Journal of Cellular Biochemistry 101:1567–1587 (2007)

PD98059 Enhanced Insulin, Cytokine, and Growth Factor Activation of Xanthine Oxidoreductase in Epithelial Cells Involves STAT3 and the Glucocorticoid Receptor

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Abstract PD98059 and U0126 are organic compound inhibitors frequently used to block the activity of the MEK-1/2 protein kinase. In the present work, promoter activation analyses of xanthine oxidoreductase (XOR) in epithelial cells uncovered the unexpected opposite effect of these inhibitors on activation of XOR. Activation of an XOR-luciferase fusion gene was studied in stably transfected epithelial cells. The XOR reporter gene was activated by the epidermal growth factors (EGF), prolactin, and dexamethasone and by the acute phase cytokines (APC) IL-1, IL-6, and TNFα as previously reported for its native gene, and insulin further stimulated activation induced with acute phase cytokines or growth factors. Activation of the proximal promoter was blocked by inhibitors of the glucocorticoid receptor (GR), p38 MAP kinase, and U0126. Unexpectedly, PD98059 activated the promoter and significantly enhanced expression induced by insulin, APC, or growth factors. Analysis of the XOR upstream DNA and proximal promoter revealed primary roles for the GR and STAT3 in mediating the effects of PD98059 on XOR activation and protein complex formation with the promoter. STAT3 phosphotyrosine-705 was rapidly induced by PD98059, dexamethasone, and insulin. XOR activation by PD98059, dexamethasone, or insulin was superinduced by a constitutively active derivative of STAT3, while a dominant negative derivative of STAT3 blocked the enhancing effect of PD98059 on XOR activation. These data demonstrate a previously unrecognized effect of PD98059 on STAT3 and the GR that could have unanticipated consequences when used to infer the involvement of the MEK-1/2 protein kinase. J. Cell. Biochem. 101: 1567–1587, 2007.

Key words: xanthine oxidoreductase; mammary epithelial cell; MEK-1/2 inhibitor; PD98059; insulin; glucocorticoid receptor; STAT3

The pharmacological inhibitors, PD98059 and U0126, have been widely used to infer a physiological role for the MEK-1/2 MAP kinase and its downstream effector MAP kinase, ERK-1/2 [Favata et al., 1998; Davies et al., 2000]. Both drugs are routinely used in cell culture experiments at concentrations from 15 to 50 μM, and although differences in their mechanisms of action have been reported [Favata et al., 1998; Davies et al., 2000] they are commonly used to implicate MEK-1/2 or ERK-1/2 signaling in many different settings.

Xanthine oxidoreductase (XOR) is a molybdoflavoenzyme traditionally recognized as a household gene product that catalyzes the formation of uric acid from xanthine and hypoxanthine as the terminal step in purine

Abbreviations used: XOR, xanthine oxidoreductase; LH, lactogenic hormones; EGF, epidermal growth factor; GR, glucocorticoid receptor; ER, estrogen receptor; PR, progesterone receptor; EMSA, electrophoretic mobility shift assay; C/ EBP, CCAAT/ enhancer binding protein; STAT, signal transducer and activator of transcription; APC, acute phase cytokine; DIP, dexamethasone–insulin–prolactin differentiation medium.

Grant sponsor: National Institutes of Health; Grant number: HL52509 and HL45582; Grant sponsor: Robert and Helen Kleberg Foundation.

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Received 26 September 2006; Accepted 20 December 2006
DOI 10.1002/jcb.21272

degradation. While XOR has been intensively studied as a source of reactive oxygen or nitrogen species [Davies et al., 2000] (ROS/RNS) implicated in ischemia/reperfusion injury and inflammatory disorders [Harrison, 2002; Garattini et al., 2003], XOR was also found to play a critical but poorly understood role in early development and in the biology of the mammary gland. Although not well understood, XOR gene expression, mRNA content, and protein level exhibit biphasic induction by pregnancy and lactation in the mouse mammary gland in parallel with regulation of β-casein, another protein of the MFG [Kurosaki et al., 1996; McManaman et al., 1999; McManaman et al., 2000]. XOR was induced in mammary gland epithelial cells early in pregnancy, further stimulated by lactation, and then precipitiously lost upon the cessation of lactation [Kurosaki et al., 1996; McManaman et al., 1999; McManaman et al., 2000]. In cultured HC11 mammary epithelial cells XOR was induced by the lactogenic hormones (LH) dexamethasone (or cortisol), insulin, and prolactin [Kurosaki et al., 1996; McManaman et al., 2000]. Induction of XOR by prolactin was blocked by the tyrosine kinase inhibitor, genistein, but was stimulated by genistein in untreated cells. Vanadate, an inhibitor of both phosphatase activity and the glucocorticoid receptor (GR) [Modarress et al., 1994; Yang et al., 1997] blocked LH induced XOR expression as did the GR antagonist, RU38486, suggesting a mode of XOR regulation by LH that is mediated by the GR in conjunction with prolactin induced JAK2/STAT5 and tyrosine kinases [Rosen et al., 1999].

XOR gene expression is also regulated in mammary epithelial cells by low dose cycloheximide stress [Kurosaki et al., 1996; Seymour et al., 2006], epidermal growth factors (EGF) and the acute phase cytokines (APC) TNFα, IL-1, IL-6, and IFNγ [Page et al., 1998]. Regulation of XOR by APCs may be of particular significance to mammary gland development and BC since these factors also appear to contribute to epithelial cell proliferation, differentiation, and tumorigenesis [Shea-Eaton et al., 2001; Ben-Baruch, 2003; Parrinello et al., 2005]. Since relatively little is known about XOR activation induced by any of these factors, we have initiated analyses of the rat XOR upstream DNA in stably transfected epithelial cells. Previous detailed analysis of the rat XOR regulatory DNA had identified a proximal promoter and transcription factors mediating basal activation in HeLa and NIH3T3 cells [Chow et al., 1994; Clark et al., 1998a,b], but these experiments did not characterize expression induced by APCs, growth factors, or insulin. Analysis of the XOR reporter and proximal promoter revealed the anticipated activation by APC, growth factors, and insulin but demonstrated a previously unrecognized enhancement of XOR expression by PD98059. While these data suggest a role for STAT3 and the GR in XOR activation by insulin, APC, and growth factors, they also revealed STAT3 and the GR to be involved in PD98059 induced activation.

MATERIALS AND METHODS

Materials and Reagents

Most reagents, buffers, substrates, and PAGE supplies were purchased from Sigma Chemical Company (St. Louis, MO). Media for cell culture were obtained from Gibco/BRL (Bethesda, MD). Antibodies to the glucocorticoid receptor (M-20, P-20) and STAT3 (H-190) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). An additional antibody to the GR (BuGR2) was purchased from Abcam, Inc. (Cambridge, MA). STAT3-phospho-Tyr705 specific antibody (9131) and STAT3-phospho-Ser727 specific antibody (9134) were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Oligonucleotides were synthesized by Gibco/BRL or Integrated DNA Technology (Coralville, IA). The pGL3 luciferase fusion plasmids and beta galactosidase expressing plasmid were obtained from Promega (Madison, WI). The Topo-II T: a cloning vector was obtained from Invitogen (Carlsbad, CA). The Rat Genome Walker Kit (PT1116-2) was purchased from Clontech Laboratories (Palo Alto, CA). O-nitrophenyl beta-D-galactopyranoside, poly dI:dC, and restriction endonucleases were obtained from Roche Molecular Biochemicals (Indianapolis, IN). PD98059, U-0126, insulin, prolactin, and dexamethasone, were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Fetal bovine serum was from Gemini Bioproducts (Woodland, CA).

Cell Culture and Inducer Treatment

L2 rat lung epithelial cells (ATCC # CCL-149) were grown Ham's F12K with 10% fetal calf

serum and 1× of antibiotic/antimycotic solution according to the supplier's instructions. HC-11 mammary epithelial cells were grown in RPMI 1640 containing 2 mM L-glutamine, 2 g/L sodium bicarbonate, pH 7.4, 1× of antibiotic/antimycotic solution, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 10% fetal calf serum [Hynes et al., 1990]. Cells were maintained at 37°C in 95% air/5% CO_2, fed every 2 days, and split 1:4 when at, or near, confluency. Cells were routinely grown to confluency in the presence of insulin and EGF and were then shifted into the above medium in the absence of insulin or EGF and with 2% heat inactivated fetal calf serum. Cells were exposed to cytokine and growth factors or the appropriate vehicles after 2 days of growth in insulin and EGF free medium, or differentiated in dexamethasone, insulin, prolactin (DIP) medium as described [Hynes et al., 1990].

SDS–PAGE and Western Immunoblot Analysis

HC11 cells were grown in six-well plates until confluent, switched to 2% heat inactivated serum in the absence of EGF for 48 h and then treated as indicated. Cells were washed once with ice-cold PBS, resuspended in cell lysis buffer (2 mM DTT, SDS 2.0%, 25 mM β-glycerophosphate, 20 mM Tris-HCl pH 7.5, 10 mM EDTA, and 1:5,000 dilution of the Calbiochem Protease Inhibitor Cocktail set III), and kept on ice. The protein concentration in the supernatant was determined by using the bicinchoninic acid assay (Sigma). Aliquots containing 50 μg of protein were incubated with equal amounts of loading buffer (5% β-mercaptoethanol, 95% Laemmli loading dye) for 10 min at 37°, then boiled for 5 min. Samples were then separated by electrophoresis on 7.5% SDS–PAGE or 4% to 20% gradient SDS–PAGE gels for 40 min at 100 V, transferred to PVDF membranes (Whatman, Inc., Albama). Membranes were blocked overnight at 4° in 5% nonfat dried milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween. Membranes were then incubated with antibodies as indicated. Antigen–antibody complexes were detected by reaction with an ECL Western blotting detection kit according to manufacturer's instruction (Amersham Life Sciences, Piscataway, NJ). Each experiment was run in triplicate, and representative immunoblots are shown.

Rat XOR Upstream DNA Cloning, Deletion Construction, and Expression

The Rat Genome Walker Kit (Clontech) was used to clone upstream DNA from the XOR genomic locus. PvuII cleaved and adaptor ligated Sprague Dawley rat genomic DNA was amplified by polymerase chain reaction (PCR) in two stages as described by the supplier. The gene specific primer used in the first round PCR was 5′-CCTGCTTACCTTTTTGCCATTCAC-3′ and in the second round it was 5′-CACAAAGAA GACCAACTCATCCGC-3′. Sequences were derived from Chow et al. [1994] that were deposited as GenBank AC #U08120. The largest of four PCR products was gel purified and then amplified using the 5′ primer ENDPVU 5′-CATCGACAGCTGACTATAGGGCACGCG-TGGT-3′ which incorporates an adaptor primer with a nested Pvu II cleavage site. This was paired to the primer RXDHCON 5′-GACCAA-CTCATCCGCAGCCATGG TGGCTGCTGGA-GTCAC-3′ which produces two nucleotide substitutions in the rat XOR DNA that convert the translational start site CGATGAC into an Nco1 site CCATGG(C) and allow subsequent fusion to the translational start site of the luciferase gene in the vector pGL3-Basic. The resulting PCR product was cloned into the TopoII vector and sequenced from both directions. The resulting DNA, comprising 2,311 bp has been deposited into GenBank under the accession number DQ104432. Potential transcription factor binding sites were identified by the TESS multifactorial detection software (http://www.cbil.upenn.edu/cgi-bin/tess/) and the TRANSFAC 4.0 database.

Twelve in-frame deletion fusions to the luciferase translational start site in the luciferase expression vector pGL3-Basic (pGL3-B; Promega) were constructed in steps of 200 bp as indicated previously [Clark et al., 1998a,b; Wright et al., 2000]. Briefly, upstream DNA to be cloned in pGL3-B was amplified by PCR using the rat XOR upstream DNA, cloned as pRXORTopoII-5A as a template for PCR. Upstream primers were paired to 3′ primer, RXDHCON. The resulting PCR products were cleaved with XhoI and Nco1 and cloned in the forward orientation in pGL3-B. The resulting deletions have been designated here by the location of each 5′ end relative to the "A" (+1) in the translational start ATG for rat XOR, and the clones designated pRXD-B2 to pRXD-B11. An

Nco1 site present upstream of the B2 terminus at −2,106 bp was used to clone pRXD-B1, and orientation of the resulting plasmid was determined by sequence analysis. A functional proximal promoter was identified previously between +1 and −200 bp and corresponds to pRXD-B11. This clone contains the primary (−59 nt) and subsidiary transcription initiation sites (INRs) and binding sites for the C/EBP, YY-1, Oct-1, and NF-1 transcription factors [Chow et al., 1994; Clark et al., 1998a,b]. Luciferase fusion constructs were confirmed by DNA sequence analysis as described [Wright et al., 2000]. All sequences were determined from both directions and sequence data were compiled manually. Oligonucleotides used as PCR or sequencing primers are available upon request.

Transfection and Luciferase Reporter Assay

Cells to be transfected were grown to 50–70% of confluency in six-well plates and shifted to 2 ml of fresh medium 1 h prior to transfection. Transfections were conducted using Fugene6 (Roche, Nutley, NY) essentially as described by the supplier. 1.7 μg of total plasmid were mixed with Fugene6 in 0.1 ml of RPMI 1640/sodium bicarbonate medium with or without 10% fetal bovine serum as indicated below. After mixing, the transfecting solution was held at room temperature for 30 min and than applied to cells in a single well of a six-well plate. Cells were harvested for analysis after 24 or 48 h of incubation. Cytoplasmic extracts were analysed for luciferase activity (Promega CCLR kit) using a BMG Lab Technologies (Durham, NC) Lumistar luminometer. As reported by others, uniformity of data and transfection efficiency were determined by a minimum of six independent transfections because in all cases β-galactosidase co-transfecting plasmids suppressed activity of the XOR reporter [Clark et al., 1998a,b; Xu et al., 2000]. Luciferase activity was normalized to total cytoplasmic protein as determined spectrophotometrically using the Lowry assay. Each individual transfection was assayed in quadruplicate, and each individual transfection was repeated six times, thus each value reported represents 24 biochemical assays for each parameter. Luciferase values represent arbitrary light units/mg protein/minute. Means and standard deviation were calculated for each group, and in most cases standard deviations were no greater than 10% of the mean value. Comparisons between groups used the Students' *t*-test where a *P*-value of <0.05 was considered significant.

To eliminate the variability observed in transient transfection and to facilitate analysis of the rat XOR upstream DNA, HC11 stable transfectants were generated for each deletion (pRXD-B1 through pRXD-B11) as previously described [Doppler et al., 1989; Doppler et al., 1995]. Briefly, HC11 cells were grown to 80% confluency and transfected with a mix of the deletion plasmid and pCI-Neo (Promega) at a 10:1 ratio (respectively) using Fugene6 as the transfecting reagent. The co-transfecting plasmid pCI-Neo carries the neomycin resistance marker that confers resistance to the antibiotic G418. Transfected cells were shifted into complete HC11 medium containing 400 μg/ml G418 24 h after transfection. After 24 h, cells were split 1:4 and colonies allowed to develop for 1 week. Approximately 400 individual colonies were pooled and reseeded in the same medium to ensure random incorporation of transgene. Cells from the pooled colonies were maintained in this medium for 2 weeks and passaged every 4 days prior to freezing. Routine growth of stably transfected cells was conducted in complete HC11 medium containing 200 μg/ml G418. Luciferase expression from stably transfected cell lines varied by less than 15% between independent cell platings for each of the individual luciferase fusion.

Preparation of Nuclei and Nuclear Proteins

Nuclei were prepared from hypotonically swelled cells by lysis in 0.1% NP40 and differential centrifugation as described previously [Wright et al., 2000; Seymour et al., 2006] with the present addition of 2 mM sodium vanadate and 1 mM NaF in each buffer. Proteins were leached from isolated and washed nuclei by incubation in 320 mM potassium buffer as described [Wright et al., 2000; Seymour et al., 2006]. Nuclear preparations were routinely followed microscopically and are estimated to contain greater than 95% nuclei with no more than 5% of cellular contamination. Following sedimentation at 10,000*g* to remove extracted nuclei, protein solutions were stored at −70°C in high salt buffer containing: 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 320 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 25% glycerol, and 1 mM NaF/2 mM Na_2VO_4.

Electrophoretic Mobility Shift Analysis (EMSA)

The 200 bp fragment of XOR upstream DNA cloned into pRXD-B11 was excised from the plasmid by cleavage with Nco1 and Xho1 followed by agarose gel purification. DNA was labeled using bacteriophage T4 DNA polymerase and α-32p-dATP as previously described [Wright et al., 2000; Seymour et al., 2006]. Probes were extracted in phenol:chloroform: isoamyl alcohol (24:24:1), precipitated in ethanol, and resuspended in TE at 0.5 ng/μl. Fifty-one microliter EMSA binding reactions contained 17.8 μg of nuclear protein, 5 μg poly dI:dC, 0.5 ng of labeled probe, and final reaction buffer conditions composed of 10 mM Tris, pH 7.9, 50 mM NaCl, 1.0 mM DTT, 1.0 mM EDTA, and 5% glycerol. Double strand oligonucleotide competitors were included at 50, or 100-fold molar excess as indicated in the figures. Supershift experiments with anti sera to C/EBPβ or the GR were conducted at a 19.6:1 dilution of antisera. Isotype antisera were used to control for non-specific affects of the antisera. Binding reactions were assembled in the order: H_2O, buffer, nuclear protein solution, poly dI:dC, competitor oligonucleotide, and labeled probe. Competitor oligonucleotides corresponding to consensus binding sites for the GR, C/EBP, and NF-1 were purchased (Santa Cruz). Double strand XOR promoter oligonucleotides were synthesized, annealed, and used in 50-fold molar excess. Competitor oligonucleotides were designed to span the entire 200 bp of the B11 clone in steps of 20 nucleotides. Where appropriate, antisera were added 5 min before probe addition. Binding reactions were conducted on ice for 45 min as described [Plevy et al., 1997]. Following addition of Ficoll dye, 15 μl of binding reaction were electrophoresed on 4% native PAGE gels in TGE buffer (25 mM Tris, pH 8.5, 0.38 M glycine, 2 mM EDTA). Gels were run in the cold at 4°C and at 125 V for approximately 4 h [Plevy et al., 1997] and were subsequently dried and exposed to Kodak XAR autoradiographic film.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described previously [Seymour et al., 2006] with the following modifications. Cells from 100-mm dishes were crosslinked, sonicated, and the entire chromatin preparation pre-cleared with proteinG-sepharose beads using the EZ ChIP kit (Upstate, Lake Placid, NY). Twenty percent of the pre-cleared input chromatin was collected for input DNA analysis. The remaining $9.6 \times 10(6)$ nuclear equivalents were divided into two fractions, one immunoprecipitated with non-specific antisera and the other immunoprecipitated with H190 anti-STAT3 antisera (Santa Cruz). Following crosslink reversal and proteinase K digestion, DNA was purified on spin columns, extracted in phenol/chloroform/ isoamyl alcohol, and analyzed by PCR using two sets of primers specific for the 200 bp proximal promoter and including 80 or 130 bp of the B11 luciferase reporter sequence. Subtle differences in non-specific background amplification were observed between primer sets, and the average of results from each set is reported.

RESULTS

APCs and Growth Factors Activate an XOR DNA-Luciferase Reporter in Stably Transfected Cells

HC11 mouse mammary epithelial cells and L2 rat lung epithelial cells were stably transfected with an XOR upstream DNA-luciferase fusion containing 2,106 bp of the rat XOR upstream DNA fused at the translational start site of the luciferase reporter (B1 fusion). Stable transfectants were tested for response to APCs and growth factors previously found to activate XOR transcription. As reported for activation of the native XOR gene in several different cultured cells, stably transfected XOR-luciferase fusions were activated by the APCs IL-1, IL-6, and TNFα, but were, in contrast, not activated by INF-γ (Fig. 1A,B). We observed that for the most part stably transfected HC11 and L2 cells activated the rat XOR promoter reporter in a similar fashion, although the response to TNFα was greater in L2 cells (Fig. 1B). Activation by LH was comparable to activation of the native XOR gene in HC11 cells, showing marked activation in insulin containing DIP medium that was significantly greater than that observed by the individual LH components (Fig. 1C). We tested the response of the stably transfected XOR reporter to insulin and EGF since these growth factors are required for either proliferation (EGF) or differentiation (insulin). Both insulin and EGF activated expression of the XOR reporter and significantly stimulated activation induced by APCs (Fig. 1D).

The MEK-1/2 Inhibitor PD98059 Stimulates XOR Expression

We observed previously that prolactin and cortisol induced XOR activity in HC11 cells was blocked by 50 μM of the MEK-1/2 inhibitor, PD98059 [McManaman et al., 2000]. To determine whether APC and growth factor activation of the rat XOR-luciferase fusion gene was also sensitive to MEK-1/2 inhibition, we treated stably transfected HC11 cells with either PD98059 or U0126, another inhibitor of MEK-1/2, and 45 min later exposed the cells to cytokine or growth factor activation. While U0126 blocked activation induced by either IL-1/IL-6/insulin (Fig. 2A) or EGF and insulin (Fig. 2B),

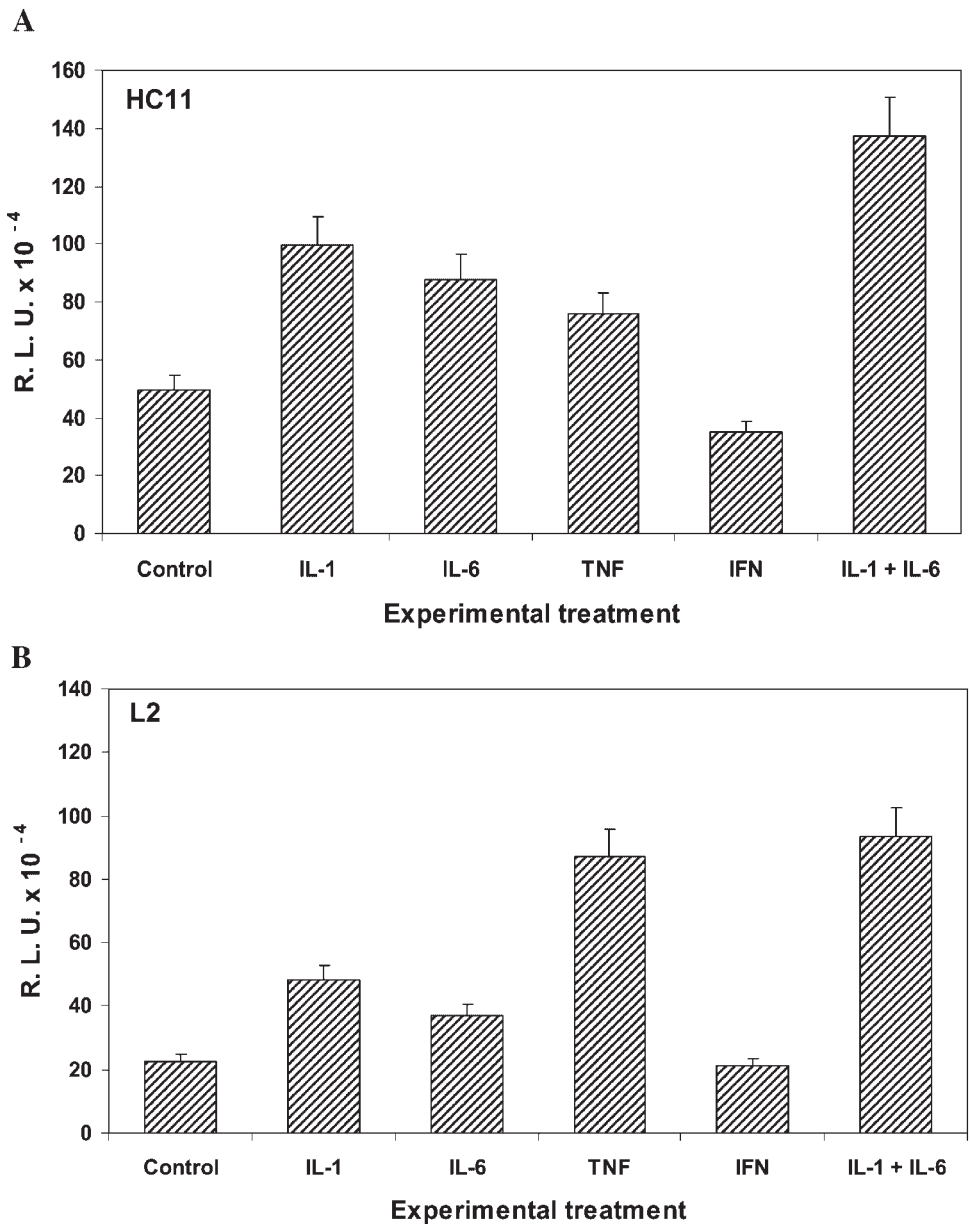


Fig. 1. A stably transfected XOR promoter–reporter is activated by APCs and growth factors: HC11-B1 (**A**,**C**,**D**) and L2-B1 (**B**) cell lines were grown to confluency as described above, shifted into EGF and insulin free medium with 2% heat inactivated FCS. After 2 days of growth, cells were exposed to cytokine and growth factors as shown and 24 h later cells were harvested and luciferase activity quantitated. Cytokine and growth factor concentrations were as follows. IL-1, 10 ng/ml; IL-6, 25 ng/ml; TNFα, 25 ng/ml; IFNγ, 25 ng/ml; dexamethasone, 10^{-7} M; prolactin, 5 μg/ml; EGF, 10 ng/ml; insulin, 5 μg/ml.

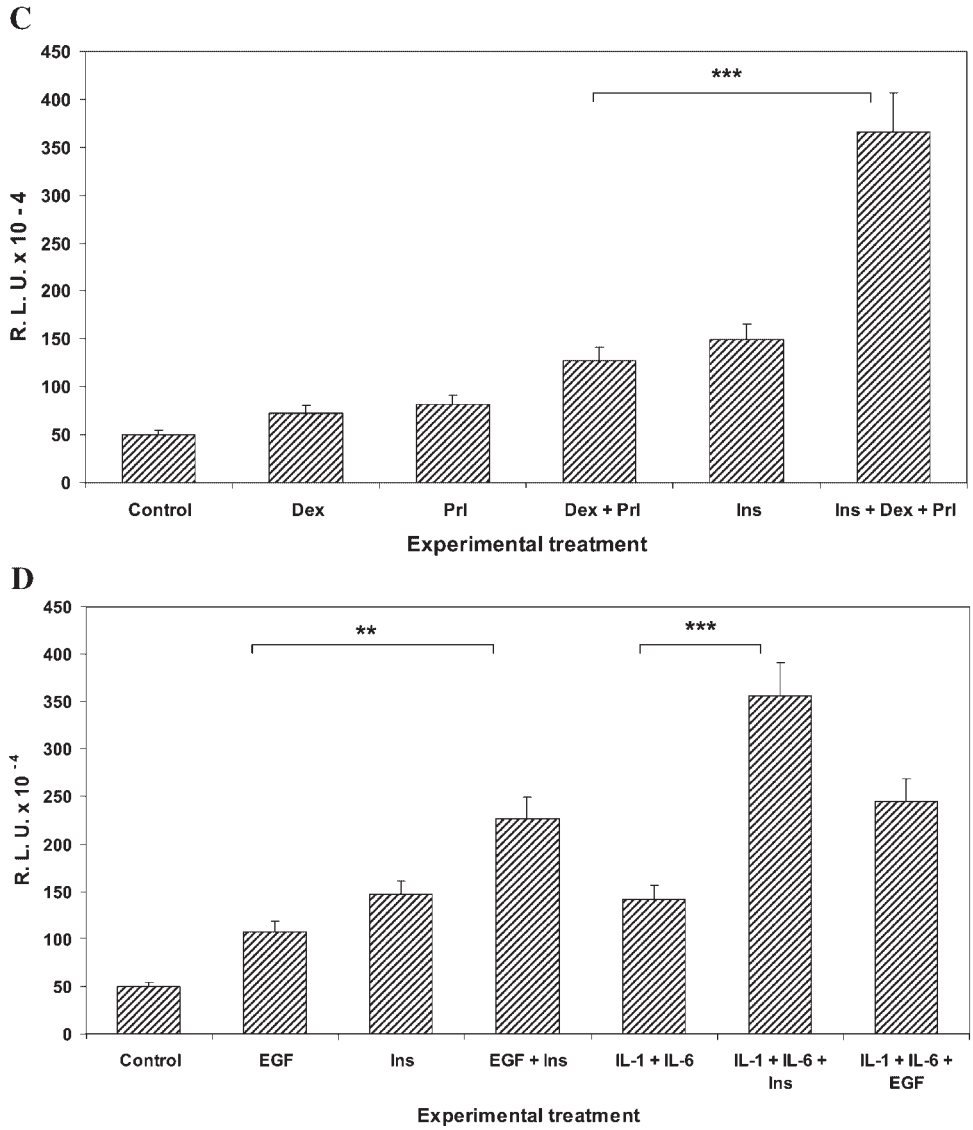


Fig. 1. (*Continued*)

PD98059 significantly enhanced expression by both inducers. This was unexpected, as the inhibitors were anticipated to block MEK-1/2 in a similar fashion [Davies et al., 2000].

To further characterize the response to PD98059, stably transfected HC11 cells were treated with PD98059 over a broad concentration range and in the absence or presence of cytokine/insulin activation. We observed that PD98059 stimulated both cytokine/insulin mediated and basal activation of XOR (Fig. 2C). Essentially identical results were obtained using the stably transfected L2 cell line as well (data not shown). Maximal enhancement of XOR expression by PD98059 occurred at 16.7 μM for both basal and cytokine/insulin treated cells.

Deletion Analysis Reveals a Proximal Promoter Activated by PD98059 and IL-1/IL-6/Insulin

To localize the response to PD98059 and cytokine/insulin stimulation on the rat XOR upstream DNA, we generated a set of 11 stably transfected HC11 cell lines containing deletions of the XOR upstream DNA (B1 through B11). Stably transfected HC11 cells containing each deletion were treated with either DMSO (vehicle control), PD98059, IL-1/IL-6/insulin, or pretreated with PD98059 for 45 min and then

treated with IL-1/IL-6/insulin and after 24 h we determined the level of reporter gene expression. We observed that the response to PD98059 and to IL-1/IL-6/insulin was preserved throughout the deletion set (Fig. 3A) and was therefore encoded by the previously identified proximal promoter comprising nucleotides from −200 to the translational start site. In addition, we observed a potential repressor region in the upstream DNA between −1,400 and −800 and

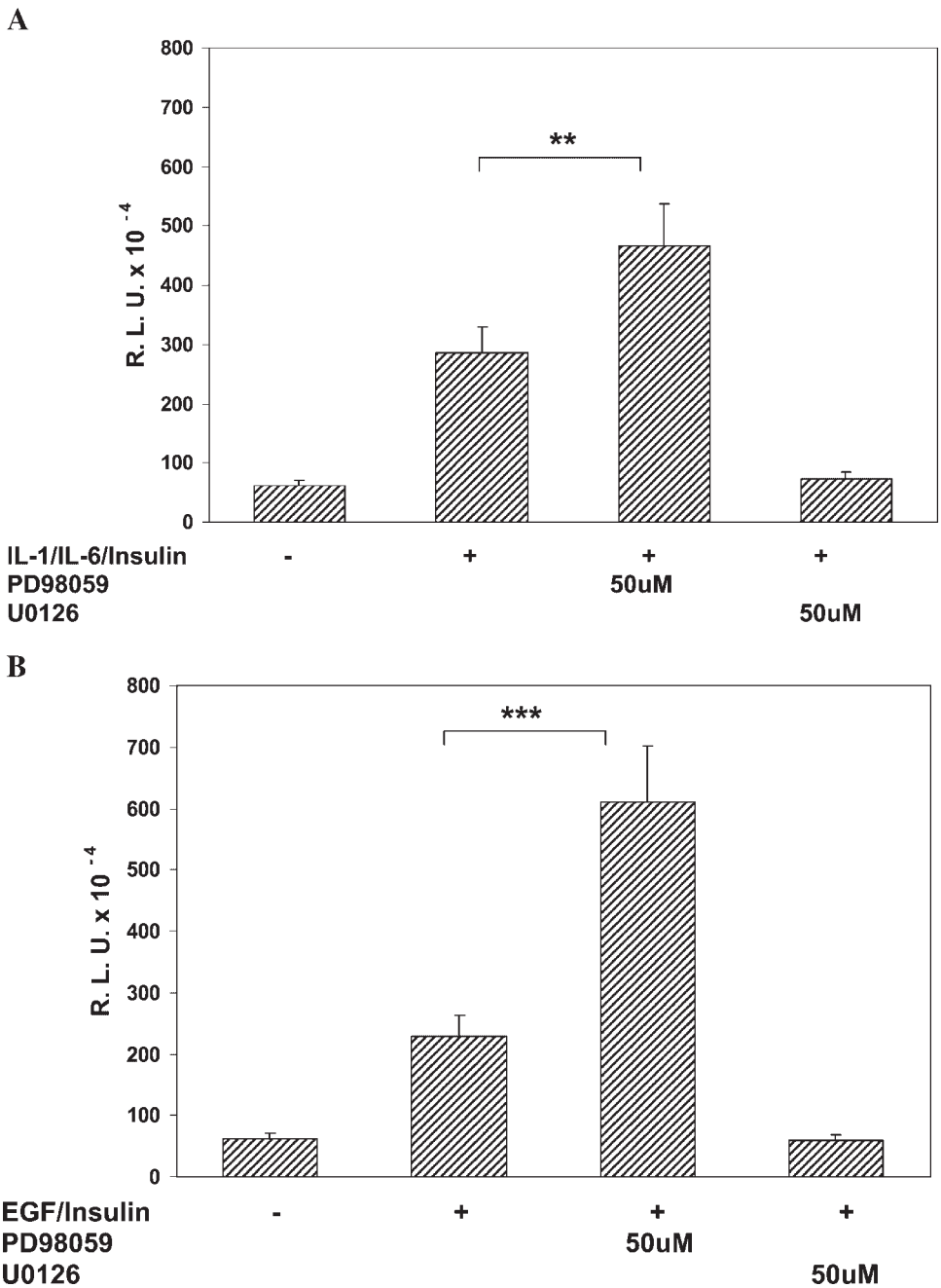


Fig. 2. The MEK-1/2 inhibitors, PD98059 and U0126, exert opposite effects on XOR activation: **Panels A**,**B**: HC11-B1 cells were grown as described in Figure 1. Cells were treated with the MEK-1/2 inhibitors PD98059 or U0126 at 50 μM and exposed to cytokine and insulin (A) or EGF and insulin (B) inducers 45 min later. Cells were harvested and luciferase quantitated from the cell free extracts 24 h later. **Panels C**,**D**: HC11-B1 cells were grown as described in Figure 1. Cells were treated with PD98059 (A) or U0126 (B) over a broad concentration range and subsequently exposed to cytokine/insulin activation (hatched bars) or the DMSO vehicle (black bars). Cells were harvested and luciferase quantitated from the cell free extracts 24 h later.

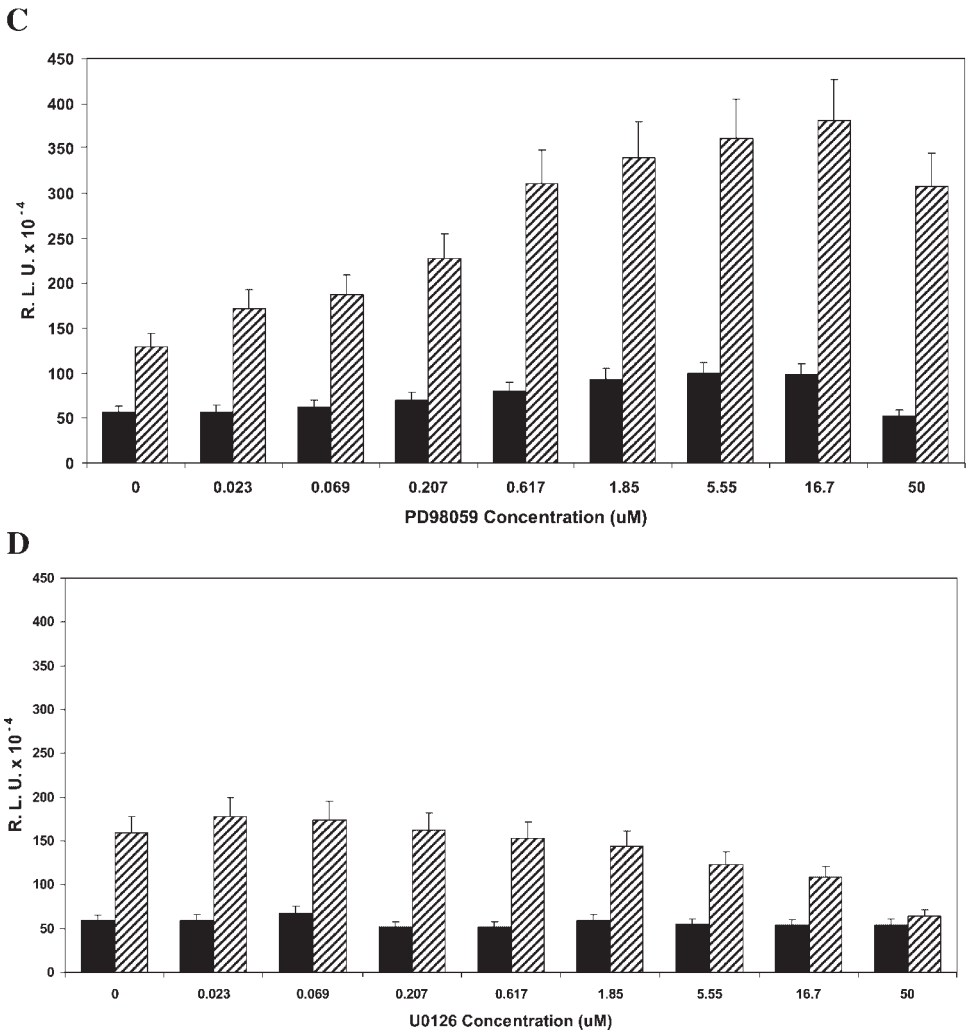


Fig. 2. (*Continued*)

a potential enhancer region between −800 and −600.

Activation of the XOR Proximal Promoter by PD98059 is Blocked by RU38486

The GR antagonist RU38486 was found previously to block LH induced XOR activation [Kurosaki et al., 1996; McManaman et al., 2000]. We observed that RU38486 blocked cytokine/insulin and EGF/insulin activation of HC11 cells stably transfected with the 2,106 bp B1 XOR deletion as well (Fig. 3B). To determine if activation produced by PD98059 was also inhibited by RU38486, we treated both the B1 and B11 cells with increasing dose of RU38486 and after 45 min exposed the cells to PD98059. Expression of the reporter gene was determined 24 h later. PD98059 activation of both the B1 and B11 deletions was dose dependently inhibited by RU38486 (Fig. 3C,D). Dexamethasone, a direct activator of the GR, stimulated both the B1 and B11 deletions, and activation of both the B1 and B11 deletions was also dose dependently inhibited by RU38486 (data not shown). Furthermore, RU38486 fully blocked activation of both B1 and B11 deletions in cells treated simultaneously with PD98059 and dexamethasone (Fig. 3E,F). Thus, both PD98059 and dexamethasone activation were blocked by RU38486, and activation by both agents was mediated by the XOR proximal promoter. PD98059 activation could arise as a result of estrogen or progesterone receptor stimulation [Lange et al., 2000; Shen et al., 2001]. To determine if XOR activation induced by PD98059 was the result ER or PR stimulation,

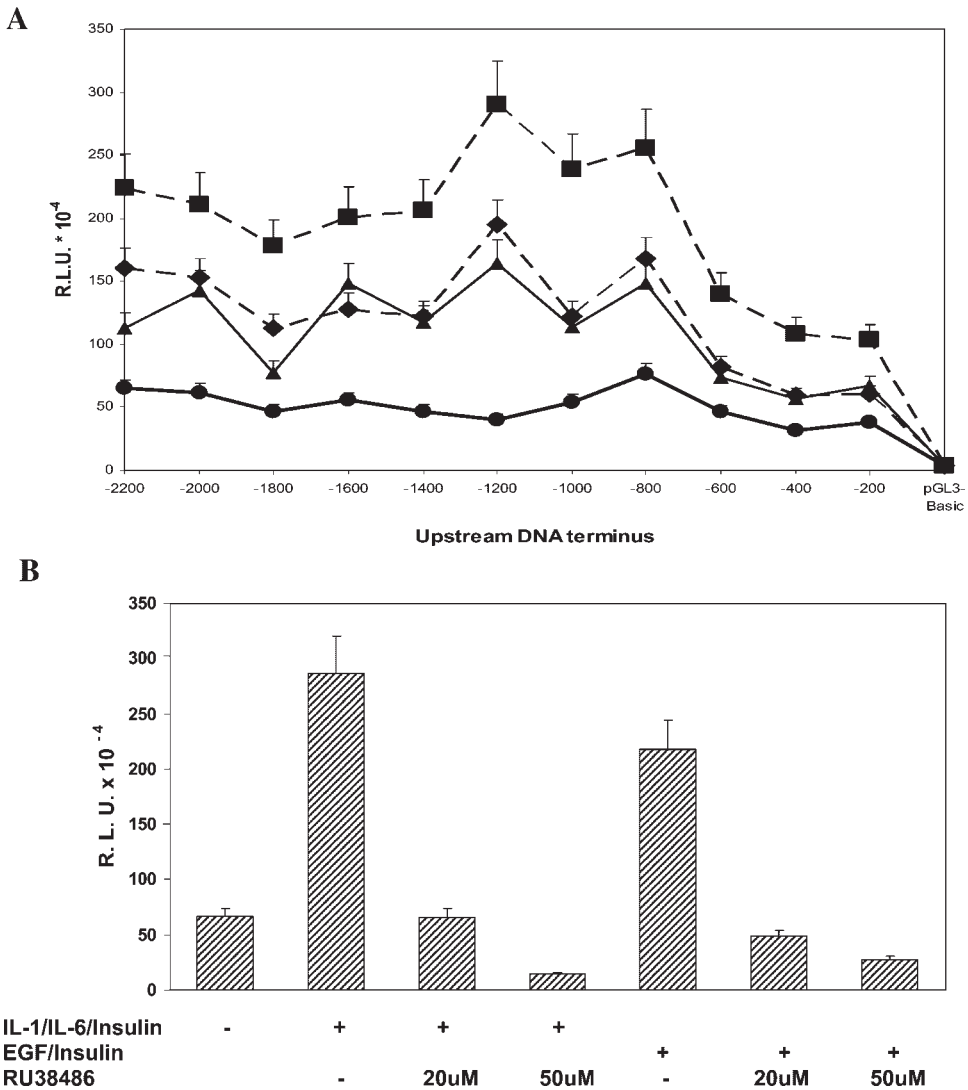


Fig. 3. PD98059 activation of the XOR proximal promoter is blocked by an inhibitor of the glucocorticoid receptor: **Panel A**: expression of an XOR-reporter deletion set in stably transfected HC11 cells. Deletion end points are specified from the translational initiation site and noted on the x-axis. Cells were grown at confluency for 2 days, shifted into low serum conditions in the absence of EGF or insulin as described in Figure 1, and were then treated with either DMSO (circles), IL-1/IL-6/insulin (triangles), PD98059 (diamonds), or were treated with PD98059 for 45 min and then treated with IL-1/IL-6/insulin (squares). Cells were harvested 24 h later, and luciferase was quantitated from three independent experiments. **Panel B**: HC11-B1 cells were grown as described in Figure 1, treated with RU38486 at 20 or 50 μM for 45 min, and were then exposed to IL-1/IL-6/insulin or EGF/insulin. Luciferase was quantitated from three independent experiments 24 h later. **Panels C**,**D**: HC11 stable transfectants containing the B1 (C) or B11 (D) constructs were grown to confluency and maintained for 48 h in medium with reduced serum, EGF and insulin free, as in Figure 1. Cells were treated with increasing dose of RU38486 and cultures were exposed to PD98059 after 45 min treatment with RU389486. Expression of luciferase was determined 24 h later. **Panels E**,**F**: Stable transfectants containing the B1 (E) or B11 (F) reporter constructs were grown to confluency as above and cells were treated with vehicle or RU38486 at 50 μM and cultures were exposed to 20 μM PD98059 in the presence of increasing dose of dexamethasone after 45 min treatment with RU389486. Expression of luciferase was determined 24 h later. **Panel G**: Stable B11 transfectants were grown to confluency as above and treated with 17β-estradiol or progesterone in the presence or absence of PD98059 in low serum and insulin/EGF free medium. Luciferase expression was determined 24 h later.

we treated B11 transfectants with 17β-estradiol or progesterone in the presence or absence of PD98059. We observed neither activation nor any further stimulation by either steroid in the presence of PD98059 (Fig. 3G). These data suggest that RU38486 sensitive PD98059 activation may be mediated by the GR acting through the XOR proximal promoter.

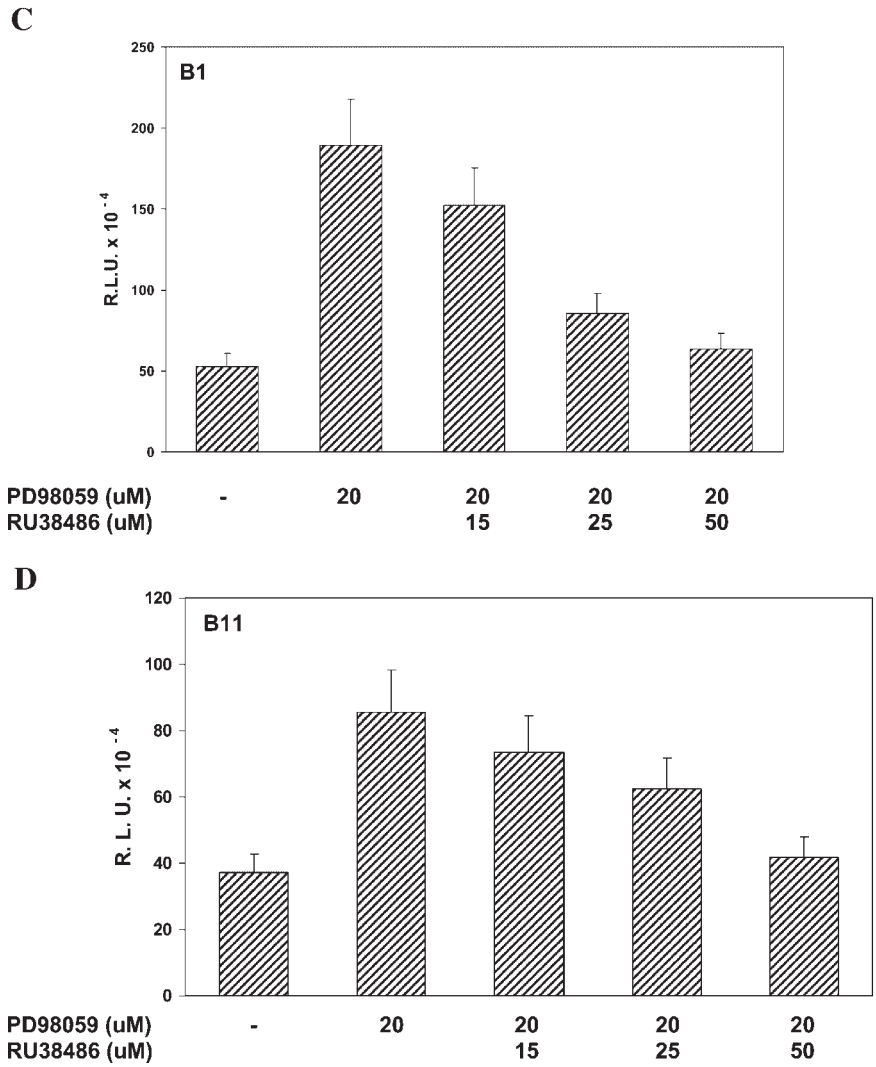


Fig. 3. (*Continued*)

PD98059, Dexamethasone, and Insulin Induce Similar Protein Interactions With the XOR Proximal Promoter

The 200 bp proximal promoter encodes activation in response to insulin, dexamethasone, and PD98059 as well as inhibition by RU38486. Furthermore, the above data suggested that the GR may mediate activation by dexamethasone and PD98059 and inhibition by RU38486. EMSA analysis of the 200 bp XOR proximal promoter (B11 DNA) revealed marked alteration in complex formation by PD98059, dexamethasone, and insulin (Fig. 4A). Seven complexes (C1–C7) were formed with the 200 bp proximal promoter in response to these activators. PD98059 markedly induced C3 and C4 and weakly induced C5 and C7 (lane 2). Dexamethasone also induced C3, C4, and weakly induced C5, C6, and C7 (lane 3). The combination of PD98059 and dexamethasone induced the same complexes but to an apparently greater extent (lane 4). Insulin induced all complexes but C6 (lane 8). C1 and C2 were formed in response to insulin, in the presence or absence of PD98059 or dexamethasone (lanes 5–8). Furthermore, while insulin induced C3, C4, C5, and C7, C4 and C7 were most prominently induced by insulin (lane 8). This pattern was largely unchanged in the presence of dexamethasone (lane 6) or PD98059 (lane 7), but the presence of both PD98059 and dexamethasone preserved high-level induction of C3.

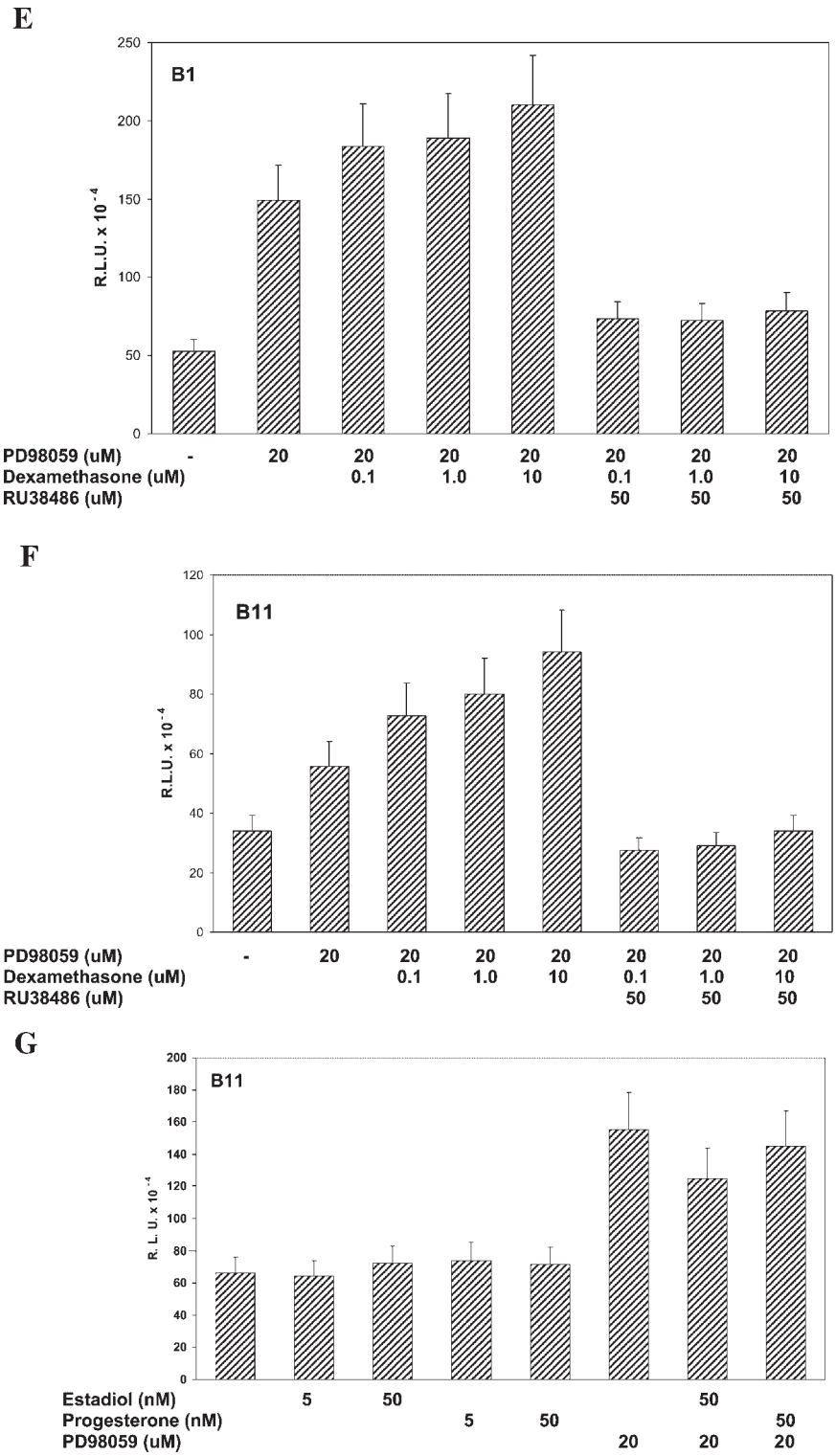
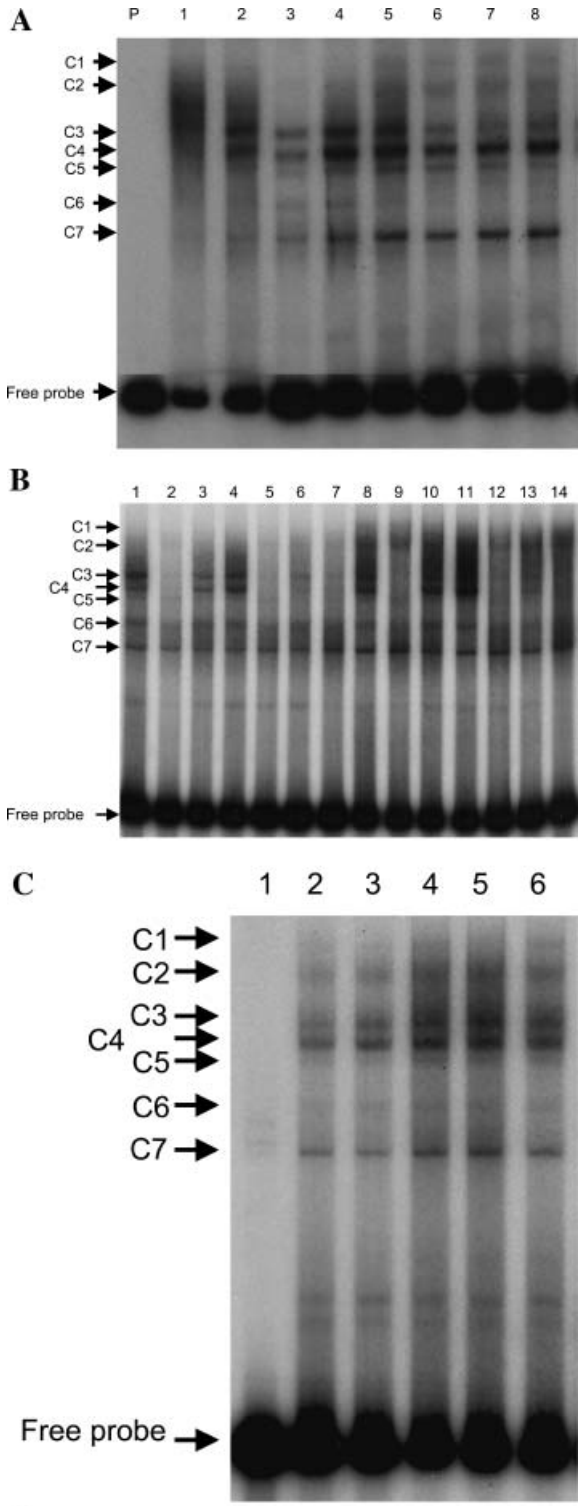


Fig. 3. (*Continued*)



The GR Contributes to Protein Complex Formation With the PD98059 Activated XOR Proximal Promoter

TESS analysis of the proximal promoter revealed good prediction for a GR binding site and a cEts-1 binding site lying from −163 to −183 bp from the translational start site. Competition EMSA was conducted with B11 DNA and nuclear extracts from cells treated with PD98059 and dexamethasone (Fig. 4B; lanes 1–7) or with insulin, dexamethasone, and PD98059 (Fig. 4B; lanes 8–14). An oligonucleotide (WT) comprising −163 to −183 bp of the XOR proximal promoter fully competed for C3, C4, and C5, with no effect evident on C1, C2, C6, or C7 (Fig. 4B; lanes 2,9). Substitution mutations M1 and M2 in the WT oligonucleotide that eliminated GR binding sequences but not cEts-1 binding sequences prevented effective competition (Fig. 4B; lanes 3,10 and lanes 4,11). Substitution mutations eliminating cEts-1 binding did not prevent effective competition (Fig. 4B; lanes 5,12). Furthermore, two related GR consensus oligonucleotides also blocked formation of C3, C4, and C5 (Fig. 4B; lanes 6,13 and 7,14). We were unable to demonstrate

Fig. 4. PD98059, dexamethasone, and insulin induced protein interactions with the XOR proximal promoter involve the GR indirectly: **Panel A** shows EMSA analysis of nuclear proteins using the 200 bp B11 proximal promoter DNA as a radio-labeled probe. Lanes are as follows: P, unreacted probe; 1, untreated control cell nuclear protein; 2, nuclear proteins from cells treated with PD98059; 3, from cells treated with dexamethasone; 4, from cells treated with PD98059 and dexamethasone; 5, from cells treated with insulin, PD98059, and dexamethasone; 6, from cells treated with insulin and dexamethasone; 7, from cells treated with insulin and PD98059; 8, from cells treated with insulin. The autoradiograph was cropped of the large band free region migrating above the probe. **Panel B** shows oligonucleotide competition EMSA analysis of B11 DNA using nuclear proteins from cells treated with PD98059 and dexamethasone (**lanes 1–7**) or insulin, dexamethasone, PD98059 (**lanes 8–14**). Lanes are as follows: 1 and 8, stimulated cell nuclear proteins, no competitor; 2 and 9, competition with WT XOR derived GR domain; 3 and 10, GR-M1 competitor; 4 and 11, GR-M2 competitor; 5 and 12, GR-M3 competitor; 6 and 13, GRcon1 competitor; 7 and 14, GRcon2 competitor. All competitor oligonucleotides were present in 20-fold molar excess relative to B11 probe DNA. **Panel C** shows supershift EMSA using three different antisera to the GR. Lanes are as follows: 1, probe alone; 2, uncompeted complexes; 3, supershift using non-specific isotype antisera; 4, supershift using M-20 antisera to the GR; 5, supershift using P-20 antisera to the GR; 6, supershift using BuGR2 antisera to the GR. **Panel D** shows the oligonucleotide sequences employed here. Nucleotides changed in the substitutions mutations M1, M2, and M3 are shown in bold type. GRcon1 and GRcon2 are commonly used consensus oligonucleotides for the GR.

any evident supershift of C1 through C7 using the Santa Cruz M-20 or P-20 antisera against the GR or using the BuGR2 antisera from Abcam, Inc. (Fig. 4C). Thus, while the GR appears to participate in complex formation with the PD98059 activated XOR proximal promoter, we were unable to detect the GR protein in these complexes.

STAT3 Contributes to Complexes Formed With the PD98059 Activated XOR Proximal Promoter

The GR could contribute indirectly to XOR activation and complex formation through interaction with several co-activator proteins [Edwards, 2000]. TESS analysis of the XOR proximal promoter revealed good predictions for binding STAT factors 1, 3, or 5 in the region from −84 to −64 bp. This region also generated good prediction for binding the factors Lyf-1, CBF-1, and GATA-1. Competition EMSA was conducted with B11 DNA as described above using nuclear extracts from cells treated with PD98059 and dexamethasone (Fig. 5A; lanes 1–7) or with insulin, dexamethasone, and PD98059 (Fig. 5A; lanes 8–14). A wild type oligonucleotide comprising the region from −84 to −64 bp fully competed for C3, C4, and C6, with no effect apparent on C1, C2, C5, or C7 (Fig. 5A; lanes 2,9). Substitution mutation M1 (Fig. 5C) that eliminated putative Lyf-1/CBF-1 binding sequences but not GATA-1 binding sequences prevented effective competition (Fig. 5A; lanes 3,10). Substitution mutations eliminating GATA-1 binding did not prevent effective competition (Fig. 5A; lanes 4,11). However, competition with consensus oligonucleotides for CBF-1, Lyf-1, and GATA-1 failed to compete for C3, C4, and C5 (Fig. 5A; lanes 5–7 and 12–14). Both CBF-1 and GATA-1 oligonucleotides had inconsistent and minor effect on C7, reflecting a potential non-specific effect of these oligonucleotides. We were unable to demonstrate any supershift of C1 through C7 using anntisera to Lyf-1, CBF-1, or GATA-1 (data not shown).

Competition EMSA using consensus oligonucleotides for STAT1, STAT3, and STAT5 (Fig. 5B) revealed specific competition by the STAT3 oligonucleotide for C3, C4, and C6 formed with B11 DNA (Fig. 5B lanes 3,6), while STAT1 or STAT5 consensus oligonucleotides showed no competition for complex formation (Fig. 5B; lanes 2,4). Antisera to STAT3 also disrupted formation of C3, C4, and C6 and may have reduced C7 as well (Fig. 5C). Furthermore, both anti-STAT3 antisera and the STAT3 consensus oligonucleotide prevented complex formation with the −84 to −64 bp DNA itself (Fig. 5D; lanes 3,6, respectively). Thus, STAT3 is specifically involved in complex formation with the XOR proximal promoter activated by PD98059, dexamethasone, or insulin.

PD98059, Dexamethasone, and Insulin Activate STAT3 Tyrosine 705 Phosphorylation

To characterize STAT3 phosphorylation status during activation of the XOR proximal promoter, western blots were analyzed using antisera specific for STAT3-phospho-Tyr705 or STAT3-phospho-Ser727. We observed marked up-regulation of STAT3-phospho-Tyr705 in cells treated with PD98059, dexamethasone, PD98059 and dexamethasone, or insulin (Fig. 6A; lanes 2,5,8,11). STAT3-phospho-Ser727 was activated by insulin but not by treatment with PD98059 or dexamethasone (Fig. 6A; lanes 11–13). RU38486 pretreatment blocked up-regulation of STAT3-phospho-Tyr705 in cells treated with PD98059 and/or dexamethasone, but not insulin (Fig. 6A; lanes 4,7,10,13). Previous work demonstrated a role for p38 MAP kinase in XOR activation [Abdulnour et al., 2006; Seymour et al., 2006], and we observed that prior treatment with the p38 inhibitor SB202190 blocked STAT3-phospho-Tyr705 up-regulation in cells subsequently exposed to PD98059, dexamethasone, PD98059, and dexamethasone, or insulin (Fig. 6A; lanes 3, 6,9,12). STAT3-phospho-Ser727 up-regulation was not blocked by SB202190 or RU38486. Steady state levels of STAT3 protein were not affected by any of these treatments.

STAT3 Contributes to PD98059 Enhanced Activation of the XOR Reporter

To determine whether STAT3 contributed to activation of the XOR promoter, HC11-B11 cells were transiently transfected with a constitutively active form of STAT3 (STAT3C) or a dominant negative derivative of STAT3 (STAT3F). STAT3C dose dependently increased XOR activation in the absence of additional stimulation while cells transfected with STAT3F showed very little response over a wide range of DNA concentration (Fig. 6B). Furthermore, STAT3C markedly enhanced activation in control cells and in cells treated with

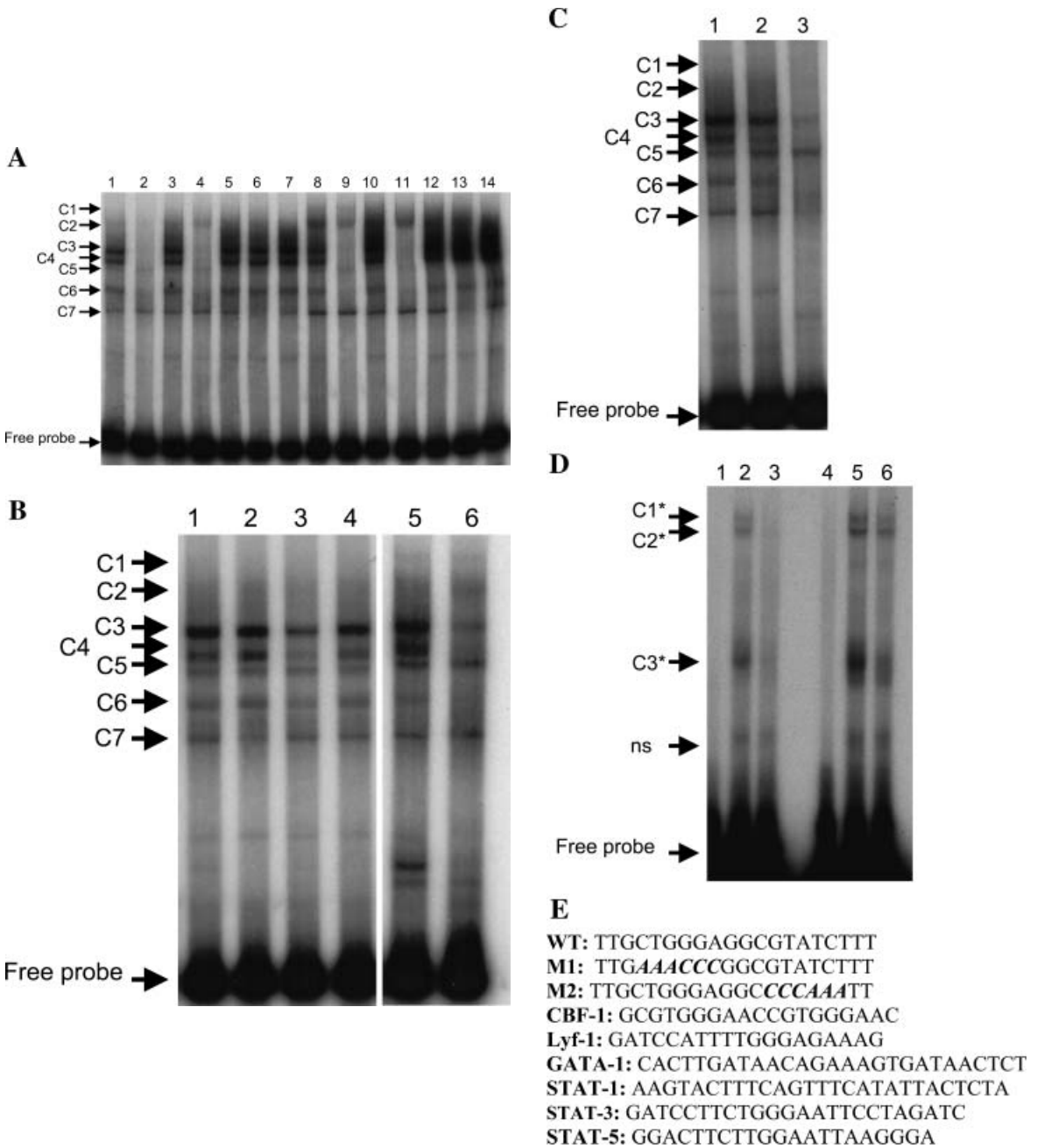


Fig. 5. STAT3 contributes to complex formation with the XOR proximal promoter activated by PD98059, dexamethasone, or insulin: **Panel A** shows oligonucleotide competition EMSA analysis of B11 DNA using nuclear proteins from cells treated with PD98059 and dexamethasone (**lanes 1–7**) or with insulin, dexamethasone, and PD98059 (**lanes 8–14**). Lanes are as follows: 1 and 8, stimulated cell nuclear proteins, no competitor; 2 and 9, competition with the WT −84 to −64 oligonucleotide; 3 and 10, M1 competitor; 4 and 11, M2 competitor; 5 and 12, CBF-1 competitor; 6 and 13, LyF-1 competitor; 7 and 14, GATA-1 competitor. **Panel B** shows oligonucleotide competition EMSA analysis of B11 DNA using nuclear proteins from cells treated with PD98059 and dexamethasone (lanes 1–4) or insulin, dexamethasone, and PD98059 (lanes 5,6). Lanes are as follows: 1 and 5, stimulated cell nuclear proteins, no competitor; 2, STAT-1 competitor; 3, STAT3 competitor; 4, STAT5 competitor; 6, STAT3 competitor. **Panel C** shows supershift analysis of complexes formed with nuclear proteins from cells treated with insulin, dexamethasone, and PD98059. Lanes are as follows: 1, no antibody; 2, non-specific isotype antisera; 3, STAT3 antibody. **Panel D** shows competition and supershift EMSA using the WT −84 to −64 oligonucleotide as a radio-labeled probe. Lanes are as follows: 1 and 4, unreacted probe; 2, no competition; 3, STAT3 competitor; 5, non-specific isotype antibody; 6, STAT3 antibody. **Panel E** shows the oligonucleotide sequences employed here. WT is the −84 to −64 oligonucleotide from the native XOR gene. Nucleotides changed in the substitutions mutations M1 and M2 of the WT sequence are shown in bold type. Commonly used consensus oligonucleotides for CBF-1, Lyf-1, GATA-1, STAT-1, STAT-3, and STAT-5 are shown.

PD98095, dexamethasone, and/or insulin (Figs. 3F and 6C). In contrast, STAT3F restrained activation to only 20% of the induced level generated in control cells or cells treated with PD98095, dexamethasone, and/or insulin (Fig. 6C,D). To confirm that STAT3 participated in B11 promoter activation, ChIP analysis was performed on HC11-B11 cells transfected with either STAT3C or STAT3F and subsequently treated with insulin, dexamethasone, and PD98059 (I/D/PD). Cells transiently transfected with the STAT3F cDNA expression plasmid showed STAT3 immunoprecipitation that was indistinguishable from that observed with non-specific immunoprecipitation (Fig. 6E). Conversely, cells transiently transfected with STAT3C showed marked STAT3 immunoprecipitation that was increased by 1 h of exposure to I/D/PD. We normalized the band intensity for each STAT3 immunoprecipitation to that obtained from the input chromatin and averaged the results from each primer set, and this indicated that after 1 h of exposure to I/D/PD STAT3 increased in the treated cells by 2.9-fold over the control cells. These data confirm that STAT3 was indeed present on the XOR B11 promoter DNA and was increased by treatment with I/D/PD.

DISCUSSION

In the present study, we used a series of stably transfected promoter–reporter constructs to examine regulation of XOR by growth factors

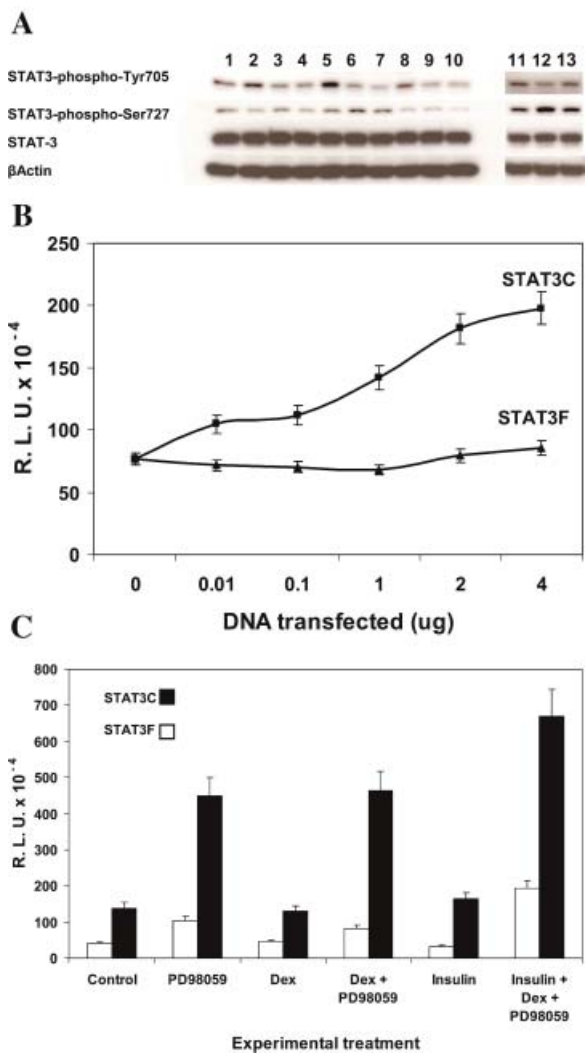


Fig. 6. STAT3 contributes to XOR activation by PD98059, dexamethasone, and/or insulin: **Panel A** shows western immunoblot analysis of STAT3-phospho-Tyr705 and STAT3-phospho-Ser727. Cells were grown as described in Figure 1, washed, and the medium was refreshed. Cells were treated with SB202190 (**lanes 3**,**6**,**9**,**12**), RU38486 (**lanes 4**,**7**,**10**,**13**), or vehicle (**lanes 1**,**2**,**5**,**8**,**11**). After 45 min of inhibitor treatment cells were treated with vehicle (lane 1), PD980569 (lanes 2,3,4), dexamethasone (lanes 5,6,7), a combination of PD89059 and dexamethasone (lanes 8,9,10), or insulin (lanes 11,12,13). Cells were harvested after 30 min of treatment and lysates prepared in RIPA buffer. Separate blots were run for STAT3-phospho-Tyr705 and STAT3-phospho-Ser727. Blots were stripped and reprobed using antisera for STAT3 peptide or βActin. Representative examples of both are shown. **Panel B**: HC11-B11 cells were transfected with cDNA expression clones for STAT3C or STAT3F over a broad concentration range. After 24 h, cells were harvested and luciferase quantitated as described above. **Panel C**: 1.0×10^6 HC11-B11 cells in six-well plates were transfected with 1.0 μg of STAT3C or STAT3F cDNA expression clones. Four hours following transfection, medium was exchanged for insulin and EGF free medium containing 2% heat inactivated serum. After 24 h, cells were treated with vehicle (control), PD98059, dexamethasone, and/or insulin as shown. Twenty-four hours following treatment cells were harvested and luciferase quantitated as described above. **Panel D**: data in panel C were normalized to the level of luciferase obtained in STAT3C transfected cells which was set at 100%. **Panel E**: ChIP analysis of HC11-B11 cells transfected with cDNA expression clones for STAT3C or STAT3F and treated or not treated with insulin, dexamethasone, and PD98059 (I/D/PD). HC11-B11 cells were transfected with STAT3C or STAT3F expression clones as described above, and shifted into insulin and EGF free medium containing 2% heat inactivated serum 4 h later. Twenty-four hours following transfection cells were exposed to DMSO (vehicle control) or I/D/PD, and 1 h later cells were fixed in formaldehyde and processed for ChIP. Two primer sets were used in PCR analysis and each pair used a common primer at the 5′ boundary of B11 DNA. For **lanes A–D** the 3′ primer generated a PCR amplified region that included 130 bp of the luciferase reporter sequence, and in **lanes F–I** the 3′ primer was translocated 50 bp upstream. **Top panels** for each amplification show immunoprecipitation performed with non-specific isotype antisera; **middle panels** for each amplification show immunoprecipitation performed with H190 anti-STAT3 antisera; **bottom panels** for each amplification show amplification of input chromatin.

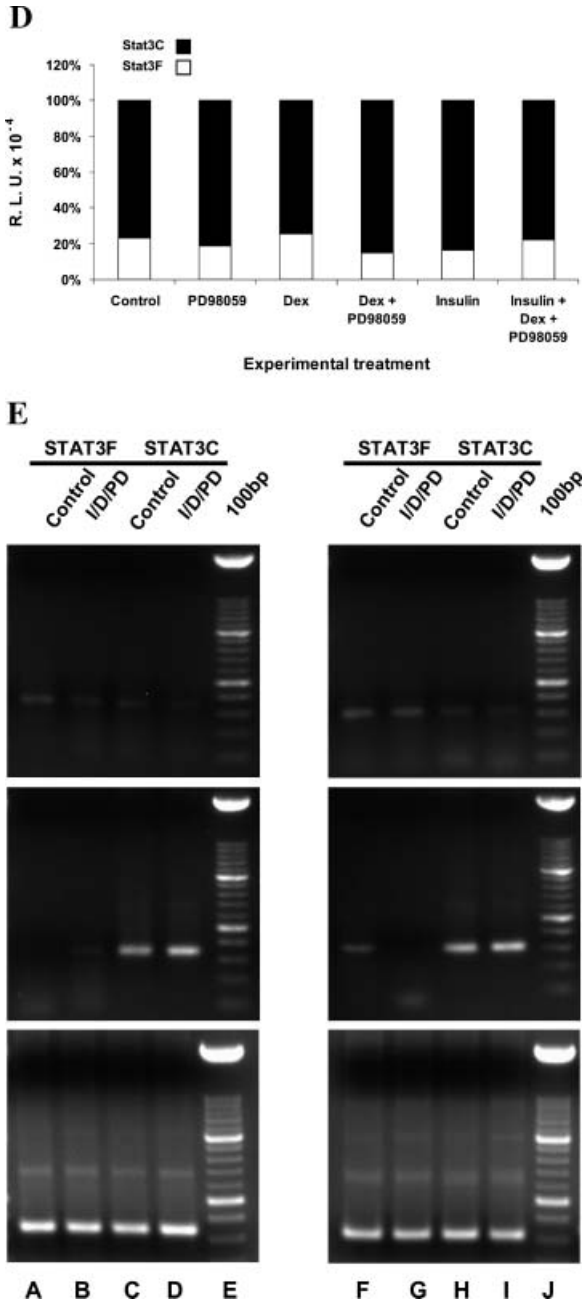


Fig. 6. (*Continued*)

mediating mammary epithelial cell growth and differentiation and by APCs found previously to activate XOR transcription in mammary epithelial cells. As anticipated, the XOR reporter was activated in mammary epithelial cells by the growth factors EGF, insulin, prolactin, and dexamethasone and by the APCs IL-1, IL-6, and TNFα as previously reported for activation of the native gene in epithelial cells of mammary or renal origin [Pfeffer et al., 1994; Kurosaki et al., 1996; Page et al., 1998; McManaman et al., 1999; McManaman et al., 2000]. Furthermore, rat lung epithelial cells, and mouse mammary epithelial cells were very similar in their pattern of activation. Deletion analysis of the XOR upstream DNA revealed that the previously identified proximal promoter from -1 to -200 bp was activated by APCs, EGF, insulin, and dexamethasone. Activation by both growth factors and APCs was blocked by the GR inhibitor (RU38486) and the MEK-1/2 inhibitor (U0126). Thus, sequences from -1 to -200 are sufficient to confer activation by APCs, EGF, insulin, dexamethasone, and inhibition by RU38486 and U0126.

While activation by both growth factors and APCs was prevented by the MEK-1/2 inhibitor, U0126, we observed unexpected activation of XOR by another MEK-1/2 inhibitor, PD98059 over a broad concentration range. Although both drugs are widely used to infer a contribution by the MEK-1/2 MAP kinase, differences in their mechanisms of action, kinetics, and efficiency are well known [Favata et al., 1998; Davies et al., 2000]. Despite the high specificity attributed to PD98059 as a MEK-1/2 inhibitor [Davies et al., 2000], PD98059 but not U0126 has been reported to possess apparent estrogenic effects [Long et al., 2001; Dang and Lowik, 2004]; to block proteolysis of the progesterone receptor [Lange et al., 2000; Shen et al., 2001]; to induce ERK-1/2 phosphorylation [Cerioni et al., 2003]; and to stimulate adipogenesis [Dang and Lowik, 2004]. The mechanism by which PD98059 promotes such different effects are presently unknown. We observed previously that PD98059 inhibited XOR activation by prolactin and cortisol, but as shown here enhanced expression induced by APC, growth factor, or insulin, and this may reflect the different signaling pathways mediating activation of XOR by these different stimuli.

Activation of XOR by PD98059 apparently required the GR. XOR was activated by dexamethasone (a GR agonist), PD98059, or the combination of dexamethasone and PD98059, and activation was in each case fully blocked by RU38486 suggesting that activation by dexamethasone and PD98059 were not functionally independent. We found no evidence for stimulation of XOR by either estrogen or progesterone, ruling out an effect of PD98059 on PR or ER stimulated expression. Furthermore, activation by both dexamethasone and PD98059 was

localized to the 200 bp proximal promoter, and nuclear protein complexes formed with the proximal promoter from cells treated with either agent, alone or in combination, were largely indistinguishable. In addition, both agents induced STAT3-tyrosine705 phosphorylation that was blocked by RU38486. Thus, our present data indicate that PD98059 enhanced XOR expression required GR activity.

Activation of XOR by both growth factors and APCs was prevented by the GR antagonist, RU38486, even in the absence of dexamethasone stimulation, suggesting a fundamental role of the GR in XOR activation. Analysis of XOR proximal promoter protein binding revealed several complexes associated with PD98059, dexamethasone, and insulin activation. Significantly, the complexes formed with nuclear proteins from cells treated with each inducer, alone or in combination, were largely very similar. While no effort was made at this point to fully characterize protein binding, the GR was anticipated to comprise a primary component. The GR inhibitor (RU38486) blocked activation by APCs, growth factors, and PD98059. Activation by the GR was carried in part on the proximal promoter, and DNA sequence analysis revealed a potential GR responsive site. The region from −163 to −183 comprises the sequence GGCTGTCCCGTCCTTCCTGG and contains the core GR responsive site TGTCCCGTCC. Competition EMSA containing the entire 20 bp region blocked formation of major complexes induced by PD98059, dexamethasone, and/or insulin, and substitution mutations that altered the GR responsive site reversed the capacity for effective competition. In addition, two related consensus GR binding oligonucleotides also blocked complex formation. Nonetheless, we were unable to demonstrate the presence of the GR in protein complexes formed with the proximal promoter using three different antibodies to the GR in supershift analyses. Since these antisera have been used previously in supershift analyses in other cell systems, we imagine that the GR antigen may be inaccessible to antibody binding or the GR may contribute indirectly to protein complex formation as reported elsewhere [Edwards, 2000; Levy and Darnell, 2002; Lerner et al., 2003].

While several co-activators could contribute to GR induced activation [Edwards, 2000], sequence analysis suggested the potential involvement of STAT3. The region from −84 to −64 produced good prediction for binding of STAT factors in general and for Lyf-1, CBF-1, and GATA-1. We found no evidence for binding of STAT1, STAT5, Lyf-1, CBF-1, or GATA-1 by either competition or supershift EMSA. On the other hand, both competition EMSA and supershift EMSA revealed the presence of STAT3 in the major complexes induced by PD98059, dexamethasone, and/or insulin. Furthermore, substitution mutagenesis of the STAT3 core binding site (CTGGGAG to AAACCCG) specifically blocked oligonucleotide competition. The prominent complexes C3, C4, C6, and C7 induced by PD98059, dexamethasone, and/or insulin were blocked by both the GR competitors and by STAT3 competition and supershift. These observations suggested that STAT3 could play a critical role in XOR activation by PD98059, dexamethasone, and/or insulin and in the formation of protein complexes associated with XOR activation.

Indeed, we observed that expression of a constitutively active STAT3 cDNA, STAT3C [Levy and Darnell, 2002; De Miguel et al., 2003], markedly induced XOR expression in cells stably transfected with a proximal promoter reporter gene, whereas a dominant negative derivative of STAT3, STAT3F [Grandis et al., 1998; Kijima et al., 2002], exerted little effect on XOR expression. Activation of the proximal promoter by PD98059, dexamethasone, and/or insulin was markedly enhanced by STAT3C. We obtained between eight and tenfold greater activation by PD98059, dexamethasone, and/or insulin in the presence of STAT3C. In contrast, expression of STAT3F blocked activation by each inducer and restrained expression to 20% of the activated state for each inducer. STAT3F also depressed the uninduced control expression to 20% as well. ChIP analysis was used confirm that STAT3 was indeed participating in XOR promoter activation. While we were unable to detect STAT3 immunoprecipitation above non-specific immunoprecipitation in cells transiently transfected with STAT3F, STAT3 was both present and inducible in immunoprecipitations from cells transiently transfected with STAT3C. Using two different primer sets for promoter PCR amplification we observed 2.9-fold induction of STAT3 in cells treated with I/D/PD for 1 h. Kinetic analyses of STAT3 assembly into a multiprotein complex in other cells showed the rapid induction of nuclear

STAT3 that was maximal in 1 h. This was followed by the clearance of STAT from nuclear protein complexes that was largely complete 2 h following stimulation with dexamethasone [Lerner et al., 2003]. How PD98059 alone or in conjunction with dexamethasone or insulin affects this time course is unknown. However, we were able to observe both the presence and inducibility of STAT3 in XOR promoter complexes 1 h following I/D/PD treatment in HC11 mammary epithelial cells. We conclude that STAT3 is an important mediator of XOR activation by PD98059, dexamethasone, and/or insulin and that the GR contributes to this activation as well.

The mechanism by which STAT3 contributes to XOR activation by insulin, dexamethasone, and PD98059 is unknown. Although both EMSA and ChIP analyses revealed that STAT3 was involved in protein complex formation with the XOR promoter and was present in protein complexes with the XOR promoter DNA, the interaction of STAT3 with XOR DNA may be indirect. Furthermore, competition analyses with the STAT5 oligonucleotide failed to inhibit STAT3 binding, although the STAT5 binding sequence present in the STAT5 oligonucleotide may be expected to displace STAT3 binding as well. Several scenarios are envisioned that may explain these observations. As shown elsewhere, STAT3 can form a number of mixed heteromeric protein complexes with several different proteins including the GR, P300, PIAS3, SOCS3, etc. The presence of STAT3 in a heteromeric protein complex may alter its DNA binding characteristics, and even preclude its binding to the STAT5 oligonucleotide. Indeed, such interactions with STAT co-activator proteins may underlie the unique properties that differentiate the biological function of STAT3 and STAT5.

Our unpublished data do suggest the requirement for additional proteins involved in XOR activation, and it is certainly possible that the identity of the protein binding to the STAT3 region is itself unknown or perhaps interacting with STAT3. Additional analysis of proteins mediating XOR activation will be required to determine whether STAT3 directly activates XOR, whether activation involves the formation of a STAT3 and GR dependent multiprotein complex as observed elsewhere [Lerner et al., 2003], or whether some presently unknown indirect interaction mediates the role of STAT3. However, it is noteworthy that previous studies uncovered binding of NF-1 and Oct-1 in the XOR proximal promoter in unstimulated HeLa cells [Chow et al., 1994; Clark et al., 1998a,b], and these proteins may contribute to STAT3 mediated activation as they do in the GR/STAT3 enhanceosome found in rat hepatoma cells [Lerner et al., 2003].

Synergy with STAT3 has been recognized to potentiate the action of the GR in response to several inducers in leukocytes, melanoma cells, and hepatocytes [Zhang et al., 1997; Krasil'nikov and Shatskaya, 2002; Levy and Darnell, 2002; De Miguel et al., 2003]. In co-transfection analyses of CV-1 cells synergy with STAT3 was found to be a general characteristic not only of the GR but of four different steroid hormone receptors including the ER, PR, and androgen receptor [De Miguel et al., 2003]. While the mechanism responsible for STAT3/GR synergy is not fully understood, models have emerged that provide a framework for interaction between the STAT3 transactivation domain and co-activator proteins that is sensitive to phosphorylation status and other post-translational modifications [Levy and Darnell, 2002]. Such synergistic and regulated interactions of this nature between STAT3 and the GR may provide a critical component of XOR expression in mammary epithelial cells that is regulated by steroid and by insulin.

While the mechanism by which PD98059 enhances insulin, APC, or growth factor activation of the XOR reporter is unknown, both STAT3 and the GR appear to be involved. The involvement of such broadly acting transcription factors may have unanticipated consequences. For example, STAT3 exhibits complex and often contradictory roles in the biology of the mammary gland [Bromberg, 2000; Clarkson et al., 2006]. STAT3 can mediate the effects of stress, growth factor stimulation, inflammatory cytokine stimulation, and participate in apoptotic involution [Marti et al., 1999; Clarkson et al., 2006]. The GR itself may also participate in numerous interactions with STAT factors, p300, p160 proteins, and provide a critical function in multiprotein complexe regulation [Edwards, 2000; Lerner et al., 2003]. Although the mechanism by which PD98059 affects STAT3 is unknown, present data suggest that p38 MAPK may be involved. These observations are consistent with previous demonstrations of the contribution of p38

MAPK to stress activation of XOR [Abdulnour et al., 2006; Seymour et al., 2006], but they also suggest additional points at which PD98059 may be acting. The unexpected activation of the XOR promoter by STAT3 in response to PD98059, dexamethasone, or insulin suggests a potential role for STAT3 in the physiological induction of XOR in the mammary gland by insulin, APC, or growth factors, and additional analyses will determine whether STAT3 contributes in a fundamental way to the integration of diverse signals mediating XOR activation in the mammary gland.

ACKNOWLEDGMENTS

The authors are grateful to Dr. James E. Darnell, Jr. (NYU School of Medicine, NY) and Dr. Allen Gao (Roswell Park Cancer Institute, NY) for the STAT3C cDNA expression clone. Dr. Jennifer Grandis (University of Pittsburgh, PA) kindly provided the STAT3F cDNA expression clone. These studies were supported in part by grants from the National Institutes of Health (HL52509 and HL45582) and the Robert and Helen Kleberg Foundation.

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