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Inhibition of extracellular signal-regulated kinase (ERK) signaling participates in repression of nuclear factor (NF)-κB activity by glucocorticoids

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ABSTRACT

Glucocorticoid (GC) effects are mediated via the GC-receptor (GR), which either stimulates or represses gene expression. Repression of target genes often involves negative cross-talk between the GR and other transcription factors e.g. NF-κB, important for gene activation. Using HEK293 cells we here describe that repression of NF-κB requires functions of the GR that are dependent on the signaling pathways employed to activate NF-κB. While a GR mutant was able to repress NF-κB activity following activation by TNFα, it did not so following activation by the phorbol ester TPA. In these cells, TPA stimulation but not TNFα, activated extracellular signal-regulated kinase (ERK). We demonstrated that the ability of the dexamethasone activated GR mutant to repress TPA-induced NF-κB activity was restored in conjunction with ERK1/2 inhibition. Previous reports have shown GC-mediated inhibition of ERK1/2 phosphorylation to involve GC induction of MAPK phosphatase-1 (MKP-1). Here, we demonstrated that the GR_{R488Q} mutant was incapable of inducing gene expression of endogenous MKP-1 following dexamethasone treatment, in contrast to the GR_{wt}. However, TPA treatment alone resulted in much stronger MKP-1 expression in both GR_{wt} and GR_{R488Q} containing cells than that of dexamethasone suggesting that the inability of GR_{R488Q} to inhibit TPA-induced NF-κB activity did not involve a lack of MKP-1 expression. In line with this, RNAi targeted towards MKP-1 did not abolish or inhibit the ability of the GR_{wt} to repress NF-κB activity. Importantly, we observed no difference in activated ERK1/2 (phospho-ERK1/2) expression over time between GR_{wt} and GR_{R488Q} containing cells following co-treatment with TPA and dexamethasone. Based on these results we suggest that GR_{wt} does not directly regulate ERK1/2 but rather alters ERK1/2-mediated effects allowing it to repress NF-κB activity, a capacity lacked by the GR_{R488Q} mutant.

1. Introduction

The cell is constantly exposed to a number of signals that simultaneously trigger several separate signal transduction pathways. Activation of cellular signaling cascades ultimately leads to changes in the activity of nuclear transcriptional regulators that modulate gene transcription. In order for the cell to handle multiple transduction pathways activated simultaneously, sometimes signaling opposite effects, the cell utilizes highly controlled ways to integrate the different transduction cascades. This communication between different signaling pathways is referred to as "cross-talk". This usually involves a physical interaction between proteins belonging to the individual signal transduction pathways or enzymatic modification e.g. phosphorylation/dephosphorylation that ultimately will change the activity of the effector proteins. This cross-talk does not only integrate multiple signals but also contributes to the generation of cell-specific responses, dependent on which signal transduction pathways are activated at a given time and the "menu" of available active factors present in the responsive cell.

One physiologically important example of cross-talk between separate signaling pathways is the repression of NF-κB activity by glucocorticoids (GCs). This cross-talk constitutes a key component for the anti-inflammatory effect exerted by GCs and may also be of importance in the anti-proliferative activity of GCs [1–4]. NF-κB is a transcription factor and a key regulator of a variety of genes involved in e.g. inflammatory processes, growth control and apoptosis [5]. In its inactive form, NF-κB resides in the cytoplasm through its interaction with the inhibitory protein, IκB, which masks the nuclear localization signal of the NF-κB complex. A variety of stimuli trigger activation of NF-κB, including proinflammatory cytokines e.g. tumor necrosis factor α (TNFα), phorbol esters and lipopolysaccharides. Activation by these agents leads to phosphorylation of IκB, targeting it to ubiquitin dependent degradation by the 26S proteasome, thus, allowing the NF-κB complex to translocate to the nucleus, interact with NF-κB binding sites in target genes and to stimulate gene transcription [6]. Dependent on the signal used to stimulate NF-κB and the cell type

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analyzed a variety of additional signal transduction pathways may simultaneously be activated in the cell e.g. the mitogen-activated protein kinase (MAPK) signaling cascade. These additional pathways may directly influence NF-κB activity or they may regulate a third pathway that in turn influences NF-κB activity [7,8].

GCs exert their effects following binding to the GC-receptor (GR). The GR functions as a ligand (GC) dependent transcription factor. In the absence of ligand, the receptor resides in the cytoplasm and upon ligand binding the GR homodimerizes, becomes hyper-phosphorylated and translocates to the nucleus [4,9]. The GC activated GR acts at the level of gene transcription, where it either activates or represses expression of target genes. Transcriptional activation usually occurs when GR binds to so called GC-responsive elements (GREs) in promoter regions of target genes. Repression of transcription by the GR is often GRE-independent and takes place via a physical interaction between the GR and other transcription factors or co-activators without the GR directly contacting the DNA. One such transcription factor targeted by the GR is the NF-κB. In this case, the GR is thought to inactivate NF-κB without displacing NF-κB from the DNA [10,11]. This protein-protein interaction between GR and NF-κB on the DNA (tethering) is a key step in the cross-talk mechanism by which GCs repress NF-κB activity [2,4]. Furthermore, it has been shown that this interaction leads to the displacement or blockage of recruitment by NF-κB of co-activators important in transcriptional activation by NF-κB [12–15]. GR mediated interference with NF-κB activity has also been suggested in some cells to involve up-regulation of the inhibitory protein, IκBα that may lead to retention of NF-κB in the cytoplasm (see [1,2,4] for refs). It has also been demonstrated that GCs can inhibit the activity of the mitogen-activated protein kinase (MAPK) signaling cascades, including the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway [16–21]. ERK1/2 is activated by a series of up-stream phosphorylation events following stimulation with e.g. growth factors and phorbol esters [22]. ERK1/2 activation involves dual phosphorylation at conserved tyrosine and threonine residues by the immediate up-stream kinases, MEK1/2. Negative regulation of ERK1/2 takes place, at least in part, through dephosphorylation of ERK1/2 by dual-specificity phosphatases, e.g. MAPK phosphatase-1 (MKP-1) also called dual specificity phosphatase 1 (DUSP1) [23–26]. We and others have shown that GCs can induce MKP-1 expression in a wide variety of cell types [18,19,27–33], which could explain GC inactivation of the ERK signaling pathway. However, reports demonstrating the effects of MKP-1 inactivation for GR dependent responses are contradictory [34,35]. More specifically, the role of GC-induced MKP-1 expression in NF-κB repression by GCs is unclear.

We have previously demonstrated that a point mutation (R→Q) at position 488 (rat nomenclature) in the GR DNA binding domain (DBD) impairs the capacity of the receptor to repress TPA-induced NF-κB activity [36]. Interestingly, GR_{R488Q} failed to repress NF-κB activity despite still being able to physically interact, similarly to the wild type GR (GR_{wt}), with NF-κB. However, during our further investigations of the mechanism responsible for the failure of GR_{R488Q} to repress NF-κB activity, we observed that this was dependent on the signal used to activate NF-κB. In contrast to the inability of GR_{R488Q} to repress NF-κB activity following TPA administration, GR_{R488Q} was able to repress NF-κB activity when activated by TNFα (presented in this report). Thus, the inability of GR_{R488Q} to repress NF-κB activity involved separate GR functions dependent on the signaling transduction pathways activated in the cells. The aim of this study was to evaluate the importance of ERK MAPK signaling in GC-mediated repression of NF-κB activity and a possible role of GC-induced up-regulation of MKP-1 expression for this response. We here show that the ability of GR to efficiently repress NF-κB in HEK293 cells requires inhibition of ERK1/2 signaling. However, GR-mediated NF-κB repression does not depend on GC-induced up-regulation of MKP-1 expression or GC inhibition of ERK itself, but rather involves the alteration of effects mediated by activated ERK.

2. Materials and methods

2.1. Reagents and chemicals

Dexamethasone (Dex) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma (St Louis, MO, USA). TNFα and U0126 were purchased from Boehringer Mannheim (Indianapolis, IN, USA) and Cell Signaling Technology (Beverly, MA, USA), respectively. The culture media DMEM (high glucose) and F12 (HAM), penicillin/streptomycin, zeocin, hygromycin, L-glutamine, and Lipofectin® and Lipofectamin® 2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). FuGENE® 6 reagent was purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA). Fetal bovine serum (FBS) was purchased from Saveen Werner AB (Malmö, Sweden) and the chemiluminescence reagents used for measurement of alkaline phosphatase and luciferase activity were purchased from Perkin Elmer Life Sciences (Boston, MA, USA) and BioThema (Handen, Sweden), respectively. Primers were obtained from DNA Technology A/S (Aarhus, Denmark) and all reagents used for real-time PCR were purchased from Applied Biosystems (Foster City, CA, USA).

2.2. Reporter and expression plasmids

The luciferase reporter plasmid 3x(NF-κB)tk-Luc [37], the dominant-negative MEK1 expression plasmid S222A [38], the rat GR expression and 2x(GRE)tk-Luc plasmids [39] have been described previously. CMV-ALP was used as internal control to normalize for differences in transfection efficiency.

2.3. Cell culture

Maintenance of HEK293 Flp-In cells (Invitrogen, Carlsbad, CA, USA) and generation of pooled clones of HEK293 Flp-In cells stably transfected with wild type GR (GR_{wt}) or the GR mutant GR_{R488Q} have previously been described [36]. All clones express similar amounts of GR protein (~80,000 receptors per cell).

2.4. Transfection

Lipofectin®, FuGENE® 6 or Lipofectamin® 2000 (for RNAi experiments) was used in all transfection experiments according to the manufacturer's instructions. The GR expression plasmids and the reporter gene plasmids were used at a concentration of 25 ng/well and 200 ng/well, respectively. The plasmid CMV-ALP was used as an internal control at a concentration of 1 ng/well. Briefly, 30,000 cells/well were seeded in 24-well plates 24 h prior to transfection. Twenty hours post transfection, cells were exposed to treatment, 100 nM dexamethasone ± 5 ng/ml TPA or ± 200 U/ml TNFα in the presence or absence of 10 μM U0126, for 20 h and the cell medium was collected and cell extract was prepared for measuring alkaline phosphatase and luciferase activities as described [36].

2.5. RNAi experiments and real-time PCR (RT-PCR)

RNAi for human MKP-1 (also called DUSP1) was obtained from Invitrogen. The target sequence (sense strand) for the MKP-1 validated StealthTM RNAi duplex 1 is 5′-GCCUUGAUCAACGUCUCAGCCAAUU-3′ and for duplex 2, 5′-CCACCAUCUGCCUUGCUUACCUUAU-3′. Control RNAi with the same GC content was also obtained from Invitrogen (Cat. No. 12935-300). 300,000 cells/well of HEK293 cells stably expressing the GR_{wt} were plated in six-well plates and transfected the next day using Lipofectamin® 2000 (Invitrogen) according to the protocol provided by the manufacturer. Cells were transfected with 400pmol of RNAi in a volume 0.5 ml for 24 h after which the cells were exposed to an additional 0.5 ml of complete medium containing vehicle, 100 nM Dex, 5 ng/ml TPA (final concentrations) or a

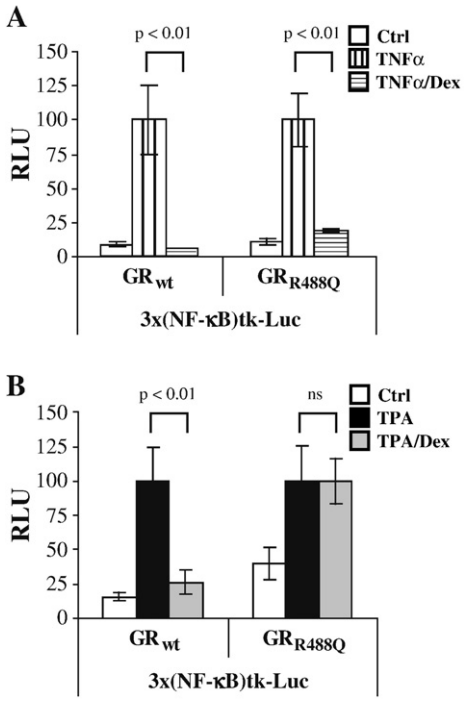

Fig. 1. GR_{R488Q} has the ability to repress NF-κB activity stimulated by TNFα but not TPA. (A) Both GR_{wt} and GR_{R488Q} repress TNFα-stimulated NF-κB activity. Pooled clones of HEK293 cells stably expressing GR_{wt} and GR_{R488Q}, respectively, were transiently transfected with an NF-κB-regulated reporter gene (3x(NF-κB)tk-Luc) and the internal control vector CMV-ALP. 20 h post transfection cells were exposed to vehicle (open bars), 200 U/ml TNFα (vertical striped bars) or 200 U/ml TNFα + 100 nM Dex (horizontal striped bars) for 20 h. (B) GR_{wt}, in contrast to GR_{R488Q} mutant, represses TPA-stimulated NF-κB activity. Same protocol as in "A" with the exception that 5 ng/ml TPA (black bars) or 5 ng/ml TPA+100 nM Dex (gray bars) were used. The luciferase activity was normalized to the activity of the internal control (alkaline phosphatase). The luciferase activity following TNFα and TPA stimulation, respectively, was given the nominal value of 100 in each experiment and the results for the other conditions were expressed relative to this nominal value. RLU, relative light units. Data represent mean ± SD. ns, not significant. Each experiment was performed in triplicate and repeated three times.

combination thereof for an additional 16 h without removing the RNAi. Cells were harvested, total RNA isolated and mRNA expression was analyzed by RT-PCR as described [36]. Forward primer for MKP-1 is 5′-CGAGGCCATTGACTTCATAGACT-3′ and the reverse primer is 5′-CAAACACCCTTCCTCCAGCAT-3′. Primers for analysis of Cox-2, JunB and GAPDH mRNA, the latter used to normalize all qRT-PCR results, have been given before [36]. In the case of the studies of the effect of MKP-1 RNAi on NF-κB-Luc activity, 3x(NF-κB)tk-Luc and CMV-ALP reporter plasmids were first transfected with FuGENE® 6 reagent for 6 h according to the manufacturer's description. After allowing the cells to recover for 18 h, cells were then retransfected with RNAi using Lipofectamin® 2000 as described above.

2.6. Western blot analysis

Whole cell extract was prepared from cells cultured to subconfluence by lysing the cells in ice-cold Nonidet P-40 (NP-40) buffer (0.5% NP-40, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA) for 20 min. Cell debris was removed by centrifugation at 14,000 ×*g* for 15 min at 4 °C and an equal volume of 2×SDS loading buffer was added to the supernatant and the mixture was boiled for 2 min. Protein concentrations were determined with the BioRad protein assay kit according to the instructions from the manufacturer (BioRad, Hercules, CA, USA). Samples were separated by 9% SDS-polyacrylamide gel electrophoresis and electroblotted onto a Hybond C-extra membrane (Amersham Biosciences, UK). The immunoblot was probed with an antibody against MKP-1 (sc-1104, Santa Cruz Biotechnology, CA, USA). ERK1/2 or a phospho-specific ERK1/2 antibody (Cell Signaling Technology, MA, USA) or β-actin (Abcam, Cambridge, UK) followed by a secondary horseradish peroxidase-labeled anti-rabbit antibody (Amersham Biosciences, UK). The immunoreactivity of ERK, MKP-1 and β-actin was visualized using the enhanced chemiluminescence kit (Amersham Biosciences, UK) according to the manufacturer's instructions.

2.7. Statistics

Data were analyzed using a two-tailed Student's *t*-test. The results were expressed as means ± SD.

3. Results

3.1. TNFα- but not TPA-induced NF-κB activity is repressed by GR_{R488Q}

The previously described GR mutant, GR_{R488Q}, despite physically interacting with NF-κB, lacks the ability to repress NF-κB activity following activation by phorbol ester (TPA) [36]. This suggests that physical interaction between GR and NF-κB is not sufficient to impair NF-κB activity. To search for other mechanisms that are important for GC-mediated repression of NF-κB activity, we tested if the impaired ability of GR_{R488Q} to repress NF-κB activity was dependent on the stimuli used to activate NF-κB. For this purpose we activated NF-κB using TNFα, another potent NF-κB activator, in HEK293 cells. Transient transfection of the reporter gene 3x(NF-κB)tk-Luc was used to measure NF-κB activity in pooled HEK293 clones stably expressing GR_{wt} and GR_{R488Q}, respectively. The results showed that, similar to GR_{wt}, dexamethasone activated GR_{R488Q} was able to repress TNFα-induced NF-κB activity (Fig. 1A). This was in contrast to TPA-induced NF-κB activity, which only was repressed by GR_{wt} and not by GR_{R488Q} (Fig. 1B). This demonstrated that this mutation impaired the NF-κB repressive property of the GR during defined, but not all, conditions. This also showed that the inability of GR_{R488Q} to repress NF-κB activity was not due to *bona fide* alterations in the receptor protein. Accordingly, this provided evidence for that GR-mediated repression of activated NF-κB required separate functions of the GR, functions that were dependent on the signaling pathway employed to activate NF-κB.

3.2. TPA, but not TNFα , activates ERK1/2 in HEK293 cells and TPA-induced NF-κB activity is repressed by GR_{R488Q} in conjunction with ERK1/2 inhibition

The reason for the different ability of GR_{R488Q} to repress NF-κB activity following TPA and TNFα stimulation respectively, may correlate to different signaling pathways being activated following treatment with respective stimuli. We observed that the mitogen-activated protein kinase (MAPK) pathway was differentially activated following TPA versus TNFα stimulation in HEK293 cells. Immunoblotting following TPA and TNFα treatment showed that TPA treatment but not TNFα treatment resulted in ERK1/2 activation (Fig. 2). This differential profile of ERK1/2 activation in response to a given stimuli could explain why GR_{R488Q} was able to repress NF-κB activity following TNFα stimulation but not after TPA stimulation.

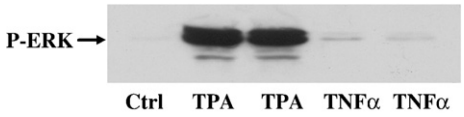

Fig. 2. TPA but not TNFα induces phosphorylation of ERK1/2 in HEK293 cells. Western blot analysis of phosphorylated ERK1/2 in HEK293 cells following 15 min exposure to vehicle (Ctrl), 5 ng/ml TPA or 200 U/ml TNFα. ERK1/2 phosphorylation was assessed using a phospho-specific ERK antibody.

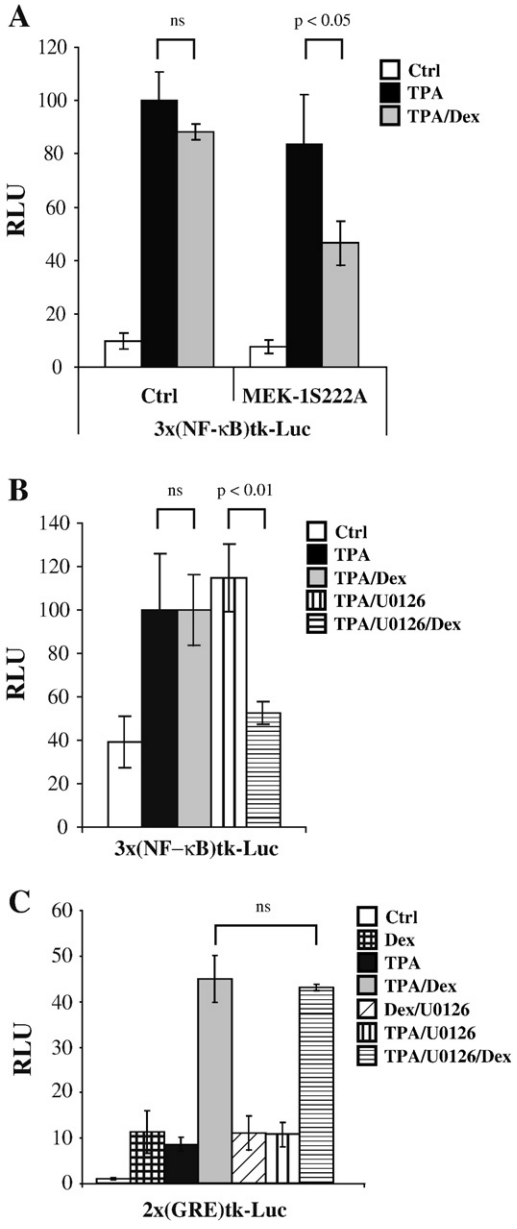

Fig. 3. MEK-1 inhibition by the dominant-negative MEK-1 S222A mutant or the MEK-1 inhibitor U0126 restores the ability of GR_{R488Q} to repress TPA-induced NF-κB activity. (A) HEK293 cells stably expressing GR_{R488Q} were transiently co-transfected with an NF-κB-regulated reporter gene (3x(NF-κB)tk-Luc), the internal control vector CMV-ALP and the dominant-negative MEK-1 expression plasmid MEK-1 S222A or empty expression vector as control. In (B) the MEK-1 inhibitor U0126 was used instead of the MEK-1 S222A expression plasmid. 20 h post transfection cells were exposed to vehicle (open bars), 5 ng/ml TPA (black bars) or 5 ng/ml TPA + 100 nM Dex (gray bars) and 10 μM U0126 when indicated for 20 h. U0126 was added 1 h prior to the other treatments. RLU from TPA treatment alone in the absence of MEK-1 S222A or U0126 was given the nominal value of 100 and the results for the other conditions were expressed relative to this nominal value. (C) HEK293 cells stably expressing GR_{R488Q} were transiently co-transfected with the 2x(GRE)tk-Luc reporter gene and the internal control vector CMV-ALP and treated with dexamethasone (100 nM), TPA (5 ng/ml) or a combination thereof in the presence or absence of 10 μM U0126 for 20 h. U0126 was added 1 h prior to the other treatments. RLU from untreated samples were given the nominal value of 1 and the results for the other conditions were expressed relative to this nominal value. Data represent mean ± SD. The experiments were performed in triplicate and repeated three times. ns, not significant.

A hypothesis that absence of ERK1/2 activity or ERK-mediated responses is required for GC-induced repression of NF-κB activity would predict that inhibition of ERK1/2 will restore the ability of GR_{R488Q} mutant to inhibit NF-κB following TPA stimulation. To test this prediction, we inhibited ERK activation by blocking the upstream ERK-activating kinase MEK-1 using a dominant-negative MEK-1 mutant S222A [38]. HEK293 cells stably expressing GR_{R488Q} were transfected with an NF-κB-regulated reporter gene and an expression vector for MEK-1 S222A followed by TPA treatment in the presence or absence of dexamethasone. In line with the hypothesis, MEK-1 inhibition by the dominant-negative MEK-1 S222A restored the ability of GR_{R488Q} to repress TPA stimulated NF-κB activity in the presence of dexamethasone (Fig. 3A). The same result was seen when ERK1/2 was inhibited by the MEK-1 inhibitor U0126 (Fig. 3B). Co-transfection of the dominant-negative MEK-1 mutant S222A resulted in a small but

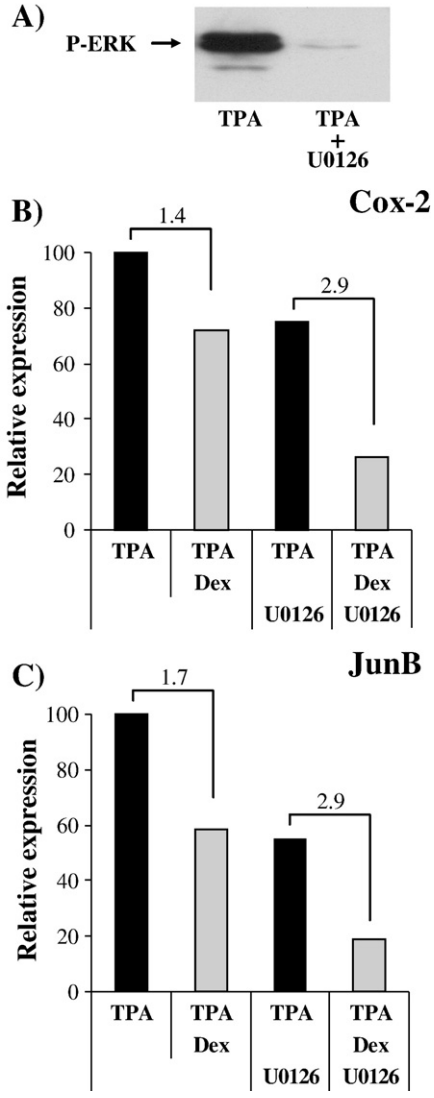

Fig. 4. MEK-1 inhibition by the specific metabolic inhibitor U0126 enhances the ability of GR_{R488Q} to repress TPA-induced Cox-2 and JunB expression. (A) Western blot analysis of ERK phosphorylation following stimulation by TPA. HEK293 cells were treated with 5 ng/ml TPA (TPA) or 5 ng/ml TPA + 10 μM U0126 (TPA + U0126). U0126 was added 1 h prior to the TPA treatment (15 min). ERK1/2 phosphorylation was assessed using a phospho-specific ERK antibody. (B and C) HEK293 cells stably expressing GR_{R488Q} were treated with 5 ng/ml TPA (black bars) or 5 ng/ml TPA + 100 nM Dex (gray bars) for 2 h in the presence or absence of 10 μM U0126. The U0126 was added 1 h prior to the other treatments. Total RNA was extracted from the cells and Cox-2 (B) and JunB (C) mRNA expression was determined by RT-PCR. Results were related to GAPDH mRNA expression. TPA treatment alone was given the nominal value of 100 and the results from the other conditions were expressed relative to this value. Data shows the mean of two experiments.

non-significant reduction of TPA-stimulated NF-κB activity (Fig. 3A), while U0126 treatment resulted in small but non-significant increase (Fig. 3B). Similarly, no significant change in NF-κB activity after stimulation by TNFα in the presence of the ERK inhibitor U0126 was observed (data not shown). These results clearly showed that GC-mediated NF-κB repression was dependent on the absence of activated ERK1/2 or ERK1/2-mediated effects following stimulation by TPA and that ERK1/2 inhibition had no or only a minor effect on TPA or TNFα stimulated NF-κB activity in the absence of dexamethasone.

Previous reports have suggested that ERK1/2 may directly inhibit GR activity [40–42]. Thus, the ability of GR_{R488Q} to repress NF-κB activity following ERK1/2 inhibition may be due to a relief of the inhibitory effect by the ERK1/2 inhibitors. To test this, HEK293 cells stably transfected with GR_{R488Q} were transiently transfected with the 2x(GRE)tk-Luc reporter gene in the absence or presence of the ERK1/2 inhibitor U0126. As can be seen in Fig. 3D, dexamethasone induced GR_{R488Q} to activate the 2x(GRE)tk-Luc reporter gene 11-fold in the presence or absence of U0126. As previously demonstrated, this was markedly less as compared to the $GR_{wt,}$ which induced GRE-Luc activity 80-fold ([36] and data not shown). TPA alone induced the reporter gene 8- and 11-fold in HEK293 GR_{R488Q} cells in the absence and presence of U0126, respectively. Co-treatment with TPA + dexamethasone resulted in a slight synergistic activity (Fig. 3D). More importantly, no difference in the response in the absence or presence of U0126 was seen, demonstrating no enhanced GR activity in the presence of ERK1/2 inhibition. This showed that TPA and ERK1/2 activation in itself did not block GR activity in the HEK293 cells.

3.3. Inhibition of ERK1/2 activity enhances the ability of GR_{R488Q} to repress endogenous NF-κB-regulated genes following dexamethasone administration

To test whether ERK1/2 inhibition also affects repression of endogenous NF-κB-regulated genes, GR_{R488Q} containing HEK293 cells were treated with the MEK-1 inhibitor U0126 in conjunction with TPA activation. Previous results have shown that GR_{R488Q}, in contrast to the GR_{wt}, is impaired with regard to its capacity to repress the endogenous NF-κB-regulated genes Cox-2 and JunB following activation by TPA [36]. HEK293 cells stably expressing the GR_{R488Q} mutant were treated with TPA and dexamethasone in the presence or absence of U0126 followed by analysis of Cox-2 and JunB expression by real-time PCR (RT-PCR). The ability of U0126 to inhibit ERK1/2 activation in HEK293 cells was confirmed by Western blot analysis (Fig. 4A). Fig. 4B demonstrates that inactivation of ERK in HEK293 GR_{R488Q} cells by U0126 enhanced fold-repression by dexamethasone of the endogenous NF-κB-regulated gene Cox-2. Similar results were seen for JunB gene expression (Fig. 4C). The ability of the ERK1/2 inhibitor U0126 to enhance fold repression of Cox-2 and JunB following GC treatment supported the conclusion that absence of ERK1/2 signaling or effects also was important for efficient GC inhibition of endogenous NF-κB-regulated genes following TPA stimulation in HEK293 cells. Furthermore, U0126 seemed to repress TPA-stimulated Cox-2 and JunB expression by ~30–40%, indicating a role of ERK in TPA activation of Cox-2 and JunB expression. However, since ERK inhibition had no or only a minor effect on NF-κB activity

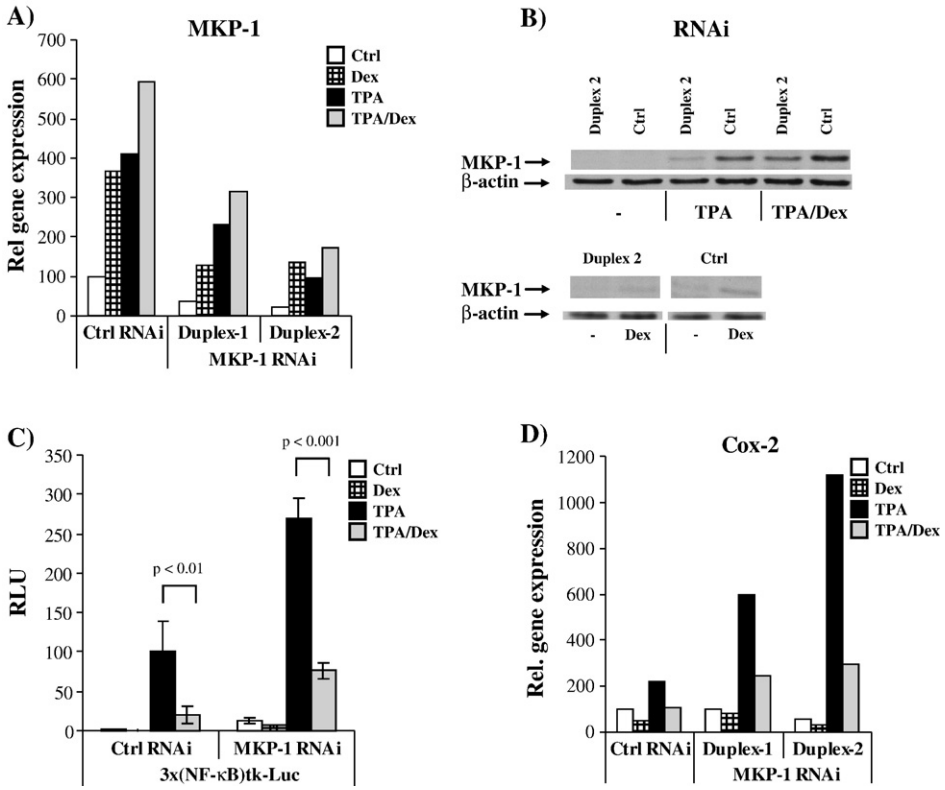

Fig. 5. Reducing MKP-1 expression by RNAi does not impair the ability of the GR_{wt} to repress TPA-stimulated NF-κB activity or endogenous Cox-2 expression. HEK293 cells stably expressing the GR_{wt} were transfected with RNAi's oligos targeting MKP-1 mRNA or control RNAi. Twenty-four hours post transfection, cells were exposed to vehicle (open bars), 100 nM Dex (squared bars), 5 ng/ml TPA (black bars) or 5 ng/ml TPA + 100 nM Dex (gray bars) for 16 h (without removing the RNAi's). Total RNA was extracted and mRNA expression for MKP-1 (A) and Cox-2 (D) was analyzed by RT-PCR. All values are related to GAPDH mRNA expression which was unaffected by the MKP-1 RNAi's or control RNAi treatment. The relative mRNA expression are all expressed in relation to the mRNA expression obtained in cells transfected with control RNAi and no treatment and given the nominal value of 100. Panels A and D represent the results obtained from one out of two experiments with similar results. (B) Protein levels of MKP-1 following RNAi transfection was assessed by Western immunoblotting (C) HEK293 cells were transiently co-transfected with GR_{wt} expression vector, the NF-κB regulated reporter gene (3x(NF-κB)tk-Luc) and the internal control vector CMV-ALP. After recovering for 18 h the cells were re-transfected with control or MKP-1 RNAi (100 nM). Twenty-four hours later cells were treated as above for 16 h where after cell extracts were prepared and luciferase activity measured. The RLU from cells transfected with control RNAi in the presence of TPA was given the nominal value of 100 and the results for the other conditions were expressed relative to this nominal value. Data represent mean ± SD (n = 3).

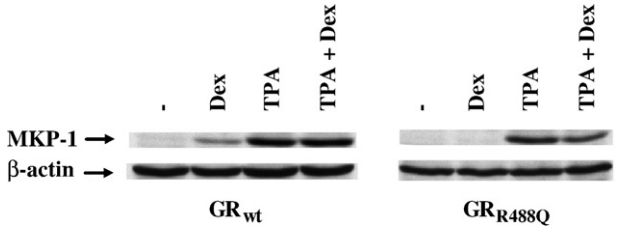

Fig. 6. TPA induces MKP-1 expression in HEK293 cells stably transfected with GR_{wt} or GR_{R488Q} while dexamethasone induces MKP-1 expression only in HEK293 GR_{wt} cells. No evidence for a synergistic effect. HEK293 cells stably transfected with GR_{wt} or GR_{R488Q} were treated with dexamethasone (100 nM), TPA (5 ng/ml) or a combination thereof for 16 h and analyzed for MKP-1 expression by Western immunoblotting. Equal protein loading in each lane was determined by re-probing the filters with a β-actin antibody.

following TPA stimulation (cf. Fig. 3A and B), it is more likely that the effect of U0126 on TPA-stimulated Cox-2 and JunB expression was mediated via other regulators of Cox-2 and JunB expression than NF-κB.

3.4. The dual-specificity phosphatase MKP-1 is not involved in GC-mediated repression of NF-κB activity in the presence of activated ERK

Negative regulation of the ERK1/2 MAPK pathway can take place through dephosphorylation of ERK1/2 by phosphatases, e.g. MAPK phosphatase-1 (MKP-1). Previous reports have shown GC-mediated inhibition of ERK1/2 phosphorylation concomitant with a GC-stimulated up-regulation of MKP-1 expression [18,19,43] and it has been suggested that MKP-1 may participate in the anti-inflammatory actions of GCs [44]. However, the effect of MKP-1 on NF-κB is not known. Unlike the GR_{wt}, the GR_{R488Q} mutant is not able to induce MKP-1 gene expression following dexamethasone treatment (see Fig. 6). This suggests the possibility that lack of MKP-1 induction by GR_{R488Q} was responsible for the inability of the mutant to inactivate ERK1/2 and as a consequence was non-productive in GC-mediated down-regulation of NF-κB activity (cf. Fig 3A and B). This implies that inhibition of MKP-1 expression would result in a loss of the ability of the GR_{wt} to repress NF-κB activity. To evaluate the importance of GC-stimulated MKP-1 expression in relation to GR-mediated NF-κB repression, MKP-1 mRNA was targeted for degradation by RNA interference. For this purpose, HEK293 cells expressing the GR_{wt} were transfected with RNAi and the NF-κB reporter gene. Cells transfected with MKP-1 RNAi displayed reduced MKP-1 mRNA and protein expression as compared to transfection with control RNAi following TPA stimulation alone or in the presence of dexamethasone (Fig. 5A and B). Following reduction of MKP-1 expression by MKP-1 RNAi, the expression of the NF-κB reporter gene revealed an increased response to TPA administration (Fig. 5C). However, fold repression of NF-κB activity by dexamethasone in the presence of TPA and MKP-1 RNAi was not affected in comparison to when using control RNAi. The same results were seen on the endogenous NF-κB regulated Cox-2 gene (Fig. 5D). These results showed that GC-induced MKP-1 expression is not involved in GR-mediated repression of NF-κB activity or Cox-2 expression in the presence of activated ERK1/2. Furthermore, since TPA in itself was much more efficient in inducing MKP-1 expression in comparison to dexamethasone in both GR_{wt} and GR_{R488Q} containing cells as assayed by Western immunoblotting (Fig. 6), this further suggested that the relatively weak induction of MKP-1 expression by dexamethasone in GR_{wt} containing cells did not contribute to the NF-κB repression by GCs. Additionally, no synergistic activation of MKP-1 expression following a combined treatment with TPA and dexamethasone was observed (Fig. 6).

3.5. The differential ability to repress TPA-induced NF-κB activity by GR_{wt} vs. GR_{R488Q} does not involve a difference in ERK1/2 dephosphorylation

To evaluate whether there was a difference in the ability of GR_{wt} and GR_{R488Q} containing cells to differentially affect ERK1/2 activity independent of MKP-1, we analyzed by Western immunoblotting, the presence of activated ERK1/2 (phospho-ERK1/2) expression over time in both GR_{wt} and GR_{R488Q} containing cells in the presence of both TPA and dexamethasone. As can be seen in Fig. 7, activated ERK1/2 declined in both GR_{wt} and GR_{R488Q} containing cells. Importantly, no difference in the presence of activated ERK1/2 over time (corresponding to the time of TPA plus dexamethasone treatment in the transfection experiments) between the cells was seen. In addition, the change in activated ERK1/2 over time was not dependent on whether dexamethasone was present or absent during TPA stimulation (data not shown). Considering that ERK1/2 inhibition restored the ability of GR_{R488Q} to repress NF-κB activity (Fig. 3A and B), but no difference in the ability of GR_{wt} and GR_{R488Q} to directly repress ERK1/2 activity was seen, suggested that the GR_{wt}, in contrast to GR_{R488Q}, acted by inactivating or reversing ERK1/2-mediated effects allowing NF-κB repression to occur.

4. Discussion

Cross-talk between the GR and NF-κB signaling is well recognized. Also negative influence of GCs on MAPK pathway signaling has been demonstrated (for reviews, see [4,17]). However, the importance of a functional inactivation of ERK1/2 MAPK signaling by GCs in relation to GR-mediated responses is not well established.

The GR_{R488Q}, despite physically interacting with NF-κB, has an impaired capacity to repress NF-κB activity following activation by the phorbol ester TPA [36]. Interestingly, we here show that this is not the case when NF-κB is activated by TNFα. This implies that mechanisms additional to a physical GR interaction with NF-κB are important for GC repression of NF-κB activity, mechanisms that are dependent on concomitant signaling pathways activated in the cells. Using the

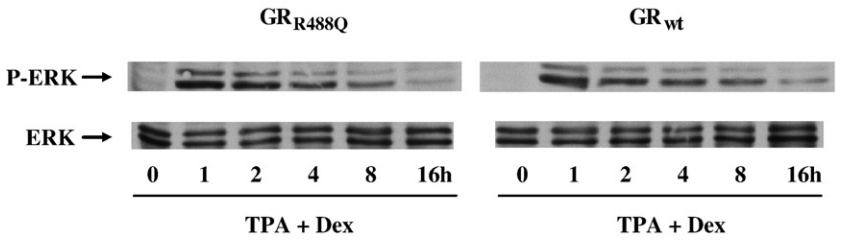

Fig. 7. No difference in phospho-ERK1/2 levels over time in GR_{wt} and GR_{R488Q} containing cells following co-treatment with TPA and dexamethasone. HEK293 cells stably transfected with GR_{wt} or GR_{R488Q} were co-treated with dexamethasone (100 nM) and TPA (5 ng/ml) and analyzed for phospho-ERK1/2 activity (upper panel) from 0 to 16 h by Western immunoblotting. Lower panel, the membrane was stripped and reprobed with a phosphorylation state-independent ERK1/2 antibody.

GR_{R488Q} mutant as an investigative tool, we here provide evidence that efficient GC repression of NF-κB requires inhibition of ERK1/2 signaling under conditions when this MAPK signaling pathway is activated.

The importance of ERK1/2 MAPK signaling pathway inactivation for GR repression of NF-κB activity in the HEK293 cells was directly demonstrated using specific ERK1/2 inhibitors (dominant-negative MEK-1 and U0126) that restored or enhanced the ability of GR_{R488Q} to repress NF-κB activity. This was also the case for endogenous NF-κB regulated genes as demonstrated for Cox-2 and JunB. Furthermore, GR_{R488Q} maintains its capacity to repress AP-1 activity following TPA stimulation [36]. These results together with the ability of the GR_{R488Q} to repress NF-κB following activation by TNFα, demonstrated that the inability to repress NF-κB following activation by TPA was not intrinsic to the GR_{R488Q} mutant but dependent on concomitant signaling pathways activated in the cells. Why inhibition of ERK1/2 activity restored or enhanced GR_{R488Q}'s ability to repress NF-κB activity and endogenous NF-κB-regulated genes in the HEK293 is unclear, but is unlikely to be related to an impaired GR function due to ERK1/2 mediated GR phosphorylation as previously suggested in other cells [40,42]. This conclusion is based on that addition of U0126 did not enhance the ability of GR_{R488Q} to activate from a GRE in the presence of activated ERK (Fig. 3C). Secondly, GR_{R488Q} is still functional in repression of AP-1 activity in HEK293 cells following stimulation by TPA [36] and thirdly, the GR mutation does not involve a ERK1/2 phosphorylation site. Furthermore, ERK does not seem to influence NF-κB activity following TPA or TNFα stimulation, since expression of the dominant negative MEK-1 plasmid or addition of the ERK inhibitor U0126 had only minor or no effect on NF-κB activity (Fig. 3A and B and data not shown). Instead, the ability of ERK1/2 to influence GR activity may rather relate to the phosphorylation of other proteins e.g. coactivators preventing GR to inhibit their activities. Indeed, Rogatsky et al. have suggested that ERK influences GR_{wt} transcriptional activity indirectly, likely through a co-factor, in contrast to c-Jun N-terminal kinase (JNK), which seems to mainly act via receptor phosphorylation of Ser-246 [41]. In the absence of activated ERK1/2, as seen following TNFα stimulation of HEK293 cells, the necessity of inactivation of ERK1/2 effects is not required, allowing also GR_{R488Q} to repress NF-κB activity (Fig. 1). Why TNFα does not activate ERK1/2 MAPK signaling in HEK293 cells is unclear.

Several studies have demonstrated an induction of MKP-1 in a number of cell lines following GC administration [18,19,27–33]. However, the importance of MKP-1 induction by GCs for dephosphorylation of ERK and for GC-regulated responses is contradictory [35,44–46]. Furthermore, an effect specifically on NF-κB activity by GC-stimulated MKP-1 expression has not been established. Although both Abraham et al. and Maier et al. in experiments using macrophages or mast cells with ablated MKP-1 expression presented contradictory results with regard to the role of MKP-1 for GC repression of target genes and anti-inflammatory effects *in vivo*, they both showed that ablated MKP-1 expression impaired GC-mediated repression of JNK and p38 MAPKs but had no effect on ERK activation and its response to GCs [34,35]. While Maier et al. [35] reported no impairment of the ability of GCs to repress proinflammatory cytokines or chemokines in bone marrow derived mast cells with ablated MKP-1 expression, Abraham et. al. [34] showed impaired GC repression of a subset of NF-κB regulated inflammatory mediators in bone marrow derived macrophages from MKP-1 knockout mice. This differential response of NF-κB regulated genes implies that MKP-1 effects on NF-κB are not responsible for the differential response to GCs. In line with this suggestion, we in the present report were unable to demonstrate involvement of MKP-1 in the regulation of ERK activity and GC repression of NF-κB activity in the HEK293 cells. First, TPA treatment alone of HEK293 cells stably expressing GR_{R488Q} efficiently induced MKP-1 expression and this induction was much more pronounced than the MKP-1 induction seen following dexamethasone treatment of HEK293 GR_{wt} cells (Fig. 6). Thus, the contribution of MKP-1 expression by GCs in the presence of TPA is minor. Secondly, although down-regulation of MKP-1 expression using RNAi enhanced NF-κB activity and expression of the Cox-2 gene following TPA stimulation of HEK293 GR_{wt} cells, no effect on the fold repression by GCs was demonstrated (Fig. 5). These results support our conclusion that GC-stimulated expression of MKP-1 is not involved in the regulation of ERK and NF-κB activity in the HEK293 cells and that the inability of GR_{R488Q} to repress ERK and NF-κB activity does not relate to the inability of $GR_{R488Q,}$ in contrast to $GR_{wt,}$ to induce MKP-1 expression in response to GCs. Importantly, no difference in levels of activated ERK1/2 was seen overtime between GR_{wt} and GR_{R488Q} containing cells following co-treatment of TPA and dexamethasone (Fig. 7). Also considering that inhibition of activated ERK1/2 by a dominant negative MEK-1 mutant or the MEK inhibitor UO126 (Fig. 3A and B) restored the ability of GR_{R488Q} to repress NF-κB activity, suggests that GR_{wt} instead has the capacity, in contrast to $GR_{R488Q,}$ to alter ERK1/2-mediated effects required for NF-κB repression to occur. In addition, since ERK inhibition did not affect TPA-stimulated NF-κB activity (Fig. 3A and B), it is unlikely that the enhancing effect of MKP-1 RNAi on TPA-stimulated NF-κB activity and Cox-2 expression is mediated via enhanced ERK activity. Rather, this may be mediated through effects on other MKP-1 targets as p38 and JNK MAPKs. This is in line with a preferred preference of MKP-1 for p38 and JNK over ERK ([47], see also above). However, cell specific differences may exist because blocking MKP-1 expression by RNAi in breast epithelial cell lines hampers GC-mediated ERK dephosphorylation and cell survival following paclitaxel induced cytotoxicity [26]. Similarly, regulation of ERK by MKP-1 controls osteoblast proliferation in response to GCs [45]. As an alternative, GCs may also block ERK activation independent of MKP-1 expression as it has previously been shown that ERK2 activation can be repressed by dexamethasone already at the level of Raf-1 [16].

Our results using GR_{R488Q} showed that it was unable to repress NF-κB activity following stimulation by the phorbol ester TPA, but did so following activation by TNFα. This seemed to relate to the differential activation of ERK1/2 in the HEK293 cells. This result stresses that the nature of the inducer of NF-κB activity will have profound effects on the mechanism by which the GR represses NF-κB. This is in line with previous results from the laboratory of Glass and co-workers, who showed that the GR only represses NF-κB dependent target genes following activation by the Toll receptors TLR4 and TLR9 but not following NF-κB activation by TLR3 [14, 15]. In this case, the different signal transduction pathways used to activate NF-κB resulted in differential co-activator utilization by NF-κB, which constituted the basis for the differential GC response of NF-κB target genes [14,15].

In summary, our experiments demonstrate that GR-mediated inhibition of ERK signaling is involved in GC repression of NF-κB activity in HEK293 cells when this signaling pathway is activated by TPA. However, this does not involve GC inhibition of ERK itself and instead seem to be dependent on GR_{wt} inhibition of ERK1/2-mediated effects, a capacity lacked by the GR_{R488Q} mutant. Our results also provided evidence for that GR-mediated repression of activated NF-κB requires separate functions of the GR, functions that are dependent on the signaling pathway employed to activate NF-κB. Finally, our results underscore the complexity and diversity in the mechanisms involved in GC repression of NF-κB activity.

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