Repression of interleukin-6 gene expression by 17β-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-κB by the estrogen receptor

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Abstract The cytokine interleukin-6 (IL-6), a key mediator of immune and acute phase responses of the liver, has also been implicated in uterine functions. Estrogens are potent repressors of IL-6 production by uterine stromal cells. In the endometrial adenocarcinoma cell line Ishikawa, phorbol ester-induced activation of the IL-6 promoter was inhibited to basal levels by 17β-estradiol (E_2) in a wild-type receptor-dependent fashion. Although tamoxifen has been shown to have estrogenic effects on the endometrium, it did not inhibit induction of the IL-6 promoter. We previously showed that inhibition of *IL-6* gene expression by E_2 does not involve high-affinity binding of the estrogen receptor (ER) to IL-6 DNA. We now report that the ER can directly interact with the transcription factors NF-IL6 and NF-κB and can inhibit their ability to bind DNA which might be the molecular basis for repression of *IL-6* gene expression by estrogens.

Key words: Estrogen; Tamoxifen; Interleukin-6; Gene expression; Protein–protein interaction

1. Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine produced in response to infection, injury or trauma by a wide variety of cells including monocytes/macrophages, fibroblasts, epithelial cells and endothelial cells [1]. In turn, IL-6 acts on a wide variety of cells regulating immune responses, acute phase responses of the liver, hematopoiesis, neuronal functions and osteoclastogenesis [1,2]. Although IL-6-deficient mice do not display any obvious defects in development, absence of IL-6 perturbs immune and acute phase responses in these mice [3,4]. Strikingly, ovariectomy in the IL-6-deficient mice ablated the osteoporosis that was seen in normal mice suggesting an important role for IL-6 in mediating bone loss in an estrogen-depleted state [4]. IL-6 has also been implicated in uterine functions [1,3–6]. Both uterine stromal fibroblasts and epithelial cells produce IL-6 which can be inhibited by 17β-estradiol (E_2) [5,6]. This is of considerable interest since particular cytokines are now known to have either positive or negative effects on blastocyst implantation and embryo development. For example, it has been suggested that IL-6 via its induction of chondroitin sulfate proteoglycans inhibits balstocyst implantation [6]. Deficiency of a related cytokine leukemia inhibitory factor (LIF) in female mice leads to failure of blastocyst implantation and development [7]. Inhibition of IL-6 production by estrogens suggests that the hormonal control of these processes may be, at least partly, regulated via modulations of local cytokine production.

Our earlier studies showed that 225 bp of the IL-6 5′-flanking region is sufficient for conferring responsiveness of the IL-6 promoter to cytokines such as IL-1 and activators of protein kinase C (phorbol esters) and protein kinase A (forskolin) [8–11]. We and others showed that the activation of the IL-6 promoter involves synergism between the transcription factors NF-IL6 (also called CAAT enhancer binding protein/β or C/EBPβ) and NF-κB [11,12]. We also showed that while multiple agents activate *IL-6* gene transcription, glucocorticoids and estrogens are potent repressors of *IL-6* gene expression [5,9–11,13]. It appears that negative feedback by steroid hormones on endogenous IL-6 levels is an important regulator of physiological homeostasis. Disruption of this control, as in an estrogen-depleted state in post-menopausal women, is believed to be the major reason for uninhibited IL-6 production in the post-menopausal phase that ultimately precipitates osteoporosis [14,15]. Indeed, E_2 down-modulates IL-6 production in osteoblasts and in bone marrow stromal cells [14–17]. Understanding the molecular mechanisms that control steroid regulation of key cytokine genes, therefore, represents an important facet of endocrinology/immunobiology.

The DNA elements in the IL-6 5′ flanking sequence that respond to inhibition by steroid hormones in functional assays do not bind either the glucocorticoid receptor (GR) or the estrogen receptor (ER) [10,11,13,18]. Our investigations of the mechanisms by which glucocorticoids inhibit *IL-6* gene transcription revealed direct physical association and functional antagonism between the GR and NF-κB [11]. Negative cross-talk between the GR and NF-κB was also reported by Caldenhoven et al. and Scheinman et al. [19,20].

In these studies, we investigated the effects of E_2 and tamoxifen on phorbol ester-induced activation of the IL-6 promoter in the endometrial adenocarcinoma cell line Ishikawa that was created by Nishida et al. as a model for studies of effects of E_2 on endometrial tumors [21]. Our studies suggest that estrogens repress *IL-6* gene expression through inhibition of the DNA-binding activities of the transcription factors NF-IL6 and NF-κB by the ER.

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2. Materials and methods

2.1. Cell culture and transfections

Ishikawa cells were provided by Drs. Erlio Gurpide and Stephanie DeGrandis. The cells were cultured in minimal essential medium (MEM) with Earle's salt and 10% fetal bovine serum (FBS). While stock cells were grown in MEM containing regular FBS, cells used in experiments were maintained for at least 8 days in phenol red-free MEM supplemented with dextran-coated charcoal-treated serum with change of medium every 3 days. Cells were transfected by the calcium phosphate coprecipitation procedure as previously described [8–11]. Cells were transfected with pIL225 (2 μg) in the presence or absence of an expression vector for wild-type or mutant ER (2 μg) together with the carrier DNA pGEM7Zf(+) to make up the total DNA to 10 μg per 60 mm petri dish. Luciferase assays were performed using a kit (Promega) following instructions of the manufacturer and measured in a Lumat LB9501 luminometer (Berthold, Germany). In each experiment, equal amounts of protein, as measured by the Bradford method (BioRad), were assayed for luciferase activity. Each experiment was repeated at least 3 times.

2.2. Plasmid constructs

The construct pIL225 containing *IL-6* gene sequences from −225 to +13 linked to the *luciferase* gene has been described previously [22]. The expression vectors for wild-type and mutant ERs were gifts of Dr. Pierre Chambon.

2.3. Preparation of His-tagged ER

The ER cDNA was derived from the expression vector HE0 and was cloned in frame into the plasmid pET15b (Novagen) for protein expression. The recombinant plasmid was transformed into host bacteria (BL21) harboring a *T7 polymerase* gene in a λ lysogen (λDE3) under the control of a *lacUV5* promoter. His-ER was purified by affinity chromatography on Ni^{2+}-nitriloacetic acid agarose beads. The beads were extensively washed and bound protein was eluted in a buffer containing 80 mM imidazole. His-β-gal was expressed from a pET15b-β-gal plasmid construct. The purification protocol was identical to that described above except the elution buffer contained 200 mM imidazole. The purified proteins were applied to Centricon 10 columns (Amicon) to remove imidazole and further concentrate the proteins.

2.4. Co-immunoprecipitation experiments

The cDNAs for ER, p65, bzipNF-IL6 (containing amino acids 141–296 of murine NF-IL6) and IκBR were translated in vitro using wheat-germ extracts following instructions of the manufacturer (Promega) with one modification. At the end of the incubation period, the reactions were terminated by the addition of RNase A to a final concentration of 200 μg/ml followed by a brief incubation at 30°C. Individual extracts were appropriately mixed and incubated in a buffer containing 20 mM Hepes, pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 10% glycerol for 45 min at room temperature. To rule out the possibility of co-immunoprecipitation of proteins due to adventitious binding to template DNA provided in the in vitro transcription/translation reactions, ethidium bromide at a concentration of 12.5 μg/ml was included in the reactions. DNA-dependent protein associations are selectively inhibited by ethidium bromide in immunoprecipitation reactions without adversely affecting DNA-independent protein associations [23]. The mixtures were diluted to 200 μl with NET buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 0.25% gelatin and 1 mM PMSF. The protein mixture was precleared by the addition of 30 μl of protein-A Sepharose beads for 1 h at 4°C. The beads were spun down, appropriate antisera were added to the supernatants and the beads were left on a rotary shaker overnight at 4°C. The beads were washed 2 times with NET buffer and once with a buffer containing 10 mM Tris-HCl, pH 7.5 and 0.1% NP-40 (1 ml each time). The immune complexes were boiled in 2× SDS sample buffer containing 10% β-mercaptoethanol and analysed by SDS-PAGE, fluorography and autoradiography. The anti-p65 antiserum was an affinity-purified rabbit polyclonal antibody raised against a peptide mapping with the amino terminal *rel*-related region of the p65 subunit of human NF-κB (Santa Cruz Biotechnology). The anti-NF-IL6 and the anti-IκBR antisera were rabbit polyclonal antisera raised by us against the glutathione S-transferase (GST) fusion proteins GST-NF-IL6 and GST-IκBR [24] respectively.

2.5. Electrophoretic mobility shift assays (EMSA)

EMSAs were done using His-tagged ER, in vitro-translated (using wheat-germ extract, Promega) p65 or NF-IL6 or recombinant p50 (Promega). Conditions for EMSAs were essentially as described previously [24] with slight modifications. Briefly, for NF-IL6, 1 μl of in vitro-translated protein was pre-incubated in the presence or absence of antibodies or competitor proteins for 30 min on ice in a buffer containing 10 mM Hepes, pH 7.9, 50 mM NaCl, 1 mM DTT, 1 mM $MgCl_2$, 0.1 mM EDTA and 10% glycerol, 5 μg/ml poly dI-poly dC, 200 μg/ml of BSA and a protease inhibitor cocktail (Boehringer Mannheim) before addition of the labeled probe and further incubation at room temperature for 15 min. In the case of p50 and p65, the conditions were the same except no EDTA or $MgCl_2$ was added. Probes used were a ^{32}P-labeled double-stranded oligonucleotide containing the NF-κB sequence derived from the IL-6 promoter (nucleotides −64 to −73) or a commercial (Santa Cruz Biotechnology, Inc.) oligonucleotide containing the consensus core NF-IL6 binding sequence (TTGCGCAA) which is only different by one base from the NF-IL6 site (TTGTGCAA) present between nucleotides −148 and −155 in the IL-6 promoter [11]. All antisera were purchased from Santa Cruz Biotechnology, Inc., except the anti-Fos antibody which was purchased from Oncogene Science. The binding reactions were analyzed by electrophoresis on 6% native polyacrylamide gels (acrylamide/bisacrylamide=30:1). Electrophoresis was carried out at 200 V in 0.5×TBE (1×=0.05 M Tris base, 0.05 M boric acid and 1.0 mM EDTA) at 4°C. Gels were dried and subjected to autoradiography.

3. Results

3.1. Inhibition of phorbol ester-induced activation of the IL-6 promoter by ER/E_2 combination: absence of inhibition by Tam

The IL-6 promoter containing 225 bp of 5′ flanking sequences is activated by diacylglycerol/activators of protein kinase C in many different cell types including fibroblasts, epithelial cells and osteoblasts [8,18,25]. In Ishikawa cells, we observed a 6–10-fold activation of the IL-6 promoter upon stimulation with the phorbol ester phorbol 12-myristate 13-acetate (PMA) (Fig. 1). Ishikawa cells were previously reported to be ER positive [26]. However, after maintenance in culture for prolonged periods of time, particularly under certain culture conditions, they become unresponsive to E_2 (ref. [27] and E. Gurpide, personal communication). The lack of responsiveness of these cells to E_2 could be due to down-regulation of ER expression in more differentiated cells [28]. Indeed, in cells transfected with pIL225 alone, PMA-induced expression from the IL-6 promoter was repressed only between 20 and 40% in the presence of E_2 but upon cotransfection with HE0, an expression vector containing the wild-type human ER [29], the induction was inhibited to almost basal levels (Fig. 1). HE0 has been extensively used previously in studies of estrogen-induced promoters [30,31]. Earlier, we and others demonstrated the ability of HE0 to also efficiently repress IL-6 promoter activity [13,18]. In these studies we also investigated the ability of the anti-estrogen tamoxifen (Tam) to repress IL-6 promoter activity in the presence of HE0 (Fig. 1) or mutant receptors (Fig. 2) essentially for two reasons: (i) the effect of Tam on inhibition of gene expression has not been adequately investigated in any estrogen-responsive cell including Ishikawa cells and (ii) Tam has been shown to have partial agonist activity in the endometrium; several recent studies have suggested a possible link between tamoxifen use and the development of endometrial carcinoma [32,33]. In our studies, used at either 10^{-7} M or even higher concentrations, Tam did not display any inhibitory effect on IL-6 promoter activity thus

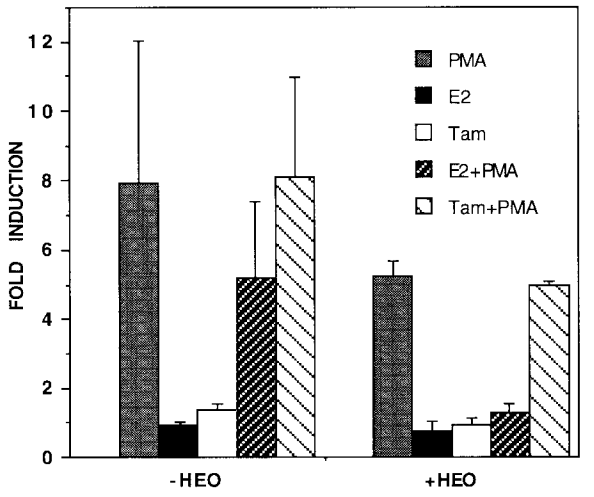

Fig. 1. Effect of E_2 and Tam on PMA-induced IL-6 promoter activity in Ishikawa cells. Monolayer cultures of Ishikawa cells were transfected with the IL-6/luciferase construct pIL225 (2 μg) and a carrier DNA pGEM7Zf(+) (to make up to 10 μg/60 mm petri dish) in the presence or absence of an expression vector for wild-type (HE0) (2 μg). Cells were transfected by the calcium phosphate co-precipitation procedure [45] as previously described [8–11]. At 16–18 h post-transfection, the precipitate was washed off and the plates were replenished with serum-free medium in the presence or absence of PMA (100 ng/ml), E_2 or Tam (both at 10^{-7} M). Identical data were obtained with 10-fold lower concentrations of E_2 or Tam. The cells were harvested 6 h later and cell extracts prepared by freezing and thawing of the cells were assayed for protein amounts. Extracts containing equal amounts of protein were assayed for luciferase activity. The fold-inductions represent luciferase activities in induced cultures relative to those in uninduced control cultures. Values are mean ± SE of three independent experiments.

demonstrating the strict estrogen-dependence of ER for repression of IL-6 promoter activity (Fig. 1).

3.2. *Structural requirements in ER for repression of IL-6 promoter activity*

The ER, like other hormone receptors, has a modular structure with distinct functions for each domain, as was previously determined using estrogen-stimulated promoters [30,31]. For example, domain C is the DNA-binding domain, domains B and E encompass two non-acidic independent transcriptional activation domains called TAF-1 and TAF-2 respectively and domain E is the hormone-binding domain (Fig. 2A). Also, the ER was shown to bind to estrogen response elements as a ligand-induced dimer [31]. We used expression vectors for different mutant ERs (all derived from HE0) in transfection experiments to evaluate the role of some of the domains in repression of phorbol ester-induced IL-6 promoter activity (Fig. 2B). A deletion extending from the hinge region D into the hormone-binding domain E (HE5), previously shown to abolish estrogen binding was unable to repress IL-6 promoter activation. This was similar to our previous observations in HeLa cells [13]. However, a deletion of the hinge region alone (HE12) did not impair the ability of ER to inhibit IL-6 promoter activation. The mutant HE11 containing a deletion in the DNA-binding domain was unable to behave as a repressor (Fig. 2). Curiously, this mutant slightly augmented the phorbol ester induced stimulation of IL-6 promoter function (Fig. 2B). The mutant HE14 containing an intact hormone-binding domain but devoid of TAF-1 and the DNA-binding domain (deletion of amino acids 1–281) was not able to repress the IL-6 promoter. HE15 containing TAF-1 and the DNA-binding domain, but lacking TAF-2 and the hormone-binding domain also did not repress the IL-6 promoter. Conversely, HE19, lacking the TAF-1 domain but with intact DNA-binding domain and hormone-binding domain containing the hormone-inducible transactivating function-2 (TAF-2), repressed activation of the IL-6 promoter by up to 80–90%. Taken together, these data establish a clear dependence of the ER on the DNA-binding domain (mutants HE11, HE14) and the hormone binding domain (HE5, HE15) for repression of the IL-6 promoter in Ishikawa cells.

3.3. *Direct interactions between ER and transcription factors NF-IL6 and NF-κB*

We and others previously established the importance of the NF-κB site and the NF-IL6 site in the IL-6 promoter for its activation by multiple agents including cytokines, viruses and activators of protein kinase C (diacylglycerols/phorbol esters) and protein kinase A (forskolin, cAMP analogues) [9,11,25,34–37]. Furthermore, the p65 subunit of NF-κB (and also the p50 subunit, albeit less efficiently) and NF-IL6 were found to synergize in the activation of the IL-6 promoter [11,12]. The PMA-induced activation of the IL-6 promoter in Ishikawa cells was also found to be dependent on intact NF-κB and NF-IL6 sites in the promoter (data not shown). In our initial investigations of repression of IL-6 promoter activation by E_2, we were unable to detect binding of the ER to the promoter region between –225 and +13 that could be functionally repressed in transfection experiments [13]. This suggested that inhibition of NF-IL6- and p65-mediated activation of this region of the promoter by E2 may be a consequence of physical interaction between the ER and the transcription factors involved in activation of the IL-6 promoter [13,18]. To examine this possibility, we investigated direct interactions of the ER with both p65 and NF-IL6 by co-immunoprecipitation methods. Full length p65 and the ER and a region of NF-IL6 comprising amino acids 141–296 of NF-IL6 containing the basic amino acid-leucine zipper (bzip) domain of the protein (amino acids 206–296) (bzipNF-IL6) were synthesized in vitro using wheat-germ extracts. To distinguish between the ER and p65, which are of identical molecular size (65 kDa), the ER was synthesized in the presence of [^{35}S]methionine while p65 was synthesized in the presence of unlabeled amino acids. For the sake of uniformity, bzipNF-IL6 was also synthesized in the presence of unlabeled amino acids. Interactions of the ER with NF-IL6 or NF-κB were found to be estrogen-independent just as the GR can interact with these transcription factors in the absence of its ligand [11,19,20,38]. As a negative control, we used an unrelated protein IκBR, a member of the IκB-family of proteins, whose cDNA was recently cloned by us [24]. IκBR was also synthesized in the presence of unlabeled amino acids. The translation mixtures were briefly treated with RNase following the recommended incubation period, to ensure that no labeled p65 or NF-IL6 or IκBR would be made once it was mixed with extract containing labeled ER and unreacted [^{35}S]methionine. Also, ethidium bromide was included in the incubation mixtures to inhibit adventitious DNA-mediated protein interactions. The presence of unlabeled p65 or NF-IL6 proteins in the respective programmed wheat-germ extract was verified by assaying the

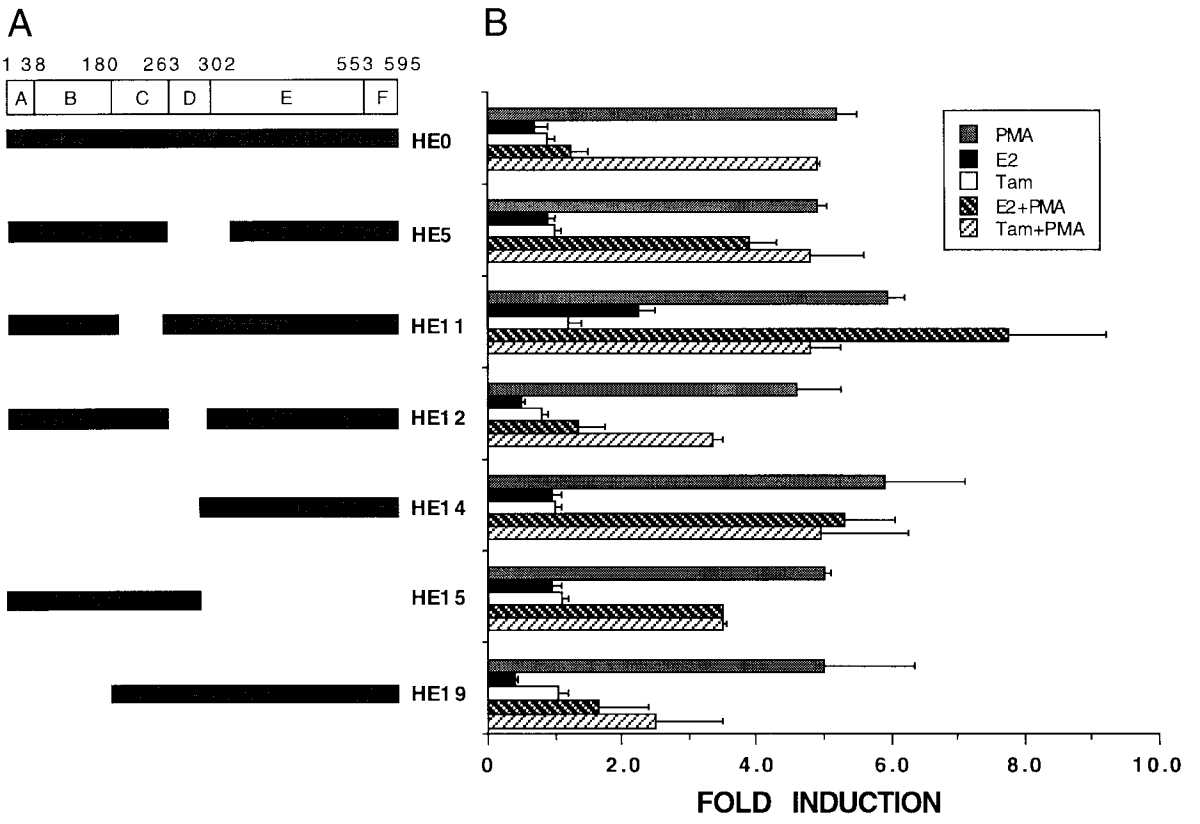

Fig. 2. Structural requirements in the ER for repression of IL-6 promoter activity in the presence of E_2. Cells were transfected with pIL225, in the presence or absence of HE0 or expression vectors for mutant ERs. All other conditions were as described in the legend to Fig. 1. Data shown are mean ± SE of three independent experiments.

ability of the proteins to bind to target sites in DNA in EMSAs and the presence of IκBR in the extract was verified by Western blotting (data not shown).

^{35}S-labeled ER was incubated in buffer in the presence or absence of unlabeled p65 or NF-IL6 or IκBR. Subsequently, these reactions were immunoprecipitated either with an anti-p65 antibody, an anti-NF-IL6 antibody, an anti-IκBR antibody or with control immunoglobulin. The immunoprecipitated material was analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The anti-p65 antibody did not precipitate labeled ER in the absence of p65 (Fig. 3, lane 2). Likewise, anti-NF-IL6-antibody did not precipitate labeled ER in the absence of NF-IL6 (lane 5). However, in the presence of the respective proteins, both the antibodies precipitated ER (lanes 3 and 6) indicating interactions of the ER with both p65 and NF-IL6. In parallel reactions, control immunoglobulin did not precipitate ER (lanes 2 and 4). No immunoprecipitation of ER was detected by either control serum or specific antiserum in the reactions containing IκBR (lanes 7–9).

3.4. *ER inhibits the DNA-binding ability of NF-IL6 and NF-κB subunits*

We investigated whether direct association between the ER and the transcription factors NF-IL6 and NF-κB affects the binding of these proteins to their target sites in the IL-6 promoter in EMSAs. The NF-IL6 protein and the p65 subunit of NF-κB were obtained by in vitro translations of the respective full-length cDNAs. The ER that we used in these assays was a recombinant His-tagged receptor. To discount any effects of the His tag in ER, we used His-tagged β-gal as a control. As illustrated in Fig. 4A (lanes 4–6), the DNA-binding activity of NF-IL6 was significantly inhibited by His-ER. His-β-gal had no effect on the binding of NF-IL6 indicating that the effects of His-ER were mediated by the ER part of the fusion protein.

We next investigated the effect of the ER on the DNA-binding ability of the p65 subunit of NF-κB. The molecules smaller than p65 appear to be truncated forms of in vitro translated p65. The ER minimally affected the DNA-binding ability of p65 (Fig. 4B, lanes 4–6). Our inability to detect a significant inhibition in the DNA binding ability of p65 in the presence of the ER, despite evidence for interaction between the two proteins by co-immunoprecipitation techniques, may be due to lack of optimal conditions for detection by EMSA. Similarly, in the case of the GR, while an interaction between the GR and NF-IL6 was demonstrated by co-immunoprecipitation techniques, evidence for such an interaction could not be demonstrated by EMSA [38]. However, in the same experiment and under similar conditions, the ER strongly inhibited the ability of the p50 homodimer to bind DNA (Fig. 4C, lanes 4–6). The control protein His-β-gal did not influence the binding of p65 (Fig. 4B, lanes 7–9) but surprisingly increased the binding activity of p50 and by itself formed a slower migrating complex with the NF-κB probe (Fig. 4C, lanes 7–9). We and others have shown that NF-IL6 can efficiently synergize with NF-κB to activate the IL-6 promoter [11,12]. Therefore, an inhibition of the DNA-binding activity of p50 by the ER,

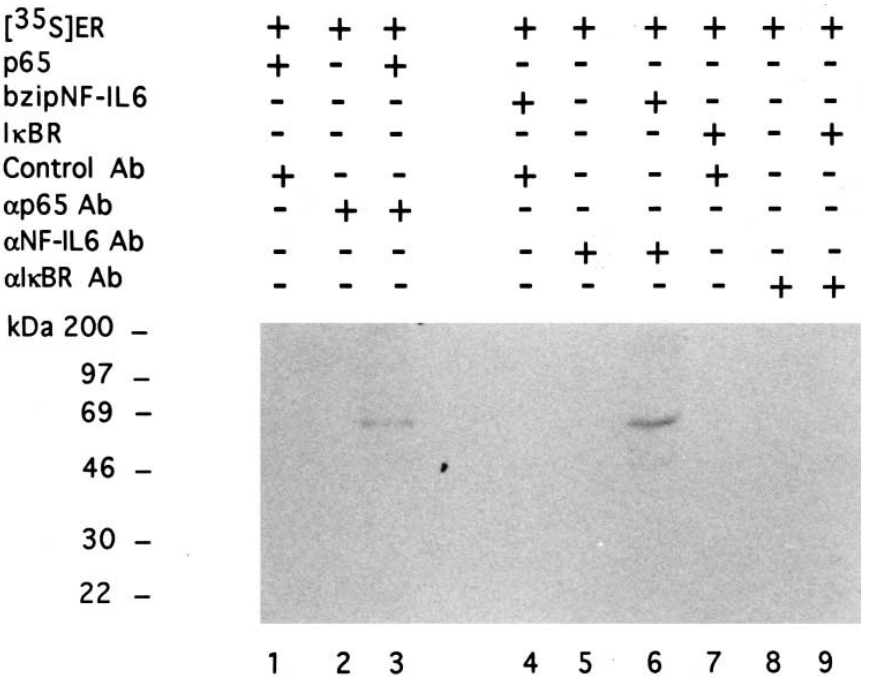

Fig. 3. Co-immunoprecipitation of ER-NF-IL6 and ER-p65 complexes. ^{35}S-labeled ER and unlabeled p65, bzipNF-IL6 or IκBR were translated in wheat germ extracts. 10 μl aliquots of the programmed extract containing labeled ER (treated with RNase to prevent any further synthesis of proteins when mixed with extracts containing unlabeled proteins) were incubated in the presence or absence of p65 (10 μl), NF-IL6 (10 μl), IκBR (10 μl), anti-p65 antibody, anti-NF-IL6 antiserum, anti-IκBR antiserum or control immunoglobulin. The relative amounts of the translated proteins p65, bzipNF-IL6 and IκBR present in the wheat germ extracts were predetermined by analysing ^{35}S-labeled proteins on SDS-gels and normalizing the radioactivity incorporated in the proteins relative to the content of methionine in each protein. Equal volumes of the extracts were found to contain approximately similar amounts of the respective proteins. The proteins were immunoprecipitated as described under Section 2 and the resulting complexes were analyzed by fluorography following separation on an 10% SDS-polyacrylamide gel. The mobilities of the protein standards are indicated.

which in all likelihood is also through direct association between the two proteins, will considerably diminish occupancy of the NF-κB site in the IL-6 promoter by the active NF-κB complex. Also, since the NF-IL6 and NF-κB sites are both crucial for IL-6 promoter function [11,12], inhibition of the DNA-binding activity of NF-IL6 alone would disrupt activation of the promoter.

4. Discussion

Repression of *cytokine* gene expression, such as that of IL-6, by endogenous estrogens and corticosteroids represents an important immunoregulatory feature of steroid hormones. Steroid receptors mediate the specific response of cells to their respective ligands, in many instances, by virtue of their ability to bind *cis*-acting enhancer sequences termed steroid response elements. Although activation of genes by glucocorticoids and estrogens typically is mediated by binding of the ligand-activated receptor to the respective response element(s) present upstream of or within target genes, negative regulation by these hormones cannot be adequately explained by receptor–DNA interactions. Therefore, the molecular targets that determine inhibition of gene expression by steroid hormones is a subject of intense investigation in many laboratories. Our studies are the first to show the ability of the ER to compromise the DNA-binding activities of NF-IL6 and NF-κB. Although transcriptional interference between ER and Fos/Jun (AP-1) was described previously using synthetic AP-1 oligonucleotide-driven reporter genes [39], functional antagonism between the ER and NF-IL6/NF-κB demonstrate negative cross-talk in the context of a biologically important molecule which regulates normal physiological responses such as endometrial functions [5,6] and bone metabolism [2,14].

Although the anti-estrogen Tam has been shown to have estrogen-like activities in the endometrium, it was ineffective in repressing IL-6 promoter function in the presence of wild-type ER. This suggests that adjuvant Tam therapy for ER-positive breast cancers in post-menopausal women may not alleviate the elevated systemic IL-6 level that has been suggested to contribute to osteoporosis in these women [2].

In our previous studies on the mechanisms by which glucocorticoids inhibit *IL-6* gene expression, we found no evidence for interactions between the GR and IL-6 DNA using a variety of techniques [9,10]. We subsequently demonstrated functional antagonism between the p65 subunit of NF-κB and the GR [11]. Specifically, we showed mutual interference in transactivation functions between the GR and the p65 subunit of NF-κB in functional assays and direct physical association between the two proteins in co-immunoprecipitation experiments [11]. In other studies, interactions between the GR and NF-IL6 were also demonstrated [38].

In our initial investigations of the mechanisms of repression of IL-6 promoter activity by estrogens, we and others showed that like the GR, the ER also does not bind with high affinity to IL-6 DNA [13,18]. However, mutations in the DNA-binding domain of the ER was unable to repress IL-6 promoter activity in HeLa cells [13]. In Ishikawa cells too, deletion of the DNA-binding domain (with intact TAF-1 and TAF-2

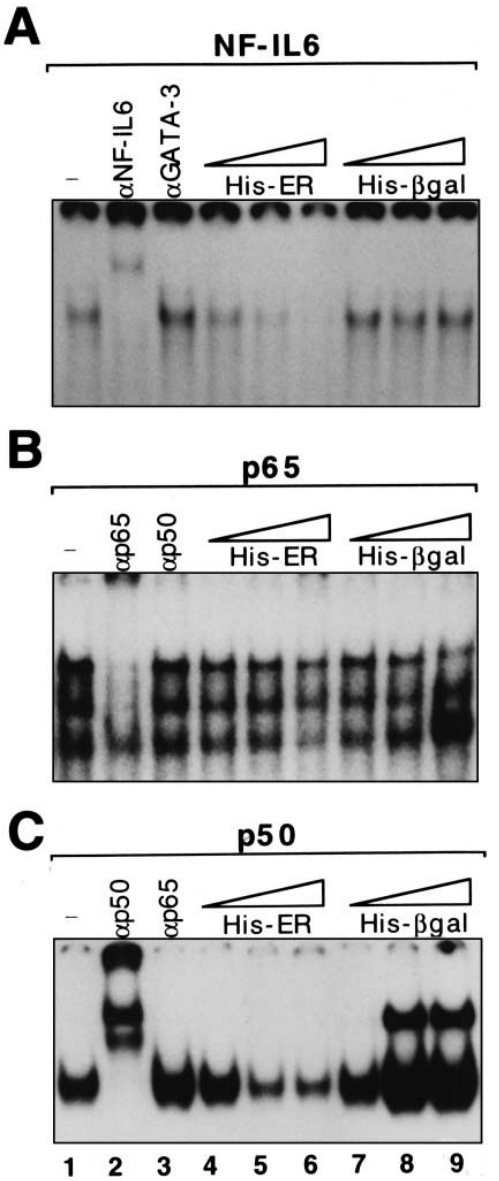

Fig. 4. Effect of the ER on the DNA-binding abilities of different transcription factors. A: In vitro translated (1 μl of translation mix) NF-IL6 was incubated in the presence or absence of anti-NF-IL6 antibody or anti-GATA3 antibody (used as a negative control) or 5, 50 or 250 ng of His-ER or equimolar amounts of His-βgal protein. The samples were incubated with labeled probe before electrophoresis. The probe was a ^{32}P-labeled commercial (Santa Cruz Biotechnology, Inc.) double-stranded oligonucleotide containing the consensus core NF-IL6 binding sequence (TTGCGCAA) which is only different by one base from the one (TTGTGCAA) present between nucleotides −148 and −155 in the IL-6 promoter [11]. B,C: Effect of the ER on the DNA-binding abilities of the individual subunits of NF-κB. The DNA-binding reaction was carried out with (B) in vitro-translated p65 or (C) purified p50 protein (Promega) in the presence or absence of antibodies to the proteins as indicated, or in the presence of His-ER or equimolar amounts of His-βgal. The probe used was a ^{32}P-labeled oligonucleotide containing the NF-κB sequence from the IL-6 promoter. The binding reactions were analyzed by electrophoresis on 6% native polyacrylamide gels. Gels were dried and subjected to autoradiography.

functions) totally abrogated repressor function of the receptor. It is curious that this mutant receptor actually potentiated the activation of the promoter by PMA. This is reminiscent of our previous findings with first Zinc (Zn) finger mutants of the GR in which mutations in the first Zn-finger (but not the second Zn finger) of the GR caused a repressor to activator switch in the behavior of the receptor toward IL-6 promoter activity, which was even more obvious with suboptimal concentrations of IL-1 [10]. Furthermore, we found that specific promoters, otherwise unaffected by glucocorticoids and the wild-type GR were also activated by these mutant receptors suggesting that mutations in the DNA-binding domain of GR may unleash aberrant expression of genes which are not normally regulated by glucocorticoids [10]. Whether similar mutations in the ER can also aberrantly activate specific genes remains to be determined.

Small deletions in the ligand binding domain which are known to impair binding of estrogen to the ER, as in HE5, eliminated the ability of the receptor to inhibit IL-6 promoter activity. However, the hormone-binding domain alone containing an intact TAF-2 function (which is ligand-inducible) was unable to cause repression. What appears clearly dispensable for repression is the region (D) adjacent to the DNA-binding domain of the ER. This is not particularly surprising since deletion or insertion mutations in this domain were previously shown to have little effect on the stimulatory effects of the receptor on some promoters. This region, which is conserved neither in length nor in amino acid composition in different nuclear hormone receptors, and also poorly conserved across species in the ER has been postulated to act as a hinge between the DNA- and hormone-binding domains [30,40]. The TAF-1 domain alone (in HE15) was demonstrated to have constitutive transactivational functions in a promoter- and cell-specific fashion [41]. Furthermore, this activity strongly correlated with the agonistic activity of OH-Tam in combination with the intact ER on the same genes in the same cells [41]. In our experiments the Tam/ER combination did not repress IL-6 promoter function which correlated with the inability of HE15 to repress.

The data in Figs. 3 and 4 indicate that E_2 is not required for interactions of the ER with NF-IL6 or NF-κB. These results are in agreement with previous reports demonstrating the ability of in vitro-synthesized ER to interact in vitro, in the absence of hormone, with a variety of proteins such as c-Jun [42], the TBP associated factor (TAF) $TAF_{II}30$ [43] and the co-activator SPT6 [44]. This is not a unique ability of the estrogen receptor since the structurally related glucocorticoid receptor synthesized in vitro also interacts with multiple transcription factors in vitro in an hormone-independent fashion as has been shown by us [11] and other investigators [19,20,38]. It has been suggested that in vitro-synthesized steroid receptors fold into conformations that are permissive for protein–protein interactions in vitro and therefore these interactions are not influenced by hormone [20]. It is important to note, however, that although protein–protein interactions with in vitro-synthesized receptors are hormone-independent, these interactions occur only with specific proteins.

Thus we have defined novel molecular targets for inhibition by estrogen that now provide a basis for the initial description of inhibition of *IL-6* gene expression by E_2 in freshly explanted human endometrial stromal cells, osteoblasts and uterine epithelial cells [5,6]. It can be speculated that other cytokine genes that are regulated by a combination of NF-IL6 and NF-κB, such as *IL-8*, may also be subject to negative regulation by estrogens by similar mechanisms. Protein–protein

interactions between hormone receptors and transcriptional activators resulting in inhibition of the DNA-binding functions of the transactivators are likely integral to the complex interplay between hormones and the immune system.

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