**Forschungszentrum Karlsruhe**
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FZKA 7321

# Inhibition of c-Jun/ATF2 Phosphorylation, Trans-repression of c-Jun/c-Fos: Two Modes of Regulation of the Transcription Factor AP-1 Activity by Glucocorticoid Receptor

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**Inhibition of c-Jun/ATF2 phosphorylation, Transrepression of c-Jun/c-Fos: Two modes of regulation of the transcription factor AP-1 activity by Glucocorticoid Receptor**

# ABSTRACT

Changes in the environment lead to the activation of multi-component signaling networks. The intracellular Mitogen-Activated Protein Kinase (MAPK) signaling cascades transfer the signal from the cell membrane to the nucleus, leading to the regulation of gene expression. Expression of genes in response to the signaling is mediated through the activation of transcription factors, such as Activator Protein 1 (AP-1).

AP-1 is a family of transcription factors made of different dimers from Jun, Fos and ATF families of proteins. Different AP-1 dimers, such as c-Jun/c-Fos and c-Jun/ATF2 regulate different subset of target genes whose products regulate different aspects of cell function like proliferation, inflammation or cell death.

c-Jun/c-Fos and c-Jun/ATF2 are activated by different mechanisms. For example the activation of the Erk MAPK cascade leads to an increase in the expression of Fos, and consequently to an activation of c-Jun/c-Fos. The activation of JNK cascade induces the phosphorylation of c-Jun and ATF2 leading to an increased activity of c-Jun/ATF2.

The function of c-Jun/c-Fos and c-Jun/ATF2, in order to prevent the overexpression of the target genes which might lead to pathological changes, must be negatively regulated. One mechanism of negative regulation is the transrepression of AP-1 by the Glucocorticoid receptor (GR). Previous results show that in transrepression GR is recruited to the promoter bound c-Jun/c-Fos through an interaction with nTrip6, a nuclear isoform of the LIM domain protein Trip6. This interaction is essential for transrepression.

Results presented in this work show that the transcriptional activity of c-Jun/ATF2 is not regulated by GR in this manner. Indeed, c-Jun/ATF2 does not interact with nTrip6, and is not transrepressed by GR. However, GR represses c-Jun/ATF2 function by inhibiting the JNK-mediated phosphorylation of c-Jun.

An increased activity of AP-1, through expression of its target genes can lead to pathological conditions, like a sustained inflammation. Two mechanisms of AP-1 repression by GR can act in concert to block the overexpression of AP-1 target genes, consequently preventing an excessive inflammatory response.

**Die Inhibition der Phosphorylierung von c-Jun/ATF2 sowie die Transrepression von c-Jun/c-Fos: zwei unterschiedliche Formen der Regulation der Aktivität des Transkriptionsfaktors AP-1 durch den Glukokortikoidrezeptor**

## ZUSAMMENFASSUNG


Veränderungen in der Umwelt aktivieren Mehrkomponenten-Signalnetzwerke. Signalkaskaden, die über intrazelluläre Mitogen-Aktivierte Proteinkinasen (MAPK) laufen, übertragen das Signal von der Zellmembran zum Nukleus und führen dort zur Genregulation. Die Genexpression als Antwort auf diese Signale wird über die Aktivierung von Transkriptionsfaktoren – wie z.B. dem Aktivierungsprotein 1 (AP-1) – vermittelt.

AP-1 bildet eine Familie von Transkriptionsfaktoren, die aus Dimeren von Jun-, Fos- und ATF-Familienproteinen besteht. Unterschiedliche AP-1 Dimere wie c-Jun/c-Fos und c-Jun/ATF2 regulieren unterschiedliche Untergruppen von Zielgenen. Deren Genprodukte regulieren wiederum unterschiedliche Aspekte der Zellfunktion wie Proliferation, Entzündung und Zelltod.

C-Jun/c-Fos und c-Jun/ATF2 werden über unterschiedliche Mechanismen aktiviert. Die Aktivierung der Erk-MAPK-Kaskade führt zum Beispiel zu einer Steigerung der Expression von Fos und damit zu einer Aktivierung von c-Jun/c-Fos. Die Aktivierung der JNK-Kaskade induziert die Phosphorylierung von c-Jun und ATF2; dieses führt zu einer erhöhten Aktivität von c-Jun/ATF2.

Um die zu starke, zu pathologischen Änderungen führende Expression der Zielgene zu vermeiden, muss die Funktion von c-Jun/c-Fos und c-Jun/ATF2 negativ reguliert werden. Ein Mechanismus der negativen Regulation ist die Transrepression von AP-1 durch den Glukokortikoidrezeptor (GR). Vorherige Resultate zeigten, dass bei der Transrepression GR durch die Interaktion mit nTrip6 an Promoter-gebundenes c-Jun/c-Fos rekrutiert wird – bei nTrip6 handelt es sich um eine nukleäre Isoform des LIM-Domänenproteins Trip6. Diese Interaktion ist für die Transrepression essentiell.

Die Ergebnisse dieser Arbeit zeigen, dass die Transkriptionsaktivität von c-Jun/ATF2 nicht in dieser Weise reguliert ist. In der Tat interagiert c-Jun/ATF2 nicht mit nTrip6 und wird nicht durch GR transreprimiert. Hingegen unterdrückt GR die c-Jun/ATF2 Funktion durch Inhibition der JNK vermittelten Phosphorylierung von c-Jun.

Eine übermäßige Aktivität von AP-1 kann über die Expression der Zielgene zu pathologischen Zuständen wie anhaltender Entzündung führen. Die zwei Mechanismen der Unterdrückung von AP-1 durch GR können bei der Blockade der übermäßigen, AP-1-vermittelten Expression von Zielgenen zusammenwirken und dadurch eine unverhältnismäßige Entzündungsreaktion vermeiden.

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| | |
|---|---|
| AP-1 | Activator Protein 1 |
| bp | base pair |
| BSA | bovine serum albumin |
| bZIP | basic leucine zipper domain |
| cmp | count per minute |
| DBD | DNA-binding domain |
| DEX | dexamethasone |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethylsulfoxide |
| dNTP | deoxynucleotide triphosphate |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediamine-N,N-tetraacetate |
| EGTA | Ethylenguanindine-N,N-tetraacetate |
| FCS | Foetal calf serum |
| GAPDH | glyceraldehydes-3-phophate dehydrogenase gene |
| GC | glucocorticoid hormone |
| GR | Glucocorticoid Receptor |
| HRP | Horseradish peroxidase |
| JNK | Jun-N-terminal kinase |
| LB | Luria-Bertani |
| µ | micro |
| MOPS | 4-morpholinepropanesulfonic acid |
| OD | optical density |
| SDS | Sodium-lauryl-sulphate |
| TRE | 12-o-tetradecanoylphorbol 13-acetate response element |
| Tris | Tris-(hydroxymethyl)-aminomethane |
| UV | Ultra-violet light |
| uPA | urokinase plasminogen activator |

# 1. INTRODUCTION

Changes in the physical and the chemical properties of the environment lead to the activation of multi-component signaling networks. Intracellular signaling cascades transfer the signal from the cell membrane to the nucleus, leading to the regulation of gene expression. Expression of genes in response to the signaling is mediated through the activation of transcription factors.

Activator Protein 1 (AP-1) was one of the first transcription factors to be identified. AP-1 modulates the expression of genes whose products regulate many aspects of cell function including migration, proliferation, differentiation, inflammation and cell death.

Changes in extracellular conditions also induce mechanisms that limit the response. Such brake mechanisms are essential to maintain homeostasis. One of the main pathways maintaining homeostasis is the neuroendocrine hypothalamic-pituary-adrenal axis. Stress, like inflammation, leads to the induction and release of glucocorticoid (GC), cortisol. Released GC act through binding to the glucocorticoid receptor (GR) to inhibit the expression of pro-inflammatory genes. One mechanism by which GR inhibits pro-inflammatory gene expression is the repression of the activity of AP-1, therefore preventing an excessive inflammatory response.

## 1.1 AP-1 transcription factors family

AP-1 was one of the first mammalian transcription factors identified, but its mechanisms of regulation are still being unraveled. The transcription factor AP-1 can be composed of either homodimers or heterodimers between members of the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2) and ATF/CREB (ATF2, ATF3) sub-families (Angel and Karin, 1991). They belong to the class of basic zipper (bZip) family of sequence-specific dimeric DNA-binding proteins. They share the same structural domains for DNA binding and dimerization: a basic region (b) and a leucine zipper (Zip), respectively. The formation of each dimer depends on their relative dimerization affinities,

and on the abundance of each of the Jun, Fos and ATF proteins available in the cell at a given time.

Fos members of the family cannot form stable homodimers but heterodimerize with Jun members. c-Jun/c-Fos heterodimers are more stable than c-Jun/c-Jun homodimers. ATF family members can dimerize with c-Jun as well as with ATF/CREB family members (Benbrook and Jones, 1994; Chatton et al., 1994; Hai and Curran, 1991).

The ability of the AP-1 transcription factor to control many different biological processes stems primarily from its structural and regulatory complexity. Different AP-1 dimers regulate different sets of target genes, which in turn play roles in a variety of biological processes. For example, studies on combinatorial variants of AP-1 complexes, using Jun mutants that preferentially heterodimerize with either c-Fos or ATF2 revealed opposing roles of c-Jun/c-Fos and
c-Jun/ATF2 dimers in transformation (van Dam et al., 1998). Jun-dependent cell transformation can be resolved into at least two distinct and independent processes: anchorage independence triggered by c-Jun/c-Fos and growth factor independence triggered by c-Jun/ATF2. These differences in the roles of Jun/Fos and Jun/ATF make these dimers particularly interesting to study.

### 1.1.2 Sequence-specificity of Jun/Fos and Jun/ATF transcription factors

The DNA binding domain determines the spectrum of genes that are controlled by AP-1. The dimeric structure and different dimerization partners can significantly modulate the specificity of DNA targeting. c-Jun/c-Fos heterodimers preferentially bind to the heptamer consensus 5'-TGA (C/G) TCA-3', known as the phorbol ester 12-O-tetradecanoate-13-acetate (TPA) response element (TRE), based on their ability to mediate transcriptional induction in response to TPA (Angel et al., 1987). c-Jun/ATF2 dimers, on the other hand, bind the octameric cyclic-AMP response element (CRE) 5'-TGACGTCA-3'. These binding sites have been identified in the regulatory regions of a wide range of genes, including transcription factors such

as c-Jun and ATF3, matrix-degrading enzymes like collagenase I and urokinase plasminogen activator (uPA), cytokines like IFN-γ or adhesion molecules such as E-selectin (Angel et al., 1988; Brostjan et al., 1997; Cippitelli et al., 1995; De Cesare et al., 1995; Liang et al., 1996).

### 1.1.3 Regulation of Jun/ Fos and Jun / ATF activity

Activity of AP-1 can be regulated by *de novo* synthesis of *c-fos* gene induced by activation of the Extracellular-Signal-Regulated Kinase (ERK) signaling pathway.

ERK1/2 subgroup of MAPKs is mediating the response of cells to growth factors and hormones. The MAP3K and MAP2K that activate ERK MAP kinase include c-Raf-1 and MEK-1, respectively. Once activated, ERK1/2 translocate to the nucleus and phosphorylate, and thereby potentiate, the transcriptional activity of the ternary complex factor (TCFs) that bind to c-*fos* promoter (Hill et al., 1994). The resulting increased synthesis of c-Fos, leads to the formation of stable dimers with pre-existing Jun proteins, and activation of c-Jun/c-Fos target genes.

In *in vitro* cell culture studies potent ERK activation is achieved upon treatment with 12-*O*-Tetradecanoylphorbol-13-acetate (TPA). TPA is known to induce signaling events mimicking those triggered by activated growth-factor receptors. Members of the Protein Kinase C (PKC) family serve as key meditors of phorbol-ester actions (Nishizuka, 1986). The activation of PKC results in activation of the Raf / MEK1/2 / ERK1/2 cascade (Schonwasser et al., 1998) but the exact mechanism of the cross-talk between PKC and ERK signaling pathways is still controversial.

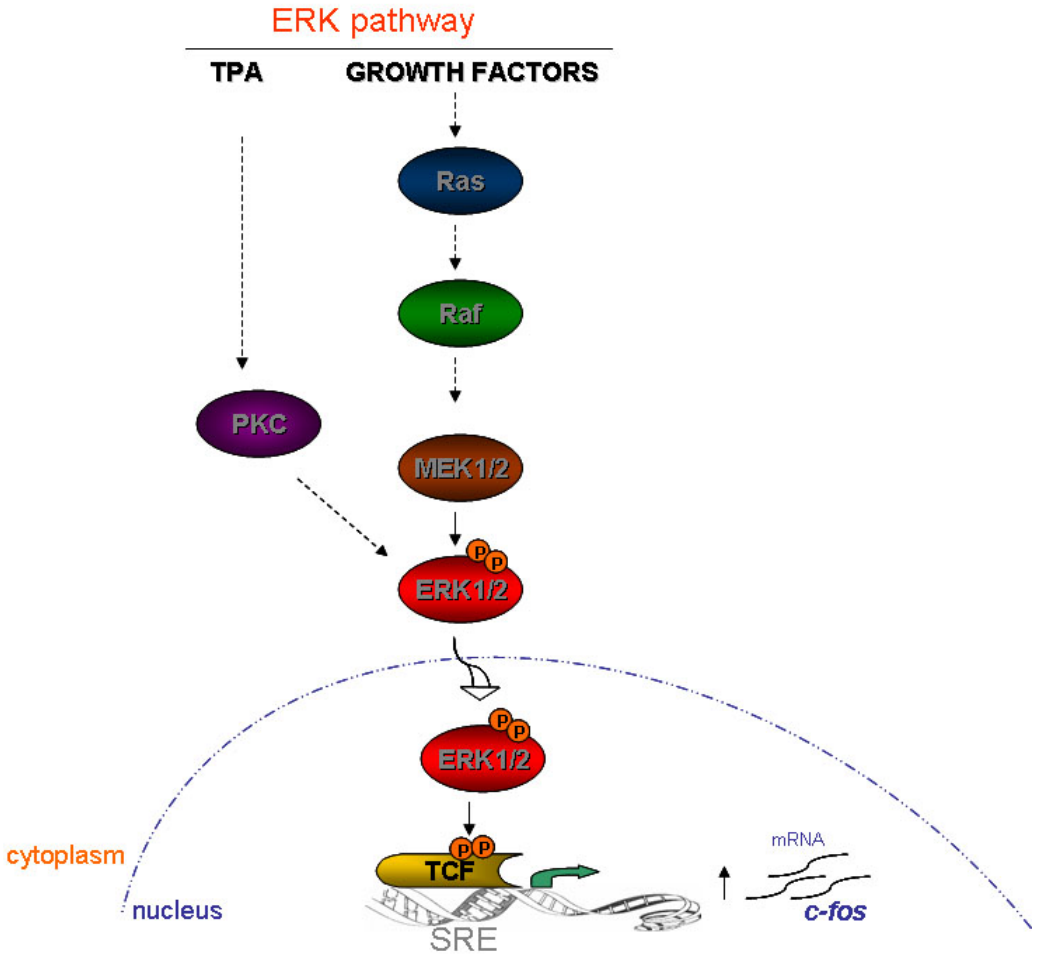


Figure 1.1 Induction of *c-fos* expression through activation of the ERK signaling pathway. Growth factors stimulates activation of Ras. Subsequently, the serine/threonine protein kinase Raf-1 binds to Ras and becomes activated, leading to phosphorylation and activation of the dual-specificity ERK-kinase, MEK. MEK activates the MAPKs ERK1 and ERK2, which once activated translocate to nucleus. In the nucleus, the ERKs phosphorylated the transcription factor TCF, which is bound to the serum response element (SRE) of the *c-fos* promoter. Phosphorylation of TCF at a cluster of sites located next to its carboxyl terminus stimulates its transactivation function which leads to rapid activation of *c-fos* transcription.
TPA activates PKC which through a crosstalk with ERK stimulates the phosphorylation of TCF which regulates the expression of *c-fos*. Adapted from (Edmunds and Mahadevan, 2004).

The activity of c-Jun/ATF2 is upregulated by phosphorylation.

The phosphorylation of c-Jun and ATF2 proteins is mediated by Jun-N-terminal kinase (JNK), a member of Mitogen Activated Protein Kinases (MAPK) which is regulated at multiple levels to ensure the specificity, timing, and strength of action (Davis, 2000; Karin, 1995).

Jun-N-terminal kinase (JNK) is activated by environmental stress, such as UV radiation, heat shock and by proinflammatory cytokines, including interleukin 1 (IL-1) and tumor necrosis factor alpha (TNFα) (Chang and Karin, 2001).
The complexity in the mechanism of JNK activation comes from the involvement of many MAP3 kinases that contribute to stress-activated signaling.

UV–induced DNA damage in the nuclear compartment is an essential component that acts in concert with membrane-anchored proteins to mediate c-Jun phosphorylation by JNK. Upon UV activation of MAPK cascade, JNK can be activated directly by MAP kinase kinases such as MKK4/SEK1 and MKK7. Once activated, JNK translocates to the nucleus (Cavigelli et al., 1995), where it phosphorylates c-Jun and ATF2. c-Jun is phosphorylated by JNK at serines 63 and 73 (Hibi et al., 1993), whereas ATF2 at threonine 69 and 71 (Livingstone et al., 1995). Phosphorylation is increasing the transcriptional activity of promoter bound cJun/ATF2 dimers, and induces the expression of their target genes (van Dam et al., 1995).

Another stimulus strongly activating JNK is treatment with the bacterial compound anisomycin. Anisomycin inhibits translation by binding to the 60S ribosomal subunits, and by blocking peptide bond formation, thereby preventing elongation and causing polysome stabilization (Grollman, 1967). Anisomycin at concentrations below those required for inhibiting translation (sub-inhibitory concentrations) has been shown as an extremely potent activator of kinase cascades in mammalian cells, especially the stress-activated mitogen-activated protein kinase subtypes, JNK (Mahadevan and Edwards, 1991). Anisomycin was proposed to activate MKK7 and MKK4 which stimulate JNK activation (Hazzalin et al., 1998). JNK in turn phosphorylates and activates c-Jun and ATF2which regulate expression of their target genes.

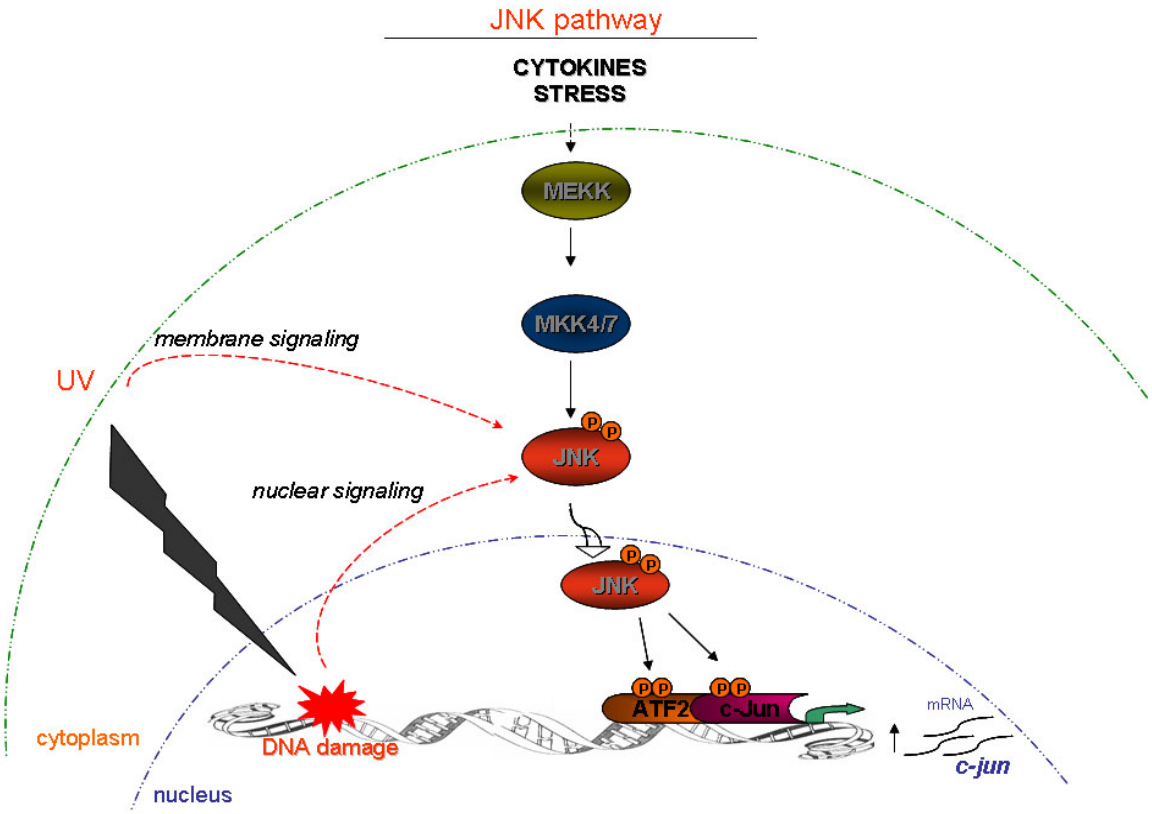


Figure 1.2. Regulation of c-Jun and ATF2 through activation of JNK signaling pathway.
Cytokines or stress stimulate the activation of MEKK1 which activates MKK4/7. Both protein kinases can activate JNK by dual phosphorylation. Activated JNK can phosphorylate AP-1 proteins, like c-Jun and ATF-2. Phosphorylation of AP-1 stimulates its transactivation function which leads to the activation of the transcription of target genes, here on example of *c-jun*..
UV–induced DNA damage in the nuclear compartment and signaling from the membrane-anchored proteins mediate activation of JNK and subsequent phosphorylation of AP-1. Adapted from (Edmunds and Mahadevan, 2004).

AP-1 complexes are active in many biological processes like tissue remodeling, tumorgenesis, inflammation or apoptosis. Phosphorylation-induced transcriptional activity of AP-1 dimers leads to the expression of many genes encoding for transcription factors, adhesion molecules, cytokines. One of the important negative feedback mechanisms, which protects the organism from overexpression of these genes is the activation of another transcription factor, the glucocorticoid receptor (GR).

## 1.2 Glucocorticoid Receptor (GR)

The glucocorticoid hormone regulates many biological processes through the intracellular glucocorticoid receptor (GR). GR, a member of the superfamily of ligand-regulated nuclear receptors (Hollenberg et al., 1985), is a hormone-dependent transcription factor that mediates a variety of biological responses including gluconeogenesis, antiinflammation and antiproliferation (Barnes, 2006; De Bosscher et al., 2003; Necela and Cidlowski, 2004; Newton, 2000).

The glucocorticoid receptor participates in numerous physiological processes required for many facets of cell homeostasis. These physiological actions of glucocorticoids occur in diverse cell types regulating numerous genes. The glucocorticoid receptor regulates the expression of these genes by three basic modes of action: (*1*) binding to glucocorticoid response elements (GREs) in target genes to activate gene transcription, (*2*) inhibition of target gene transcription through direct DNA binding at negative GRE (nGREs), and (*3*) gene regulation by physical interaction with other transcription factors (Necela and Cidlowski, 2004). These properties are reflected in the protein structure of GR: a central DNA binding domain (Luisi et al., 1991), a C-terminal ligand- and transcriptional activation domain (Giguere et al., 1986; Hollenberg and Evans, 1988).

In the absence of hormone, GR resides in the cytoplasm as a multiprotein complex composed of chaperone proteins hsp90 and hsp70, immunophilin p59, and phosphoprotein p23 (Pratt and Toft, 1997). This complex retains GR in the cytoplasm and maintains GR in a favorable conformational state required for high-affinity ligand binding.

Released corticosteroids diffuse readily across cell membranes and bind to GR in the cytoplasm. Binding of hormone to the GR induces the release of hsp90, resulting in a conformational change that unmasks the nuclear localization signal. The receptor then translocates to the nucleus, where it can act through several modes of action, as discussed below.

### 1.2.1 Regulation of Gene Activation by DNA-dependent Mechanisms

#### Activation of Gene Expression

In the nucleus, the activated hormone-bound GR dimerizes and binds in the major groove of the DNA through its central zinc finger DNA-binding domain. The DNA-binding domain recognizes distinct palindromic DNA sequences termed glucocorticoid response elements (GREs), usually located in the promoter of GR-regulated genes like IκB (Auphan et al., 1995; Scheinman et al., 1995), MKP-1 (Kassel et al., 2001; Lasa et al., 2002) or osteocalcin (Morrison and Eisman, 1993) (Figure ). Binding of the GR to the response element in the promoter, results in an allosterically induced conformational change within the receptor (Starr et al., 1996). The allosteric interaction promotes the recruitment of several coactivator complexes critical for the remodeling of chromatin structure. The GR interacts with cAMP response element–binding protein (CREB)–binding protein/p300 and p/CAF, both of which contain intrinsic histone acetylase activity (Deroo and Archer, 2001; Smirnov, 2002). These coactivators acetylate lysine residues in core histones to induce nucleosomal rearrangement and DNA unwinding. Other coactivator complexes such as steroid receptor coactivator-1, p/CIF, SWI/SNF, and GRIP1/TIF2/NcoA-1 contribute to the chromatin-remodeling process (Deroo and Archer, 2001; Jenkins, 2001 ; Smirnov, 2002). Nucleosomal rearrangement leads to promoter accessibility and to the recruitment of the basal transcriptional machinery, including TATA box-binding protein (TBP), TBP-associated factors, and RNA polymerase II. The concerted assembly of these factors results in the stimulation of gene transcription.

#### Inhibition of target gene transcription

Direct transcriptional repression by GCs can be achieved by the interaction of GR with a site on the DNA, designated nGRE for negative GRE, of which the actual sequence is poorly defined (Dostert and Heinzel, 2004). This mechanism of action was proposed to account for repression of the proopiomelanocortin gene (Drouin et al., 1993), prolactin (Sakai et al., 1988).

and proliferin genes (Hoeppner et al., 1995), as well as the vitamin D-induced osteocalcin gene (Meyer et al., 1997). In addition, for some of these genes the mechanism was also found to involve GR-dependent displacement of another factor (for example TATA-binding protein, TBP) (Meyer et al., 1997).

### 1.2.2 Regulation of Gene Activation by DNA-independent Mechanisms

**Transrepression**

Because no nGRE could be detected in the majority of inflammatory genes whose transcription is repressed by GR, transcriptional interference was discovered to mostly result from protein-protein interactions between the GR and other transcription factors such as AP-1: c-Jun/c-Fos (Jonat et al., 1990; Schule et al., 1990a; Yang-Yen et al., 1990), CREB/ATF (Akerblom et al., 1988; Cippitelli et al., 1995), NF-κB (Brostjan et al., 1997; McKay and Cidlowski, 1999; Reichardt et al., 2001), Oct-1 (Kutoh et al., 1992) or GATA-1 (Chang et al., 1993). This mechanism is referred to as trans-repression, as opposed to trans-activation. In contrast to GR activation of transcription, trans-repression by GR is exerted by monomers (Heck et al., 1994).

DNA- binding independent transrepression of transcription factors by the GR is required for survival (Reichardt et al., 1998). $GR^{dim/dim}$ mice, with mutations in the DNA-binding domain known to impair dimerization and DNA binding of GR, are viable in contrast to $GR^{-/-}$ mice (Cole et al., 1995). This reveals the in vivo relevance of DNA-binding-independent activities of the GR.

GR - transcription factors transrepression mechanism can be reciprocal. This AP-1 / GR interaction also results in the repression of GR-mediated gene activation (Reik et al., 1994). A similar mutual antagonism also occurs between glucocorticoid receptor and NF-κB (McKay and Cidlowski, 2000).

Transrepression is mediated through the recruitment of GR to the DNA-bound transcription factors (Kassel et al., 2004; Konig et al., 1992; Nissen and Yamamoto, 2000; Rogatsky et al., 2001). For example, repression of collagenase I, an AP-1 target gene, occurs with promoter-bound AP-1 (Kassel et al., 2004). It does not affect AP-1 binding to DNA (Konig et al., 1992) and is also not mediated by competition for nuclear coactivators (De Bosscher et al., 2001).

Recent findings showed that a LIM-domain protein, nTrip6 is necessary for GR-mediated repression of AP-1 and NF-κB (Kassel et al., 2004). Trip6 (*t*hyroid *r*eceptor-*i*nteracting *p*rotein 6) has been first isolated as a protein of unknown function interacting with thyroid receptor (TR) and retinoid X receptor (RXR) (Lee et al., 1995). Trip6, also known as ZRP-1 (*z*yxin-*r*elated *p*rotein 1), is a member of the zyxin family (Wang et al., 1999; Yi and Beckerle, 1998). Trip6 and other zyxin family members like LPP (lipoma preferred partner), and Ajuba, possess a proline-rich region and nuclear export signals at their N terminus, and three LIM domains at their carboxyl terminus. The LIM domains (named by the initials of three homeodomain proteins, *L*in-11, *I*sl-1, and *M*ec-3) contain two cysteine-rich zinc finger motifs, which are critical for protein-protein interaction (Dawid et al., 1998). Trip6 is localized in the cytosol due to the presence of a nuclear export signal (NES) at the N-terminus (Wang and Gilmore, 2001) and has been implicated in the regulation of actin dynamics and signal transduction involved in cell adhesion and migration (Murthy et al., 1999; Xu et al., 2004; Yi et al., 2002).

A nuclear isoform of Trip6, nTrip6, was identified. nTrip6 lacks NES and is involved in transcriptional regulation. It is a coactivator for AP-1 and NF-κB transcription factors (Kassel et al., 2004). nTrip6 interacts with c-Fos and p65 subunit of NF-κB (RelA) on their target promoters. At the same time nTrip6 is also able to interact with GR, and this interaction is essential for GR to repress AP-1 and NF-κB. Through this interaction nTrpi6 mediates the recruitment of GR to the promoter-bound AP-1 and NF-κB, as a prerequisite for transrepression.

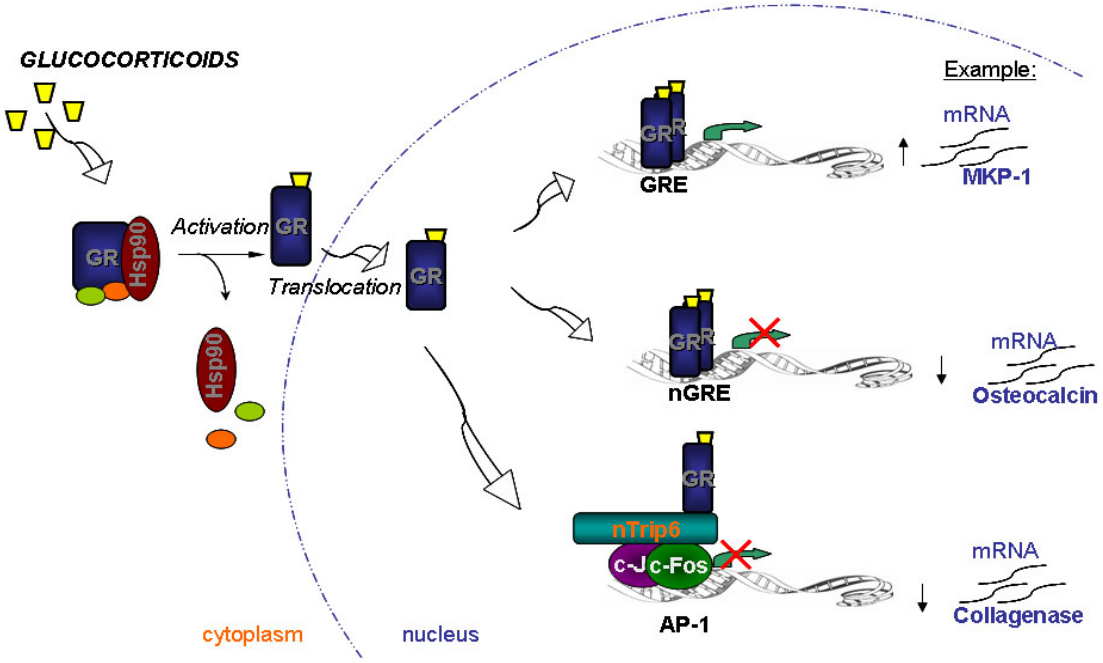


Figure 1.3 Basic mechanisms of glucocorticoid receptor (GR) action.
*Left*: The GR resides in the cytoplasm complexed with several chaperones including hsp90 and immunophilin p59. Upon binding to glucocorticoids, the activated receptor dissociates from the attached accessory proteins and translocates into the nucleus. *Right*: The GR then regulates the expression of genes by several basic modes of action. From *top* to *bottom*: The GR binds as a dimer to glucocorticoid response elements (GREs) in target genes to activate gene transcription; the GR binds to negative GREs (nGREs) and inhibits target gene transcription; the GR physically interacts with AP-1 complex through nTrip6 and inhibits AP-1-mediated gene expression. Adapted from (Necela and Cidlowski, 2004).

### Inhibition of Mitogen-Activated Protein Kinase Signaling

An alternative mechanism by which GCs might exert their antagonistic action on AP-1 is through GR-mediated interference with the signaling pathways that activate AP-1. GR can induce the expression of MAPK phosphatase 1 (MKP-1) (Kassel et al., 2001; Lasa et al., 2002) which in turn inactivate MAP kinases (Camps et al., 2000; Franklin and Kraft, 1997; Kassel et al., 2001) as well as block kinase activities (Caelles et al., 1997; Gonzalez et al., 2000; Swantek et al., 1997). This GC actions lead to the inhibition of phosphorylation, and concomitant activation, of transcriptional activators, such as c-Jun, ATF-2 or Elk-1, which are involved in the induction of AP-1 activity by different mechanisms.

MAPK subgroups such as JNK regulate the activity of the AP-1 complex required for proinflammatory gene expression. Hormone activated GR inhibits the activation of the JNK signaling pathway and prevents the phosphorylation of c-Jun on Ser-63/73. Inhibition of JNK by GR is DNA binding-independent and transcription-independent (Caelles et al., 1997). It also does not block the nuclear translocation of JNK induced by TNFα (Gonzalez et al., 2000). Instead, GR reduces the amount of JNK associated with MKK7 by promoting binding of GR to JNK (Bruna et al., 2003). This inactive GR-JNK complex was proposed to trans-represses promoter-bound AP-1 transcription factor (Bruna et al., 2003).

By interfering with JNK cascade GR might block AP-1 activation and expression of target genes and exert additional anti-inflammatory effects. But so far no data showing the contribution of this mechanism on inhibition of AP-1 transcriptional activity was shown.

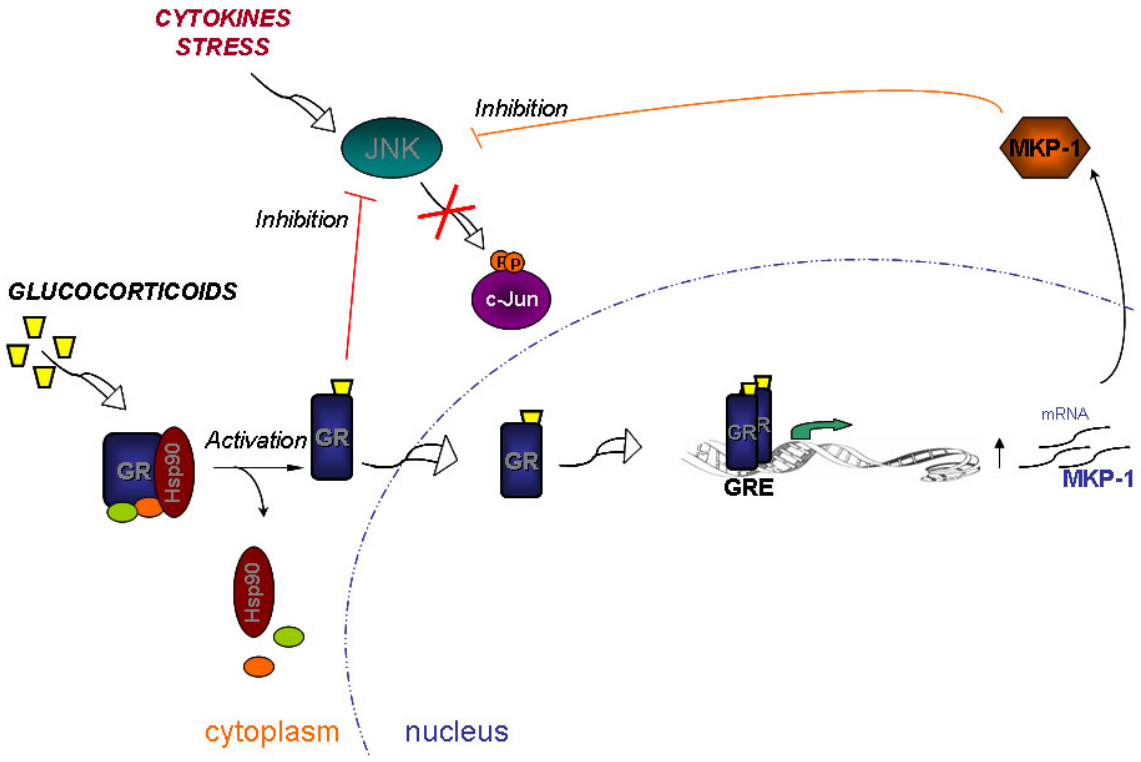


Figure 1.4 Scheme of Glucocorticoid Receptor- mediated inhibition Jun N-terminal Kinase. Cytokine or stress activated JNK phosphorylation of c-Jun can be inhibited by GR mediated induction of MKP-1 or direct inhibition of JNK activity. Adapted from (Necela and Cidlowski, 2004)

## *Aim*

The aims of this study were based on previous findings of the laboratory. Kassel et al. (2004) could show that of nTrip6 interacts with c-Fos and that this interaction is essential for the transcriptional activation by c-Jun/c-Fos.

Preliminary results in the lab suggest that nTrip6 could not interact with c-Jun. This raises the question of the specificity of nTrip6 interaction with different AP-1 dimers. Moreover, nTrip6 is essential for GR to transrepress c-Jun/c-Fos.

My hypothesis was therefore that AP-1 dimers not interacting with nTrip6 might not be transrepressed by GR.

Differences in repression of AP-1 which depend on the composition of the dimer will affect different subsets of target genes regulated by these dimers. My study on GR-medited repression of AP-1 is focusing on the regulation of c-Jun/c-Fos and c-Jun/ATF2 dimers. c-Jun/c-Fos dimer is a “prototypical” AP-1 dimer studies by many groups. Thus, aviability of data on function and regulation of c-Jun/c-Fos allowed comparative studies on ill understood regulation of c-Jun/ATF2 dimer.

# 2. MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Chemicals

All the chemicals were of the highest quality and were purchased from:

| | |
|---|---|
| Agarose | Peqlab, Erlangen, Germany |
| Anisomycin | Merck Bioscience, Nottinhgham, England |
| Bacto-agar | Otto-Nordwald KG, Hamburg, Germany |
| BSA, | PAA Laboratories GmbH, Pasching, Austria |
| DMSO | Fluka, Buchs, Germany |
| Phosphate-buffered saline (PBS) | Gibco-BRL, Karlsruhe, Germany |
| Skimmed milk powder | Saliter, Obergünzburg, Germany |
| Trypsin | Difco, Detroit, USA |

All other chemicals, unless otherwise stated, were purchased from Carl Rotch GmbH GmbH&Co, Karlsruhe, Germany; Merck, Darmstadt, Germany or Sigma, Deisenhofen, Germany.

Radiochemicals:

| | |
|---|---|
| [$\alpha^{32}$P]- dCTP tips | Amersham Pharmacia Biotech, Freiburg, Germany |

### 2.1.2 Kits

| | |
|---|---|
| Easy Pure DNA purification Kit | Biozym Diagnostik GmbH, Oldendorf, Germany |
| Qiagen Plasmid Maxi Kit | Qiagen, Hilden, Germany |
| Zero® Blunt® TOPO PCR kit | Invitrogen, Karlsruhe, Germany |
| Rediprime™ II labeling system | Amersham Pharmacia Biotech, Freiburg, Germany |
| Trizol Reagent | Gibco-BRL |
| QuickHyb hybridization solution | Stratagene |
| Passive Lysis Buffer 5x | Promega, Mannheim, Germany |

### 2.1.3 Bacteria medium

| | |
|---|---|
| LB | 10g/L tryptone |
| | 5 g/L yeast extract |
| | 5 g/L NaCl |
| SOC | 2% tryptone |
| | 0.5% yeast extract |
| | 10mM NaCl |
| | 2.5mM KCl |
| | 10mM $MgSO_4$ |
| | 10mM $MgCl_2$ |
| | 20mM glucose |

### 2.1.4 Buffers and Reagents

| | |
|---|---|
| TAE: | 40mM Tris, pH7.0 |
| | 20mM sodium acetate |
| | 1mM EDTA |
| TBS 10x: | 242g Tris base |
| | 80g NaCl |
| | Total volume 1L, pH 7.6 |
| RIPA buffer: | 30mM Tris-HCl pH7.4 |
| | 150mM NaCl |
| | 1mM EDTA |
| | 0.5% TRITON X-100 |
| | 0.05% Deoxycholate |
| | 10mM NaF |
| Blocking buffer: | 150ml TBS |
| | 150 µl Tween-20 |
| | 7.5g nonfat dry milk |
| SDS sample buffer 1x: | 62.5mM Tris-HCl ph6.8 |
| | 2% w/v SDS |
| | 50mM DTT |
| | 10% v/v glycerol |

| | |
|---|---|
| | 0.01% w/v bromophenol blue |
| Stripping buffers for: | |
| Northern blot: | 0.1x SSC |
| | 0.1% SDS |
| Western blot: | 62.5mM Tris-HCl pH6.8 |
| | 2% v/v SDS |
| | 0.385g DTT |
| | total volume 50ml |
| Glycylglycine buffer: | 25mM Glycylglycine |
| | 15mM $MgSO_4$ |
| | 4mM EGTA |
| | pH 7.8 |
| DNA loading buffer: | 0.5M EDTA |
| | 50% glycerol |
| | 0.01% bromophenol blue |
| MOPS: | 20mM MOPS, pH 7.0 |
| | 5mM Na-acetate pH 4.8 |
| | 1mM EDTA |
| RNA sample buffer: | 17.5% formaldehyde |
| | 50% formamide |
| | 1x MOPS buffer |
| | 0.01% bromophenol blue |
| SSC buffer: | 1.5M NaCl, |
| | 150mMNa-citrate, pH 7.0 |
| Bradford solution: | 100mg Coomassie Brillian Blue G-250 |
| | 50ml ethanol |
| | 100ml 85% phosphoric acid |
| | Total volume 1L |

### 2.1.5 Oligonucleotides

| | |
|---|---|
| **YC cJun_for_Sal1** | 5'-acg cgt cga cca ctg caa aga tgg aaa cg acct tct-3' |
| **YC cJun_rev _Bgl2** | 5'-gaa gat cta caa acg ttt gca act gct gcg taa g-3' |
| **YC ATF2_for_Sal1** | 5'-acg cgt cga cca gtg atg aca aac cct ttc ta-3' |
| **YC ATF2_rev_Bgl2** | 5'-gaa gat cta cac ttc ctg agg gct gtg cct ggg agg-3' |
| **YC cFos_for_Sal1** | 5'-acg cgt cg acct tct cgg gtt tca acg ccg ac-3' |
| **YC cFos_rev_Kpn1** | 5'-ggg gta ccc agg gcc agc agc gtg ggt gag ctc-3' |
| **mATF3-624_for_Kpn1** | 5'-ggg gta ccg ac acct tcc cca cac cac ag-3' |
| **mATF3+35_rev_Xho1** | 5'-ccc tcg agc tgt gca gtg cgc gcc tgg c-3' |

### 2.1.6 DNA probes for Northern blot analysis

| **Name** | **fragment** | **Origin** |
|---|---|---|
| **ATF3 probe** | Xba1-BamH1, 0.6kb | pBSSK |
| **Coll probe** | Hind3-Sal1, 2 kb | pXP |
| **GAPDH probe** | Pst1, 1.3 kb | pGAPDH-13 vector |

### 2.1.7 Plasmids

| **Name** | **Description** |
|---|---|
| **pCGcJun~cFos** | Expression of cJun~cFos fusion under the control on CMV promoter.<br>Described in Bakiri et al., 2002 |
| **pCGcJun~ATF2** | Expression of cJun~ATF2 fusion under the control on CMV promoter.<br>Described in Bakiri et al., 2002 |

| | |
|---|---|
| **pCGcJun~cJun** | Expression of cJun~cJun fusion under the control on CMV promoter.<br>Described in Bakiri et al., 2002 |
| **pcDNA HA GR** | HA tagged Glucocorticoid Receptor cDNA under the control of CMV promoter. |
| **pYC** | Expression of HA-tagged C-terminal part (YC) of yellow fluorescent protein encoding amino acids 155 to 238 under the control of CMV promoter.<br>Described in Hu et al., 2002 |
| **pYN** | Expression of FLAG-tagged N-terminal part (YN) of yellow fluorescent protein encoding amino acids 1-154 under the control of CMV promoter.<br>Described in Hu et al., 2002 |
| **pYN- nTrip6** | Expression of FLAG-tagged C-terminal part (YC) of yellow fluorescent protein in fusion with Trip6 cDNA encoding amino acids from 190 to 476 under the control of CMV promoter. |
| **pYC-cJun~cFos** | Expression of HA-tagged C-terminal part (YC) of yellow fluorescent protein in fusion with cJun~cFos under the control of CMV promoter. |
| **pYC-cJun~ATF2** | Expression of HA-tagged C-terminal part (YC) of yellow fluorescent protein in fusion with cJun~ATF2 under the control of CMV promoter. |
| **pDsRed2-N1** | *Discoma sp*. red fluorescent protein under the control of CMV promoter. |
| **pUbi-GAL4$_{DBD}$cJun** | Expression of GAL4$_{DBD}$ in fusion with c-Jun sequence encoding amino acids from 1 to256 under the control of human ubiquitin C promoter.<br>Described in Weiss et al., 2003 |

| | |
|---|---|
| **pRSV-GAL4$_{DBD}$cFos** | Expression of GAL4$_{DBD}$ in fusion with c-Fos under the control of RSV promoter. |
| **pRSV-GAL4$_{DBD}$ATF2** | Expression of GAL4$_{DBD}$ in fusion with ATF2 under the control of RSV promoter. |
| **uPA -Luc** | Luciferase gene under the control of Urokinase Plasminogen Activator (uPA) enhancer element -1993 to -1870 (relative to the transcription start site). Described in Cirillo et al., 1995. |
| **-624+35 ATF3-Luc** | Luciferase gene under the control of ATF3 promoter nucleotides -624 to +35 (relative to the transcription start site). |
| **Ubi-*Renilla*** | *Renilla reniformis* cDNA under the control of ubiquitin promoter. Described in Kassel et al., 2004. |
| **ΔMEKK1** | Expression of ΔMEKK1 under control of CMV promoter Described in Weiss et al., 2003 |

### 2.1.7.1 Construction of -624+35ATF3-Luc

To generate these minimized ATF3 promoter reporter constructs ATF3-Luc reporter was used as a template for PCR reactions. mATF3-624_for_Kpn1 and mATF3+35_rev_Xho1 primer pair was used to generate of -624+35ATF3-Luc. PCR reaction was performed as described in section 2.2.7. After PCR amplification product was subjected to agarose gel electrophoresis. PCR product was purified from the gel as described in section 2.2.9. Purified PCR products were subcloned into pCR-Blunt II-TOPO vector and amplified in E. coli DH5ά. Colonies were inoculated for mini-prep isolation. Maxi-preparation of positive clones was performed. Insert was released from pCR-Blunt II-TOPO using Kpn1 and Xho1 restriction enzymes. Insert was ligated into Kpn1 and Xho1 digested vector caring Luciferase gene. DNA from positive clones was analyzed by endonuclease digest and sequencing.

### 2.1.7.2 Construction of fusion proteins for the Bimolecular Fluorescence Complementation (BiFC) assay.

To create fusions with C-terminal part (YC) of yellow fluorescent protein (YFP) with AP-1 pseudodimers: cJun~cFos and cJun~ATF2, pCG vectors carrying these inserts were used as templates for amplification of cJun~cFos and cJun~ATF2 fragments.

pCGcJun~cFos was used to amplify cJun~cFos insert using YC cJun_for_Sal1 and YC cFos_rev_Kpn1 primer pair.

pCGcJun~ATF2 was used to amplify cJun~ATF2 inset using YC cJun_for_Sal1 and YC ATF2_rev_Bgl2 primer pair.

PCR reaction was performed as described in section 2.2.7. After PCR amplification products were subjected to agarose gel electrophoresis. PCR products were purified from the gel as described in section 2.2.9. Purified PCR products were subcloned into pCR-Blunt II-TOPO vector and amplified in E. coli DH5ά. Colonies were inoculated for mini-prep isolation. Maxi-preparation of positive clones was performed. Insert was released from pCR-Blunt II-TOPO using appropriate restriction endonucleases and inserts were ligated into Sal1 and Bgl2 or Sal 1 and Kpn1 sites of pYC vector carring sequence encoding the C-terminal fragment (YC) of YFP: amino acids 155-238. DNA from positive clones was analyzed by endonuclease digest and sequencing.

### 2.1.8 Antibodies

**Primary antibodies:**

| Name | Source | Supplier |
|---|---|---|
| **ATF2** | | |
| > ATF2 (N-96) | rabbit | Santa Cruz, Santa Cruz, USA; |
| Dilution 10:1000 | | sc-6233 |
| **ERK** | | |
| > ERK1 (K-23) | rabbit | Santa Cruz, Santa Cruz, USA; |
| Dilution 1:1000 | | sc-94 |

| | | |
|---|---|---|
| **Fos** | | |
| > c-Fos | rabbit | Upstate, New York, USA; |
| Dilution 1:1000 | | #06-341 |
| **c-Jun** | | |
| > phospho-c-Jun II | rabbit | Cell Signaling; |
| Dilution 10:1000 | | #9261 (Ser63) |
| > c-Jun | mouse | BD Biosciences, Heidelberg, Germany; |
| Dilution 1:1000 | | #558036 |
| **JNK** | | |
| > phosphor-SAPK/JNK | rabbit | Cell Signaling, #9251 |
| Dilution 10:1000 | | (Thr183/Tyr185) |
| > JNK1 (C-17) | rabbit | Santa Cruz, Santa Cruz, USA; |
| Dilution 1:1000 | | sc-474 |
| **Tubulin** | | |
| > Tubulin (TU-02) | mouse | Santa Cruz, Santa Cruz, USA; |
| Dilution 1:3000 | | sc-8035 |
| **Anti-Flag M2** | mouse | Stratagene, La Jolla, USA |
| Dilution 1:200 | | |
| **Anti-HA** | rat | Roche, Indianapolis, USA |
| Dilution 1:100 | | |

**Secondary antibodies:**

| **Name** | **Supplier** |
|---|---|
| HRP-conjugated Anti-mouse: | DacoCytomation GmbH, Hamburg. Germany |

| | |
|---|---|
| HRP-conjugated Anti-rabbit: | DacoCytomation GmbH, Hamburg. Germany |
| HRP-conjugated Anti-rat | DacoCytomation GmbH, Hamburg. Germany |
| Alexa fluor 488 Anti mouse | Invitrogen |
| Alexa fluor 546 Anti rat | Invitrogen |

### 2.1.9 Enzymes

All restriction endonucleases and other modifying enzymes were purchased from Promega (Mannheim, Germany) or New England Biolabs (Beverly, USA) unless otherwise stated.

### 2.1.10 Bacteria

*E.coli* DH5α: *sup*E44Δ*lac*U169(φ80*lac*ZΔM15)*hsd*R17*rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1

### 2.1.11 Cell lines and media

All media and other reagents for cell culture were purchased from Invitrogen GmbH (Karlsruhe, Germany). FCS was purchased from PAA laboratories GmbH (Linz, Austria). Trypsin was purchased from Difco Laboratories.

| **Name** | **Origin** | **Source** | **Medium** |
|---|---|---|---|
| **MEF** | immortalized mouse embryonic fibroblasts | Dr. P. Angel Heidelberg Germany | DMEM, 10%FCS |
| ***c-jun*$^{-/-}$MEF** | immortalized MEF isolated from *c-jun* knock-out mice | Dr. P. Angel Heidelberg Germany | DMEM, 10%FCS |

| | | | |
|---|---|---|---|
| **Hela** | human cervical carcinoma cells | M. Litfin | DMEM, 10%DBS 2mM L-glutamine |
| **Cos-7** | monkey kidney cells | M. Litfin | DMEM, 10%FCS |
| **HEK 293** | human embryonic kidney cells | C. Weiss | DMEM, 10%FCS |

> Stably transfected with GAL4 responsive luciferase gene

### 2.1.12 Other materials

| | |
|---|---|
| ECL Hyperfilm | Amersham, Freiburg, Germany |
| MP Hyperfilm | Amersham, Freiburg, Germany |
| Filter paper 3MM | Bender&Hobein, Karlsruhe, Germany |
| HybondN+ membrane | Amersham, Freiburg, Germany |
| Immobilon-P (PVDF membrane) | Millipore; Bedford, USA |
| Nick columns | Amersham Pharmacia, Uppsala, Sweden |
| Chambered coverglass | Nunc, Rochester, USA |
| Mounting medium, Immu-mount | Shandon, Pittsburgh, USA |

## 2.2 METHODS

The majority of protocols and recipes for commonly used buffers in this study were taken from the laboratory manual of Sambrook *et al*. (1989) unless otherwise stated.

### 2.2.1 Preparation of chemically competent *E. coli*

A single colony of *E. coli* DH5ά was incubated in 5ml LB medium overnight at $37^0$C with shaking (200 rpm). Than 4 ml was used to inoculate 400 ml of fresh LB medium and allowed to grow to an $OD_{590}$ of 0.4. After chilling of ice for 10 min the cells were centrifuged at 3600g for 10 min at $4^0$C. The pellet was re-suspended in 20 ml of ice cold 0.1M $CaCl_2$ and allowed to stand on ice for 10 min. Then bacteria was centrifuged once more and again re-suspended in CaCl2. This procedure was repeated once more. Finally the pellet was re-suspended in 2 ml of ice-cold $CaCl_2$ with 10% glycerol. After 5 min incubation on ice, the bacteria were aliquoted and frozen down at -$80^0$C.

### 2.2.2 Transformation of *E. coli*

Chemical transformation was used for propagation of plasmids and DNA ligation products. 1µg of plasmids of 2-7µl of a ligation mix was added to 100µl ice-thawed chemically competent *E.coli*. After mixing and incubation on ice for 15 min bacteria were heat-shocked at $42^0$C for 30 sec and incubated on ice for another 2 min. The transformed bacteria were mixed with 1ml of SOC medium and incubated at $37^0$C with shaking for 1h. Finally the bacteria were plated onto the LB agar plates supplemented with appropriate antibiotics and allowed to grow for 16-24h at $37^0$C.

### 2.2.3 Plasmid DNA preparation

Plasmid DNA was prepared on a large or small scale using QiagenPlasmidMaxi Kit according to manufacturer's instructions.

### 2.2.4 Restriction endonuclease digestion of DNA

DNA was digested in the buffer recommended by the supplier and 2-3 units of a restriction enzyme were used per µg of DNA. Reaction mix was incubated at $37^0$C for 4h. The quality of digest was controlled by agarose gel electrophoresis.

### 2.2.5 DNA ligation

Ligation reaction was performed using T4DNA ligase according to manufacturer's instructions.

### 2.2.6 Subcloning into pCR-Blunt II-TOPO vector

PCR products were cloned using Zero® Blunt® TOPO PCR kit according to manufacturer's instructions.

### 2.2.7 Polymerase chain reaction (PCR)

All PCR reactions were performed in a total volume of 50µl containing:

100ng of DNA template
250µM dNTP's mix
250ng of primers
3U of polymerase
1x polymerase buffer

The reactions were carried out in a PCR thermocycler (Perkin Elmer, Norwalk, USA) using following parameters:

$95^0$C 5 min 1 cycle
$95^0$C 30 sec
$55^0$C-$75^0$C 1-4 min } 25-35 cycles
$72^0$C 2-5 min
$72^0$C 5 min 1 cycle

Analysis of the PCR products was performed by a gel electrophoresis.

### 2.2.8 Agarose gel electrophoresis

1% to 1.5% agarose was boiled in TAE buffer. Ethidium bromide was added to a final concentration of 0.3µg/ml and the solution was poured into a horizontal electrophoresis chamber, which was fitted with a comb. The comb was removed from the solidified gel and the DNA mixed with loading buffer wad loaded into the wells left by the comb. Elecrophoresis was carried out at 50-130V at room temperature. DNA was visualized by translumination with 320nm UV light and photographed with an Eagle Eye photo camera system (Stratagene).

### 2.2.9 Purification of DNA from agarose gel

DNA fragment was isolated and purified from agarose gel using Easy Pure Purification Kit according to manufacturer's instructions.

### 2.2.10 Preparation of total RNA

Total RNA was prepared from HeLa cells. $10^6$ cells were seeded in 10cm cell culture dishes and starved for 42h in 0.5% DCS DMEM. Medium was collected and cells were washed 2x in PBS and UVC-irradiated (10J/m2) or treated with Anisomycin (50 ng/ml), TPA (80ng/ml), Dexamethasone ($10^{-6}$M). Collected medium was poured onto plates and cells were incubated for additional 4h. Cells were harvested in 1ml of Trizol solution. Isolation of RNA was performed according to manufacturer's instructions.

### 2.2.11 Radioactive labeling of DNA probes for Northern Hybridization

Radioactive labeling of DNA probes was performed with a Ready-Prime kit and purified with Nick columns according to manufacturer's instructions.

### 2.2.12 Northern Blot analysis

1% agarose was boiled in 130ml of water. The solution was cooled down to 37C and 15ml of 10x MOPS buffer was added, together with formaldehyde to final concentration of 6% was added and 3ul of ethidium bromide. The agarose solution was poured into a horizontal electrophoresis

chamber, fitted with a comb and allowed to solidify. 10µg of total RNA was mixed with an equal volume of RNA sample buffer, denatured at $65^0$C and loaded onto the gel. Electrophoresis was preformed in 1x MOPS buffer at 60V until bromophenol blue front had moved for 6cm.

The RNA was blotted overnight onto Hybond N+membrane in 10x SSC buffer and cross-linked by UV-irradiation. The membrane was pre-hybridized for 20 minutes at $68^0$C in QuickHyb buffer. Hybridization was performed for 1h at $68^0$C in the presence of radio labeled probe. Membrane was washed 2x 15 minutes at room temperature in 2xSSC buffer in presence of 0.1% SDS, and 1x 30 minutes at $65^0$C in 0.1 SCC buffer in presence of 0.1% SDS. Signal was detected using MP Hyperfilm and a phosphoimager (FLA-3000, Fuji). Quantification of the density of the bands was performed using Aida 2.11 software (Raytest, Straubenhardt, Germany).

### 2.2.13 Stripping Northern blots

To allow re-use of the Northern blots, radioactively labeled DNA probe was stripped by incubation the membranes in a stripping buffer at $95^0$C for 30 minutes. The membranes were than used for re-probing from the pre-hybridization step.

### 2.2.14 Whole cell extracts for Western blot analysis

Cells were either lysed directly in the SDS-PAGE sample buffer before sonication to break down the chromosomal DNA, or lysed in RIPA buffer and the resulting cell extracts was mixed with equal volume of 2x SDS-PAGE sample buffer. For both preparations samples were boiled for 5 min before loading on the SDS-PAGE gel.

### 2.2.15 Determination of protein concentration

Protein concentration was determined according to Bradford.

2 µl of sample was added to 1ml of Bradford and measured in ELISA plate reader at 600nm. For the calibration curve 2, 4, 8 µl of 1mg/ml BSA was added to Bradford reagent and measured in parallel.

### 2.2.16 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were elctrophoretically separated on the basis of the size using the method from Laemmli (1970). The resolving gels containing between 8-12% acryl amide depending on experiment and 5% stacking gel were cast according to Sambrook et al. (1989).Samples were run into stacking gel at 60-80V and than run at 100-120V in the separating gel with a mini gel system (Hoefer, San Francisco, USA).

### 2.2.17 Western blot analysis

Proteins in SDS-PAGE gels were transferred onto methanol soaked Immobilon-P membranes in a semi-dry blotter (H.Hötzel, Wörth/Hörlkofen, Germany) at current of 1mA/cm$^2$ for 1h. After the transfer membranes were incubated in blocking buffer at room temperature for 1h to reduce unspecific binding. Primary antibodies were diluted in TBST-BSA buffer at concentration recommended by the supplier (generally 1:500-1:3000). Membranes were incubated in the primary antibody-containing buffer for 1-2h at room temperature or overnight at 4$^0$C. The membranes were then washed three times with TBST buffer for 5 minutes each. An appropriate secondary HRP-conjugated antibody was added to a blocking buffer and membranes were incubated for additional 1h at room temperature. Membranes were washed again with TBST buffer for three times. Detection of specific proteins signals was achieved using enhanced chemiluminescence ECL Western blotting detection reagents and ECL Hyperfilm following manufacturer's instructions.

### 2.2.18 Stripping Western blot membrane

Stripping of the membranes was performed to utilize Western blots more than a single use. The membranes were incubated in a stripping

solution at $50^0$C for 30 minutes with gentle agitation. The membranes were then washed twice with TBST buffer for 5 minutes each time, and blocked in blocking buffer once again.

### 2.2.19 Cell culture

All cell lines were maintained at 37°C in Steri-Cult 200 incubator (Forma Scientific, Marietta, USA) in 5% $CO_2$ and 95% humidity. Adherent cells were grown till they reached 80-90% confluency. For trypsinization, the medium was aspirated and cells were washed once with PBS. Cells were incubated with trypsin until they detached from cell culture dish than was diluted with fresh culture medium and the cell suspension was transferred into a centrifugation tube. Cells were collected by centrifugation for 2minutes at 1200 rpm and re-plated.

### 2.2.20 Freezing and thawing of cells

For freezing, logarithmically growing cells were trypsinized as described previously and collected by centrifugation. Cells were resuspended in freezing medium (DMEM, 10%FSC, 10%DMSO) and transferred into cryotubes. After incubation on ice for 30 minutes, cells were stored at -80°C.
For re-propagation cells were thawed quickly at 37°C and transferred to fresh medium. The next day medium was replaced with fresh cell culture medium.

### 2.2.21 Transfection of cells

Cells were transfected with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) or FuGene6 (Roche Diagnostics) according to manufacturer instructions.

### 2.2.22 Treatment of cell lines

UV irradiation: the medium was collected; cells were washed with PBS -Ca/-Mg buffer and irradiated with 40J/$m^2$ UV-C light; collected medium was poured back on cells.

Dexamethasone (DEX): after starvation of cells DEX ($10^{-6}$M) was added to the medium. Stock of DEX ($10^{-2}$M) is stores at -20°C in EtOH.

Phorbol ester (TPA): after starvation TPA was added to medium to final concentration 50ng/ml. Stock of TPA (200µg/ml) is stored at -20°C.

Anisomycin: after starvation anisomycin was added to the medium to the final concentration of 50 ng/ml. Stock of anisomycin (100 mg/ml) is stored as DMSO solution at -20°C.

### 2.2.23 Luciferase assay

Cells were transfected with Lipofectamine 2000 or Fugene6 reagent in ratio 3µl of transfection reagent to 1µg of DNA. 100ng of plasmid DNA and 100 ng of reporter DNA were used for transfection. After transfection cells were incubated for 20h before starvation in 0.5%FCS containing DMEM medium. The cells were starved for 20h and then cells were not induced or induced with TPA (50ng/ml), Dexamethasone ($10^{-6}$M) or UVC (10-40J/$m^2$). Cells were incubated for additional 6-24h after treatment, than washed with 1ml ice cold PBS –Ca/-Mg and lysed on ice in 100µl of 1x lysis buffer (Promega). 20µl of cell lysate was used to measure Firefly luciferase activity. Firefly luciferase activities were normalized to *Renilla* luciferase activity (Ubi-*Renilla*). Measurements were performed on Luminescence Counter (Perkin Elmer).

### 2.2.24 Bimolecular Fluorescence Complementation (BiFC) Analysis

BiFC analysis has been developed for visualization of protein interactions in living cells. This approach is based on the complementation between two fragments of a fluorescent protein when they are brought together by interaction between proteins fused to the fragments (Figure 2.1.). It enables visualization of the subcellular locations of protein interactions in the normal cellular environment.

The advantage of the BiFC approach over other complementation methods is that the reconstituted fluorescent protein has strong intrinsic fluorescence. This allows direct visualization of the protein complex.

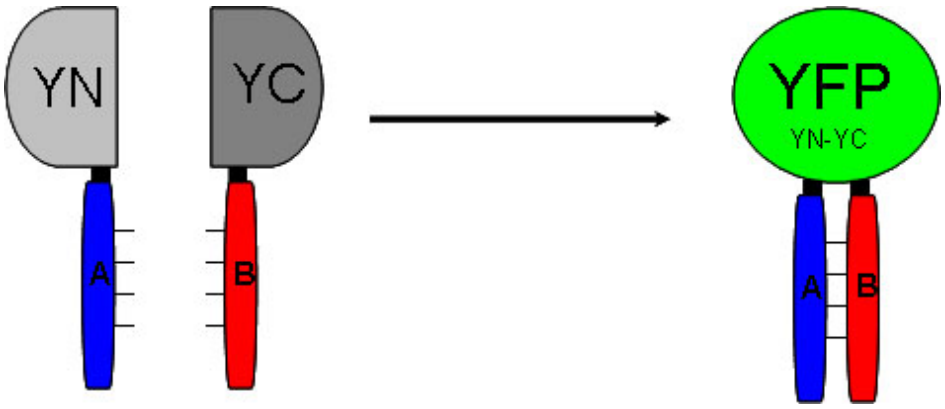


Figure 2.1. Schematic principle of the Bimolecular Fluorescence Complementation (BiFC) assay. Two non-fluorescent fragments (YN and YC) of the yellow fluorescent protein (YFP) are fused to putative interaction partners (A and B). The association of the interaction partners allows formation of the bimolecular fluorescent complex.

The complex can therefore be visualized without exogenous fluorogenic or chromogenic agents, resulting in minimal perturbation of cells. Using BiFC approach, living cells can be observed over a long periods and the possibility of experimental manipulations altering results is minimized.

One limitation of the BiFC method is the time required for fluorophore maturation. This prevents real-time detection of rapid changes in interactions. In addition, bimolecular fluorescent complex formation is irreversible *in vitro*. Despite these limitations, the BiFC assay was used to study interactions among a variety of structurally diverse proteins in many different cell types (Atmakuri et al., 2003; Fang and Kerppola, 2004; Hu et al., 2002). Thus, the BiFC assay is generally applicable for the visualization of a variety of protein complexes in living cells.

*Design of fusion proteins.*

Fragments of yellow fluorescent protein (YFP) truncated at residue 155 give: N-terminal residues 1-154 (YN fragment) and C-terminal residues 155-238 (YC fragment). Proteins of interest are fused to the N- and C- terminal fragments of the YFP (Figure 3.2.).

In order to determine whether any fluorescence that is observed reflects a specific protein interaction and not spontaneous re-association of two halves

of YFP, it is necessary to include negative controls in the experiment. This is essential since the fluorescent protein fragments are able to form fluorescent complexes with low efficiency, even in the absence of a specific interaction. This non-specific complementation is generally reduced when the fragments are fused to proteins that do not interact with each other.

**Live imaging of interacting proteins**

For live imaging of protein complementation cos7 cells were transfected in chambered slides using FuGene6 transfection reagent with appropriate DNA in ratio 3µl Fugene6 to 1µg DNA. FuGene6 reagent was used to introduce DNA into cells because it has low background fluorescence and requires minimal manipulation of cells. 40-80ng of plasmid DNA were user for transfection. Cells were co-transfected with pDsRed expressing vector. This construct is expressing red fluorescent protein derived from *Discoma sp*. and was used to visualize cells that were successfully transfected. 20h post-transfection cells were incubated at $30^0$C for additional 2h to allow fluorophore maturation. Then cells were washed two times with 500µl PBS -Ca/-Mg. For visualization of nuclei, cells were counterstained with Draq5 (dilution 1:1000) in 200µl PBS -Ca/-Mg buffer for 10min. The cells were washed and 300µl of fresh PBS-Ca/-Mg was added to cells and complementation of fusion proteins was observed under inverted confocal laser scanning microscope (Zeiss).

**Immunocytochemistry**

To check the expression and the localization of proteins using antibodies staining procedure, cos7 cells were cultured on coverslips in 6 well cell culture dish. The cells were transfected using FuGene6 reagent with appropriate DNA in ratio 3µl Fugene to 1µg DNA. 20h post-transfection glass coverslips were transferred in 24well cell culture dish and washed 2 times in PBS -Ca/-Mg at room temperature. 10% formalin solution in PBS -Ca/-Mg was poured onto cells. Cells were fixed at room temperature for 10 min. The cells were washed 3 times in 500 µl PBS –Ca/-Mg and permeabilized in 0.1% TritoX-100 solution in PBS -Ca/-Mg for 5 min at room temperature. The cells were washed 3 times in PBS -Ca/-Mg. Than 1%BSA in PBS -Ca/-Mg blocking solution was added onto cells.

The cells were incubated in blocking solution for 30 min at room temperature with slow shaking. Then slides were incubated for 1h with 1%BSA in PBS –Ca/-Mg solution containing primary antibody in 1:100 dilution for anti-HA antibody and 1-.200 dilution for anti-FLAG antibody. The cells were washed 3 times in PBS -Ca/-Mg. Then 1%BSA in PBS -Ca/-Mg solution containing secondary antibody and Draq5 for counterstaining of nuclei in dilution 1:1000 was added onto cells. 1:1000 dilutions of Alexa fluor 546 anti-rat and of Alexa fluor 488 anti-mouse secondary antibodies were used. The cells were incubated with secondary antibody solution for 30 min. The cells were washed 3 times in PBS -Ca/-Mg; slides were mounted on object glass in mounting medium Immu-mount and dried in horizontal position over night at room temperature in darkness. Localization and expression of proteins was observed under inverted confocal laser scanning microscope (Zeiss).

**Confocal laser scanning microscopy**.

Slides were observed under inverted confocal laser microscope (LSM510; Carl Zeiss). An argon-krypton laser produced excitation bands at 495 nm for Alexa 488, 556nm for Alexa546, 556 nm for DsRed and 543nm for Draq5. Images of cells were acquired with Zeiss microscope equipped with a 60× oil immersion phase-contrast objective, with emission filters 510 to 550 nm for Alexa488 and 585 to 610 nm for Alexa546 and DsRed. Digital images were transferred to a computer equipped with Photoshop (Adobe Systems).

## 3. RESULTS

The abundance and composition of AP-1 transcription factor vary during the cell cycle and is cell type dependent. To restrict the analysis to the repression of transcriptional function of a specific AP-1 dimer by GR I used AP-1 pseudodimers as a tool. These AP-1 molecules were developed in M. Yaniv group and were used to study promoter specificity and biological activity of different dimers (Bakiri et al. 2002). AP-1 pseudodimers were constructed by fusing coding regions of AP-1 family members in frame via a synthetic oligonucleotide which encodes a glycine-rich polypeptide linker of 18 amino acids (Fig. 3.1.). The glycine residues maximize the flexibility, and the interspersed serine residues increase the hydrophilicity.


Figure 3.1. AP-1 pseudodimer design, example of c-Jun~cFos.
The sequence encoding c-Jun and c-Fos are connected in frame via a flexible linker.
b: basic DNA binding domain; ZIP: leucine zipper. A FLAG epitope tag was inserted at the C-terminus of constructs. Adapted from Bakiri *et al*. 2002.

### 3.1. Interaction between AP-1 pseudodimers and Trip6

Interaction of c-Fos with nTrip6 is necessary for the repression of c-Jun/c-Fos by GR (Kassel et al., 2004). I wanted to study another AP-1 dimer, c-Jun/ATF2 also interacts with nTrip6. This interaction would be a prerequisite for GR-mediated repression of this AP-1 dimer.

I therefore evaluated the interaction between nTrip6 and AP-1 dimers *in vivo* using the Bimolecular Fluorescence Complementation (BiFC) method. BiFC assay is based on the reconstitution of yellow fluorescent protein (YFP) by fusion of its halves to interacting partner proteins (Fig. 3.2.) (Hu et al., 2002).

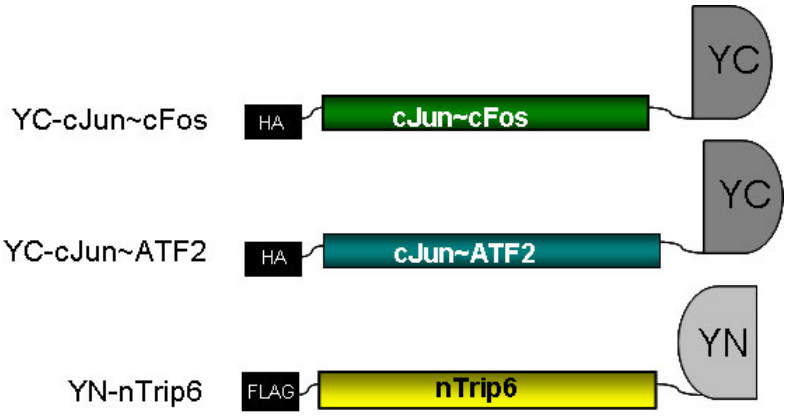


Figure 3.2. Schematic structure of YC-cJun~cFos, YC-cJun~ATF2 and YN-nTrip6. The sequences encoding cJun~cFos and cJun~ATF2 were fused in frame with the C-terminal half of yellow fluorescent protein (YC). A HA epitope tag was inserted at the N-terminus of cJun. The sequence encoding nTrip6 was fused in frame with the N-terminal half of yellow fluorescent protein (YN). A FLAG epitope tag was inserted at the C-terminus of nTrip6.

*Cos7* cells were chosen for these experiments. The presence of a big nucleus and transfection efficiency in these cells facilitate BiFC assay. Cells were additionally co-transfected with dsRed expressing vector that serves as a transfection control. Only cells expressing a dsRed protein were acquired for images. Confocal microscopy of living cells was performed 22h post-transfection.

Expression and localization of the fusion proteins was analyzed by immunocytochemistry. Cos7 cell were transfected with vectors expressing fusion proteins: YN-nTrip6, cJun~cFos-YC and cJun~ATF2-YC in 6 well plate on glass slide. 20h post-transfection cells were harvested, fixed and immunobloted.

Probing with an anti-FLAG antibody showed expression and a nuclear localization of nTrip6, as expected (Fig. 3.3, top panel). Expression of cJun~cFos-YC and cJun~ATF2-YC was detected after probing with anti-HA antibody. Proteins showed a nuclear localization (Fig. 3.3, middle and bottom panel). This confirmed that the fusion protein maintained their natural nuclear distribution.

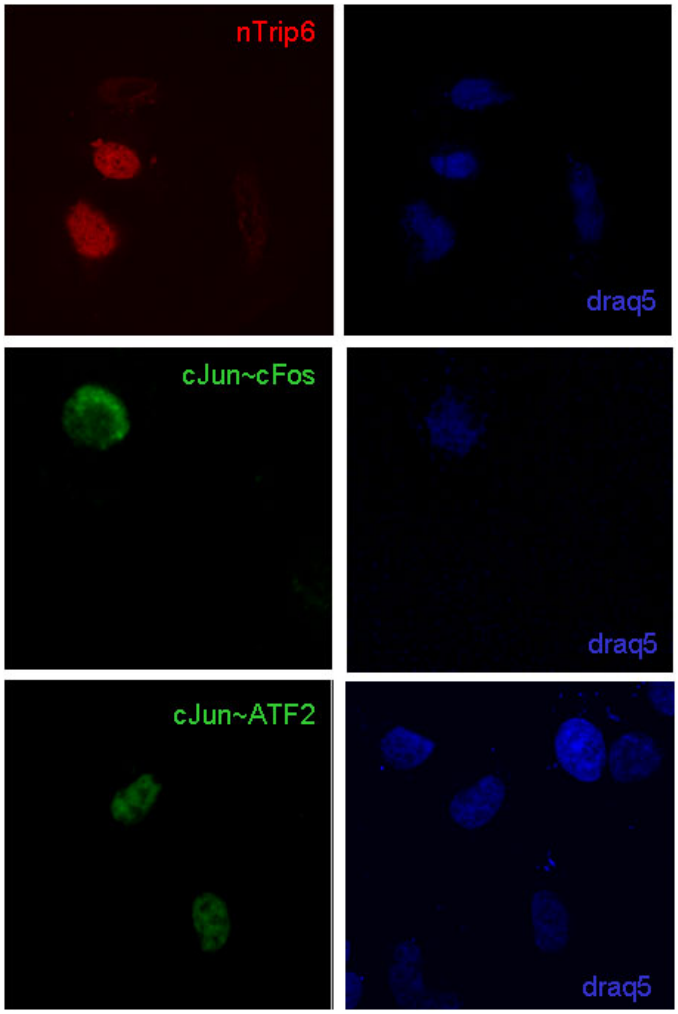


Figure 3.3. Expression of YC- and YN-fusion proteins.
Cos7 cells were transfected with cJun~cFos-YC, cJun~ATF2-YC and YN-nTrip6 expression vectors. 24h post-transfection cells were harvested. Expression of fusion proteins was analyzed by immunobloting with anti-FLAG antibody to visualize YN-nTrip6 fusion and anti-HA antibody to visualize cJun~cFos-YC and cJun~ATF2-YC fusions. Fluorescent images were acquired by confocal microscopy using filters selective for Alexa Fluor 488 (green) or 567 (red). DNA was stained with Draq5 (right panel).

It is possible that two parts of a fluorescent protein will spontaneously reassociate giving unspecific fluorescence. To check the specificity of the complementation, a control experiment was performed. Cos7 cells were transfected with control vectors expressing only YC or YN together with vectors expressing YN-nTrip6, cJun~cFos-YC or cJun~ATF2-YC. Cells expressing YC and YN-nTrip6 did not show fluorescence. This suggests that

two parts of YFP did not reassemble spontaneously. The same was observed in cells expressing YN together with cJun~cFos-YC or cJun~ATF2-YC (Fig.3.4 A, middle and bottom panel). dsRes expression shows that cells were successfully transfected. This result shows that in these experimental conditions no spontaneous re-association of the two parts of YFP fluorescent protein takes place.

In cells transfected with YN-nTrip6 together with cJun~cFos-YC complementation fluorescence was observed (Fig. 3.4.). Fluorescence was, as predicted, localized in the nucleus, where the interaction between nTrip6 and AP-1 is expected to occur. This confirms that nTrip6 interacts with c-Fos, as previously observed (Kassel et al., 2004). Cells co-transfected with YN-nTrip6 and cJun~ATF2-YC did not show fluorescence (Fig. 3.4.). dsRed expression shows that cells were transfected but still no interaction between nTrip6 and cJun~ATF2 was detected.

This result is in agreement with *in vitro* interaction data. In pull down experiments nTrip6 and c-Fos interaction was detected (Heilbock, unpublished data; Kassel et al., 2004) whereas no interaction in between nTrip6 and Jun of ATF family members was detected using this method (Heilbock, unpublished results).

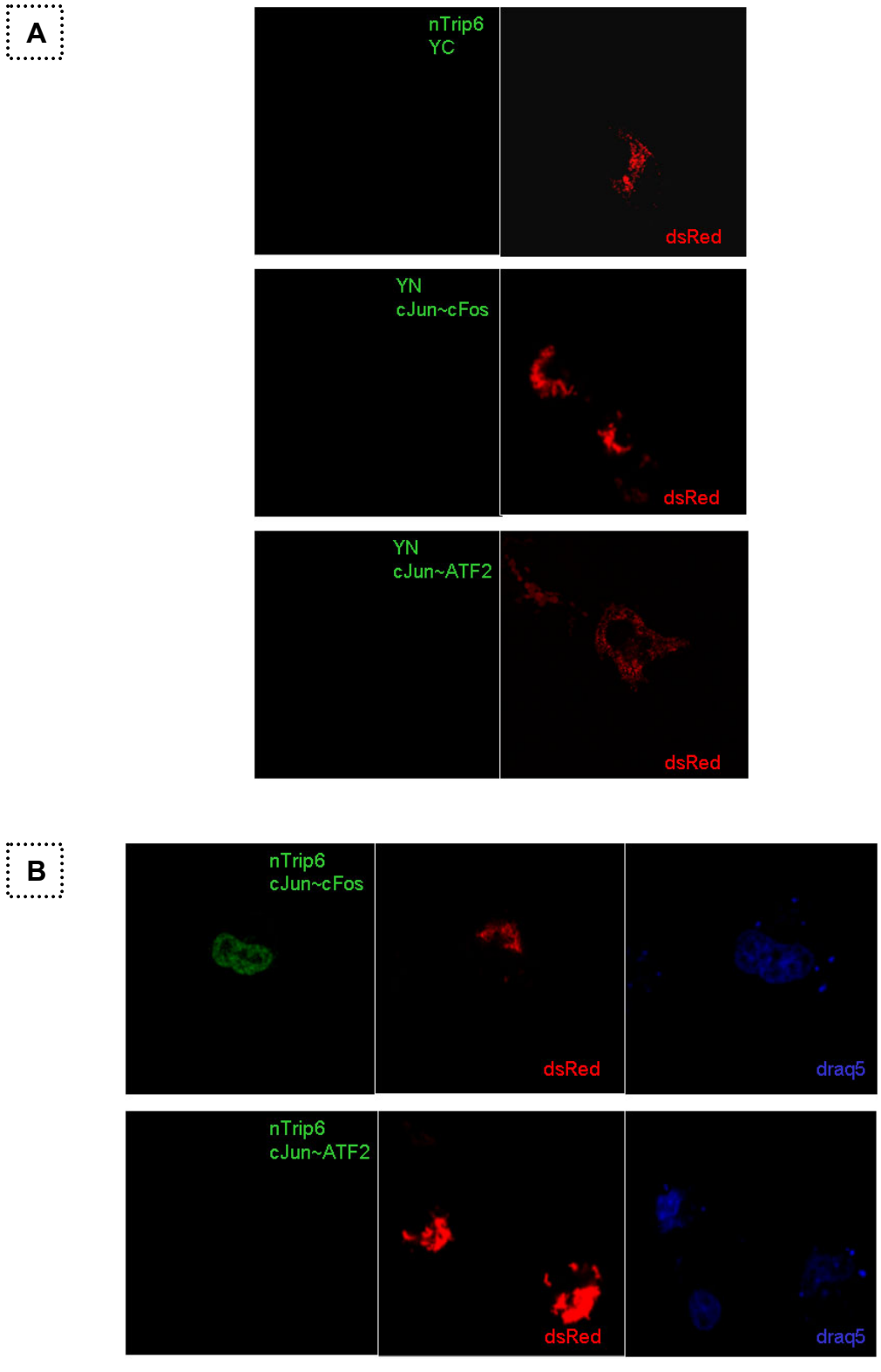


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Figure 3.4. Visualization of interaction between nTrip6 and cJun~cFos and cJun~ATF2 using BiFC assay in living cells.
A. cos7 cells were co-transcected with dsRed expressing vector together with YN-nTrip6 and cJun~cFos-YC or cJun~ATF2-YC. 20h post transfection cells were incubated at $30^0$C for 2h to allow fluorophore maturation. Fluorescence images were acquired by using filters selective for YFP (left) or dsRed (middle). DNA was stained with Draq5 (left).
B. Cos7 cells were co-transcected with dsRed expressing vector and vector controls together with YN-nTrip6 or cJun~cFos-YC and cJun~ATF2-YC. 20h post transfection cells were incubated at $30^0$C for 2h to allow fluorophore maturation. Fluorescence images were aquired by using filters selective for YFP (left) or dsRed (right).

## 3.2. Specificity of GR mediated repression for different AP-1 dimers.

nTrip6 is required for GR-dependent repression of AP-1 (Kassel et al., 2004). c-Jun~ATF2 pseudodimer did not show interaction with nTrip6 in the BiFC assay. This suggests that the c-Jun/ATF2 dimer might not be regulated by ligand-bound GR.

To evaluate the regulation of a specific AP-1 dimer by GR I used reporter gene assays in cultured cells. Transcription factor binding to the promoter region of the reporter gene results in the expression of the firefly luciferase. The amount of luciferase activity detected from the cell lysates correlates directly with the promoter activity of a reporter gene.

An ATF3 reporter construct was used to study the effect of GR on the transcriptional activity of AP-1 pseudodimers. ATF3 promoter is a known AP-1 target and contains binding sites for both c-Jun/c-Fos (AP-1/TRE) and c-Jun/ATF-2 (ATF/CRE) (Liang et al., 1996) (Fig. 3.5.).

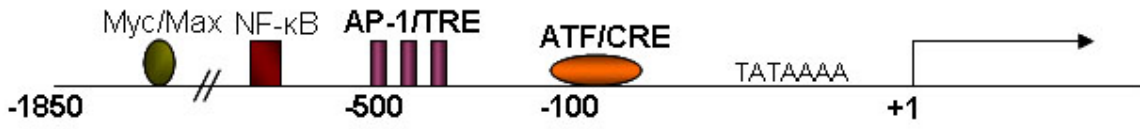



Figure 3.5. Schematic representation of the ATF-3 promoter region.
The Myc/Max, NF-κB, AP-1/TRE and ATF/CRE binding sites, located between position -1850 and -30 (relative to the transcription start site) are shown. Adapted from Liang et al., 1996.

ATF/CRE is an octameric sequence that appears to be specifically bound by the c-Jun/ATF-2 heterodimer, while AP-1/TRE is a heptameric motif, similar to the collagenase TRE, which binds c-Jun/c-Fos.

HeLa cells were transfected with cJun~cFos and cJun~ATF2 encoding vectors and ATF3-luc reporter construct. Ectopic expression of the cJun~cFos pseudodimer increased the activity of the luciferase reporter gene around 25 fold (Fig. 3.7, A). This strong response of the reporter was repressed by GR after dexamethasone treatment. Activation of the reporter by ectopically expressed cJun~ATF2 pseudodimer was slightly stronger as compared to cJun~cFos. Dexamethasone had no effect on the transactivation of the reporter gene by cJun~ATF2 pseudodimer (Fig. 3.7, A).

Next step was to evaluate if this result is not specific to ATF3 promoter. In this experiment another reporter gene, the urokinase plasminogen activator (uPA) enhancer driving the expression of luciferase gene (uPA-Luc) was used. This enhancer fragment consists of 200bp element from the uPA promoter. It contains binding sites for both c-Jun/c-Fos (AP-1/TRE) and c-Jun/ATF-2 (ATF/CRE) (De Cesare et al., 1995) (Fig. 3.6.)

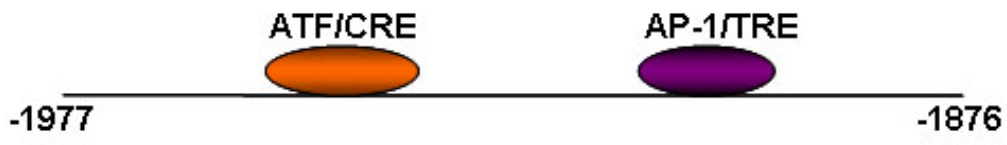



Figure 3.6. Schematic representation of the -2.0 kb human uPA enhancer. The AP-1/TRE and ATF/CRE binding elements are shown (relative to the transcription start site). Adapted from Cirillo et al. 1999.

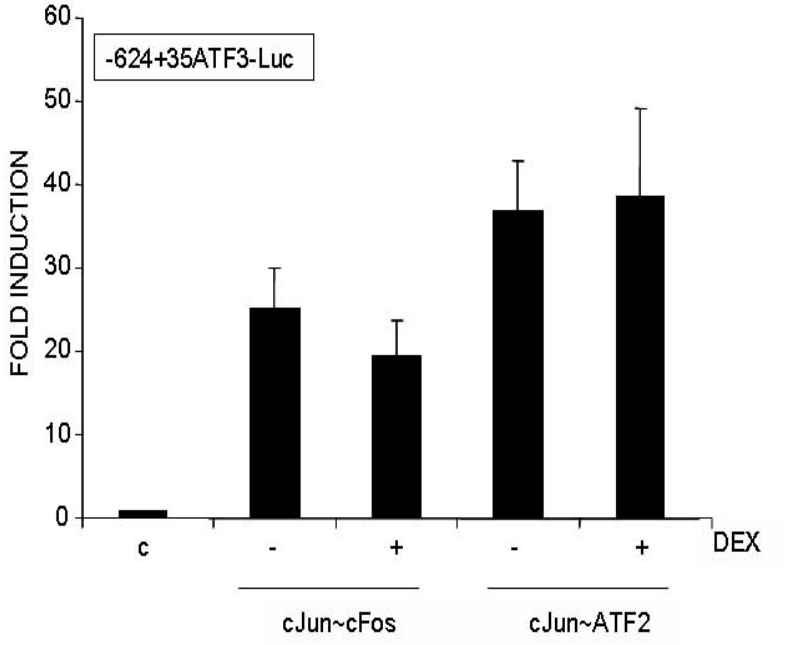
As observed for the ATF3-luc reporter, the activity of the uPA-luc reporter gene was induced by the cJun~cFos pseudodimer (Fig. 3.7, B) and repressed by dexamethasone. In cells expressing cJun~ATF2 pseudodimer the reporter gene activity was not modulated by GR after DEX treatment (Figure 3.7, B). This result suggested that the lack of dexamethasone effect on cJun~ATF2 regulated reporter activity is not promoter dependent.

Lysates were collected after the reporter assay and the expression levels of the cJun~cFos and cJun~ATF2 pseudodimers were analyzed by SDS-PAGE. Probing with specific antibodies showed that the expression of the transfected cJun~cFos and cJun~ATF2 pseudodimers is at the same level and not affected by dexamethasone treatment (Figure 3.7, C). Moreover,

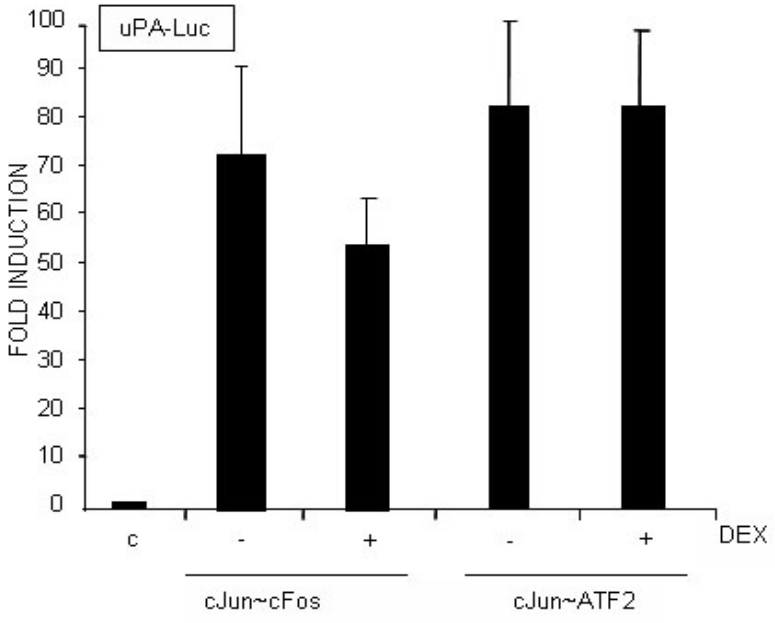
probing with an anti-c-Fos antibody showed that the levels of endogenous c-Fos are not detectable. This shows that the starvation time before dexamethasone treatment is sufficient to reduce the expression of the endogenous c-Fos protein. Thus, the measured reporter gene activity after ectopic expression of the cJun~cFos pseudodimer was due to transactivation by the pseudodimer, and not to the endogenous c-Fos-containing dimer.
Probing with anti-ATF2 antibody showed that c-Jun~ATF2 pseudodimer expression is not affected by dexamethasone (Figure 3.7, C). Expression of cJun~ATF2 pseudodimer induced the expression of endogenous ATF2. It is not surprising since ATF2 is a known AP-1 target gene (Liang et al., 1996). High level of ATF2 protein, upon heterodimerization with endogenous c-Jun, could have contributed to the activation of the reporter gene in the assay. However, this activity was not repressed after dexamethasone treatment, suggesting that neither the cJun~ATF2 pseudodimer nor the endogenous c-Jun/ATF2 dimer can be repressed by GR.

This result suggests that GR does not regulate the transcriptional activity of c-Jun/ATF2 dimer. This lack of repression by GR might due to the lack of interaction between nTrip6 and cJun or ATF2.

A




B

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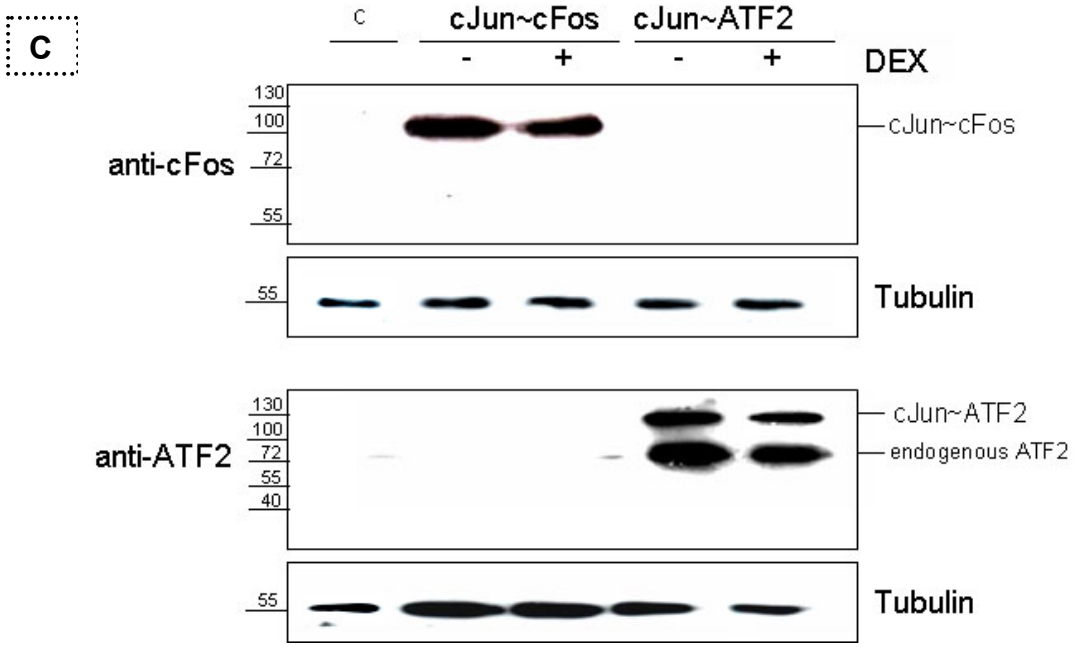


Figure 3.7. Effect of dexamethasone on c-Jun~cFos and cJun~ATF2 induced ATF3- and uPA-promoter activity.
HeLa cells were co-transfected with -624+35ATF3-Luciferase (**A**) or uPA-Luciferase reporter (**B**) (100ng) together with GR (100ng), and with empty vector (c) or pseudodimer cJun~cFos or cJun~ATF2 expressing vectors (100ng). Cells were serum starved and untreated (c) or treated with dexamethasone (DEX, $10^{-6}$M), harvested 16h later and assayed for reporter gene activities. Results are shown as fold induction relative to the activity in untreated cells transfected with the reporter construct alone (mean±SD of one representative experiment performed in triplicate).
(**C**) Expression levels of cJun~cFos and cJun~ATF2 pseudodimers.
Lysates after luciferase assay were analyzed by SDS-PAGE using an anti-ATF2 and an anti-c-Fos antibody. Membranes were stripped and re-probed with an anti-tubulin antibody, as loading control.

### 3.3. GR-mediated regulation of endogenous AP-1 target genes

Experiments presented so far suggest that c-Jun/ATF2 transcriptional activity is not repressed by GR. However it cannot be excluded that the lack of repression by GR might be an artifact. The structure of the c-Jun/ATF2 pseudodimer might not mimic the exact conformation of the natural promoter bound cJun/ATF2 dimer, and might not be repressible by ligand-bound GR.
I thus wanted to study the effect of GR on the transcriptional activity of the endogenous c-Jun/ATF2 dimer in comparison with the regulation of the well studied c-Jun/cFos dimer. The activity of endogenous c-Jun/ATF2 or c-Jun/c-Fos was stimulated through activation of different MAP kinase pathways.

JNK pathway was stimulated by UV-C to induce activity of c-Jun/ATF2 dimer by phosphorylation. To exclude effects of UV-C-induced DNA lesions, anisomycin treatment was also used to activate cJun/ATF2. At concentrations that do not inhibit protein synthesis, anisomycin activates the JNK/SAPK signal transduction pathway which leads to phosphorylation and transcriptional activity of cJun/ATF2 (Cano et al., 1994).

Activity of the ERK pathway was stimulated with the phorbol ester TPA. Activation of this pathway leads to *de novo* synthesis of c-Fos proteins which dimerize with existing c-Jun proteins and regulate the expression of target genes driven by AP-1 response elements.

As a read out for GR-mediated regulation of activated endogenous AP-1 factors, I analyzed the expression of ATF3, a known c-Jun/c-Fos and c-Jun/ATF2 target gene at the level of mRNA by Northern Blot. As a control I analyzed the expression levels of collagenase I, a known c-Jun/c-Fos target gene (Angel et al., 1987).

TPA treatment induced collagenase I and ATF3 mRNA levels, as expected. This is most probably due to transactivation by c-Jun/c-Fos dimer. Dexamethasone treatment repressed this induction (Fig. 3.8.).

UV-C treatment strongly induced the expression of ATF3 gene but had no effect on collagenase I induction. This shows that UV-C did not stimulate the activity of c-Jun/c-Fos. The induction of ATF3 expression is most probably mediated by c-Jun/ATF2 dimer. Treatment with dexamethasone surprisingly repressed the expression of ATF3.

To exclude that this effect is not at the level of c-Jun/ATF2 dimer but anywhere in the DNA damage response pathway activated by UV-C, another JNK inducer, anisomycin was used. Result of induction with anisomycin was as observed with UV-C. Collagenase I expression was not induced, whereas ATF3 expression was strongly induced and repressed upon dexamethasone treatment.

This shows that upon dexamethasone treatment, decrease of UV- or anisomycin-induced expression of ATF3 is not due to a repression of c-Jun/c-Fos dimer.

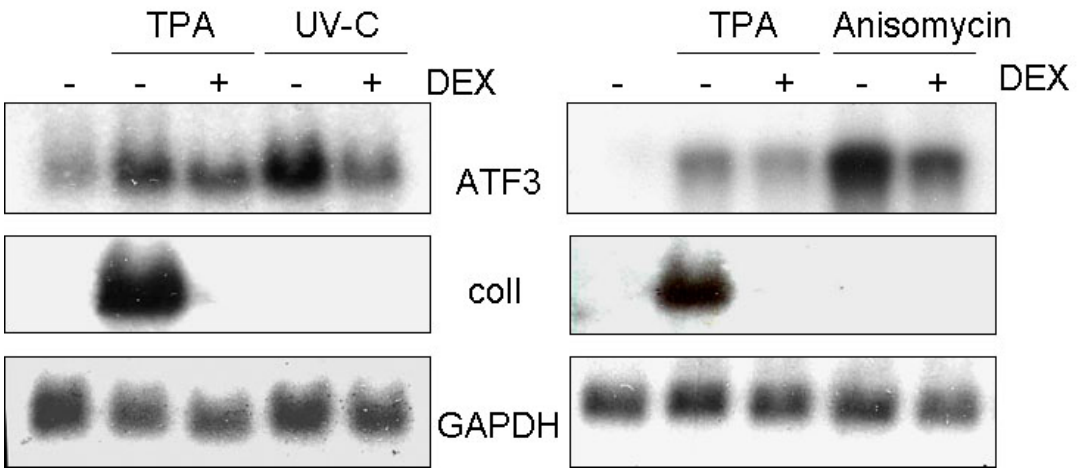


Figure 3.8. Effect of dexamethasone on phorbol ester, ultraviolet light and anisomycin induced ATF3 and collagenase 1 mRNA expression.
HeLa cells were serum starved, not treated (-) or pre-treated with dexamethasone (DEX, $10^{-6}$M) for 45 min and stimulated with phorbol ester (TPA, 50ng/ml), ultraviolet light (UV-C, 40J/$m^2$) or anisomycin (50ng/ml). 4h after treatment total RNA was extracted and subjected to Northern blotting using $^{[32}$P]-labeled cDNA probes for ATF3, collagenase 1 and GAPDH that was used as a loading control. The results are representative of three independent experiments.

Due to the complex character of the ATF3 promoter, the observed repression might also be an effect of the regulation of other transcription factors contributing to the expression of ATF3 gene upon UV or anisomycin treatment. To evaluate this possibility I studied the effect of GR on the transcriptional activity of endogenous AP-1 dimers in reporter gene assays using a promoter containing only AP-1 response elements.

I used the previously described minimal ATF3-luc reporter gene. This minimal promoter construct contains binding sites for both cJun/cFos and cJun/ATF2, which allows studying the regulation of these dimers in the very same promoter context.

TPA treatment induced the activation of the reporter gene, presumably by c-Jun/cFos dimer. Dexamethasone treatment repressed this induction, as expected (Fig. 3.9.). UV-C irradiation also induced the expression of the reporter gene. In this minimal promoter context this induction is most probably mediated by activated c-Jun/ATF2 dimer. This induction was also repressed by dexamethasone (Fig. 3.9.), confirming the results of the regulation of the endogenous ATF3 gene.

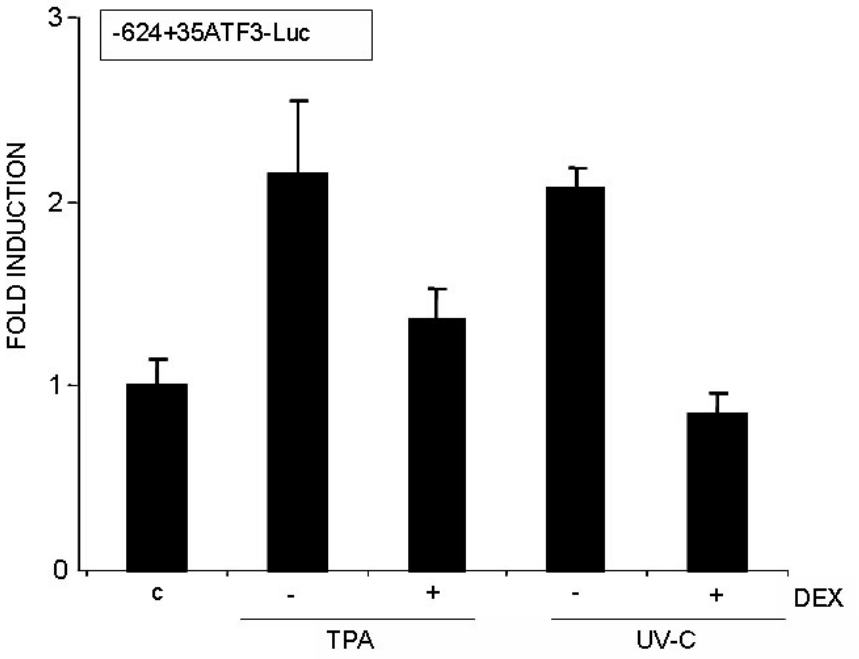


Figure 3.9. Effect of dexamethasone on phorbol ester and ultraviolet light induced ATF3-promoter activity. HeLa cells were serum starved, not treated (c) or pre-treated dexamethasone (DEX, $10^{-6}$M) for 45 min and stimulated with phorbol ester (TPA, 50ng/ml), ultraviolet light (UV-C, 40J/$m^2$) or anisomycin (50ng/ml). 4h after treatment cells were harvested and assayed for reporter gene activities. Results are shown as fold induction relative to the activity in untreated cells transfected with reporter construct alone (mean±SD of one representative experiment performed in triplicate).

These results are in contradiction with previous experiments. On the one hand, the transcriptional activity of ectopically expressed cJun~ATF2 pseudodimer was not modulated by GR. On the other hand, the induced activity of endogenous c-Jun/ATF2 dimer is repressed by GR.

In the experimental conditions used, this discrepancy could come from differences in the phophorylation status of AP-1 dimers. c-Jun~ATF2 pseudodimers, on the one hand are in a stable complex, localized in the nucleus and transcriptionaly active. On the other hand the activity of endogenous preexisting AP-1 proteins is increased by phosphorylation. Thus the repression of c-Jun/ATF2 activity by ligand-bound GR might be due to the inhibition of this phosphorylation step.

## 3.4. GR-mediated repression of c-Jun/ATF2 transcriptional activity

### 3.4.1 Inhibition of c-Jun phosphorylation

Indeed, previous reports showed that GR is interfering with MAPK signaling pathways (Caelles et al., 1997; Gonzalez et al., 1999). In particular, GR has been proposed to inhibit JNK by direct binding to the kinase through JNK docking site (Bruna et al., 2003). Inhibition of JNK activity would lead to the down regulation of the transcriptional activity of c-Jun containing AP-1 dimers. However, the contribution of such a mechanism on the repression of AP-1 activity by GR is still a matter of debate.

In the case of c-Jun/c-Fos dimer, the repression is mediated by the recruitment of GR to the promoter through an interaction with nTrip6 (Kassel et al., 2004). c-Jun/ATF2 dimer does not interact with nTrip6 and thus GR is probably not recruited to the promoter. In this case, the observed repression of endogenous c-Jun/ATF2 activity might be related to the inhibition of JNK activity by GR.

A prerequisite here was therefore to examine if GR inhibits JNK and c-Jun phosphorylation it the conditions of the assay.

In unstimulated cells the levels of phosphorylated JNK and c-Jun are low (Fig. 3.10.). Upon UV-C irradiation the phosphorylation levels of JNK and c-Jun are increased within 30 min.

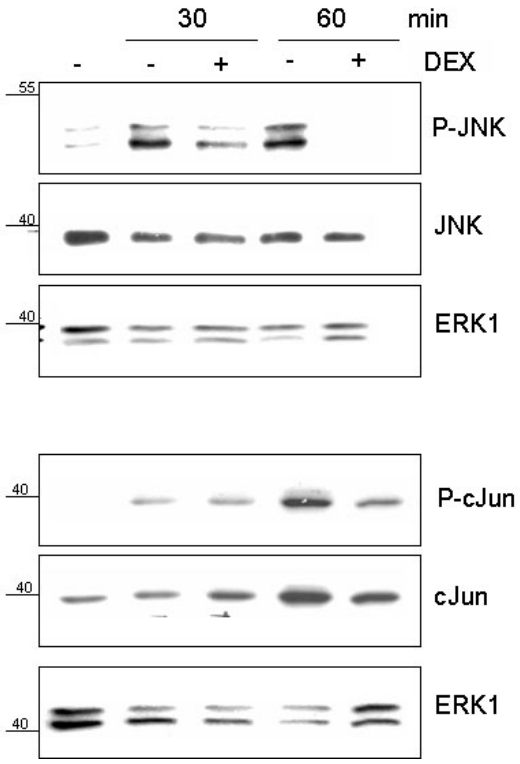


Figure 3.10. Effect of dexamethasone on ultraviolet light induced phosphorylation of JNK and c-Jun.
Fibroblasts cells were not treated (-) or pre-treated with dexamethasone (DEX, $10^{-6}$M) for 45 min and irradiated with UV-C (40J/$m^2$). Cells were harvested at indicated time points post irradiation and lysates were subjected to SDS-PAGE. JNK and c-Jun phosphorylation was assessed by immunoblots using anti-phospho-JNK and anti-phospho-c-Jun antibodies. The membranes were stripped and re-probed with phosphorylation state independent anti-JNK1 and anti-c-Jun antibodies. ERK1 antibody was used as a loading control. The result is representative of three independent experiments.

At 30 min time point dexamethasone treatment decreased the phosphorylation of of c-Jun. At 1h time point phosphorylation of JNK was completely inhibited upon dexamethasone treatment. Total levels of JNK were not affected by dexamethasone as shown after probing with an anti-JNK1 antibody.

At 1h time point the phosphorylation of c-Jun was strongly increased. Moreover, at this time point total levels of c-Jun protein are also elevated, as compared to non-irradiated cells. Dexamethasone treatment decreased the phosphorylation and total protein levels of c-Jun as compared to untreated control. This result shows that inhibition of JNK phosphorylation by dexamethasone is subsequently leading to a decrease in c-Jun phosphorylation (Fig. 3.10.).

### 3.4.2 Repression of c-Jun/ATF2 transcriptional activity by GR

These results confirm that GR inhibits JNK activity. Thus, the inhibitory action of GR on JNK signaling might contribute to the GR-mediated repression of JNK-stimulated c-Jun/ATF2 activity. To address this question, I studied the effect of GR on the transcriptional activity of the AP-1 pseudodimers upon UV-induced activation of the JNK pathway, using the uPA-luc reporter gene. To avoid any influence of endogenous c-Jun proteins activated after UV-C treatment, which could contribute to the induction of the reporter gene, *c-jun*$^{-/-}$ fibroblasts were used in this experiment. Indeed, as expected UV-C irradiation alone did not induce luciferase activity significantly (Fig. 3.11.). This also suggests that, despite elevated levels of other Jun members in this cell system, especially JunD (data not shown) they do not contribute to the UV-C induced activation of the uPA-luc reporter gene.

The transcriptional activity of transiently transfected cJun/cFos pseudodimer was repressed by dexamethasone (Fig. 3.11.), as previously seen. The cJun/cFos pseudodimer activity was further increased by 2 fold upon UV-C irradiation (Fig. 3.11.). This increase is probably due to the phosphorylation of the c-Jun moiety of the pseudodimer. This result confirms that cJun/cFos pseudodimers respond to phosphorylation like the endogenous dimer. Dexamethasone repression of cJun/cFos transcriptional activity was also observed after UV-C irradiation, and extend of the repression before and after UV-C irradiation was similar (Fig. 3.11.).

The transcriptional activity of cJun/ATF2 pseudodimer in non irradiated cells was not modulated by dexamethasone (Fig. 3.11.), as previously seen. Responsiveness of urokinase reporter to cJun/ATF2 pseudodimer was increased by 6 fold after UV-C irradiation (Fig. 3.11.). Since endogenous Jun proteins do not contribute to the activity of the reporter gene, this increase is only due to the phosphorylation of c-Jun and ATF2 in the pseudodimer.

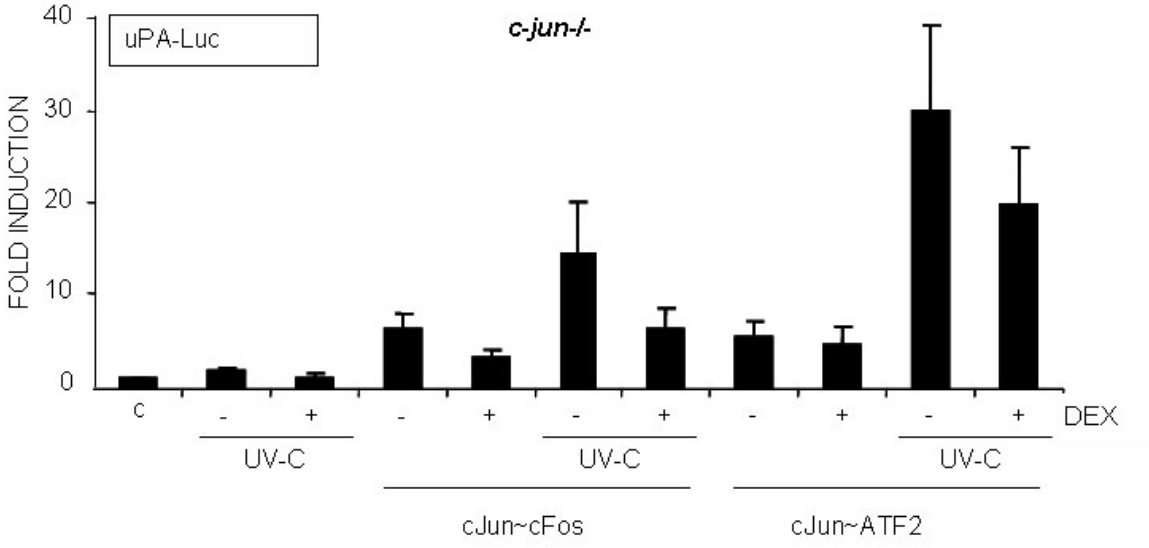


Figure 3.11. Effect of DEX on ultraviolet light induced activity of cJun~cFos and cJun~ATF2 on uPA-promoter.
*cjun-/-* MEF cells were co-transfected with uPA-Luciferase reporter (100ng) together with GR (300ng), and with empty vector (c) or pseudodimer cJun~cFos or cJun~ATF2 expressing vectors (700ng). Cells were serum starved, not treated (-) or pretreated with dexamethasone (DEX, 10-6M) and either not exposed or exposed to ultraviolet light (UV-C, 40J/$m^2$). 16h later cells were harvested and assayed for reporter gene activities. Results are shown as fold induction relative to the activity in untreated cells transfected with reporter construct alone (mean±SD of one representative experiment performed in triplicate).

This confirms that, like in the case of the cJun/cFos pseudodimers, the structure of the fusion does not affect its phosphorylation by JNK, and that the cJun~ATF2 pseudodimer responds to the activation in the same manner as the endogenous proteins. This phosphorylation induced increase in cJun/ATF2 pseudodimer transcriptional activity was partially repressed by GR upon dexamethasone treatment.

To further corroborate this result, I analyzed the effect of GR on the transcriptional function of c-Jun independent of dimerization with other AP-1 family members. c-Jun lacking the basic region/ leucine zipper (bZIP) domain, and thus unable to dimerize, was fused to the heterologous DNA-binding domain of the yeast transcription factor GAL4 ($GAL_{DBD}$) (Fig.3.12.). The activity of a reporter gene driven by GAL4-UAS-response element would

reflect only the activity of c-Jun fused to $GAL_{DBD}$, without any interference of other AP-1 transcription factors. In these experiments HEK 293 cells with a GAL4-responsive luciferase gene stably integrated into the genome were used.

JNK was activated by co-transfection with a constitutively active form of the JNK kinase kinase MEKK1 (ΔMEKK1).

As a control I used full length c-Fos and ATF2 fused to $GAL_{DBD}$ (Fig.3.12.), to ensure that the $GAL_{DBD}$ fusions of AP-1 family members do behave like their endogenous counterparts in these experimental conditions.

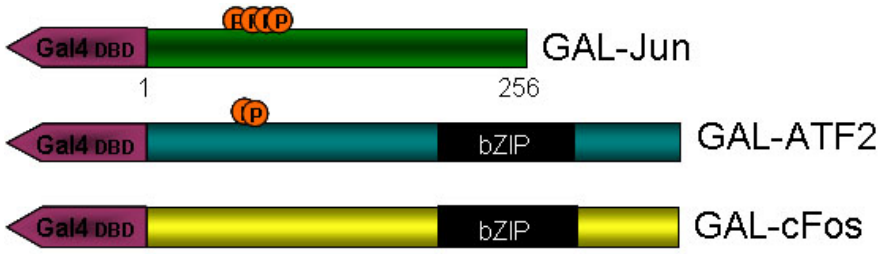


Figure 3.12. Schematic structure of GAL-Jun, GAL-ATF2 and GAL-cFos.
The DNA binding and dimerization domain (bZIP, black box) of c-Fos and ATF2 and the JNK phosphorylation sites of c-Jun (serines 63, 73 and threonines 91, 93) and ATF2 (threonine 69 and 71) are shown. Numbers indicate amino acid positions.

The basal activity of the reporter gene in control cells was neither affected by dexamethasone nor by transfection of ΔMEKK1 (Fig. 3.13). Transfection of cells with $GAL_{DBD}$c-Fos led to a strong induction of the activity of the reporter gene. Cells expressing $GAL_{DBD}$c-Fos together with the ΔMEKK1 showed a further increase in reporter activity (Fig. 3.13). This is probably due to the activation by phosphorylation of the endogenous c-Jun that dimerizes with $GAL_{DBD}$c-Fos. This confirms that $GAL_{DBD}$ fusions respond to stimulation like natural AP-1 dimers. Dexamethasone treatment, with or without activation of JNK signaling by ΔMEKK1, repressed the transcriptional activity of $GAL_{DBD}$c-Fos to the same extend (Fig. 3.13), as previously seen with the cJun~cFos pseudodimers. This result confirms that GR mediated repression of c-Jun/c-Fos activity is independent of SAPK/JNK activity, and independent of the promoter context.

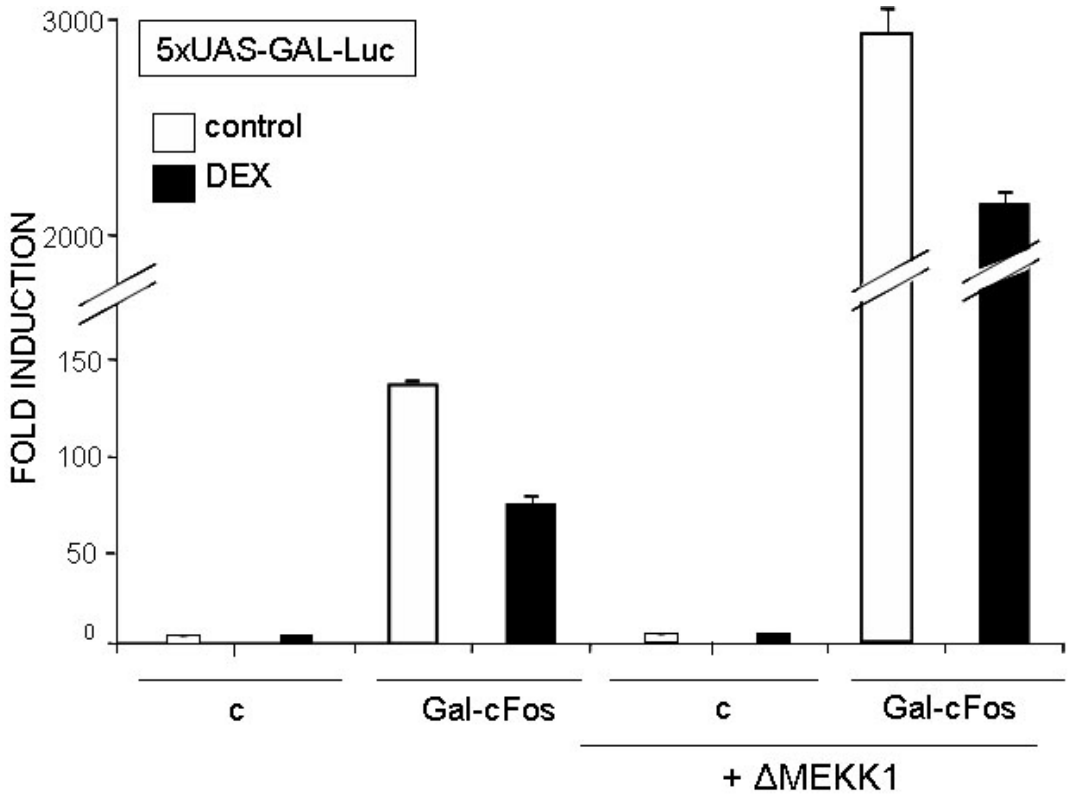


Figure 3.13. Effect of dexamethasone on Gal-cFos induced UAS-GAL-promoter activity. HEK 293 cells stably transfected with a GAL4-responsive luciferase gene were transfected with GR (200 ng) and empty vector or $GAL_{DBD}$cFos construct (200 ng) with or without ΔMEKK1 (25ng) expression vector. Cells were untreated or treated with DEX ($10^{-6}$M) for 18h and harvested and assayed for reporter gene activities. Results are shown as fold induction relative to the activity in untreated cells transfected with reporter construct alone (mean±SD of one representative experiment performed in duplicate).

The activity of the reporter was not changed in cells transfected with $GAL_{DBD}$ATF2 (Fig. 3.14.). ATF2 is a poor transactivator itself in the absence of extracellular stimulation (Li and Green, 1996). Phophorylation upon JNK activation is inducing ATF2 transactivation function. This would explain the strong induction of the reporter gene activity in cells co-transfected with $GAL_{DBD}$ATF2 and ΔMEKK1 (Fig. 3.14.). Additionally full length ATF2 in fusion with $GAL_{DBD}$, upon JNK activation can dimerize with endogenous c-Jun and regulate the expression of the reporter gene. In these conditions dexamethasone treatment repressed the transcriptional activity of $GAL_{DBD}$ATF2 (Fig. 3.14.). This result confirms that GR mediated repression of c-Jun/ATF2 activity is dependent of SAPK/JNK activity, and independent of the promoter context.

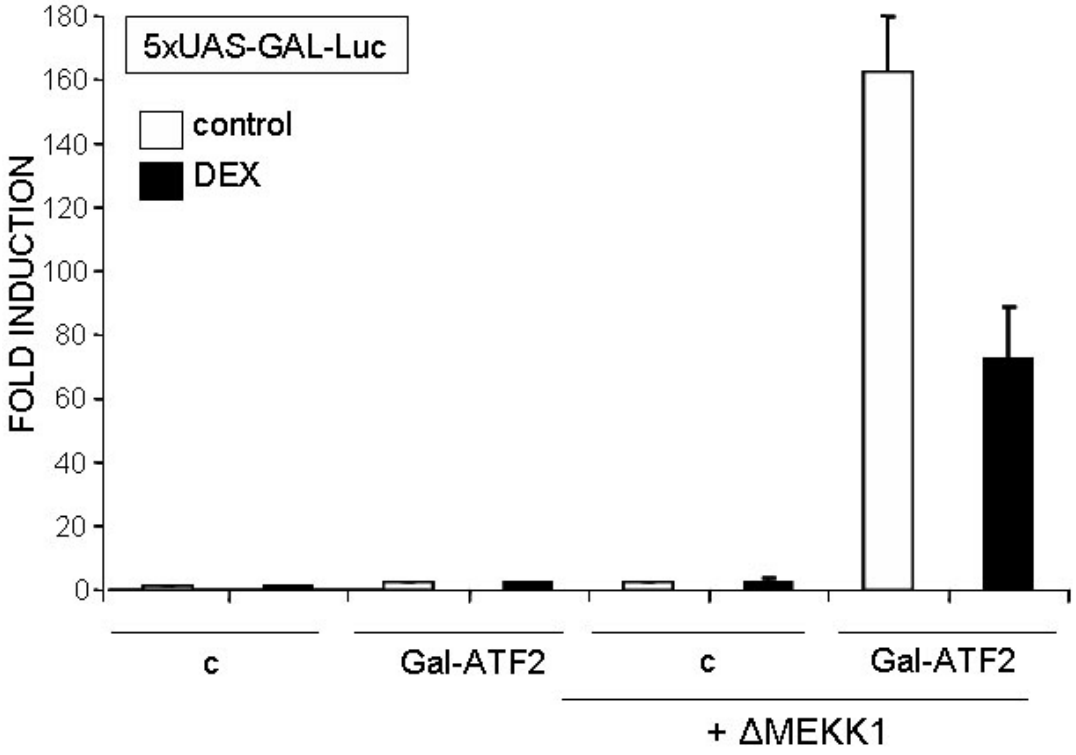


Figure 3.14. Effect of dexamethasone on Gal-cFos induced UAS-GAL-promoter activity. HEK 293 cells stably transfected with a GAL4-responsive luciferase gene were transfected with GR (200 ng) and empty vector or $GAL_{DBD}$cFos construct (200 ng) with or without ΔMEKK1 (25ng) expression vector. Cells were untreated or treated with DEX ($10^{-6}$M) for 18h and harvested and assayed for reporter gene activities. Results are shown as fold induction relative to the activity in untreated cells transfected with reporter construct alone (mean±SD of one representative experiment performed in duplicate).

c-Jun transactivation function in fusion with $GAL_{DBD}$ was not modulated after dexamethasone treatment without JNK activation (Fig. 3.15.). However, when JNK activity was stimulated by co-transfection of a constitutively active form of the JNK kinase kinase MEKK1 (ΔMEKK1), the reporter activity was strongly increased (Fig. 3.15.). Since $GAL_{DBD}$cJun is lacking the bZIP domain and cannot dimerize with other AP-1 members, this strong increase can only be due to the phosphorylation of the c-Jun moiety of the fusion. And in these conditions, phosphorylation induced activity of $GAL_{DBD}$cJun is repressed by GR (Fig. 3.15.).

This result shows that unlike c-Jun/c-Fos dimer, which is directly trans-repressed by GR, c-Jun/ATF2 dimer activity is repressed by GR only when it is phosphorylated by JNK.

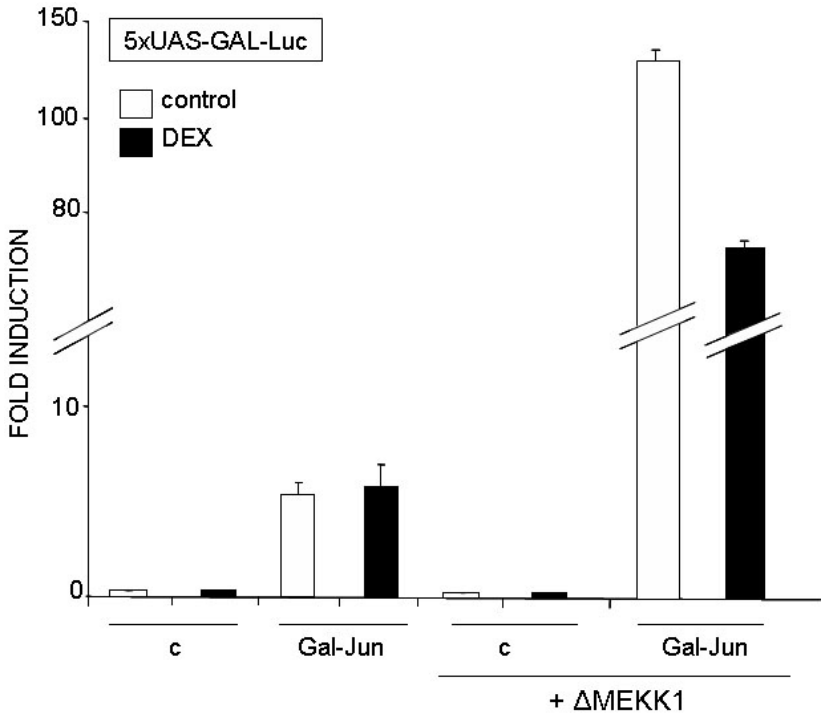


Figure 3 15. Effect of dexamethasone on Gal-c-Jun induced UAS-GAL-promoter activity. HEK 293 cells stably transfected with a GAL4-responsive luciferase gene were transfected with GR (200 ng) together with either empty vector or $GAL_{DBD}$-c-Jun construct (200 ng) with or without ΔMEKK1 (25ng) expression vector. Cells were untreated or treated with DEX ($10^{-6}$M) for 18h and harvested and assayed for reporter gene activities. Results are shown as fold induction relative to the activity in untreated cells transfected with reporter construct alone (mean±SD of one representative experiment performed in duplicate).

Taken together my results show that c-Jun/ATF2 activity is not transrepress by GR like the activity of c-Jun/c-Fos dimer. ΔMEKK1-induced activation of c-Jun/ATF2 upon phosphorylation by JNK is repressed by dexamethasone. This repression is presumably a result of the inhibition of JNK activity by GR.

# 4. DISCUSSION

This work shows that two different dimers belonging to the AP-1 family of transcription factors, c-Jun/c-Fos and c-Jun/ATF2 are repressed by Glucocorticoid Receptor by a different mechanism. This is based on the difference in the abilities of these different dimers to interact with nTrip6.
c-Jun/c-Fos does interact with nTrip6, which is essential for transrepression by GR. c-Jun/ATF2 does not interact with nTrip6 and is not transrepressed by GR. c-Jun/ATF2 is however also repressed by GR, through an inhibition of JNK-mediated phosphorylation of c-Jun.

Down regulation of c-Jun/c-Fos transcriptional activity by ligand-bound Glucocorticoid Receptor is an example of the “classical” transrepression mode of regulation. Repression of c-Jun/c-Fos transcriptional activity by GR does not involve DNA binding by GR, but the DNA binding domain of GR is necessary for efficient repression of c-Jun/c-Fos (Jonat et al., 1990). The trans-repression mechanism also does not involve competition for coactivators between c-Jun/c-Fos and GR (De Bosscher et al., 2001). Repression of c-Jun/c-Fos activity by GR is not changing promoter occupancy by this dimer (Kassel et al., 2004; Konig et al., 1992; Nissen and Yamamoto, 2000; Rogatsky et al., 2001), but involves protein-protein interaction between c-Jun/c-Fos and GR. This interaction is mediated through nTrip6, which interacts with c-Fos on the target promoter (Kassel et al., 2004). At the same time nTrip6 is also able to interact with GR. Through this interaction nTrip6 mediates the recruitment of GR to the promoter-bound AP-1 as a prerequisite for transrepression.

In the experimental conditions used the transcriptional activity of c-Jun/ATF2 was not modulated by GR. Moreover, interaction studies using BiFC assay confirmed that nTrip6 interacts with c-Jun/c-Fos *in vivo,* but no interaction between nTrip6 and c-Jun/ATF2 was detected. This confirms *in vitro* pull down experiments which did not show any interaction between nTrip6 and ATF2 or c-Jun (Heilbock, unpublished data). c-Jun/ATF2, and the activity of the dimer is not repressed. This needs to be further investigated. According to the model proposed by Kassel et al., c-Jun/ATF2 is not transrepressed by GR presumably due to the lack of interaction with nTrip6.

Without interaction with nTrip6, GR may not be recruited to the promoter bound The lack of c-Jun/ATF2 repression by GR was already reported. E-selectin expression regulated by NF-κB was strongly repressed in the presence of dexamethasone, whereas activity of c-Jun/ATF2 dimer on the same promoter was not affected by GR (Brostjan et al., 1997).
Similarly, the activity of a $GAL4_{UAS}$-driven reporter gene by $GAL_{DBD}$c-Fos was repressed by GR, whereas the activity of the same reporter by $GAL_{DBD}$c-Jun was not repressed by GR.

These results are in agreement with unpublished data from Marc Castelazzi (Lyon, France) where c-Jun mutants were used in a reporter assay in Vhicken Embryonic Fibroblasts. The transcriptional activity of a c-Jun mutant preferentially dimerizing with c-Fos was repressed by GR after dexamethasone treatment, whereas the transcriptional activation of a CRE-driven reporter gene by a c-Jun mutant dimerizing with ATF2, was not modified by GR. These experiments were performed in conditions were JNK signaling was not activated. This shows that the transrepression of c-Jun/c-Fos and the lack of transrepression of c-Jun/ATF2 by GR is independent of promoter used.

GR and AP-1 can reciprocally repress one another's transcriptional activation. Overexpression of c-Jun prevents the glucocorticoid-induced activation of genes carrying a functional glucocorticoid response element (GRE) (Jonat et al., 1990; Reik et al., 1994; Schule et al., 1990a; Yang-Yen et al., 1990) . Importantly, the region including the leucine zipper of c-Jun is required for repression of GR-mediated gene activation. This suggests that dimerization of c-Jun with another AP-1 family member might be necessary for the transrepression of GR activity. Indeed, in unpublished work of the lab, c-Fos-mediated repression of GR activity was observed. This transrepression of GR also requires interaction with nTrip6. Due to the lack of interaction between nTrip6 and c-Jun/ATF2, one may speculate that this dimer would not transrepress GR activity. This seems to be confirmed by experiments performed in Chick Embryo Fibroblasts (CEF). The transcriptional activity of GR target promoter induced with dexamethasone was repressed by an

ectopically expressed c-Jun mutant, which preferentially dimerizes with c-Fos, but not by a c-Jun mutant preferentially dimerizing with ATF2 (Caserlazzi et al., unpublished results).

However, upon stimulation of JNK activity, the phosphorylation-increased transcriptional activity of c-Jun/ATF2 was repressed by GR. This suggests the presence of a mechanism that would regulate the activity of c-Jun/ATF2 by GR in a different manner. In agreement with previous observations, my results show that GR inhibits JNK activity. Inhibition of TNFα- or UV-stimulated activation of JNK cascade after dexamethasone treatment was already published (Caelles et al., 1997; De Bosscher et al., 2001), and the involvement of JNK signaling inhibition by GR in the repression of AP-1 transcriptional activity was proposed (Caelles et al., 1997; De Bosscher et al., 2001; Gonzalez et al., 2000). However the relevance of this mechanism was not clear. My results show that through inhibition of JNK-mediated c-Jun phosphorylation, GR is repressing the transcriptional activity of c-Jun/ATF2 dimers.

A question that remains open is what is the exact molecular mechanism of GR-mediated inhibition of c-Jun phosphorylation? This effect of GR can be mediated on several levels.

Inhibition of JNK activity by direct binding of GR to phosphorylated JNK was proposed by Bruna and co-workers (2003). Binding of GR would lead to nuclear translocation of JNK. In the nucleus inactive JNK could bind to the promoter bound c-Jun/ATF2 dimers through c-Jun δ-domain, which serves as a docking site for JNK (Kallunki et al., 1996). Through this interaction JNK would exhibit a dominant negative effect on the promoter bound complex. This way GR could inhibit JNK activity not only in the cytoplasm, but also block the phosphorylation and activation of the promoter bound c-Jun/ATF2 complexes. The decrease in c-Jun protein level observed after dexamethasone treatment could also contribute to the repression of c-Jun/ATF2 activity. This decrease of c-Jun protein could be due to proteosomal degradation. The activation of JNK leads to phosphorylation of c-Jun and protection from degradation (Fuchs et al., 2000).

Dephosphorylation of c-Jun leads to its targeting for ubiquitination and subsequent degradation. Protection of the activated substrate from degradation is maintained as long as the protein remains phosphorylated. A similar mode of regulation was also reported for Bcl2 (Dimmeler et al., 1999), which is protected from ubiquitination and degradation by extracellular signal-regulated kinase 2-mediated phosphorylation in human endothelial cells.
GR-mediated inhibition of phosphorylation of c-Jun and ATF2 could result in de-protection and ubiquitin-dependent degradation. Additionaly, the inhibition of c-Jun/ATF2 activity by GR may also result in a decreased expression of *c-jun*, a known c-Jun/ATF2 target gene.

It cannot be excluded that upon phosphorylation of c-Jun and ATF2, they might interact with nTrip6. In such a hypothesis, nTrip6 would be recruited to the promoter bound phosphorylated c-Jun/ATF2 complex. This recruitment of nTrip6 could lead to the binding of glucocorticoid receptor that would transrepress c-Jun/ATF2. This needs further investigation.

Apart from GR, other nuclear hormone receptors also repress the activity of AP-1. Both mechanisms of AP-1 regulation might also be executed by these receptors. Indeed, retinoic acid receptor (RAR) (Schule et al., 1990b; Yang-Yen et al., 1991) and thyroid receptor (TR) (Pfahl, 1993; Zhang et al., 1991) which transrepress AP-1 activity, also interact with nTrip6 (Lee et al., 1995). The involvement of nTrip6 in the repression of c-Jun/c-Fos activity by these nuclear receptors needs to be addressed. Nevertheless, it is plausible to speculate that due to the lack of interaction of c-Jun/ATF2 with nTrip6, the activity of this dimer would not be transrepressed by these receptors. However, RAR and TR also inhibit JNK-mediated phosphorylation of c-Jun (Caelles et al., 1997; Gonzalez et al., 1999; Lee et al., 1999). This suggests that RAR and TR receptors may also repress the transcriptional activity of cJun/ATF2 by inhibiting the activity of JNK.

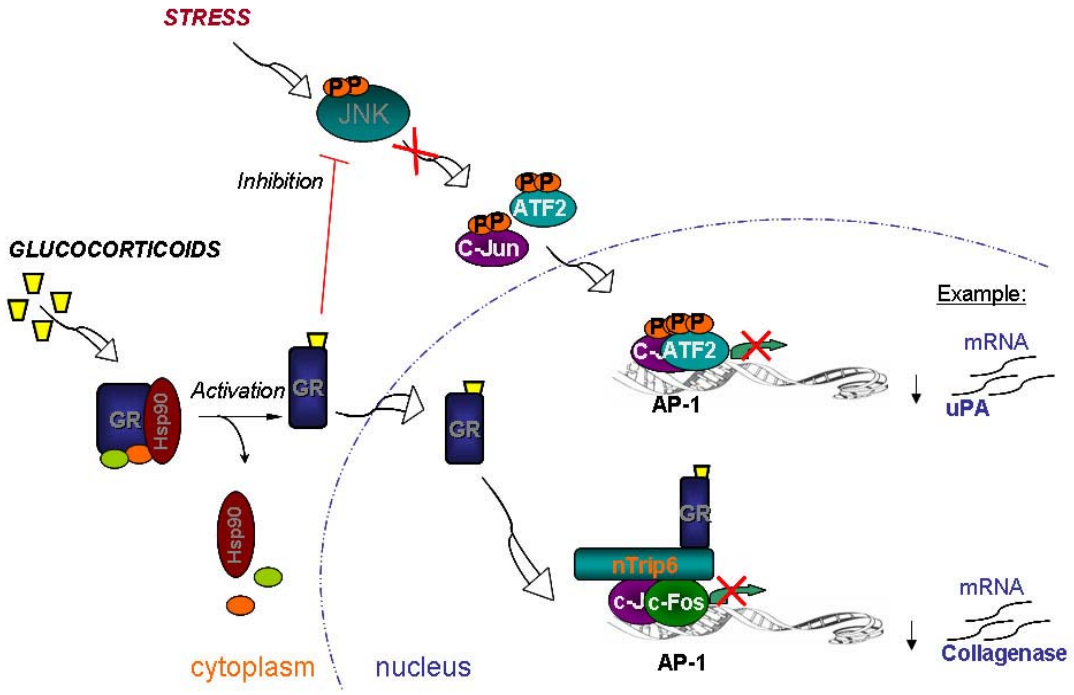


Figure 4.1. Model of GR-mediated repression of c-Jun/c-Fos and c-Jun/ATF2 activity.

In summary, this work shows that c-Jun/c-Fos and c-Jun/ATF2 dimers, belonging to the AP-1 family of transcription factors, are repressed by Glucocorticoid Receptor via a different mechanism.

The repression of c-Jun/c-Fos activity by ligand-bound GR involves protein-protein interaction mediated by nTrip6. nTrip6 interacts with c-Fos on the target promoter. At the same time nTrip6 is also able to interact with GR. Through this interaction nTrip6 mediates the recruitment of GR to the promoter-bound AP-1 as a prerequisite for transrepression.

c-Jun/ATF2 dimer activity is repressed by GR through the inhibition of JNK signaling. Blocking of JNK activity by GR leads to the inhibition of c-Jun phosphorylation and subsequent repression of c-Jun/ATF2. The detailed mechanism of JNK inhibition by GR and the involvement of nTrip6 in the repression of Jun/ATF2 upon activation of JNK signaling needs to be further investigated. Both mechanisms of GR repression of AP-1 might have important implications in the suppression of inflammatory response mediated by AP-1 dimers.

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