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T K Kerppola, D Luk and T Curran
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Fos Is a Preferential Target of Glucocorticoid Receptor Inhibition of AP-1 Activity In Vitro

TOM K. KERPPOLA, DANIEL LUK, AND TOM CURRAN*

Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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Several regulatory interactions between the AP-1 and the nuclear hormone receptor families of transcription factors have been reported. However, the molecular mechanisms that underlie these interactions remain unknown, and models derived from transient-transfection experiments are contradictory. We have investigated the effect of the purified glucocorticoid receptor (GR) DNA-binding domain (GR residues 440 to 533 [GR440–533]) on DNA binding and transcription activation by Fos-Jun heterodimers and Jun homodimers. GR440–533 differentially inhibited DNA binding and transcription activation by Fos-Jun heterodimers. Inhibition of Jun homodimers required a 10-fold-higher concentration of GR440–533. An excess of Fos monomers protected Fos-Jun heterodimers from inhibition by GR440–533. Surprisingly, regions outside the leucine zipper and basic region were required for GR inhibition of Fos and Jun DNA binding. The region of GR440–533 required for inhibition of Fos-Jun DNA binding was localized to the zinc finger DNA-binding domain. However, inhibition of Fos-Jun DNA binding was independent of DNA binding by GR440–533. GR440–533 also differentially inhibited Fos-Jun heterodimer binding to the proliferin plfG element. Differential inhibition of DNA binding by different AP-1 family complexes provides a potential mechanism for the diverse interactions between nuclear hormone receptors and AP-1 family proteins at different promoters and in different cell types.

Transcription regulation requires the integration of signals from multiple signal transduction pathways that mediate responses to diverse extracellular signals. Such signals can interact either positively, to augment the transcriptional response, or negatively, to block the response at many different levels in the signal transduction cascade. Interactions at an early stage are likely to produce generic effects, causing many genes to respond identically to the same combination of signals, whereas interactions at later stages allow independent regulation of different target genes. Direct interactions between transcription factors that respond to different signal transduction pathways provide the most immediate control of the response of a gene to a given combination of signals. Such interactions have been proposed to account for the regulatory interplay between phorbol ester and steroid hormone signals mediated by the Fos/Jun and the nuclear hormone receptor families of transcription factors.

Both positive and negative regulatory interactions between Fos/Jun and nuclear hormone receptors have been described (reviewed in reference 12). The collagenase and stromelysin genes are activated by Fos/Jun and repressed by the glucocorticoid receptor (GR) and retinoic acid receptor in cotransfection assays (7, 15, 19, 20, 24, 25). Conversely, the activation of several nuclear-hormone-responsive genes is repressed by overexpression of Fos and Jun (7, 11, 16, 19, 21, 23, 24). In contrast, cotransfection of Fos/Jun with the estrogen receptor causes synergistic activation of the ovalbumin gene (6). The human proliferin gene is activated by phorbol esters and repressed by dexamethasone in several cell lines (13). However, a reporter gene linked to a proliferin regulatory element (plfG) is synergistically activated by phorbol esters and dexamethasone in HeLa cells (3). Dexamethasone also potentiates transcription activation by transfected c-*jun* but represses activation by cotransfected c-*fos* and c-*jun* in F9 cells (3). Cotransfection of different combinations of c-*fos* or c-*jun* and glucocorticoid, progesterone, or androgen receptor expression vectors has different effects on the expression of hormone-responsive genes, depending on the promoter and the cell line (22). Whereas c-*jun* can either inhibit or stimulate, c-*fos* can only inhibit receptor-induced transcription.

The molecular basis for the multiple regulatory interactions between Fos/Jun and nuclear hormone receptors at different promoters and in different cell types remains unknown. The inhibitory effect of GR on transcription activation by Fos/Jun has been most intensely investigated for the human collagenase gene. The collagenase gene promoter is activated by phorbol esters and repressed by dexamethasone in the presence or absence of protein synthesis inhibitors (7). The sequences required for repression have been delimited to the AP-1 binding site (7, 19, 24). From the results of cross-linking and coimmunoprecipitation experiments, GR has been proposed to interact directly with Fos or Jun (3, 7, 24). However, others have not detected complexes between Fos or Jun and nuclear receptors (11, 19, 22), suggesting that any interaction between the proteins may be weak or indirect. Two distinct mechanisms have been proposed for GR inhibition of AP-1 activity. GR may block DNA binding by Fos and Jun or repress their transcriptional activation potentials. Yang-Yen et al. (24) reported that purified GR could partially inhibit DNA binding by Jun. They (24) and Schüle et al. (19) also reported that Jun could inhibit DNA binding by GR. In contrast, Jonat et al. (7) reported that dexamethasone had no effect on AP-1 binding activity in nuclear extracts. In vivo footprinting experiments indicated that the AP-1 site remained occupied even when transcription from the collagenase promoter was repressed by dexamethasone

* Corresponding author.

(10). Thus, the effect of GR on DNA binding by Fos and Jun remains controversial.

The regulatory interactions between Fos/Jun and GR at the human proliferin gene suggest that there may be a more complex association between these proteins (3, 13). The plfG element from the proliferin gene mediates the antagonistic effects of phorbol esters and dexamethasone in some cell lines but synergistic activation in others (3). Cotransfection experiments with c-*fos* and c-*jun* expression vectors suggest that the relative levels of Fos and Jun determine the effect of dexamethasone on transcription regulation by this element (3). In cells containing Jun alone, dexamethasone causes synergistic activation of transcription, whereas in cells containing both Fos and Jun, dexamethasone represses transcription. In cells containing little or no Fos or Jun, dexamethasone has no effect on transcription, suggesting not only that Fos and Jun function in concert with GR, but that the effect of GR at this site may be mediated by Fos and Jun.

Many models have been proposed to explain the different interactions between Fos/Jun and nuclear hormone receptors at various promoters and in different cell types (reviewed in reference 12). Direct protein-protein interactions in the absence of DNA may cause mutual inhibition of DNA binding (24). Fos and Jun may interact with GR in a manner that allows DNA binding by one or both components, tethering the other component to DNA (7). Fos/Jun and GR may co-occupy a regulatory element by binding to adjacent or overlapping recognition sites and alter the transcription-regulatory properties of each other (3). Competitive interactions with other proteins involved in DNA binding or transcription activation by Fos/Jun and nuclear hormone receptors may also be involved (22). Conceivably, several of these mechanisms could operate at different promoters or in different cell types. However, in its simplest form, mutual inhibition of DNA binding is inconsistent with either tethering or co-occupancy of elements in the same cell.

It is difficult to reconcile the results obtained in different studies, since they have generally been conducted with different cell types and under different conditions. Most of these studies have been performed with transiently transfected cells or unfractionated extracts. In these experiments, different members of the nuclear hormone receptor family can be conveniently distinguished by their dependence on different ligands. In contrast, different AP-1 family complexes are more difficult to distinguish in vivo, as virtually all cells express one or more members of this family and the transfection procedure itself can induce their expression (18). It is also difficult to determine from these experiments whether the interactions are direct or mediated by other factors. The regions of GR that were required for repression of AP-1 activity varied between different cotransfection experiments. However, the zinc finger DNA-binding domain was generally required along with additional regions that affected the efficiency of repression (7, 11, 19, 24). Therefore, we have investigated the effect of the purified GR DNA-binding domain on DNA binding and transcription activation by Fos-Jun heterodimers and Jun homodimers in vitro.

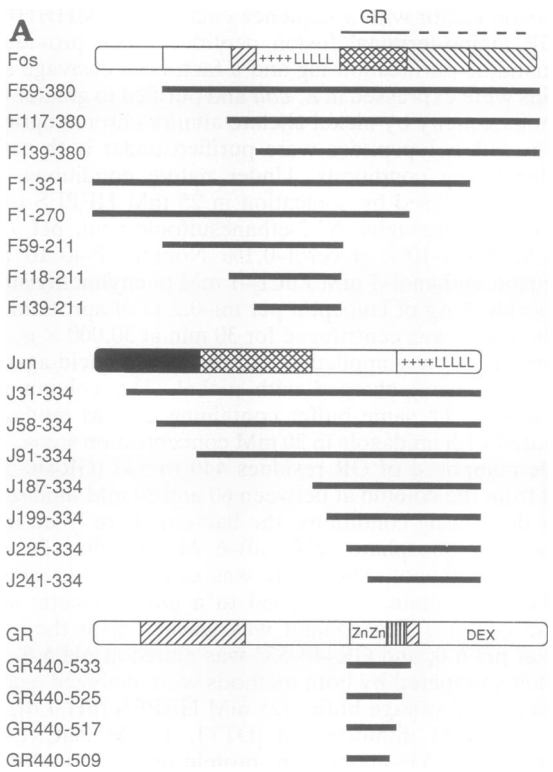
MATERIALS AND METHODS

Protein expression and purification. Synthetic genes encoding GR residues 440 to 533, 440 to 525, 440 to 517, and 440 to 509 were constructed by polymerase chain reaction with overlapping oligonucleotides and *Escherichia coli* codon usage. The open reading frames were cloned into the pDS56 expression vector with a sequence encoding an MHHHHH-HIDGR amino-terminal fusion peptide, which provides a hexahistidine purification tag and a factor Xa cleavage site. Proteins were expressed in *E. coli* and purified to greater that 95% homogeneity by nickel chelate affinity chromatography (1). The GR polypeptides were purified under both native and denaturing conditions. Under native conditions, the bacteria were lysed by sonication in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.6)–500 mM NaCl–10% glycerol–0.1% Nonidet P-40–10 mM β-mercaptoenthanol–1 mM $ZnCl_2$–1 mM phenylmethylsulfonyl fluoride–5 μg of leupeptin per ml–0.2 U of aprotinin per ml. The lysate was centrifuged for 30 min at 30,000 × *g*, and the supernatant was applied to a nitrilotriacetic acid-agarose column (Quiagen) charged with nickel. The column was washed with the same buffer containing 20 mM imidazole and eluted with imidazole in 20 mM concentration steps. The peptide composed of GR residues 440 to 533 (GR440–533) eluted from the column at between 60 and 80 mM imidazole. Under denaturing conditions, the bacteria were lysed in 25 mM sodium phosphate (pH 8.0)–6 M guanidine–20 mM β-mercaptoenthanol. The lysate was centrifuged as above, and the supernatant was applied to a nitrilotriacetic acid-agarose column. The column was washed with the same buffer at pH 6.0, and GR440–533 was eluted at pH 5.0.

Proteins prepared by both methods were dialyzed against six changes of storage buffer (25 mM HEPES [pH 7.6], 5% glycerol, 1 mM dithiothreitol [DTT], 1 mM $ZnCl_2$). To prepare GR440–533 apoprotein, protein prepared under denaturing conditions was first dialyzed against five changes of 25 mM HEPES (pH 7.6)–5% glycerol–1 mM DTT–1 mM EDTA and then against 25 mM HEPES (pH 7.6)–5% glycerol–1 mM DTT treated with imidoacetic acid chelating resin to remove trace zinc. Incubation of the apoprotein with 1 mM $ZnCl_2$ restored full DNA-binding activity. To cleave the fusion peptide, 100 μg of protein was incubated with 5 μg of factor Xa protease (New England Biolabs) for 16 h at 23°C under the conditions recommended by the manufacturer. Fos and Jun proteins and their derivatives were prepared as described previously (1). Protein concentrations were measured by the Bradford method (Bio-Rad).

DNA-binding assays. Electrophoretic mobility shift assays were performed essentially as described previously (1). Oligonucleotides (Fig. 1C) were designed with single-stranded *Xba*I and *Sal*I 5′ overhangs, which were filled in with avian myeloblastosis virus reverse transcriptase and radioactive deoxynucleotides. Fos and Jun or their derivatives were incubated for 15 min at 30°C to allow dimerization. Protein dimers were incubated with the indicated concentrations of GR derivatives or GR storage buffer for 10 min at room temperature (RT). Oligonucleotide probe and dI:dC competitor (100 μg/ml) were added, and the reaction mix was incubated for 5 min at RT. Complexes were analyzed by electrophoresis through a 5% polyacrylamide gel in 25 mM Tris–195 mM glycine buffer and detected by autoradiography. The complexes were quantitated with a Betascope radioanalytic imager.

In vitro transcription. Nuclear extracts were prepared from Namalwa and HeLa cells by a modification (1) of the procedure of Dignam et al. (4). AP-1 activity was depleted from HeLa nuclear extracts by incubation with an oligonucleotide containing an AP-1 site as described previously (1). Namalwa nuclear extracts contained low endogenous AP-1 activity, obviating the need for depletion. Transcription reactions were performed with templates prepared by linearizing plasmids $pCol^{-73/+63}CAT$ (2), $pCol^{-60/+63}CAT$, p(AP-



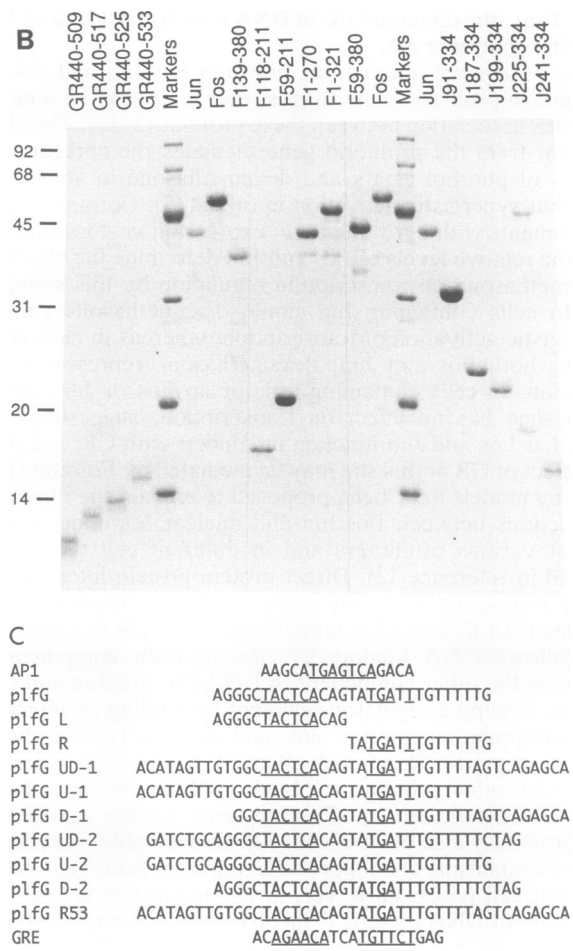


FIG. 1. Expression of truncated Fos, Jun, and GR proteins in *E. coli* and oligonucleotides used in DNA-binding assays. (A) Diagram of Fos, Jun, and GR polypeptides. Positions of the leucine zippers (LLLLL) and basic regions (++++) are indicated, as are regions that activate transcription (1) (▨), regions that amplify DNA bending (8, 9, 9a) (▧), and regions that stimulate both transcription and DNA bending (■). The solid region in Jun is a complex regulatory domain that includes regions that repress and activate transcription and modulate DNA bending. The GR zinc fingers (Zn) and ligand-binding domain (DEX) are indicated, as is a putative amphipathic α helix (▥), which has been proposed to be involved in GR interactions with other proteins (12). The regions included in the truncated polypeptides are indicated by bars below each protein, and the amino acid residues are indicated by the polypeptide designations. GR is drawn to a different scale than Fos and Jun. The region required for GR inhibition of Fos-Jun DNA binding is indicated by a solid line above Fos. The region indicated by a dashed line increases the efficiency of inhibition. (B) Fos, Jun, and GR polypeptides were expressed in *E. coli* as hexahistidine fusion proteins and purified by nickel chelate affinity chromatography. The purified proteins were resolved on a 15% polyacrylamide–SDS gel and visualized by staining with Coomassie brilliant blue. Markers (Bio-Rad) were phosphorylase B, 92 kDa; bovine serum albumin, 68 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 20 kDa; and lysozyme, 14 kDa. (C) Duplex oligonucleotides used for DNA-binding assays. AP-1 corresponds to sequences between −77 and −62 in the human collagenase gene (2). plfG corresponds to sequences between −254 and −230 in the human proliferin gene (3, 13). plfG L and plfG R are nonoverlapping subsequences from this element. plfG UD-1, plfG U-1, and plfG D-1 contain additional upstream and/or downstream flanking sequences from the proliferin promoter (13). plfG UD-2, plfG U-2, and plfG D-2 contain additional upstream and/or downstream flanking sequences from the Adh promoter context in which this element has been studied in transient-transfection experiments (3). The AP-1 consensus sequence and AP-1-like sites in the plfG element are underlined, as is the consensus GRE recognition sequence.

1)$_6$FosCAT, and p(mAP-1)$_6$FosCAT (1) at the *Eco*RI site. Between 100 and 500 nM protein dimers were incubated with the indicated concentrations of GR440–533 or GR storage buffer for 10 min at RT. Between 0.25 and 1 μg of template was added, and the solution was adjusted to 80 mM HEPES (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 8% glycerol, and 16,000 U of RNasin per ml. After 5 min of incubation at RT, between 20 and 50 μg of nuclear extract protein was added. After 5 min of incubation at 30°C, ATP, CTP, and GTP were added to 500 μM each and [α-^{32}P]UTP (10 μCi) was added to 10 μM in a 25-μl final volume. After between 30 and 60 min, transcription was stopped by the addition of EDTA to 2.5 mM, sodium acetate to 25 mM, sodium dodecyl sulfate (SDS) to 0.1%, ammonium acetate to 2.5 M, and carrier RNA to 40 μg/ml. The reaction mixes were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, washed, and resuspended in 80% deionized formamide buffer. The transcripts were separated in a 5% polyacrylamide–7 M urea gel and detected by autoradiography. The transcripts were quantitated with a Betascope radioanalytic imager.

RESULTS

GR DNA-binding domain differentially inhibits Fos-Jun heterodimer DNA binding. To investigate the interplay between Fos, Jun, and the GR DNA-binding domain (GR440–533), we expressed these proteins in *E. coli* cells, purified

them to apparent homogeneity (Fig. 1B) and tested the effect of GR440–533 on DNA binding by Fos and Jun. Fos-Jun heterodimers and Jun homodimers were incubated with GR440–533, and their DNA-binding activities were determined by electrophoretic mobility shift analysis with a collagenase AP-1 site probe. Incubation of Fos-Jun heterodimers with GR440–533 blocked subsequent binding to the collagenase AP-1 site (GR inhibition) (Fig. 2A). In contrast, incubation of Jun homodimers with GR440–533 under the same conditions caused a slight increase in DNA binding. At higher concentrations of Fos-Jun heterodimers, a proportionally larger amount of GR440–533 was required to inhibit DNA binding, suggesting that GR440–533 inhibited Fos-Jun binding through a stoichiometric interaction with the complex. A high molar ratio of GR440–533 to Fos-Jun heterodimers was required for complete inhibition of Fos-Jun DNA binding. However, partial inhibition was observed at a molar ratio of 10, and half-maximal inhibition occurred at a molar ratio of between 50 and 100 (see below, Fig. 5C). The concentration of GR440–533 required for inhibition of Fos-Jun heterodimer DNA binding was similar to that required for occupancy of a consensus glucocorticoid response element (GRE) site (Fig. 2A), suggesting that the concentration of active GR440–533 molecules used in these experiments was within the physiological range.

Incubation of Fos-Jun heterodimers with a fraction purified in parallel with GR440–533 from an isogenic *E. coli* strain that lacked the expression plasmid had no effect on DNA binding (Fig. 2A, lanes 29 to 36). GR440–533 purified by two different methods (see Materials and Methods) inhibited Fos-Jun DNA binding with similar efficiencies. To confirm that inhibition of Fos-Jun DNA binding was due specifically to the GR, we preincubated GR440–533 with monoclonal antibodies directed against the GR DNA-binding domain (5) and analyzed the effect on the inhibition of Fos-Jun DNA binding (Fig. 2B). Preincubation with anti-GR antibodies significantly reduced GR440–533 inhibition of Fos-Jun DNA binding. A 10-fold-higher concentration of GR440–533 was required to inhibit Fos-Jun DNA binding in the presence of anti-GR antibodies. Consequently, the inhibition of Fos-Jun DNA binding was caused specifically by the GR DNA-binding domain.

In contrast to the inhibition of Fos-Jun heterodimer binding, Jun homodimer binding was slightly stimulated at low to moderate concentrations of GR440–533. High concentrations of GR440–533 partially inhibited Jun homodimer DNA binding. Inhibition of Jun homodimer DNA binding required a 10-fold-higher concentration of GR440–533 than did inhibition of Fos-Jun heterodimer binding (see below, Fig. 5C). The specificity of GR440–533 inhibition for Fos-Jun heterodimer but not Jun homodimer DNA binding is even more significant in light of the lower DNA-binding affinity of Jun homodimers and their higher sensitivity to nonspecific inhibitors. We have therefore focused on GR440–533 inhibition of Fos-Jun heterodimer DNA binding.

Excess Fos monomers protect Fos-Jun heterodimers from GR inhibition. The differential inhibition of Fos-Jun heterodimer but not Jun homodimer DNA binding suggested that Fos might be the primary target of GR440–533. To investigate whether GR440–533 could interact with Fos monomers, we incubated GR440–533 with Fos in the absence of Jun and tested the effect on the inhibition of Fos-Jun DNA binding (Fig. 3). Since Fos alone is unable to bind DNA, there was no effect of excess Fos on Fos-Jun DNA binding in the absence of GR440–533. Preincubation of

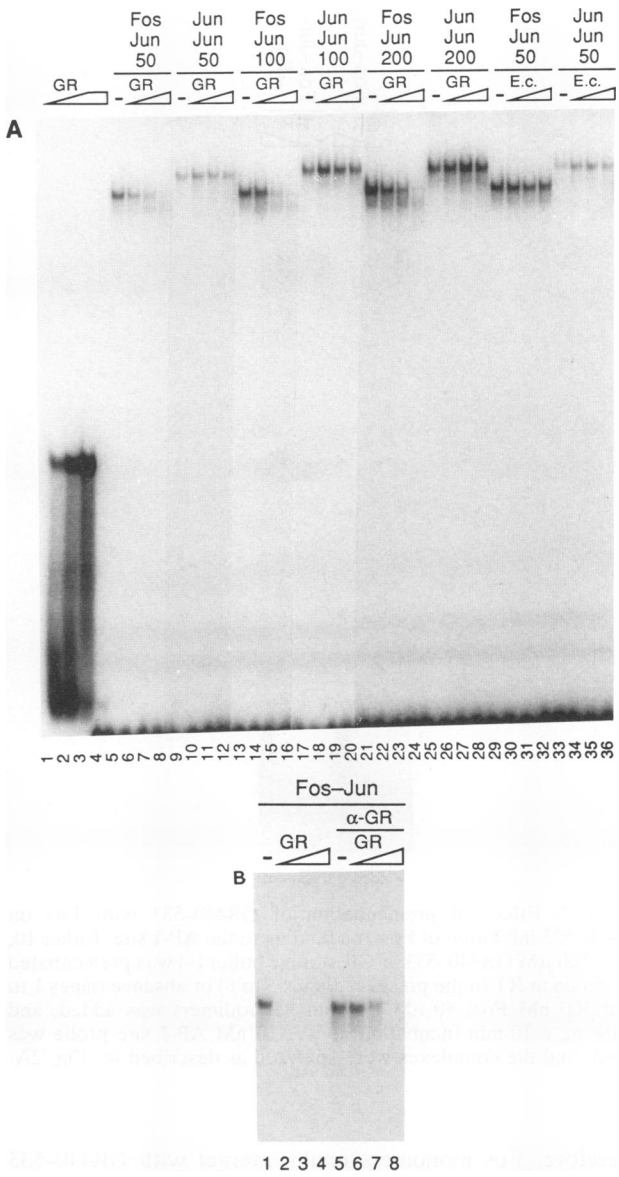


FIG. 2. GR440–533 inhibition of Fos-Jun binding to the AP-1 site. (A) The concentrations of Fos-Jun heterodimers and Jun homodimers indicated above the lanes (nanomolar) were incubated with 10, 20, or 40 μM purified GR440–533 or with a fraction purified in parallel from an *E. coli* strain that lacked the expression plasmid (E.c.) with GR storage buffer (–) (GR) (lanes 5 to 36). The same concentrations of GR440–533 were also incubated in the absence of Fos and Jun (lanes 1 to 4). After 10 min of incubation at RT, 10 nM GRE (lanes 1 to 3) or AP-1 site (lanes 4 to 36) probe was added, and following a 5-min incubation at RT, the complexes were resolved on a 5% polyacrylamide gel and detected by autoradiography. (B) Either 25, 50, or 100 μM purified GR440–533 or GR storage buffer (–) was preincubated for 15 min at RT with 1 μl of BuGR monoclonal antibody (α-GR; lanes 5 to 8) or in the absence of antibody (lanes 1 to 4); 50 nM Fos-Jun heterodimers was added, and following a 10-min incubation, 10 nM AP-1 site probe was added, and the complexes were analyzed as described for panel A.

GR440–533 with Fos significantly reduced the inhibition of Fos-Jun DNA binding. A 10-fold-higher concentration of GR440–533 was required for inhibition of Fos-Jun DNA binding in the presence of a 10-fold excess of Fos monomers.

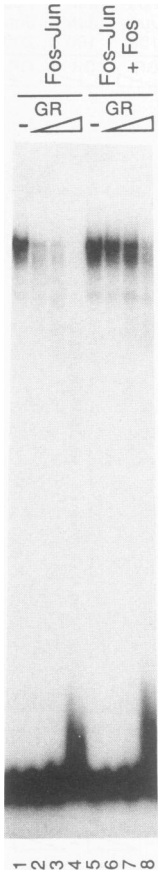


FIG. 3. Effect of preincubation of GR440–533 with Fos on GR440–533 inhibition of Fos-Jun binding to the AP-1 site. Either 10, 30, or 100 μM GR440–533 or GR storage buffer (–) was preincubated for 15 min at RT in the presence (lanes 5 to 8) or absence (lanes 1 to 4) of 500 nM Fos; 50 nM Fos-Jun heterodimers was added, and following a 10-min incubation at RT, 10 nM AP-1 site probe was added, and the complexes were analyzed as described for Fig. 2A.

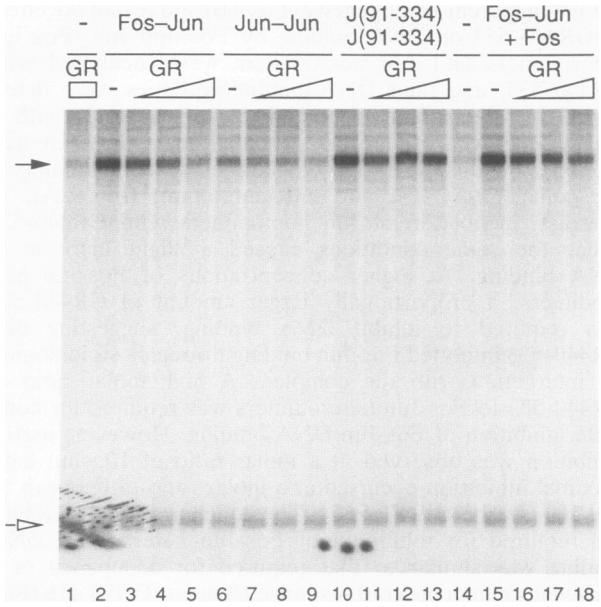


FIG. 4. Differential inhibition of Fos-Jun heterodimer and Jun homodimer transcription activation by GR440–533. A 200 nM concentration of Fos-Jun heterodimers, Jun or Jun91–334 homodimers, or Fos-Jun heterodimers supplemented with 1,000 nM Fos monomers was incubated with 20, 60, or 200 μM GR440–533 or with GR storage buffer (–) (lanes 2 to 13 and 15 to 18); 200 μM GR440–533 was also incubated in the absence of Fos and Jun (lane 1). After 10 min of incubation at RT, 1 μg of $pCol^{-73/+63}CAT$ template and transcription buffer were added. After 5 min of incubation at RT, 10 μl of Namalwa nuclear extract was added. After 5 min of incubation at 30°C, transcription was initiated by the addition of ATP, CTP, and GTP to 500 μM each and [α-^{32}P]UTP (10 μCi) to 10 μM. Transcription was stopped after 30 min, and the transcripts were isolated and analyzed on a denaturing 5% polyacrylamide gel. The solid arrow indicates the specific transcript from the collagenase promoter. The open arrow indicates an internal control RNA, which confirms equal recovery and loading of samples.

Therefore, Fos monomers could interact with GR440–533 and block the inhibition of Fos-Jun DNA binding.

GR DNA-binding domain differentially inhibits transcription activation by Fos-Jun heterodimers. Both Fos-Jun heterodimers and Jun homodimers can activate transcription of reporter genes containing AP-1 sites in vitro (1). Fos-Jun heterodimers and Jun homodimers also activated transcription from the collagenase promoter in vitro, although Fos-Jun heterodimers were much more potent activators (22-fold versus 8-fold activation). To investigate whether GR440–533 would affect transcription activation by Fos and Jun, we analyzed transcription activation of the collagenase promoter by Fos-Jun heterodimers and Jun homodimers in the presence and absence of GR440–533 in vitro (Fig. 4). GR440–533 inhibited transcription activation by Fos-Jun heterodimers at concentrations similar to those required for inhibition of DNA binding. Inhibition of transcription activation by Jun homodimers was observed at high GR440–533 concentrations. Since full-length Jun homodimers were weaker transcription activators than Fos-Jun heterodimers, we also investigated the effect of GR440–533 on transcription activation by Jun91–334 homodimers, which lack the amino-terminal repression domain (1) and are as potent transcription activators as Fos-Jun heterodimers (24-fold activation). There was no significant effect of GR440–533 on transcription activation by Jun91–334, consistent with the lack of GR440–533 inhibition of Jun91–334 DNA binding (see below). Transcription activation by Fos-Jun heterodimers in the presence of GR440–533 could be partially restored by an excess of Fos monomers, consistent with the ability of Fos monomers to protect Fos-Jun heterodimers from GR440–533 inhibition of DNA binding (see Fig. 3). There was little effect of GR440–533 on the low basal-level transcription from the collagenase promoter. Fos and Jun did not activate transcription of a collagenase promoter deleted to position −60 upstream, nor was there any effect of GR440–533, indicating that the AP-1 site located between positions −73 and −60 in this promoter was the target of both transcription activation by Fos and Jun and repression by GR440–533. GR440–533 also inhibited Fos-Jun transcription activation of a synthetic promoter containing six tandem AP-1 sites, confirming that no additional sequences from the collagenase promoter were required for inhibition by GR440–533.

To investigate the possibility that the functional interactions between GR440–533 and Fos or Jun might differ in extracts from different cell types, the effect of GR440–533 on transcription activation by Fos and Jun was investigated in both Namalwa and HeLa nuclear extracts. The effect of

GR440–533 on Fos and Jun transcription activation was similar in extracts of both cell types, suggesting that the interaction between Fos and GR440–533 was not dependent on the cell type. These results indicate that the differential inhibition of Fos-Jun heterodimer DNA binding by GR440–533 is functionally relevant and may mediate the distinct effects of the GR on transcription in cells containing different AP-1 complexes.

Regions outside the DNA-binding domains of Fos and Jun are required for GR inhibition. DNA binding by Fos and Jun is mediated by leucine zipper dimerization and basic DNA-binding domains, although additional regions of the proteins influence DNA-binding affinity (1) as well as DNA bending (8, 9). To determine the regions of Fos and Jun that were required for GR440–533 inhibition of DNA binding, we tested the effect of GR440–533 on DNA binding by truncated peptides encompassing the leucine zipper and basic regions of Fos and Jun as well as combinations of full-length proteins and peptides (Fig. 5A). Surprisingly, DNA binding by homodimers and heterodimers composed of the truncated peptides was not inhibited by GR440–533. Therefore, regions outside the dimerization and DNA-binding domains of Fos and Jun were required for GR440–533 inhibition of DNA binding. Heterodimers containing full-length Fos and truncated Jun were inhibited at lower GR440–533 concentrations than were heterodimers containing full-length Jun and truncated Fos. Thus, the differential sensitivity of Fos-Jun heterodimers and Jun homodimers to GR440–533 inhibition was not due to a difference between heterodimers and homodimers, but was a property of the full-length Fos protein.

To confirm the differential sensitivity of various Fos and Jun complexes to inhibition of DNA binding by GR440–533, we incubated mixtures containing several complexes in the presence of GR440–533 and analyzed their DNA-binding activities (Fig. 5B). In a mixture of Fos-Jun heterodimers and Jun homodimers, Fos-Jun heterodimer binding was inhibited at the same time that Jun homodimer binding was stimulated by GR440–533. Full-length Fos conferred a higher sensitivity to GR440–533 than full-length Jun when dimerized with the corresponding truncated peptides at the same time that binding by complexes containing both truncated peptides was stimulated. Quantitation of DNA binding by the various Fos and Jun complexes in several independent experiments demonstrated that the extent of GR440–533 inhibition of Fos-Jun heterodimer and Jun homodimer DNA binding was significantly different at all GR440–533 concentrations tested ($P < 0.005$) (Fig. 5C). GR440–533 inhibition of Fos-Jun241–334 and Fos118–211-Jun complexes was also significantly different at all GR440–533 concentrations ($P < 0.05$). Thus, different Fos and Jun complexes exhibit significantly different sensitivities to GR440–533 inhibition.

To map the regions of Fos and Jun that were required for GR440–533 inhibition, we investigated the effect of GR440–533 on DNA binding by complexes composed of various truncated Fos and Jun polypeptides (Fig. 1A). DNA binding by all complexes containing Fos residues 211 to 270 was inhibited by GR440–533, whereas DNA binding by Fos-Jun complexes that lacked these residues was slightly stimulated, suggesting that this region was necessary for GR inhibition (data not shown). The adjacent region (270 to 321) increased the efficiency of inhibition, and there was a small effect of the carboxy-terminal region (321 to 380) as well. Jun complexes that lacked the amino-terminal 30 residues (Fig. 1A) were not inhibited by GR440–533 (data not shown), suggesting that this region was required for the inhibition observed at high concentrations of GR440–533. These regions correspond to domains that we have shown to influence both transcription regulation (1) and DNA bending by Fos and Jun (8, 9).

Interactions between Fos, Jun, and the GR DNA-binding domain at the proliferin plfG element. A DNA sequence element (plfG) that mediates the regulatory effects of Fos, Jun, and GR has been identified upstream of the proliferin gene. It has been proposed that Fos and Jun co-occupy this element with the GR (3). To investigate the interaction between Fos, Jun, and the GR DNA-binding domain at this element, we have studied the binding of Fos and Jun to this element in the absence and presence of GR440–533. Fos-Jun heterodimers bound to the plfG element with a higher apparent affinity than Jun homodimers. Both Fos-Jun heterodimers and Jun homodimers formed two specific complexes with the plfG oligonucleotide (Fig. 5A). The plfG element contains two AP-1-like sequences (Fig. 1C), and Fos-Jun heterodimers could bind to oligonucleotides containing either of these sites (plfG L and plfG R; Fig. 1C) with similar apparent affinities. The two complexes therefore likely correspond to plfG elements with one or both AP-1-like sites occupied. There was no apparent cooperativity in Fos-Jun binding to the two sites in the plfG element. GR440–533 bound the plfG element with an apparent affinity that was at least 10-fold lower than that for the consensus GRE (Fig. 5A).

The effect of GR440–533 on the binding of various Fos and Jun complexes to the plfG element was virtually identical to its effect on their binding to the collagenase AP-1 site (Fig. 5A). Fos-Jun heterodimer binding was inhibited by GR440–533, whereas Jun homodimer binding was stimulated at low concentrations of GR440–533 and inhibited at higher concentrations. The same regions of Fos and Jun were required for GR440–533 inhibition of binding to the plfG and AP-1 sites. There was no evidence for co-occupancy of GR440–533 with full-length Fos-Jun heterodimers or Jun homodimers at the plfG site. At high concentrations of GR440–533, in the absence of dI:dC competitor, the mobility of complexes formed by truncated Fos and Jun was retarded. However, titration with dI:dC competitor caused a gradual increase in the mobility of these complexes, suggesting that no stable complex was formed. To investigate the effect of sequences flanking the plfG element on Fos, Jun, and GR440–533 binding, we tested oligonucleotides containing flanking sequences from the proliferin gene (13) (plfG UD-1, plfG U-1, and plfG D-1; Fig. 1C) and the Adh promoter (3) (plfG UD-2, plfG U-2, and plfG D-2; Fig. 1C). The same inhibition of Fos-Jun DNA binding by GR440–533 and weak binding by GR440–533 alone were observed with these oligonucleotides (data not shown).

Binding to the AP-1 site protects Fos-Jun heterodimers from GR inhibition. DNA binding by Fos-Jun could be inhibited by blocking dimerization, by preventing dimer binding to the AP-1 site, or by destabilizing the Fos-Jun-AP-1 complex. To distinguish between these possibilities, we investigated the effect of adding GR440–533 to different intermediates in the formation of the Fos-Jun-AP-1 complex (Fig. 6). GR440–533 was added to Fos and Jun prior to dimerization, to the Fos-Jun heterodimer prior to binding to the AP-1 site, or to the Fos-Jun-AP-1 complex. There was no difference between the level of inhibition caused by GR440–533 added prior to dimerization and that caused by GR440–533 added to the dimeric complex prior to the AP-1 site. However, addition of GR440–533 after the AP-1 site did not disrupt Fos-Jun-AP-1 complexes. Therefore, GR440–533 was able to

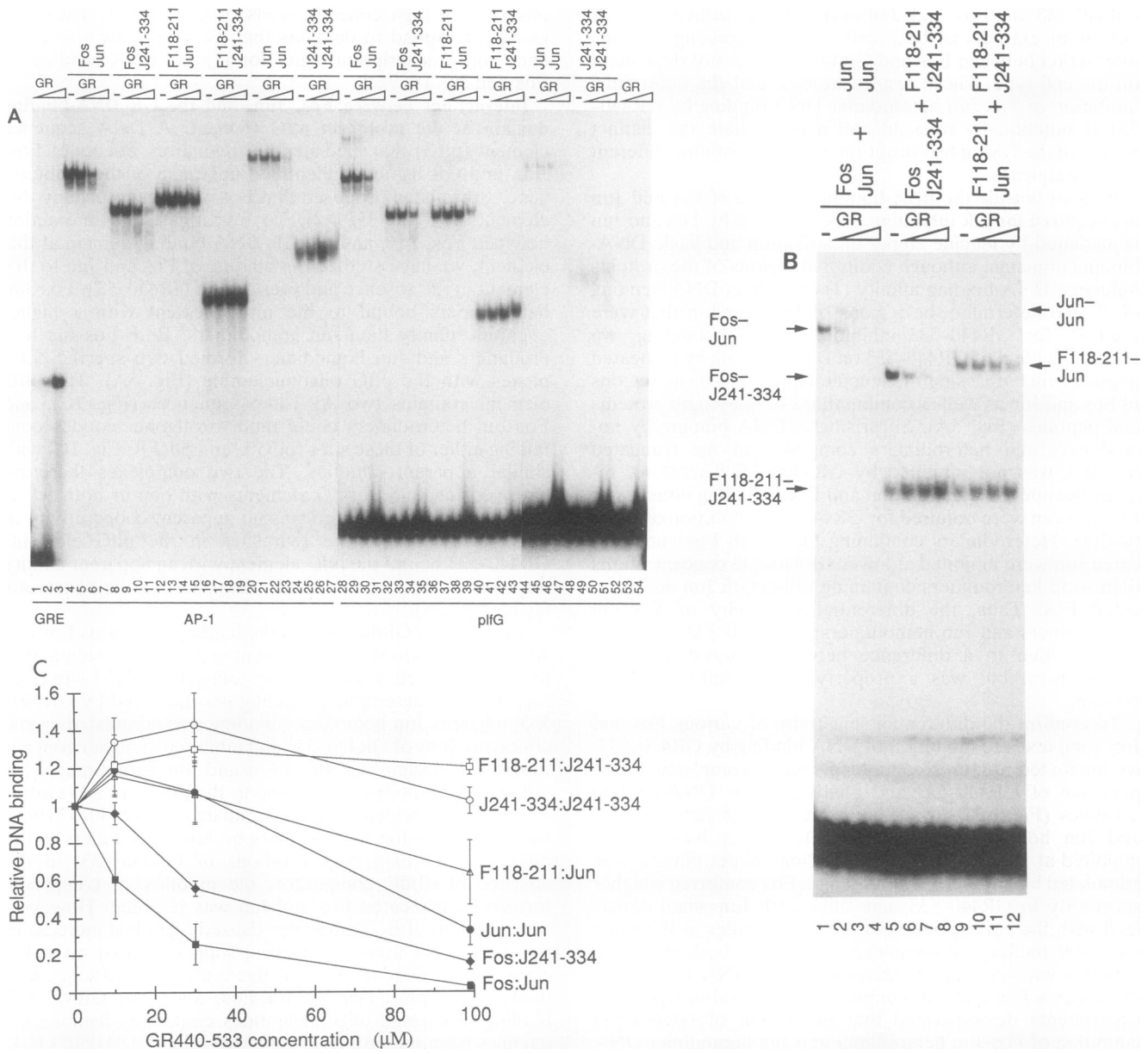


FIG. 5. GR440–533 inhibition of DNA binding by complexes containing Fos and Jun polypeptides encompassing the basic region and the leucine zipper. (A) A 100 nM concentration of the complexes indicated above the lanes was incubated with 10, 30, or 100 μM GR440–533 or with GR storage buffer (–) (lanes 4 to 54). The same amounts of GR440–533 were also incubated in the absence of Fos and Jun (lanes 1 to 3). After 10 min of incubation at RT, 10 nM GRE (lanes 1 to 3), AP-1 (lanes 4 to 27), or plfG (lanes 28 to 54) probe was added, and the complexes were analyzed as described for Fig. 2A. A three-times-longer exposure of lanes 44 to 54 on the same gel is shown to compensate for the lower affinity of Jun homodimers and GR440–533 for the plfG element. The shorter AP-1 probe was run off the gel. (B) Mixtures of the complexes indicated above the lanes (50 nM each) were incubated with 10, 30, or 100 μM GR440–533 or with GR storage buffer (–). After 10 min of incubation at RT, 10 nM AP-1 site probe was added, and the complexes were analyzed as described for Fig. 2A. (C) Quantitation of DNA binding by different Fos and Jun complexes in the presence of different concentrations of GR440–533. The amounts of the different complexes were quantitated by radioanalytic imaging. Binding in the presence of various concentrations of GR440–533 was normalized to binding in the absence of GR440–533. The data represent the averages from between three and eight experiments, and bars indicate standard deviations.

block preformed dimers from binding to the AP-1 site, but Fos-Jun complexes bound to the AP-1 site were protected from disruption by GR440–533.

Zinc finger domain of GR inhibits Fos-Jun DNA binding. The GR440–533 peptide encompasses the two GR zinc fingers and a short putative amphipathic helix on the carboxy-terminal side of the zinc fingers. This putative amphipathic helix has been proposed to be involved in many protein-protein interactions, including those with Fos and Jun (12). To investigate the role of the zinc fingers and the putative α helix in the inhibition of Fos-Jun DNA binding, we prepared a series of deletion derivatives; GR440–525, GR440–517, and GR440–509, and assayed them for inhibition of Fos-Jun heterodimer and Jun homodimer DNA binding

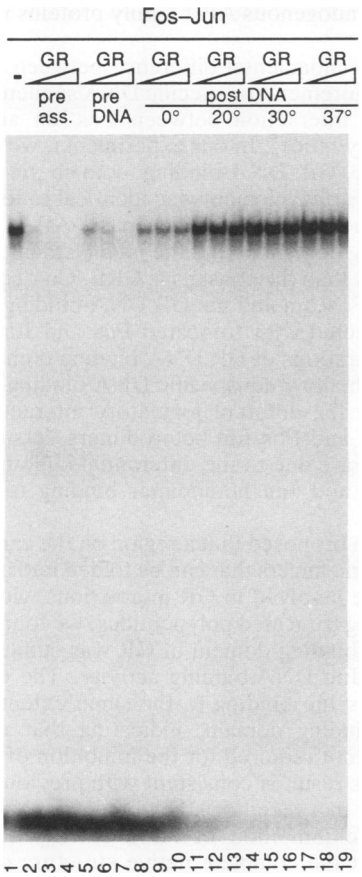


FIG. 6. GR440–533 inhibition of intermediates in the formation of the Fos-Jun-AP-1 complex. Fos (50 nM) and Jun (50 nM) were incubated with 10, 30, or 100 μM GR440–533 or with GR storage buffer (–) either prior to association (pre ass.; lanes 2 to 4), after 15 min of incubation at 30°C to allow dimerization (pre DNA; lanes 5 to 7), or after an additional 5 min of incubation with 10 nM AP-1 site probe to allow DNA binding (post DNA; lanes 8 to 19), which was followed by a 10-min incubation in the presence of GR440–533 at the indicated temperatures. Following the addition of GR440–533, dimerization and/or DNA binding was performed, and complexes were analyzed as described for Fig. 2A. The lower yield of complexes incubated at 0°C is due to the lower DNA-binding activity of Fos-Jun heterodimers at this temperature and was not affected by GR440–533.

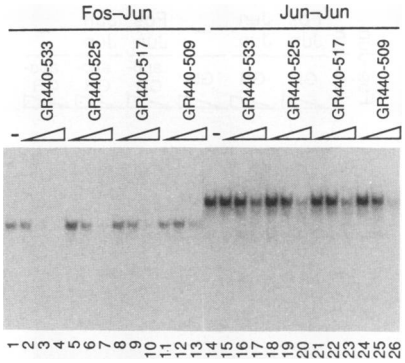


FIG. 7. Mapping of the GR region required for inhibition of Fos and Jun DNA binding. Fos-Jun heterodimers or Jun homodimers (100 nM) were incubated with 10, 30, or 100 μM GR440–533, GR440–525, GR440–517, or GR440–509. After 10 min of incubation at RT, 10 nM AP-1 site probe was added, and the complexes were analyzed as described for Fig. 2A.

(Fig. 7). All of these derivatives inhibited Fos-Jun heterodimer and Jun homodimer binding. The differences in the efficiencies of inhibition by different deletion derivatives were similar to the differences observed between different preparations of any one deletion derivative. To exclude the possibility that the fusion peptide might be involved in the inhibition of binding, we treated GR440–533 with factor Xa and assayed the cleavage product for inhibition of Fos-Jun DNA binding. The cleaved peptide inhibited Fos-Jun DNA binding to the same extent as the uncleaved peptide. Therefore, the region of GR440–533 that inhibited Fos-Jun DNA binding overlapped the zinc finger DNA-binding domain.

GR inhibition of Fos-Jun DNA binding does not require DNA binding by GR. Since GR inhibition of Fos-Jun DNA binding was mediated by the zinc finger DNA-binding domain, it was important to exclude the possibility that GR440–533 might block Fos-Jun binding by occluding the AP-1 site through nonspecific DNA binding. The amount of GR440–533 required for half-maximal inhibition of Fos-Jun DNA binding coincided with the amount required to bind 50% of an oligonucleotide containing a consensus GRE binding site (see Fig. 2A). In addition, preincubation of GR440–533 with a consensus GRE oligonucleotide prevented the inhibition of Fos-Jun DNA binding (data not shown). GR440–533 did not bind to the AP-1 site in electrophoretic mobility shift assays. However, weak binding might not be detected in these experiments.

To investigate the possible involvement of DNA binding by GR440–533 in the inhibition of Fos-Jun binding to the AP-1 site, we tested the effect of the GR440–533 apoprotein on DNA binding by Fos-Jun heterodimers and Jun homodimers (Fig. 8). The GR440–533 apoprotein inhibited Fos-Jun heterodimer and Jun homodimer binding as effectively as did native GR440–533. The AP-1 site concentration and the concentration of dI:dC competitor had no effect on GR440–533 inhibition of Fos-Jun binding. Therefore, DNA binding by GR440–533 was clearly not involved in the inhibition of Fos-Jun binding to the AP-1 site.

DISCUSSION

Several members of the Fos/Jun and the nuclear hormone receptor families of transcription factors display regulatory interactions at promoters containing AP-1 sites or hormone response elements (3, 6, 7, 11, 15, 19, 20, 23–25). The molecular mechanisms that have been proposed for these interactions from the results of cotransfection experiments are contradictory. One fundamental difference between these models is the effect of these interactions on DNA binding. The mutual-inhibition model suggests that the proteins interact in solution and block the DNA-binding activities of each other, whereas other models propose that the proteins interact when one or both of them are bound to DNA and alter the transcription-regulatory activities of each other. Studies of the effects of partially purified GR and Jun as well as retinoic acid receptor and Jun on the DNA-binding activities of each other support the former model (19, 20, 24, 25). However, in vivo footprinting indicates that the AP-1 site remains occupied during dexamethasone repression of collagenase gene expression (10), consistent with the latter class of models.

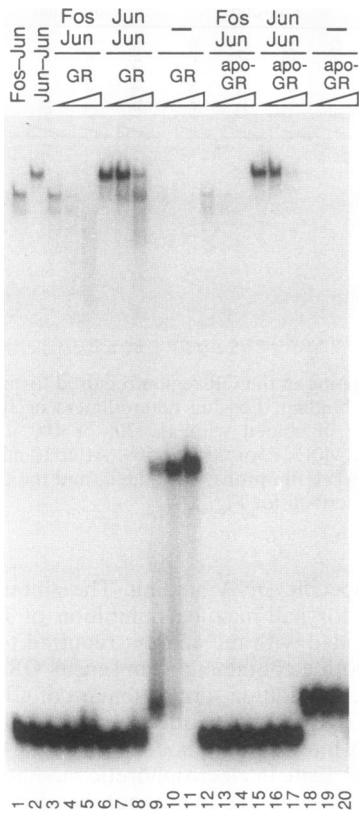


FIG. 8. GR440–533 apoprotein inhibition of Fos and Jun binding to the AP-1 site. (A) Fos-Jun heterodimers or Jun homodimers (50 nM) were incubated with 10, 30, or 100 μM GR440–533 (GR; lanes 3 to 8), the same concentrations of GR440–533 apoprotein (apo-GR; lanes 12 to 17), or GR storage buffer (lanes 1 and 2). The same concentrations of GR440–533 and GR440–533 apoprotein were also incubated in the absence of Fos and Jun (lanes 9 to 11 and 18 to 20). After 10 min of incubation at RT, 10 nM GRE (lanes 9 to 11 and 18 to 20) or AP-1 site (lanes 1 to 8 and 12 to 17) probe was added, and the complexes were analyzed as described for Fig. 2A.

Our studies of the effect of the GR DNA-binding domain on DNA binding and transcription activation by Fos and Jun can explain some of the apparent contradictions between these results. The DNA-binding domain of GR differentially inhibited DNA binding by Fos-Jun heterodimers and Jun homodimers. It is therefore possible that GR shifts the occupancy of AP-1 sites from Fos-Jun heterodimers, which bind DNA with higher affinity in the absence of GR, to Jun homodimers, which are less sensitive to GR inhibition. Since Jun homodimers are less potent transcription activators than Fos-Jun heterodimers at the collagenase promoter, this could repress transcription without reducing AP-1 site occupancy. Since Fos and Jun make very similar DNA contacts (14), it is unlikely that binding by Fos-Jun heterodimers and Jun homodimers could be distinguished by in vivo footprinting. The differential inhibition of Fos-Jun heterodimer and Jun homodimer DNA binding and transcription activation that we have observed in vitro is also consistent with the stronger GR inhibition of Fos-Jun heterodimer activation of reporter genes observed in cotransfection experiments. Fos is a more potent inhibitor of receptor activity than Jun (22, 24, 25). Thus, the cell type specificity of nuclear receptor inhibition of AP-1 activity (7, 22) may be due to the presence of different endogenous AP-1 family proteins in different cell types.

A second fundamental difference between different models is the requirement for specific DNA sequence elements to mediate the interaction between Fos/Jun and GR in the co-occupancy model. In our experiments, we found that the effect of the GR DNA-binding domain on Fos and Jun binding to the plfG element was identical to its effect on Fos and Jun binding to the collagenase AP-1 site. The GR DNA-binding domain bound the plfG element with a 10-fold-lower affinity than the consensus GRE. Co-occupancy of this element by Fos/Jun and the GR DNA-binding domain could only be detected with truncated Fos and Jun proteins and high concentrations of GR DNA-binding domain under conditions which allow nonspecific DNA binding. We therefore propose that the different regulatory interactions observed between GR and Fos-Jun heterodimers versus GR and Jun homodimers are due to the differential inhibition of Fos-Jun heterodimer and Jun homodimer binding to the plfG element.

It has been proposed that a region on the carboxy-terminal side of the zinc fingers that can be folded into an amphipathic helix may be involved in GR interactions with Fos and Jun (12). By using truncated polypeptides, we found that the zinc finger DNA-binding domain of GR was sufficient for inhibition of Fos-Jun DNA-binding activity. The GR apoprotein inhibited Fos-Jun binding to the same extent as the native GR DNA-binding domain, indicating that receptor DNA binding was not required for the inhibition of Fos-Jun DNA binding. This result is consistent with previous observations that indicate that receptor DNA-binding specificity does not influence the inhibition of AP-1 activity (19). This also suggests that the complete native structure of the GR zinc fingers may not be necessary for interaction with Fos and Jun, but that some more limited secondary or primary structural element may be the target for interaction. Thus, although the DNA-binding and Fos/Jun interaction surfaces of GR overlap intimately, they are clearly functionally independent.

The differential inhibition of Fos-Jun heterodimer but not Jun homodimer DNA binding implicated Fos as the primary target of GR inhibition. Fos monomers were able to rescue Fos-Jun heterodimers from GR inhibition, suggesting that Fos may have biological functions that are independent of dimerization with Jun family proteins. The GR DNA-binding domain did not disrupt the Fos-Jun heterodimer, since no Jun homodimer complex was formed when Fos-Jun heterodimer DNA binding was inhibited. Thus, the GR DNA-binding domain inhibited Fos-Jun DNA binding through an interaction with Fos which did not disrupt dimerization and is therefore unlikely to involve the leucine zipper.

Analysis of the effect of the GR DNA-binding domain on DNA binding by truncated Fos and Jun polypeptides indicated that regions outside the dimerization and DNA-binding domains of Fos and Jun were required for GR inhibition. An amino-terminal region of Jun was required for inhibition by high concentrations of GR polypeptide. This region contains a negative regulatory domain, which represses Jun transcriptional activity in vitro (1) and modulates DNA bending by Jun (8, 9). The requirement for this region in the inhibition of Jun homodimer DNA binding by the GR DNA-binding domain differs from previous results, in which binding by a truncated Jun protein was partially inhibited by GR (24). For Fos, a region on the carboxy-terminal side of the dimerization domain was required for inhibition by the GR DNA-binding domain. This region corresponds to a domain that

activates transcription (1) and increases DNA bending by Fos (8, 9).

The role of the same regions in both GR inhibition of Fos-Jun DNA binding and AP-1 site bending is intriguing, since both imply a change in the conformation of the DNA-binding surface. We propose that Fos-Jun exists in two conformational states; one is capable of binding DNA but not GR, and the other can bind GR but not DNA. When bound to the AP-1 site, Fos-Jun is trapped in one conformational state, incapable of interacting with GR, whereas in the presence of GR, Fos-Jun is trapped in another conformational state, incapable of binding DNA. This model is consistent with the inability of the GR DNA-binding domain to disrupt Fos-Jun complexes bound to the AP-1 site. We have shown previously that DNA binding by Fos and Jun induces a conformational change (17), and we suggest that this conformational change may influence Fos and Jun interactions with GR and other proteins.

The region of Fos required for GR inhibition is not conserved in any of the Fos-related proteins (Fra1, Fra2, and FosB). The region in Jun is partially conserved in JunB but not in JunD. Therefore, these proteins with closely related dimerization and DNA-binding specificities may differ in their interactions with GR, providing diversity in the regulatory interactions between these transcription factor families. The interaction of a subset of AP-1 complexes with a particular hormone receptor may function to restrict the population of complexes that can bind to AP-1 sites at any one time. Depending on the relative transcriptional activities of these complexes, this interaction may lead to repression or activation of transcription. These differential interactions and different transcription-regulatory properties may underlie the complex interplay between the signal transduction pathways mediated by nuclear hormone receptors and AP-1 family transcription factors.

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