilizing the calcium phosphate method with 10 μ g of the ADA gene promoter-derived constructs and 5 μ g of wild-type

or variant ER expression plasmids; in the absence of cotransfected wild-type ER, no hormone-responsiveness was observed and this was due to overexpression of the ADA promoter-derived constructs. β -Galactosidase-lacZ plasmid (5·0 μ g) obtained from InVitrogen was cotransfected in studies determining differences in basal CAT activities with constructs containing ADA gene promoter inserts; activities were corrected for transfection efficiencies. pCDNA3-Neo (InVitrogen) was utilized as an empty vector (control) and was also added in some experiments to maintain uniform levels of added DNA. Transfection efficiency was high and no additional shock was required. After 18 h, media were changed and cells were treated with vehicle (0·1 M acetic acid), different concentrations of EGF, IGF or TGF α alone or in combination with inhibitors PD98059 (50 nM) or LY294002 (50 μ M) for 44 h. Cells were then washed with PBS and scraped from the plates. Cell lysates were prepared in 0·15 ml of 0·25 M Tris-HCl (pH 7·5) by three freeze-thaw-sonication cycles (3 min each). Protein concentrations were determined using BSA as a standard and analysis for CAT activity in cell lysates utilized a constant amount of protein from each treatment group. Lysates were incubated at 56 °C for 7 min to remove endogenous deacetylase activity. CAT activity was determined by incubating aliquots of the cell lysates with 0·2 mCi D-threo-[dichloroacetyl-1-¹⁴C]chloramphenicol and 4 mM acetyl CoA. Acetylated products were visualized and quantitated using an Instant Imager System (Packard Instruments). CAT activity in various treatment groups was calculated as fraction of that observed in cells treated with vehicle alone (arbitrarily set at 100) and results are expressed as means $\pm$ s.d. At least three separate experiments were carried out for each treatment group.

Western immunoblotting

Nonphosphorylated and phosphorylated Erk-1, and Akt antibodies were purchased from New England Biolabs (Beverly, MA, USA). MCF-7 cells were incubated in serum-free media for 36 h and were then treated with 10 nM IGF in the presence or absence of 50 nM PD98059 or 50 μ M LY294002 for 10 min. Cells were washed once in ice-cold PBS and collected by scraping in 0·3 ml ice-cold lysis buffer (50 mM HEPES, pH 7·5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1·5 mM MgCl₂, 1 mM EGTA, 10 μ g/ml aprotinin, 50 mM phenylmethylsulfonylfluoride, 50 mM sodium orthovanadate). The lysates were incubated on ice for 1 h with intermittent vortexing followed by centrifugation

(15 000 g, 5 min). Equal amounts of protein from each treatment group were separated by SDS-PAGE (7.5% gel, 150 V) and electrophoresed (15 V, overnight) to a PVDF membrane using a BioRad Trans-Blot Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA); the transfer buffer was 48 mM Tris, 39 mM glycine, 0.025% SDS. Subsequent procedures followed the manufacturer's recommendations (New England Biolabs).

Statistics

Results are expressed as means $\pm$ s.d. for three separate determinations for each data point. Statistical significance ($P < 0.05$) was determined by performing ANOVA and Scheffé's *post hoc* test.

RESULTS

Activation of ADA by growth factors in MCF-7 cells

Initial studies showed that IGF induced ADA gene expression (>2.50 -fold) in MCF-7 cells, thus confirming that both growth factors and E₂ (Xie *et al.* 1999) induce this gene (Fig. 1A). Previous studies have demonstrated that a 232 bp region encompassing the -211 to $+11$ region of the ADA gene promoter confers high basal activity on reporter genes after transfection in several different cell lines including MCF-7 cells and in transgenic mouse studies (Aronow *et al.* 1992, Dusing & Wiginton 1994, Xie *et al.* 1999). In transfection studies in MCF-7 cells using pADA211 (containing the -211 to $+11$ ADA gene promoter insert), 10 nM concentrations of E₂, EGF, IGF and TGF α alone did not induce CAT (reporter gene) activity. Previous studies showed that E₂-responsiveness of this construct was dependent on cotransfection with ER α in MCF-7 cells due to high overexpression of the construct in transfected cells resulting in insufficient amounts of endogenous ER α . Similar results have been observed with other E₂-responsive genes containing functional GC-rich sites for ER α /Sp1 interactions (Porter *et al.* 1996, Duan *et al.* 1998, Sun *et al.* 1998, F. Wang *et al.* 1998, W. Wang *et al.* 1999, Dong *et al.* 1999, Qin *et al.* 1999, Xie *et al.* 1999, 2000) and also constructs derived from nonconsensus ERE-dependent genes including pS2, c-myc, cathepsin D, retinoic acid receptor α 1, and the progesterone receptor (PR) (Savouret *et al.* 1991, Dubik & Shiu 1992, Cavailles *et al.* 1993, Krishnan *et al.* 1994, Zacharewski *et al.* 1994, Rishi *et al.* 1995, Wang *et al.* 1997). Treatment of MCF-7 cells with 10 nM E₂ and IGF after cotransfection with pADA211 plus ER α expression

plasmid resulted in induction of CAT activity whereas, at this concentration, a significant induction response was not observed for TGF α or EGF. Previous studies reported a similar differential growth factor (IGF vs TGF α /EGF) induction response using E₂-responsive constructs containing cathepsin D gene promoter inserts (Vignon *et al.* 1987, Freiss *et al.* 1990, Wang *et al.* 2000). However, the results summarized in Fig. 1C show that over a wider range of doses all three growth factors activate pADA211 in MCF-7 cells (cotransfected with ER α), but IGF is the most potent inducer.

The -211 to $+11$ region of the ADA gene promoter contains multiple GC-rich sites, and only one of these sites at -79 to -73 (site IV) is required for hormone-induced ER α /Sp1 action. IGF induces CAT activity in MCF-7 cells transfected with pADA211, pADA81 and pADA56 (Fig. 2A), and only the former two constructs contain GC-rich site IV that is required for E₂-responsiveness, suggesting that IGF-induced, ligand-independent ER α /Sp1 action involves multiple GC-rich sites within the proximal region of the ADA gene promoter. The importance of GC-rich sites and Sp1 protein were also investigated using pADASp1, a construct containing a single GC-rich sequence (i.e. site IV) and a second plasmid mutated in the GC-rich sites. IGF induced CAT activity only with pADASp1, indicating that an induction response required an intact Sp1 binding site. The importance of Sp1 protein in mediating IGF-induced transactivation of pADA211 was confirmed by showing that the induced response in MCF-7 cells transfected with a dominant negative form of Sp1 (pEBG-Sp1) was significantly decreased (Fig. 2B). Thus, growth factor-induced transactivation of pADA211 requires both ER α and Sp1 proteins and one or more GC-rich promoter elements.

Role of IGF-induced phosphorylation of ER α

The role of growth factor-induced MAPK signaling on ligand-independent ER α /Sp1 action was determined in MCF-7 cells transfected with ER α expression plasmid, pADA211 and treated with the MAPK kinase inhibitor PD98059 (Fig. 3A) or cotransfected with dominant negative Ras or MAPK expression plasmids (Fig. 3B). The results show that PD98059 and both dominant negative expression plasmids inhibit IGF-induced reporter gene activity and confirm the importance of MAPK activation. Previous studies showed that phosphorylation of Ser¹¹⁸ and Ser¹⁶⁷ are important for ligand-independent activation of ER α by growth

FIGURE 1. Transcriptional activation of ADA in MCF-7 cells. (A) Induction of ADA mRNA levels. MCF-7 cells were treated with 10 nM IGF for 6, 12 and 24 h, and ADA mRNA levels were determined by Northern blot analysis as described in the Materials and Methods. Both bands were used for determining mRNA levels in each treatment group. Significant induction ($P < 0.05$) was observed at all time points. (B/C) Activation of pADA211 by growth factors. MCF-7 cells were transfected with pADA211 in the presence or absence of ER α expression plasmids, treated with 10 nM E₂ or growth factors (B) or different concentrations of growth factors (C), and CAT activity was determined as described in the Materials and Methods. Significant induction ($P < 0.05$) compared with vehicle control is indicated with an asterisk. Results are expressed as means $\pm$ s.d. for three separate experiments for each treatment group. * $P < 0.05$.

FIGURE 2. Induction of ADA promoter constructs by IGF. (A) Deletion analysis. Different constructs were transfected into MCF-7 cells, treated with 10 nM IGF and CAT activity determined as described in the Materials and Methods. Significant induction ($P<0.05$) was observed for all constructs except pADA Δ Sp1m. (B) Effects of dominant negative Sp1 expression (pEBG-Sp1). MCF-7 cells were transfected with pADA211 and empty vector (pEBG) or dominant negative Sp1 expression plasmid (pEBG-Sp1), treated with 10 nM IGF, and CAT activity was determined as described in the Materials and Methods. IGF significantly ($P<0.05$) induced CAT activity and this response was blocked by dominant negative Sp1. Results are expressed as means $\pm$ S.D. for at least three separate experiments for each treatment group. * $P<0.05$.

factors and are required for ERE-dependent transactivation (Kato *et al.* 1995, Joel *et al.* 1998). The roles of Ser¹¹⁸ and Ser¹⁶⁷ in IGF activation were determined in MCF-7 cells transfected with pADA211, wild-type ER α , or the serine mutant mER α -S118, mER α -S167 and mER α -S118/167 (both serines mutated) expression plasmids. The results showed that IGF-mediated transactivation was observed only with wild-type ER α and that mutation of either Ser¹¹⁸ or Ser¹⁶⁷ resulted in loss of ligand-independent ER α /Sp1 action (Fig. 3C). In contrast, all three Ser mutants of ER α activated the human C3-luc construct in MCF-7 cells treated with 10 nM E₂ (Fig. 3D).

IGF activation of Akt and MAPK and induction of pADA211

The interplay between IGF and activation of phosphatidylinositol-3 kinase (PI3-K), Akt and MAPK kinase pathways in breast cancer cells has previously been reported (Dufourny *et al.* 1997,

Ahmad *et al.* 1999, Zimmermann & Moelling 1999) and the results in Fig. 4A,B summarize the effects of IGF on phosphorylation of Erk-1, Erk-2 and Akt. Ten nM IGF alone induces phosphorylation of Erk-1, Erk-2 and Akt and, in cells cotreated with IGF plus the MAPKK inhibitor PD98059, there was significant inhibition of Erk-1 and Erk-2 phosphorylation, whereas levels of immunoreactive Akt phosphoprotein were unchanged. In contrast, cotreatment with IGF and the PI3 kinase inhibitor LY294002 resulted in enhanced phosphorylation of Erk-1 and Erk-2 and inhibition of Akt phosphorylation. Enhanced phosphorylation of Erk-1 and Erk-2 in breast cancer cells by LY294002 has previously been reported by Zimmerman & Moelling (1999) who also showed that this was accompanied by increased Raf kinase-dependent activity. The results in Fig. 4 indicate that IGF stimulation of Akt phosphorylation is not required for activation of pADA211 and the results also show that levels of Erk-1/-2 phosphorylation (Fig. 3A) do not correlate with pADA211 activation (Fig. 3C).

FIGURE 3. IGF activation of pADA211: effects of kinase inhibitors and Ser¹¹⁸ and Ser¹⁶⁷ ER α mutants. MCF-7 cells were transfected with ER α expression plasmid, pADA211, and treated with 10 nM IGF and PD98059 (A) and dominant negative ras (dnRas) or inhibitory MAPK (iMAPK) expression plasmids (B), and CAT activity was determined as described in the Materials and Methods. IGF-induced activity was significantly inhibited ($P < 0.05$) after treatment with PD98059, dominant negative ras, or inhibitory MAPK expression plasmids. (C) Activation using Ser mutants of ER α . Activation of pADA211 was carried out as described above except that cells were cotransfected with ER α expression plasmids mutated at serines 118 and/or 167. Induction was not observed using mutant ER α expression plasmids. (D) Activation of pC3-luc. Using the same protocol as described above, E₂ significantly activated pC3-luc in MCF-7 cells cotransfected with ER α or serine mutants of ER α , demonstrating that these ER α mutants were active in this cell line. Results are expressed as means $\pm$ s.d. * $P < 0.05$.

For example, in MCF-7 cells transfected with pADA211, treatment with 50 μ M LY294002 plus IGF induced CAT activity similar to that observed after treatment with IGF alone (Fig. 4C), even though the cotreatment resulted in significantly higher Erk-1/-2 phosphorylation (Fig. 4A). Nevertheless, these data indicate that ligand-independent activation of pADA211 by IGF is primarily dependent on the ras-MAPK pathway and phosphorylation of the AF1 domain of ER α (Fig. 5).

DISCUSSION

Growth factor polypeptides, insulin and E₂ induce proliferation of ER-positive cancer cells lines, and pure antiestrogens such as ICI 182 780 can inhibit both E₂- and growth factor-mediated cell proliferation (Wakeling *et al.* 1989, Freiss *et al.* 1990, de Cupis *et al.* 1995, Ahmad *et al.* 1999, Zimmermann & Moelling 1999). Peptide growth factor activation of ER α and E₂-responsive genes has been observed

FIGURE 4. Modulation of kinases and pADA211 by IGF. Activation of Erk-1/-2 (A) and Akt (B) by IGF. Whole cell extracts were obtained from MCF-7 cells treated with solvent control (Ctrl), 10 nM IGF in the presence or absence of 50 nM PD98059 or 50 μ M LY294002, and immunoblot analyses of phosphorylated ERK-1/-2 and Akt, and their total proteins were carried out as described in the Materials and Methods. PD98059 inhibited IGF-induced phosphorylation of Erk-1/-2 but not Akt; LY294002 enhanced IGF-induced phosphorylation of Erk-1/-2 but inhibited Akt phosphorylation. Total immunoreactive Akt and Erk-1/-2 were not affected by the various treatments. (C) Effects of LY294002 on pADA211 activation by IGF. MCF-7 cells were transfected with pADA211 and 10 nM IGF, 50 μ M LY294002, and their combination significantly ($P<0.05$) induced CAT activity; results are expressed as means $\pm$ s.d. for three separate determinations. * $P<0.05$.

FIGURE 5. Pathways for growth factor activation of ER α and ADA expression in MCF-7 cells.

in several studies (Cavaillès *et al.* 1989, Ignar-Trowbridge *et al.* 1992, 1993, 1996, Aronica & Katzenellenbogen 1993, Kato *et al.* 1995, Hafner *et al.* 1996, El-Tanani & Green 1997, Lee *et al.* 1997, Joel *et al.* 1998). Growth factor activation of ER α and subsequent ligand-independent transactivation of ERE-dependent constructs is associated with phosphorylation of Ser¹¹⁸ and/or Ser¹⁶⁷ in activation function 1 (AF1) (Kato *et al.* 1995, Joel *et al.* 1998). A recent study in this laboratory demonstrated that in MCF-7 cells transiently transfected with E₂-responsive constructs derived from the cathepsin D gene promoter, IGF activated gene expression through different response elements including at least four GC-rich sites (Wang *et al.* 2000). Moreover, IGF activated ER α /Sp1 (ligand-independent) through binding GC-rich elements that were not functional for ligand-dependent activation of the same constructs in the MCF-7 cells (Krishnan *et al.* 1994, F. Wang *et al.* 1997, F. Wang *et al.* 1998).

The proximal region (−211 to +11) of the ADA gene promoter contains six (I–VI) GC-rich sites that bind Sp1 protein and are required for high basal gene expression (Aronow *et al.* 1992, Dusing & Wiginton 1994, Xie *et al.* 1999). E₂ induces ADA gene expression and analysis of the ADA gene promoter showed that only GC-rich site IV (−79 to −73) was important for functional ER α /Sp1 interactions (Xie *et al.* 1999). IGF, EGF and TGF α induced reporter gene activity in MCF-7 cells transfected with pADA211 (Fig. 1B); IGF was the most active growth factor in the transient transfection assays, and IGF also induced ADA mRNA levels (Fig. 1A). Growth factor induction in cells transfected with pADA211 was dependent on cotransfection with ER α (Fig. 1B), and the results obtained with PD98059 (a MAPKK inhibitor) (Fig. 3A), dominant negative Ras, and MAPK expression plasmids (Fig. 3B) are consistent with growth factor activation of ER α through the Ras–MAPK pathway. E₂ activates functional ER α /Sp1 interactions

only at site IV in the ADA gene promoter in MCF-7 cells (Xie *et al.* 1999), whereas results of this study (Fig. 2A) suggest that multiple GC-rich sites within the -211 to +11 region of the promoter are functional for growth factor-induced ER α /Sp1 action (ligand-independent). Moreover, mutation of Ser¹¹⁸ or Ser¹⁶⁷ in ER α resulted in loss of growth factor-induced activity, confirming previous reports showing the importance of phosphorylation of these amino acid side chains for ligand-independent ER α action (Kato *et al.* 1995, Joel *et al.* 1998).

The mechanisms of growth factor activation of E₂-responsive genes have not been extensively investigated. IGF induces the ras-MAPK and PI3-K pathways in breast cancer cells, and these responses are blocked not only by specific kinase inhibitors but also by the pure ICI antiestrogens (Ahmad *et al.* 1999, Razandi *et al.* 1999). Dufourny and co-workers (1997) reported that the growth stimulatory effects of IGF are accompanied by increased cyclin D1 synthesis and hyperphosphorylation of retinoblastoma protein, and these responses are strongly inhibited by the PI3-K inhibitor LY294002. IGF activation of ras also increases Akt (protein kinase B) phosphorylation in breast cancer cells, and cotreatment with PI3-K inhibitors not only blocked Akt phosphorylation but induced phosphorylation of MAPK. This latter response has been linked to Akt-dependent phosphorylation of Raf-Ser²⁵⁹ to give a deactivated form of Raf (Zimmermann & Moelling 1999) and, thus, inhibition of PI3-K enhanced the MAPK pathway. The results in Fig. 3 demonstrate that transactivation of pADA211 in MCF-7 cells treated with IGF is inhibited by overexpression of dominant negative ras and MAPK and by PD98059 (a MAPKK inhibitor) suggesting that the ras-MAPK pathway plays an important role in ligand-independent activation of ER α /Sp1. Kinase inhibition studies indicate that Akt phosphorylation is not important for IGF activation of ER α /Sp1; however, increased phosphorylation of Erk-1/-2 does not correlate with increased transactivation of pADA211, and this was particularly evident in cells cotreated with IGF and LY294002. Moreover, the PI3-K inhibitor alone enhances transactivation in cells transfected with pADA211 and current studies are investigating pathways associated with this response.

Previous studies indicated that IGF-induced cell proliferation is blocked by PI3-K inhibitors but not by PD98059 (Dufourny *et al.* 1997, Ahmad *et al.* 1999, Zimmermann & Moelling 1999). This suggests that growth factor activation of ADA in MCF-7 cells through the ras-MAPK pathway may

not be critical for cell proliferation. Since genes activated through the PI3-K pathway may be more directly related to growth factor-induced proliferation of MCF-7 cells, we are currently investigating the relative contributions of Ras-PI3-K and Ras-MAPK pathways for growth factor activation of several E₂-responsive genes as a marker for their roles in the mitogenic response.

ACKNOWLEDGEMENTS

The financial assistance of the National Institutes of Health (ES09253 and ES09106) and the Texas Agricultural Experiment Station is gratefully acknowledged. S Safe is a Sid Kyle Professor of Toxicology.

REFERENCES

- Ahmad S, Singh N & Glazer RI 1999 Role of AKT1 in 17 β -estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. *Biochemical Pharmacology* **58** 425-430.
- Aronica SM & Katzenellenbogen BS 1993 Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Molecular Endocrinology* **7** 743-752.
- Aronow BJ, Silbiger RN, Dusing MR, Stock JL, Yager KL, Potter SS, Hutton JJ & Wiginton DA 1992 Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability to generate position-independent transgene expression. *Molecular and Cellular Biology* **12** 4170-4185.
- Bates SE, Davidson NE, Valverius EM, Freter CE, Dickson RB, Tam JP, Kudlow JE, Lippman ME & Salomon DS 1988 Expression of transforming growth factor- α and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance. *Molecular Endocrinology* **2** 543-555.
- Cavaillès V, Garcia M & Rochefort H 1989 Regulation of cathepsin D and pS2 gene expression by growth factors in MCF-7 human breast cancer cells. *Molecular Endocrinology* **3** 552-558.
- Cavaillès V, Augereau P & Rochefort H 1993 Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription. *PNAS* **90** 203-207.
- Conrad KE, Oberwetter JM, Vaillancourt R, Johnson GL & Gutierrez-Hartmann A 1994 Identification of the functional components of the Ras signaling pathway regulating pituitary cell-specific gene expression. *Molecular and Cellular Biology* **14** 1553-1565.
- de Cupis A, Noonan D, Pirani P, Ferrera A, Clerico L & Favoni RE 1995 Comparison between novel steroid-like and conventional nonsteroidal antioestrogens in inhibiting oestradiol- and IGF-I-induced proliferation of human breast cancer-derived cells. *British Journal of Pharmacology* **116** 2391-2400.
- Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF & Korach KS 1996 Physiological coupling of growth factor and steroid receptor signaling pathways:

- estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *PNAS* **93** 12626–12630.
- Das SK, Tsukamura H, Paria BC, Andrews GK & Dey SK 1994 Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse uterus. *Endocrinology* **134** 971–981.
- Dickson RB & Lippman ME 1988 Control of human breast cancer by estrogen, growth factors, and oncogenes. In *Breast Cancer: Cellular and Molecular Biology*, pp 119–166. Eds ME Lippman & RB Dickson. Boston: Kluwer.
- Dickson RB & Lippman ME 1991 Growth regulation of normal and malignant breast epithelium. In *The Breast*, pp 363–394. Eds KI Bland & EM Copeland. Philadelphia: WB Saunders.
- Dickson RB & Lippman ME 1995 Growth factors in breast cancer. *Endocrine Reviews* **16** 559–589.
- Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Kladde M, Vyhldal C & Safe S 1999 Mechanisms of transcriptional activation of *bcl-2* gene expression by 17 β -estradiol in breast cancer cells. *Journal of Biological Chemistry* **174** 32099–32107.
- Duan R, Porter W & Safe S 1998 Estrogen-induced *c-fos* protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* **139** 1981–1990.
- Dubik D & Shiu RPC 1992 Mechanism of estrogen activation of *c-myc* oncogene expression. *Oncogene* **7** 1587–1594.
- Dufourny B, Alblas J, van Teeffelen HA, van Schaik FM, Van der Burg B, Steenbergh PH & Sussenbach JS 1997 Mitogenic signaling of insulin-like growth factor I in MCF-7 human breast cancer cells requires phosphatidylinositol 3-kinase and is independent of mitogen-activated protein kinase. *Journal of Biological Chemistry* **272** 31163–31171.
- Dusing MR & Wiginton DA 1994 Sp1 is essential for both enhancer-mediated and basal activation of the TATA-less human adenosine deaminase promoter. *Nucleic Acids Research* **22** 669677.
- El-Tanani MK & Green CD 1997 Two separate mechanisms for ligand-independent activation of the estrogen receptor. *Molecular Endocrinology* **11** 928–937.
- Fernandez P, Burghardt R, Smith R, Nodland K & Safe S 1994 High passage T47D human breast cancer cells: altered endocrine and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin responsiveness. *European Journal of Pharmacology* **270** 53–66.
- Foster JS & Wimalasena J 1996 Estrogen regulates activity of cyclin-dependent kinases and retinoblastoma protein phosphorylation in breast cancer cells. *Molecular Endocrinology* **10** 488–498.
- Freiss G, Rochefort H & Vignon F 1990 Mechanisms of 4-hydroxytamoxifen anti-growth factor activity in breast cancer cells: alterations of growth factor receptor binding sites and tyrosine kinase activity. *Biochemical and Biophysical Research Communications* **173** 919–926.
- Furlanetto RW & DiCarlo JN 1984 Somatomedin C receptors and growth effects in human breast cancer cells maintained in long term tissue culture. *Cancer Research* **44** 2122–2128.
- Gannon F, Katzenellenbogen B, Stancel G & Gorski J 1976 Estrogen-receptor movement to the nucleus: discussion of a cytoplasmic-exclusion hypothesis. *Symposium of the Society for Developmental Biology* **34** 137–149.
- Hafner F, Holler E & von Angerer E 1996 Effect of growth factors on estrogen receptor mediated gene expression. *Journal of Steroid Biochemistry and Molecular Biology* **58** 385–393.
- Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA & Korach KS 1992 Coupling of dual signaling pathways – epidermal growth factor action involves the estrogen receptor. *PNAS* **89** 4658–4662.
- Ignar-Trowbridge DM, Teng CT, Ross MJ, Parker MG, Korach KS & McLachlan JA 1993 Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. *Molecular Endocrinology* **7** 992–998.
- Ignar-Trowbridge DM, Pimentel M, Parker MG, McLachlan JA & Korach KS 1996 Peptide growth factor cross-talk with the estrogen receptor requires the A/B domain and occurs independently of protein kinase C or estradiol. *Endocrinology* **137** 1735–1744.
- Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J & Lannigan DA 1998 pp90^{sk1} regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Molecular and Cellular Biology* **18** 1978–1984.
- Karey KP & Sirbasku DA 1988 Differential responsiveness of human breast cancer cell lines MCF-7 and T47D to growth factors and 17 β -estradiol. *Cancer Research* **48** 4083–4092.
- Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E & Kawashima H 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** 1491–1494.
- Kato S, Kitamoto T, Masuhiro Y & Yanagisawa J 1998 Molecular mechanism of a cross-talk between estrogen and growth-factor signaling pathways. *Oncology* **55** 5–10.
- Krishnan V, Wang X & Safe S 1994 Estrogen receptor–Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. *Journal of Biological Chemistry* **269** 15912–15917.
- Lee AV, Weng CN, Jackson JG & Yee D 1997 Activation of estrogen receptor-mediated gene transcription by IGF-I in human breast cancer cells. *Journal of Endocrinology* **152** 39–47.
- Musgrove EA, Hamilton JA, Lee CS, Sweeney KJ, Watts CK & Sutherland RL 1993 Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D human breast cancer cell cycle progression. *Molecular and Cellular Biology* **13** 3577–3587.
- Norris JD, Fan DJ, Wagner BL & McDonnell DP 1996 Identification of the sequences within the human complement 3 promoter required for estrogen responsiveness provides insight into the mechanism of tamoxifen mixed agonist activity. *Molecular Endocrinology* **10** 1605–1616.
- Petz LN & Nardulli AM 2000 Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. *Molecular Endocrinology* **14** 972–985.
- Planas-Silva MD & Weinberg RA 1997 Estrogen-dependent cyclin E-cdk2 activation through p21 redistribution. *Molecular and Cellular Biology* **17** 4059–4069.
- Porter W, Wang F, Wang W, Duan R & Safe S 1996 Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. *Molecular Endocrinology* **10** 1371–1378.
- Prall OWJ, Sarcevic B, Musgrove EA, Watts CKW & Sutherland RL 1997 Estrogen-induced activation of Cdk4 and Cdk2 during G₁–S phase progression is accompanied by increased cyclin D1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-Cdk2. *Journal of Biological Chemistry* **272** 10882–10894.
- Pratt MA, Satkunaratnam A & Novosad DM 1998 Estrogen activates raf-1 kinase and induces expression of Egr-1 in MCF-7 breast cancer cells. *Molecular and Cellular Biochemistry* **189** 119–125.
- Qin C, Singh P & Safe S 1999 Transcriptional activation of insulin-like growth factor binding protein 4 by 17 β -estradiol

- in MCF-7 cells: role of estrogen receptor–Sp1 complexes. *Endocrinology* **140** 2501–2508.
- Razandi M, Pedram A, Greene GL & Levin ER 1999 Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER α and ER β expressed in Chinese hamster ovary cells. *Molecular Endocrinology* **13** 307–319.
- Rishi AK, Shao ZM, Baumann RG, Li XS, Sheikh MS, Kimura S, Bashirelahi N & Fontana JA 1995 Estradiol regulation of the human retinoic acid receptor α gene in human breast carcinoma cells is mediated via an imperfect half-palindromic estrogen response element and Sp1 motifs. *Cancer Research* **55** 4999–5006.
- Salvatori L, Ravenna L, Felli MP, Cardillo MR, Russo MA, Frati L, Gulino A & Petrangeli E 2000 Identification of an estrogen-mediated deoxyribonucleic acid-binding independent transactivation pathway on the epidermal growth factor receptor gene promoter. *Endocrinology* **141** 2266–2274.
- Savouret JF, Bailly A, Misrahi M, Rarch C, Redeuilh G, Chauchereau A & Milgrom E 1991 Characterization of the hormone responsive element involved in the regulation of the progesterone receptor gene. *EMBO Journal* **10** 1875–1883.
- Smith CL 1998 Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biology of Reproduction* **58** 627–632.
- Sun G, Porter W & Safe S 1998 Estrogen-induced retinoic acid receptor α 1 gene expression: role of estrogen receptor–Sp1 complex. *Molecular Endocrinology* **12** 882–890.
- Tanaka N, Yonekura H, Yamagishi S, Fujimori H, Yamamoto Y & Yamamoto H 2000 The receptor for advanced glycation end products is induced by the glycation products themselves and tumor necrosis factor- α through nuclear factor- κ B, and by 17 β -estradiol through Sp-1 in human vascular endothelial cells. *Journal of Biological Chemistry* **275** 25781–25790.
- Vignon F, Bouton M-M & Rochefort H 1987 Antiestrogens inhibit the mitogenic effect of growth factors on breast cancer cells in the total absence of estrogens. *Biochemical and Biophysical Research Communications* **146** 1502–1508.
- Wakeling AE, Newbould E & Peters SW 1989 Effects of antioestrogens on the proliferation of MCF-7 human breast cancer cells. *Journal of Molecular Endocrinology* **2** 225–234.
- Wang F, Porter W, Xing W, Archer TK & Safe S 1997 Identification of a functional imperfect estrogen responsive element in the 5'-promoter region of the human cathepsin D gene. *Biochemistry* **36** 7793–7801.
- Wang F, Hoivik D, Pollenz R & Safe S 1998 Functional and physical interactions between the estrogen receptor–Sp1 and the nuclear aryl hydrocarbon receptor complexes. *Nucleic Acids Research* **26** 3044–3052.
- Wang F, Duan R, Chirgwin J & Safe S 2000 Transcriptional activation of cathepsin D gene expression by growth factors. *Journal of Molecular Endocrinology* **24** 193–202.
- Wang W, Smith R & Safe S 1998 Aryl hydrocarbon receptor-mediated antiestrogenicity in MCF-7 cells: modulation of hormone-induced cell cycle enzymes. *Archives of Biochemistry and Biophysics* **356** 239–248.
- Wang W, Dong L, Saville B & Safe S 1999 Transcriptional activation of E2F1 gene expression by 17 β -estradiol in MCF-7 cells is regulated by NF-Y–Sp1/estrogen receptor interactions. *Molecular Endocrinology* **13** 1373–1387.
- Weber JD, Hu W, Jefcoat SCJ, Raben DM & Baldassare JJ 1997 Ras-stimulated extracellular signal-related kinase 1 and RhoA activities coordinate platelet-derived growth factor-induced G1 progression through the independent regulation of cyclin D1 and p27^{KIP1}. *Journal of Biological Chemistry* **272** 32966–32971.
- Weigel NL & Zhang Y 1998 Ligand-independent activation of steroid hormone receptors. *Journal of Molecular Medicine* **76** 469–479.
- Westley BR & May FEB 1994 Role of insulin-like growth factors in steroid-modulated proliferation. *Journal of Steroid Biochemistry and Molecular Biology* **51** 1–9.
- Wilding G, Lippman ME & Gelmann EP 1988 Effects of steroid hormones and peptide growth factors on protooncogene *c-fos* expression in human breast cancer cells. *Cancer Research* **48** 802–805.
- Xie W, Duan R & Safe S 1999 Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor–Sp1 interactions. *Endocrinology* **140** 219–227.
- Xie W, Duan R, Chen I, Samudio I & Safe S 2000 Transcriptional activation of thymidylate synthase by 17 β -estradiol in MCF-7 human breast cancer cells. *Endocrinology* **141** 2439–2449.
- Zacharewski TR, Bondy KL, McDonnell P & Wu ZF 1994 Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on 17 β -estradiol-induced pS2 expression. *Cancer Research* **54** 2707–2713.
- Zimmermann S & Moelling K 1999 Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286** 1741–1744.

RECEIVED 10 November 2000

ACCEPTED 19 February 2001