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Glucocorticoids Antagonize AP-1 by Inhibiting the Activation/Phosphorylation of JNK Without Affecting its Subcellular Distribution

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Abstract. The immunosuppressive and antiinflammatory actions of glucocorticoid hormones are mediated by their transrepression of activating protein-1 (AP-1) and nuclear factor-kappa B (NFκB) transcription factors. Inhibition of the c-Jun NH_2-terminal kinase (JNK) signaling pathway, the main mediator of AP-1 activation, has been described in extracts of hormone-treated cells. Here, we show by confocal laser microscopy, enzymatic assays, and immunoblotting that the synthetic glucocorticoid dexamethasone inhibited tumor necrosis factor α (TNF-α)–induced phosphorylation and activation of JNK in the cytoplasm and nucleus of intact HeLa cells. As a result, c-Jun NH_2-terminal domain phosphorylation and induction were impaired. Dexamethasone did not block the TNF-α–induced JNK nuclear translocation, but rather induced, per se, nuclear accumulation of the enzyme. Consistently with previous findings, a glucocorticoid receptor mutant (GRdim), which is deficient in dimerization, DNA binding, and transactivation, but retains AP-1 transrepressing activity, was as efficient as wild-type GR in mediating the same effects of dexamethasone on JNK in transfected Cos-7 cells. Our results show that glucocorticoids antagonize the TNF-α–induced activation of AP-1 by causing the accumulation of inactive JNK without affecting its subcellular distribution.

Key words: dexamethasone • activating protein-1 • tumor necrosis factor α • c-Jun NH_2-terminal kinase • nuclear translocation

Introduction

Glucocorticoid hormones have many important regulatory roles in the organism. Their activities are exerted through the control of genes via three mechanisms: direct transcriptional regulation, indirect transcriptional regulation through interference with other transcription factors, and posttranscriptional effects (Vayssière et al., 1997; Resche-Rigon and Gronemeyer, 1998). Glucocorticoids are widely used to treat inflammatory and autoimmune disorders. Many genes involved in immune and inflammatory responses have regulatory sites for activating protein-1 (AP-1)[1] and nuclear factor kappa B (NFκB) transcription factors. There is evidence that the immunosuppressive and antiinflammatory actions of glucocorticoid hormones are mediated by their transrepression of AP-1 and NFκB (Vayssière et al., 1997; Göttlicher et al., 1998; Karin, 1998; Resche-Rigon and Gronemeyer, 1998). Transrepression of AP-1 is also a key feature of the antitumor activity of glucocorticoids in mouse skin (Tuckermann et al., 1999). This is emphasized

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[1]*Abbreviations used in this paper:* AP-1, activating protein-1; Dex, dexamethasone; GR, glucocorticoid receptor; GRdim, GR mutant deficient in dimerization; GRwt, GR wild-type; Hsp70, heat shock protein 70; JNK, c-Jun NH_2-terminal kinase; TNF-α, tumor necrosis factor-α.

by the finding that, in contrast to glucocorticoid receptor ($GR^{-/-}$) animals, GR^{dim} mutant mice expressing a mutant hormone receptor (GRdim) that is deficient in dimerization, DNA binding, and transactivation, but retains AP-1 transrepressing activity, are viable (Reichardt et al., 1998). In addition, GRdim mediates repression of AP-1-dependent genes in mouse skin (Tuckermann et al., 1999). Moreover, defective AP-1 repression may explain resistance to the antiinflammatory effect of glucocorticoids in asthma patients (Adcock et al., 1995).

AP-1 is a group of dimeric factors constituted by members of the c-Jun and c-Fos families of protooncogenic products (Karin et al., 1997). AP-1 is activated by mitogens, oncoproteins, proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1, and ultraviolet radiation. c-Jun, the main component of AP-1, is activated by NH_2-terminal phosphorylation on serines 63 and 73 ($Ser^{63/73}$) by members of the c-Jun NH_2-terminal Kinase (JNK) family (Minden and Karin, 1997). Interactions between hormone-activated receptors and AP-1, competition for limiting amounts of common transcriptional coactivators, such as cAMP response element-binding protein (CREB)-binding protein (CBP), or binding to DNA have been proposed to explain the mutual antagonism between hormones acting through nuclear receptors and AP-1 (for a review see Göttlicher et al., 1998). In addition, we and others have recently described the inhibition of the JNK signaling pathway, and hence of c-Jun phosphorylation and AP-1 activation, in extracts of hormone-treated cells (Caelles et al., 1997; Swantek et al., 1997; González et al., 1999; Lee et al., 1999; Srivastava et al., 1999; Ventura et al., 1999). Remarkably, the generation of transgenic mice harboring a mutant allele of c-Jun with $Ser^{63/73}$ mutated to alanines has shown that NH_2-terminal phosphorylation of c-Jun is critical for stress-induced apoptosis and cellular proliferation in vivo (Behrens et al., 1999).

To gain insight into the mechanism of AP-1 interference by hormone-activated nuclear receptors, we have addressed the following questions. Does the synthetic glucocorticoid dexamethasone (Dex) inhibit JNK phosphorylation/activation in intact cells? Can Dex affect nuclear translocation of JNK? And, what is the mechanism of hormone-activated GR and what is its primary target?

Materials and Methods

Cell Culture and Transfections

HeLa and Cos7 cells were grown in DME supplemented with 10% FCS. Media, tissue culture reagents, and FCS were purchased from GIBCO BRL. Cells were serum starved by changing the culture medium to DME supplemented with 0.5% FCS 16 h before treatment. Cos-7 cells were transfected with 3 μg of plasmid encoding HA-JNK (pCDNA3-JNK1) and 0.4 μg of those encoding either GRwt (pSB-hGR) or GRdim (pSB-hGR(A458T)), or with the empty vector ($pRSh^{-}R^{-}$; donated by Dr. A.C.B. Cato, Karlsruhe, Germany). Treatments: HeLa or Cos-7 cells (24–48 h after transfection) were pretreated with Dex (1 μM) or vehicle (ethanol) for 45 min. This period was chosen because of previous studies showing that it is sufficient for maximum inhibition of JNK activation by TNF-α (Caelles et al., 1997). After Dex pretreatment, TNF-α (10 ng/ml) or its vehicle (ethanol) was added to the cells. Thus, TNF-α + Dex cells were incubated with Dex for 45 min plus the indicated period of TNF-α stimulation. Dex-alone control cells were incubated with hormone during the pretreatment and throughout for the period of stimulation.

Immunocytochemistry

HeLa and Cos7 cells were rinsed twice in PBS, fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.5% Triton X-100 for 15 min, and were then treated with 0.1 M glycine in PBS for 15 min. The nonspecific sites were blocked by incubation with PBS containing 1% BSA or goat serum for 30 min at room temperature. Cells were then washed in PBS containing 0.05% Tween-20 for 5 min and incubated with the primary antibodies diluted in PBS for 1 h at room temperature or overnight at 4°C.

The following primary antibodies were used: rabbit polyclonal or mouse monoclonal anti–c-Jun phosphorylated on serine-63 (New England Biolabs, Inc., 9261S; or Santa Cruz Biotechnology, Inc., sc-822), rabbit polyclonal anti–c-Jun (Oncogene Research, PC06), mouse monoclonal against human JNK1 (BD PharMingen, 15701A), mouse monoclonal anti-JNK1 phosphorylated on threonine-183 and tyrosine-185 (Santa Cruz Biotechnology, Inc., sc-6254), and rabbit polyclonal anti-GR (Santa Cruz Biotechnology, Inc., sc-1003). The cells were incubated for 45 min with one of the following secondary antibodies: Texas red (TxR)-conjugated goat anti–rabbit (Jackson ImmunoResearch or Vector), FITC-conjugated goat anti–rabbit (Jackson ImmunoResearch), and TxR-conjugated goat anti–mouse (Jackson ImmunoResearch). To amplify the phospho-JNK and JNK1 staining, anti-mouse Ig-digoxigenin, $F(ab')_2$-fragment, followed by antidigoxigenin-rhodamine (Boehringer), or biotinylated anti-mouse, followed by streptavidin-rhodamine (Jackson ImmunoResearch), secondary antibodies were used. Double immunofluorescence with anti-GR and antiphospho-JNK or anti-JNK1 was performed on samples of Cos7 cells transfected with wild-type GR (GRwt) or GRdim.

Confocal Microscopy and Quantification

Confocal microscopy was performed with an MRC-1024 laser scanning microscope (BioRad), equipped with an Axiovert 100 invert microscope (ZEISS), and using excitation wavelengths of 488 nm (for FITC) and 543 nm (for TxR). To measure fluorescence intensity in cell cultures treated with TNF-α or preincubated with Dex before TNF-α, 30 cells without prior selection of each culture were analyzed by laser spectroscopy performed with the confocal microscope and using a Plan-Apochromat 63x/1.4 oil immersion objective. The selection of rectangular regions of interest in the nucleus, excluding nucleoli, the adjustment of the confocal settings (iris, gain, black level), the same for each cell series, and the quantification of fluorescence intensities on a grey scale (0–255) was performed following the procedure of Leclerc et al. (1998). After correcting nuclear (N) and cytoplasmic (C) fluorescence intensities for background, the nucleocytoplasmic ratio ($R = (N - C)/(N + C)$) for the JNK signal was calculated as described (Leclerc et al., 1998). Data were analyzed by Microsoft Excel on a Macintosh computer.

Subcellular Fractionation

To prepare whole-cell extracts, the monolayers were washed twice in PBS and the cells were lysed by incubation in RIPA buffer (150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 10% glycerol, 4 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 50 mM Hepes, pH 7.4, plus PPIM [25 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin]) for 15 min on ice followed by centrifugation at 13,000 rpm for 10 min at 4°C. For subcellular fractionation, cells were lysed in nuclear precipitation buffer (NPB: 10 mM Tris HCl, pH 7.4, 2 mM $MgCl_2$, 140 mM NaCl, plus PPIM) supplemented with 0.1% Triton X-100 by incubating on ice for 10 min. The lysate was layered onto 50% wt/vol sucrose/NPB and centrifuged at 13,000 rpm for 10 min. Supernatants were taken as cytosolic fraction. Pellets (nuclei) were washed with NPB and extracted with Dignam C buffer (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, plus PPIM) to obtain the nuclear fraction.

Immune Complex Kinase Assay

JNK was immunoprecipitated from each subcellular fraction using 0.4 μg of an anti-JNK antibody (sc-474 from Santa Cruz Biotechnology, Inc.). JNK activity in the immunocomplexes was assayed by incubation with 1 μg glutathione S–transferase–c-Jun1-79 (GST-c-Jun) as substrate in the presence of 20 μM cold ATP and 1 μCi of $\gamma[^{32}P]$ATP as described (Caelles et al., 1997).

Western Blotting

JNK, phosphorylated JNK, and MEK-1 were detected in subcellular extracts by immunoblotting using the specific antibodies sc-474 (Santa Cruz Biotechnology, Inc.), V7931 (Promega), and sc-219 (Santa Cruz Biotechnology, Inc.), respectively. Histone $H1^0$ was detected using an mAb donated by Prof. A. Alonso (Deutches Krebsforschung Zentrum, Heidelberg). Western blots were performed and developed using the ECL detection system (Amersham Pharmacia Biotech) using either HRP-conjugated anti-rabbit (for anti-JNK, antiphospho-JNK, and anti-MEK-1) or HRP-conjugated anti-mouse (for antihistone $H1^0$) antibodies (ICN).

Results

Glucocorticoids Inhibit both Nuclear Increase in NH_2-terminal Phosphorylated and Total c-Jun, and in Phosphorylated/Activated JNK in HeLa Cells

To study whether glucocorticoids inhibit phosphorylation of c-Jun NH_2-terminal domain in intact cells in vivo, we analyzed by confocal laser scanning microscopy the immunofluorescence of HeLa cells incubated with an antibody against c-Jun protein phosphorylated on serine-63 (hereafter referred to as phospho-c-Jun) upon treatment with TNF-α. No signal over background was found in cells treated with vehicle or Dex alone (Fig. 1 A, a and b). Upon TNF-α treatment, an intense punctate staining against a more diffuse signal appeared throughout the nucleus (except the nucleoli), whereas the cytoplasm remained negative (Fig. 1 A, c). The increase in phospho-c-Jun induced by 30-min treatment with TNF-α was efficiently inhibited in Dex-treated cells (Fig. 1 A, c and d). Dex reduced the level of nuclear phospho-c-Jun induced by TNF-α in HeLa cells by ~60% (Fig. 1 B). Unstimulated vehicle- and Dex-treated cells showed a similar low nuclear staining when an antitotal c-Jun antibody was used (Fig. 1 C, a and b). Upon TNF-α treatment, a strong nucleoplasmic immunolabeling was observed (Fig. 1 C, c). In agreement with previous data (Caelles et al., 1997), Dex also inhibited the increase in total c-Jun protein caused by the transcriptional activation of the c-*jun* gene in response to TNF-α (Fig. 1 C, c and d). Computer-assisted quantification revealed a 40% inhibition in TNF-α + Dex cells with respect to cells treated with TNF-α alone (Fig. 1 D).

c-Jun phosphorylation is a consequence of the TNF-α–induced dual phosphorylation and subsequent activation of JNK and its concomitant translocation into the cell nucleus (Karin et al., 1997; Minden and Karin, 1997). Therefore, we next examined whether the reduction in phospho-c-Jun levels could be due to the inhibition of JNK activity within the nucleus. Immunofluorescence analysis by confocal microscopy using a specific antiphospho-JNK antibody showed that Dex reduced the level of cytosolic (56%) and nuclear (71%) phospho-JNK induced by TNF-α (Fig. 2, A and B). This result was confirmed by immunoblotting analysis (Fig. 2 C). To check whether this effect of Dex correlated with a decrease in enzymatic activity in both cellular compartments, we measured JNK activity in cytosolic and nuclear fractions. In agreement with immunofluorescence and immunoblotting data, Dex pretreatment led to significant reductions in JNK activity in both cytosolic and nuclear fractions (Fig. 2 D), which were consistent with the lower levels of nuclear phospho-JNK (Fig. 2, A and B). In turn, these data are consistent with the reduc-

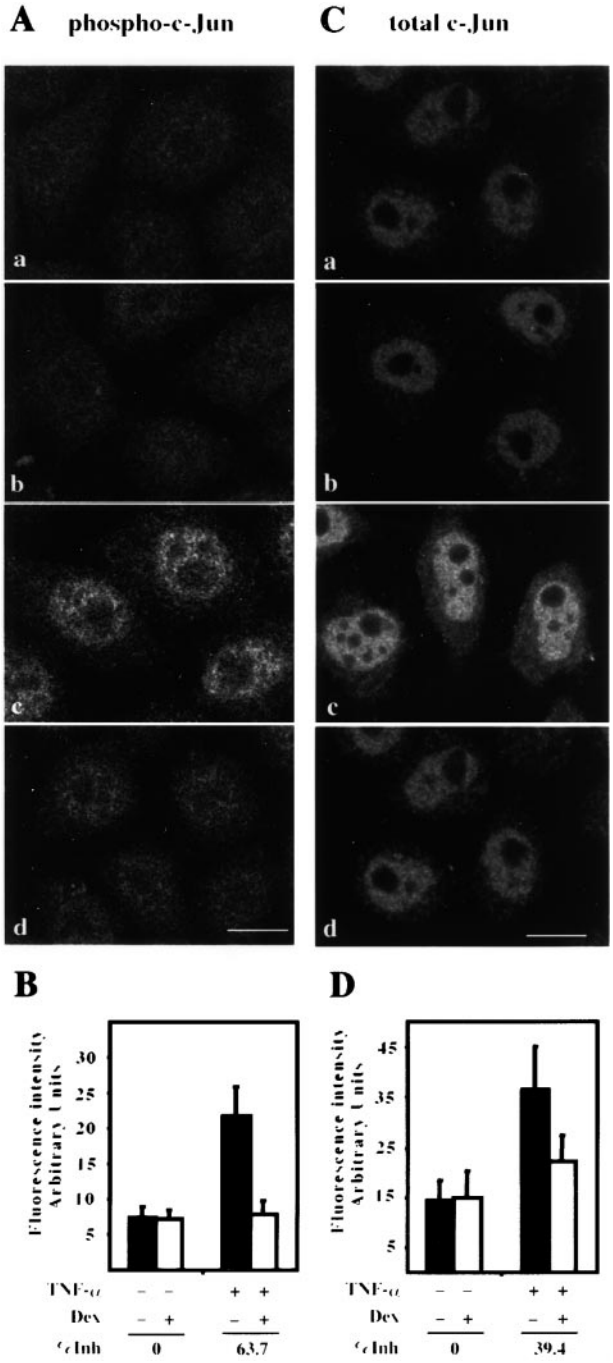


Figure 1. Inhibition by Dex of c-Jun phosphorylation and induction in TNF-α–treated HeLa cells. A, Confocal laser scanning microscopy analysis of HeLa cells incubated with a polyclonal antibody against c-Jun phosphorylated on serine-63: a, unstimulated, vehicle-treated cells; b, cells treated with 1 μM Dex alone; c, TNF-α cells, preincubated with vehicle for 45 min and then treated with 10 ng/ml TNF-α for 30 min; d, TNF-α + Dex cells, preincubated with Dex for 45 min and then treated also with 10 ng/ml TNF-α for an additional 30 min. B, Quantification of the inhibition by Dex of the TNF-α–induced nuclear phospho-c-Jun average fluorescence intensity in HeLa cells. C, Confocal microscopy analysis of the immunofluorescence of HeLa cells incubated with an anti–c-Jun antibody: a–d are as in A. D, Quantification of the inhibition by Dex of the TNF-α–induced nuclear c-Jun average fluorescence intensity in HeLa cells. Bars, 15 μm.

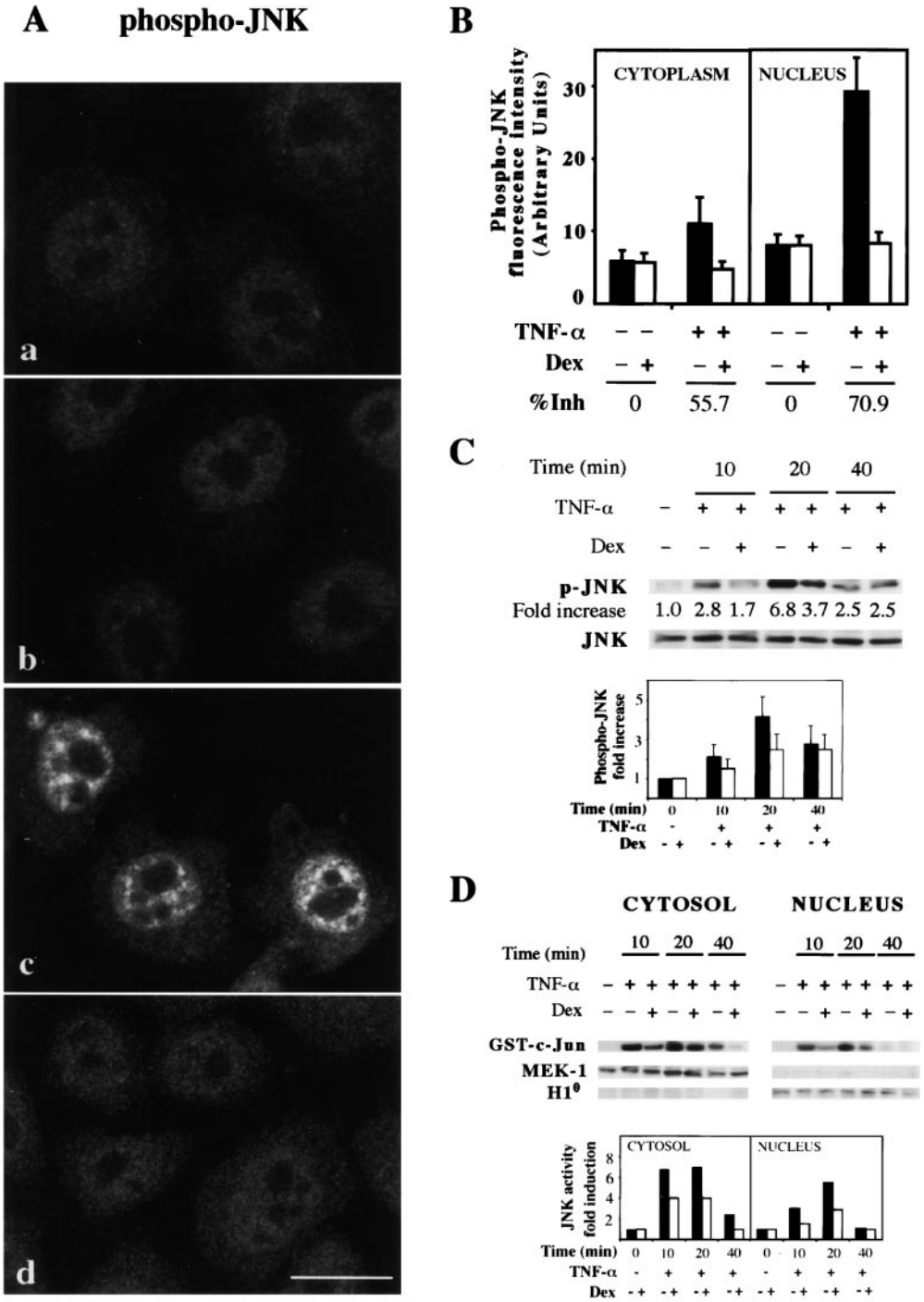
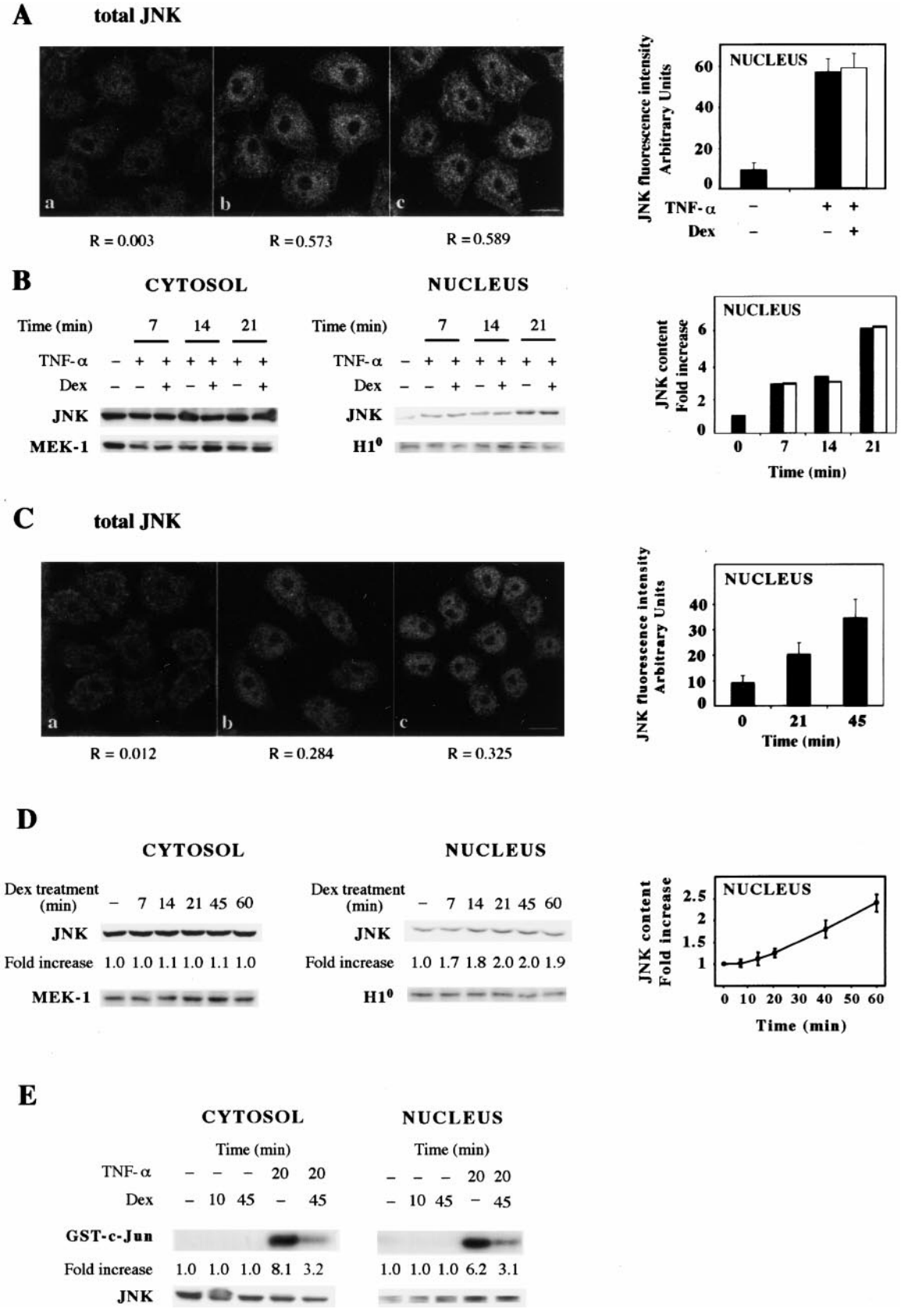


Figure 2. Inhibition by Dex of TNF-α–induced JNK phosphorylation and enzymatic activity. A, Confocal microscopy analysis of HeLa cells incubated with an mAb against JNK1 phosphorylated on threonine-183 and tyrosine-185: a–d are as in Fig. 1 A, and are described in Materials and Methods. Bar, 15 μm. B, Quantification of the inhibition by Dex of the TNF-α–induced phospho-JNK average fluorescence intensity in the cytoplasm and nucleus of HeLa. C, Western blot analysis of the amount of phosphorylated JNK present in HeLa cells upon TNF-α (10 ng/ml) addition to cultures preincubated with vehicle (−) or 1 μM Dex. A rabbit polyclonal antiphospho-JNK antibody was used. The effect of Dex on the increase of phosphorylated JNK caused by TNF-α is indicated. Filters were reincubated with a rabbit polyclonal antibody against total JNK for loading control. Quantification of the results obtained in three independent experiments (mean values ± SD) is indicated. D, Inhibition by Dex of JNK activity in nuclear and cytosolic fractions of TNF-α–treated HeLa cells. Cells were treated with vehicle (−) or 1 μM Dex 45 min before addition of 10 ng/ml TNF-α. At the indicated times after TNF-α treatment subcellular fractionation was carried out. JNK activity in each fraction was estimated by the immune complex kinase assay. Purity of subcellular fractions was ensured by incubation with polyclonal anti-MEK-1 and monoclonal antihistone $H1^0$ antibodies. Quantification of the results (mean values) obtained in two experiments is shown below.

Figure 3. Lack of effect of Dex on the TNF-α–induced nuclear accumulation of JNK in HeLa cells. A, Confocal microscopy analysis of HeLa cells incubated with an mAb against total JNK. The signal was amplified by using a biotin–streptavidin system as described in Materials and Methods. Cells were treated with: a, vehicle; b, TNF-α; and c, TNF-α + Dex, as described in Fig. 1 A. Bar, 15 μm. The values of the ratio $R = (N - C)/(N + C)$, as defined by Leclerc et al. (1998), estimating the change in subcellular distribution are shown below the micrographs. Quantification of the TNF-α–induced nuclear accumulation of JNK in cells treated or not with Dex (right). B, Western blot analysis of the amount of total JNK present in cytosol or nucleus of HeLa cells upon TNF-α (10 ng/ml) addition to cultures preincubated with vehicle (−) or 1 μM Dex for 45 min. A rabbit polyclonal anti-JNK antibody and a secondary HRP-conjugated anti-rabbit antibody were used. Antibodies against MEK-1 and histone $H1^0$ were used to ensure purity of subcellular fractions. Quantification of the results (nucleus) is indicated: black columns, TNF-α cells; white columns, TNF-α + Dex cells. C and D, Dex alone induces JNK nuclear accumulation. C, Confocal microscopy analysis of HeLa cells incubated with an antibody against total JNK. Signal was amplified as before: a, vehicle-treated cells; b, cells treated with 1 μM Dex for 21 min; c, cells treated with 1 μM Dex for 45 min. Bar, 15 μm. R values are shown below the micrographs. D, Quantification of the Dex-induced nuclear accumulation of JNK is shown. Western blot anal-



ysis of the amount of total JNK present in cytosol (30 μg) or nucleus (50 μg) of HeLa cells upon incubation with vehicle (−) or 1 μM Dex for the indicated times. The relative levels of total JNK present in a representative experiment in each subcellular fraction are indicated. Purity of subcellular fractions was checked using antibodies against MEK-1 and histone $H1^0$ as before. Quantification of the results obtained in three independent experiments (mean values ± SD) is indicated. E, Lack of effect of Dex alone on JNK activity in unstimulated cells. Results obtained in cytosolic and nuclear fractions are shown. JNK activity in each subcellular fraction of HeLa cells incubated as indicated with vehicle (−), 1 μM Dex, 10 ng/ml TNF-α, or both agents was measured by the immune complex kinase assay. The amount of JNK present in each extract was estimated by Western blot using a polyclonal antitotal JNK antibody.

tion of phospho-c-Jun in Dex-treated cells when stimulated with TNF-α (Fig. 1 A). Verification that nuclear proteins did not leak into the cytosolic fractions during the fractionation process was obtained through subsequent hybridization of Western membranes to detect histone $H1^0$, which localizes exclusively in the nucleus. Conversely, MEK-1 was detected only in cytosolic fractions (Fig. 2 D).

Glucocorticoids Do Not Inhibit the TNF-α–induced Nuclear Accumulation of JNK

We used antibodies against total JNK to test the possibility that the inhibitory effect of Dex on the nuclear levels of phospho-JNK and JNK activity could be the consequence of a reduction in the nuclear content of this enzyme. In unstimulated HeLa cells, JNK immunoreactivity was diffusely distributed in the cytosol and nucleus (Fig. 3 A, a). TNF-α induced the nuclear accumulation of the enzyme, which was unchanged by Dex treatment (Fig. 3 A, b and c). Using two distinct antibodies (anti-JNK1/JNK3 sc-474 and anti-JNK1 15701A), the same increase (five- to six-fold) in fluorescence was found in the nucleus of TNF-α–treated cells, irrespective of Dex pretreatment (Fig. 3 A, right), whereas cytoplasmic fluorescence did not change significantly upon any treatment (not shown). Measurement of JNK translocation by immunoblotting of subcellular fractions revealed that JNK was predominantly cytosolic in unstimulated HeLa cells, and confirmed that TNF-α induced a similar increase in nuclear JNK content, irrespective of pretreatment with Dex (Fig. 3 B). A large amount of JNK remained in the cytosol even in TNF-α–treated cells. The lack of significant differences in cytosolic JNK can be explained by the fact that JNK is abundant in the cytosol (~30-fold higher than in the nucleus in unstimulated cells) and therefore only a small fraction of cytosolic JNK is mobilized to the nucleus. The apparent discrepancy between the levels of extranuclear JNK content observed by immunoblotting and immunofluorescence may be explained by the fact that the image obtained by confocal microscopy corresponds to a cellular slice, whereas immunoblotting of subcellular fractions allows a more precise estimation of JNK content in each fraction as takes into account their total size. It should be kept in mind that a minor degree of protein leakage during cellular fractionation and/or differences in fluorescence signal due to variations in epitope accessibility and microenvironment cannot be ruled out. Dex alone also caused a slight, but reproducible, accumulation of JNK in the nucleus (Fig. 3 C). This effect was progressive and led to a 2–2.5-fold increase in nuclear JNK content (Fig. 3 D). To rule out the possibility that Dex could affect basal JNK activity by itself, we performed JNK assays in cytosolic and nuclear fractions of HeLa cells upon hormone addition. As expected, Dex did not induce JNK activity, but inhibited the activation of this enzyme by TNF-α (Fig. 3 E). The inhibition was simultaneous in cytosol and nucleus, with kinetics compatible with that of nuclear entry of hormone-bound GR (Htun et al., 1996). These results show that Dex alone caused an accumulation of inactive JNK in the nucleus and that Dex treatment did not affect the subcellular distribution of JNK induced by TNF-α.

Mechanism of JNK Inhibition by Hormone-activated GR

We found no evidence of interaction between activated, hormone-bound GR and JNK when we coimmunoprecipitated these two proteins from HeLa cell extracts (not shown). In view of recent reports (Gabai et al., 1997; Mosser et al., 1997) that overexpression of the heat-shock protein 70 (Hsp70) inhibits JNK activity, we also studied whether Hsp70 mediates the inhibitory action of Dex-bound GR. Western blots of separate cytosolic and nuclear fractions showed that Dex treatment did not affect the cellular content of Hsp70, at least during the period relevant for the inhibition of JNK (not shown). Likewise, no changes in Hsp70 levels in either cellular fraction were found upon TNF-α addition to control or Dex-treated HeLa cells (not shown). In addition, we examined whether Dex treatment affected the amount of Hsp70 that is bound to JNK. However, the highly unspecific binding of this protein (even to agarose beads) precluded this analysis.

Next, we examined whether a mutant form of GR (GRdim; Heck et al., 1994), which is unable to homodimerize, fails to bind DNA, and cannot transactivate GR element (GRE)-dependent promoters, could mediate Dex-induced JNK inhibition. Upon hormone binding, GRdim was as efficient as GRwt in inhibiting JNK activity (Fig. 4 A). We also analyzed whether GRdim, like GRwt, accumulates in the nucleus after Dex treatment. Both ectopically-expressed GRwt and GRdim translocate into the nucleus of transfected Cos-7 cells upon Dex addition (Fig. 4 B). This result emphasizes the DNA-binding–independent activities of GRdim and the transcription-independent inhibition of the JNK pathway by Dex (Caelles et al., 1997). Furthermore, it supports the link between AP-1 transrepression and JNK inhibition, and demonstrates that monomeric hormone-bound GR can mediate this effect.

To verify that GRdim has the same effects on JNK activity and nuclear accumulation as GRwt, we analyzed the effects of Dex in GR-deficient Cos-7 cells that were transfected with either of these two genes. As seen in Fig. 5 A, Dex treatment of GRwt- or GRdim-transfected Cos-7 caused the same inhibition of the TNF-α–induced JNK phosphorylation as observed in HeLa cells. Measurement of fluorescence intensity showed a 73 and 74% inhibition of nuclear phospho-JNK in GRwt- and GRdim-transfected cells, respectively. Likewise, in both types of transfected cells, Dex inhibited the increase in phospho-c-Jun and total c-Jun (not shown). Also as in HeLa cells, the accumulation of total JNK in the nucleus was not inhibited, but even slightly increased, by the hormone in both GRwt- and GRdim-transfected Cos-7 cells (Fig. 5 B, b). In agreement with this, Dex did not change the nuclear translocation of JNK induced by TNF-α (Fig. 5 B, c and d).

Discussion

Our results show that Dex inhibits JNK phosphorylation and activity in intact cells, and that this effect is not due to inhibition of the nuclear translocation of the enzyme induced by TNF-α. Rather, Dex by itself can promote the nuclear entry of inactive JNK in noninduced cells. These results were observed in two cell types, HeLa and Cos-7,

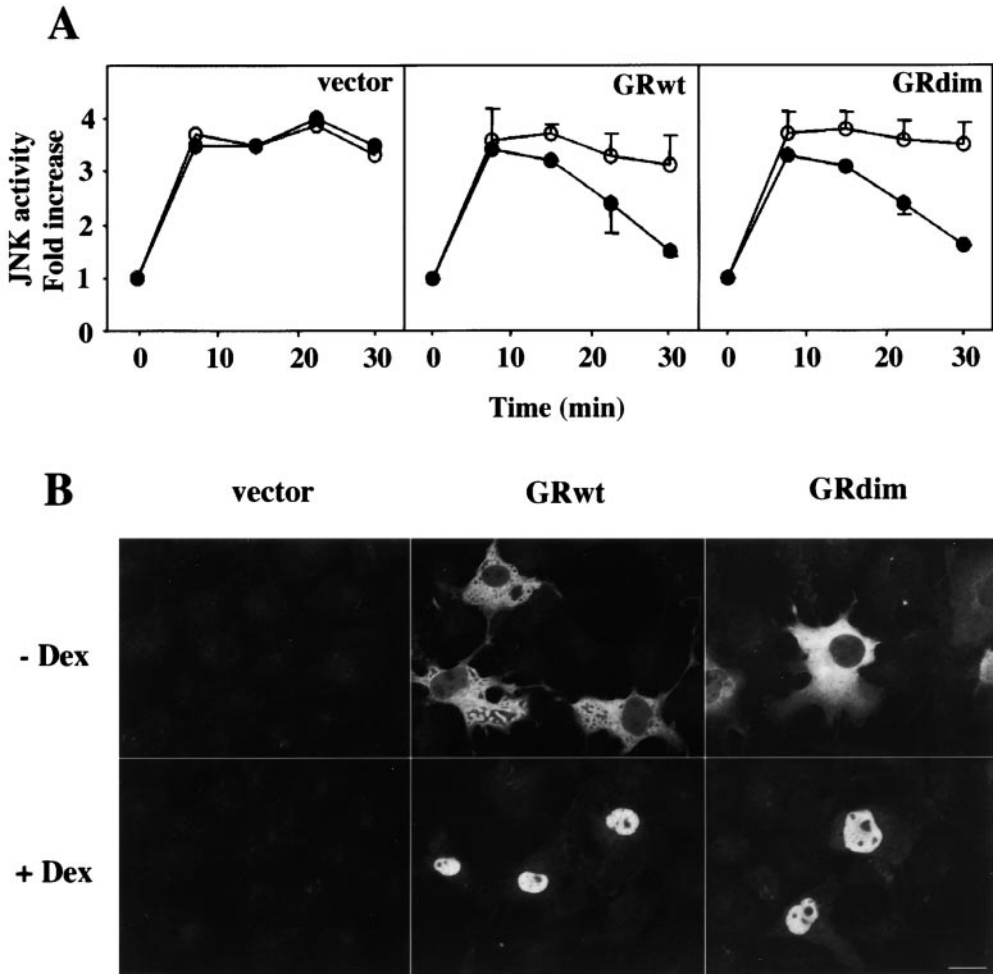


Figure 4. Inhibition of TNF-α–induced JNK activation by Dex is independent of GR dimerization, DNA binding, and gene transactivation. A, Mutant GRdim receptor mediates Dex inhibition of TNF-α–induced JNK activation in Cos-7 cells. Induction of JNK activity by TNF-α in Cos-7 cells cotransfected with 3 μg of plasmid encoding HA-JNK (pCDNA3-JNK1) and 0.4 μg of empty vector (pRSh$^-$R$^-$, left) or those encoding either GRwt (pSB-hGR, middle) or GRdim (pSB-hGR(A458T), right). 24 h after transfection, cells were treated with vehicle (open circles) or 1 μM Dex (solid circles) for 45 min. Then, TNF-α (10 ng/ml) was added with no medium change, and JNK activity was measured at the indicated times after TNF-α addition by the immune complex kinase assay. Results from duplicates obtained in two separate experiments are shown. B, GRdim translocates into the cell nucleus upon Dex treatment. Cos-7 cells were transfected with empty vector (left), or those encoding either GRwt (pSB-hGR, middle) or GRdim (pSB-hGR(A458T), right) as before. 48 h after transfection cells were treated with vehicle (top micrographs) or 1 μM Dex (bottom micrographs) for 45 min and localization of ectopically expressed GRwt or GRdim was analyzed by immunofluorescence using a rabbit polyclonal anti-GR antibody. Bar, 15 μm.

and extend previous observations that JNK activity was reduced in extracts from cells treated with glucocorticoids, retinoids, thyroid hormone, or estrogens (Caelles et al., 1997; Swantek et al., 1997; González et al., 1999; Lee et al., 1999; Srivastava et al., 1999; Ventura et al., 1999).

Our results differ from those reported for extracellular-regulated kinase (ERK), which suggested that kinase phosphorylation was necessary to induce homodimerization and subsequent nuclear translocation (Khokhlatchev et al., 1998). Differences in the mechanism of translocation between ERK and JNK, or perhaps cell type-specific differences, are also suggested by the finding that JNK1 is translocated into the nucleus of rat heart cells during ischemia, but that activation only takes place after reperfusion (Mizukami et al., 1997). Our results indicate a process other than JNK nuclear translocation for repression by hormone-bound GR. We had previously shown that 45-min pretreatment with Dex is sufficient for maximum inhibition of JNK, whereas half-maximum effect is obtained with a hormone treatment of only 10 min, which is coincident with the half-time of nuclear translocation of GR (Caelles et al., 1997). In addition, a mutant transactivation-deficient GR (LS7; Schena et al., 1989; Helmberg et al., 1995) was found to mediate JNK repression (Caelles et al., 1997). However, GR-LS7 mutant has been reported to keep residual transactivating activity (Heck et al., 1997). The finding now that GRdim is as efficient as GRwt in mediating the effects of Dex indicates rapid inhibition of JNK phosphorylation even by monomeric DNA binding-deficient, Dex-bound GR, which inactivates the enzyme in both cytosol and nucleus. This result demonstrates that the rapid inhibition of JNK does not require transcriptional activation by Dex, and also reinforces the DNA-binding independent activities of GR.

The effects of Dex have at least two possible explanations: direct or indirect inhibition of JNK activation in the cytoplasm in response to stress, and the activation of putative phosphatase(s). The negative result of the coimmunoprecipitation approach do not rule out a direct interaction between JNK and activated GR. Likewise attempts to coimmunoprecipitate JNK and its substrate c-Jun have failed, probably due to the existence of transient and/or weak interaction (Minden and Karin, 1997), JNK and GR might form unstable complexes that impede their coimmunoprecipitation by current protocols. Alternatively, they

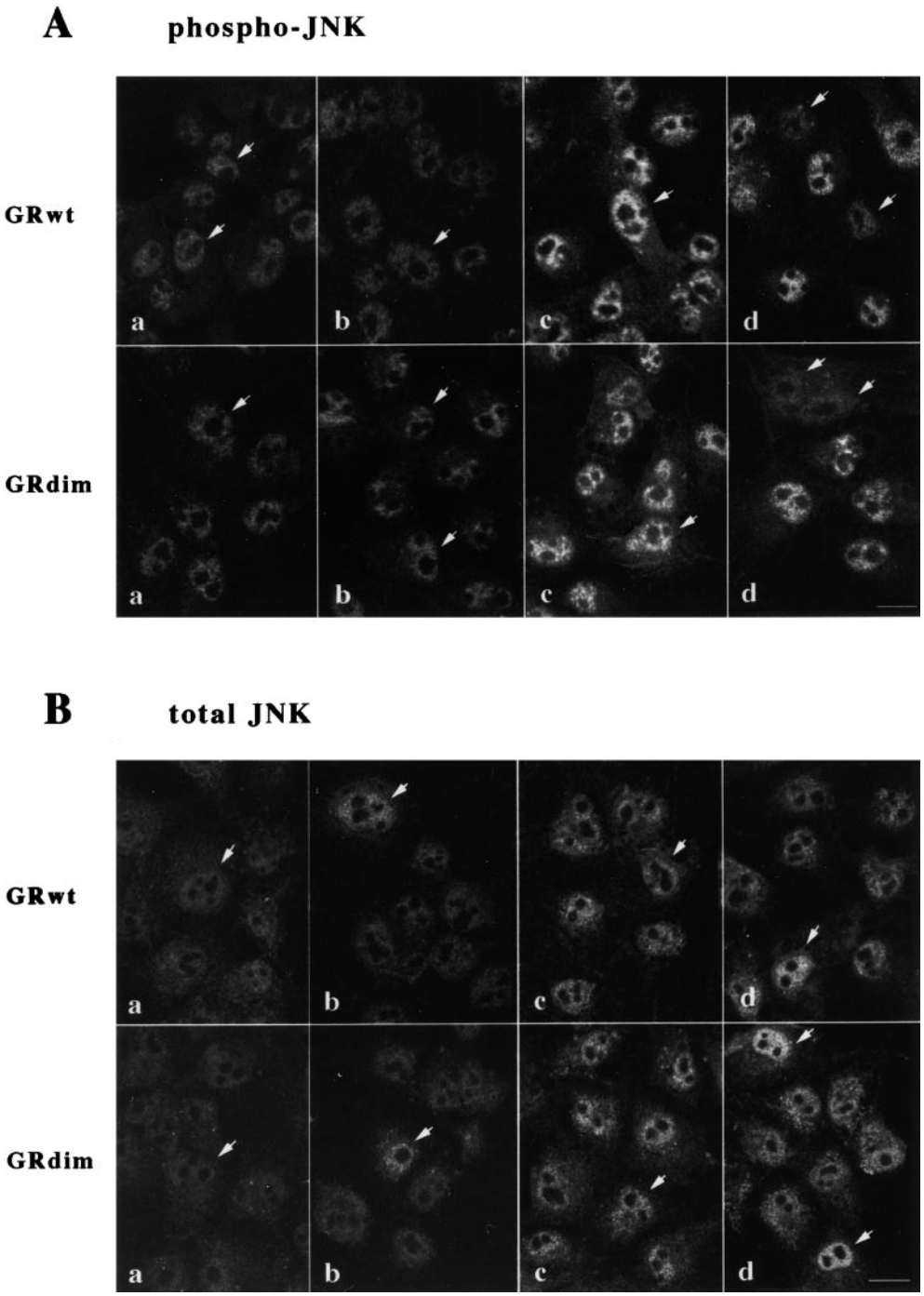


Figure 5. GRdim has the same effect as GRwt on the TNF-α–induced JNK activation/phosphorylation and accumulation into the nucleus. Cos-7 cells were transfected with plasmids encoding either GRwt or GRdim as indicated. 48 h after transfection, cells were treated with: a, vehicle; b, Dex alone; c, TNF-α; or d, TNF-α + Dex, as described in Fig. 1 A and Materials and Methods, and double-immunofluorescence was performed using anti-GR and antiphospho-JNK (A) or antitotal JNK (B) antibodies. GRwt- or GRdim-expressing cells were first identified with the anti-GR antibody (arrows) and were then analyzed for phospho-JNK or total JNK immunostaining by confocal microscopy. Bars, 15 μm.

could interact indirectly through intermediate proteins in the frame of the large multiprotein signalosome/COP9 complexes where JNK may be associated (Wei et al., 1998; Seeger et al., 1998). However, the signalosome-directed c-Jun activation is independent of JNK (Naumann et al., 1999). Regarding the second possibility, retinoids have been reported to induce MKP-1 phosphatase accumulation in nonsmall cell lung cancer cells (Lee et al., 1999). However, we have not found MKP-1 induction by Dex in HeLa cells (Caelles et al., 1997) and, furthermore, its kinetics in lung cancer cells is clearly delayed with respect to the inhibition of JNK by Dex in our system. These results, however, do not rule out the involvement of other phosphatase(s). It also remains to be determined whether JNK itself or any upstream component of the pathway is the primary target of hormone-activated GR. However, several studies suggest that Dex does not inhibit the immediate upstream kinases MEKK or SEK-1 (Caelles et al., 1997; Swantek et al., 1997; Lee et al., 1999).

Known JNK targets are mainly nuclear, such as c-Jun, ATF-2, Elk-1, and p53 (Karin et al., 1997; Minden and Karin, 1997; Fuchs et al., 1998). Only NFAT4 has been proposed to be phosphorylated by JNK in the cytosol (Chow et al., 1997). However, the presence of high levels

of active enzyme in the cytosol of stimulated cells suggests there may be other, unidentified substrates in this compartment. The inhibition of JNK activity by Dex in cytosol and nucleus also indicates the attenuation of stress-activated cytosolic pathways.

In two-hybrid experiments in yeast and in vitro, May et al. (1998) reported that the interaction between JNK and c-Jun does not require JNK catalytic activity. Interestingly, a regulated inhibitory domain in the NH_2-terminal region (δ domain) of c-Jun binding a putative δ inhibitor that could block its activation by JNK has been predicted (Baichwal and Tjian, 1990). The finding that JNK binds the δ domain in resting cells led to the hypothesis that JNK itself could be a form of this δ inhibitor (Dai et al., 1995). Our results show that Dex inhibits c-Jun phosphorylation and activation without affecting the accumulation of JNK in the nucleus. Therefore, though a direct JNK-c-Jun interaction in Dex-treated cells has not been found, our data support the hypothesis that inactive JNK may behave as a δ inhibitor, thus preventing AP-1 activation. JNK activation is believed to contribute to inflammatory responses (Ip and Davis, 1998), which, together with the recent finding that c-Jun phosphorylation is critical for stress-induced apoptosis and cellular proliferation in vivo (Behrens et al., 1999), and the implication of JNK in the control of p53 stability (Fuchs et al., 1998), suggests an important physiological role of JNK inhibition by glucocorticoids.

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