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

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ABSTRACT

Adenosine deaminase (ADA) regulates cellular levels of adenosine and deoxyadenosine, and 17β-estradiol (E2) 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 (ERα) was further investigated. IGF and other polypeptide growth factors induce reporter gene expression in MCF-7 cells cotransfected with ERα expression plasmid and pADA211, a construct containing the −211 to +11 region of the ADA gene promoter which is required for high basal and E2-inducible activity. Deletion analysis of this promoter demonstrates that IGF activates ERα/Sp1 interactions with multiple GC-rich sites in the promoter and this response is abrogated in cells transfected with ERα 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 ERα/Sp1 is dependent on the MAPK signaling pathway.

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INTRODUCTION


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. 1998, El-Tanani & Green 1997).

Research in this laboratory has identified a number of E\(_2\)-responsive genes which are ligand (E\(_2\))-activated through interaction of ER\(\alpha\)/Sp1 with specific GC-rich promoter elements. This induction process involves ER\(\alpha\)-protein (Sp1) but not ER\(\alpha\)-DNA interactions and gene promoters that are induced by this pathway include cathepsin D, E2F1, IGF binding protein 4, retinoic acid receptor \(\alpha\), thy midylate 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\(\alpha\)/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\(\alpha\)/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\(\alpha\) activation and interactions of ER\(\alpha\)/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\(\alpha\) (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\(\alpha\) mutants, HEGO-S\(^{118}\), HEGO-S\(^{167}\), 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\(\beta\) 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.
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 kg HindIII fragment of the human ADA cDNA. β-Tubulin mRNA levels were measured by using a 1·1 kg 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 × 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 µg of the ADA gene promoter-derived constructs and 5 µg 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 µg) 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 TGFα alone or in combination with inhibitors PD98059 (50 nM) or LY294002 (50 µM) 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 ± 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 µM 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 µg/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 ± 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 TGFα alone did not induce CAT (reporter gene) activity. Previous studies showed that E2-responsiveness of this construct was dependent on cotransfection with ERα in MCF-7 cells due to high overexpression of the construct in transfected cells resulting in insufficient amounts of endogenous ERα. Similar results have been observed with other E2-responsive genes containing functional GC-rich sites for ERα/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 α1, 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 ERα expression plasmid resulted in induction of CAT activity whereas, at this concentration, a significant induction response was not observed for TGFα or EGF. Previous studies reported a similar differential growth factor (IGF vs TGFα/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 ERα), 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 ERα/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 ERα/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 ERα and Sp1 proteins and one or more GC-rich promoter elements.

Role of IGF-induced phosphorylation of ERα
The role of growth factor-induced MAPK signaling on ligand-independent ERα/Sp1 action was determined in MCF-7 cells transfected with ERα 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 ERα 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 ERα 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 ± S.D. for three separate experiments for each treatment group. *$P < 0.05$. 

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factors and are required for ERE-dependent transactivation (Kato et al. 1995, Joel et al. 1998). The roles of Ser\textsuperscript{118} and Ser\textsuperscript{167} in IGF activation were determined in MCF-7 cells transfected with pADA211, wild-type ER\textsubscript{a}, or the serine mutant mER\textsubscript{a}-S118, mER\textsubscript{a}-S167 and mER\textsubscript{a}-S118/167 (both serines mutated) expression plasmids. The results showed that IGF-mediated transactivation was observed only with wild-type ER\textsubscript{a} and that mutation of either Ser\textsuperscript{118} or Ser\textsuperscript{167} resulted in loss of ligand-independent ER\textsubscript{a}/Sp1 action (Fig. 3C). In contrast, all three Ser mutants of ER\textsubscript{a} activated the human C3-luc construct in MCF-7 cells treated with 10 nM E\textsubscript{2} (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 phosphorylation, whereas levels of immunoreactive Akt phosphoprotein were unchanged. 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).
For example, in MCF-7 cells transfected with pADA211, treatment with 50 µM 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 ERα (Fig. 5).

DISCUSSION

Growth factor polypeptides, insulin and E_2 induce proliferation of ER-positive cancer cells lines, and pure antiestrogens such as ICI 182780 can inhibit both E_2- 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 ERα and E_2-responsive genes has been observed...
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 µM 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 µM LY294002, and their combination significantly (P<0.05) induced CAT activity; results are expressed as means ± s.d. for three separate determinations. *P<0.05.

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). E\textsubscript{2} 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 ERα/Sp1 interactions (Xie et al. 1999). IGF, EGF and TGFα 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 ERα (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 ERα through the Ras–MAPK pathway. E\textsubscript{2} activates functional ERα/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 ERα/Sp1 action (ligand-independent). Moreover, mutation of Ser118 or Ser167 in ERα 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 ERα 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 ERα/Sp1. Kinase inhibition studies indicate that Akt phosphorylation is not important for IGF activation of ERα/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.

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