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Regulation of glutathione *S*-transferase P1-1 gene expression by NF-kappaB in tumor necrosis factor alpha-treated K562 leukemia cells

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Abstract

Glutathione *S*-transferases (GSTs) play an important role in the protection of cells against xenobiotics and lipid hydroperoxides generated by oxidative stress. In human, the GSTP1-1 expression is commonly increased in many tumors and involved in the development of antineoplastic drug resistance. Reactive oxygen species are released at inflammation sites and oxidative stress conditions enhance the expression of genes encoding antioxidant enzymes such as GSTs. Here we investigated the regulation of the *GSTP1-1* gene expression in the K562 cell line by nuclear factor κB (NF-κB) and the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). By studying *GSTP1-1* mRNA expression and NF-κB/*GSTP1-1* promoter interactions, we showed the implication of NF-κB in the *GSTP1-1* gene expression and we described a new specific TNFα-inducible NF-κB binding site upstream of the minimal promoter. Moreover, TNFα treatment as well as cotransfection of NF-κB signaling pathway intermediates induced an activation of the *GSTP1-1* gene promoter in K562 cells. Site-directed mutagenesis of the NF-κB site strongly inhibited TNFα- and NF-κBp65-induced promoter activation. Altogether, we showed that a sequence located at −323/−314 within the *GSTP1-1* promoter bound NF-κB p50/65 and p65/p65 dimers and that this κB site was involved in the regulation of the gene by TNFα.

Keywords: GSTP1-1; TNFα; NF-κB; Leukemia; Inflammation; K562

1. Introduction

GSTs (EC 2.5.1.18) are phase II metabolizing enzymes which catalyze the conjugation of a wide range of electrophilic compounds, including carcinogens, mutagens and anticancer drugs [1–6], to the ubiquitous tripeptide glutathione (GSH). Therefore, GSTs play an important role in the protection of cells against the toxicity of xenobiotic compounds and lipid hydroperoxides generated by oxidative stress [7,8]. In leukemic lineages, studies of the expression and the activity of GST isoenzymes revealed that the human Pi class GST isoform (*GSTP1-1*) is the most represented, particularly in U937, K562 and Jurkat cells [9]. *GSTP1-1* is commonly increased in many tumors [10–14] and involved in the development of antineoplastic drug resistance [15–18]. Interestingly, *GSTP1-1* has been shown to be involved in preventing apoptosis in hematopoietic cells [19] and to protect against H_2O_2-induced cell death *via* the coordinated regulation of stress kinases [20]. On the other hand, the gene expression of inflammatory and immune responses [21–23] as well as antioxidant enzyme genes such as GSTs [24,25] are enhanced in oxidative stress conditions. However, molecular mechanisms of the *GSTP1-1* gene regulation are poorly understood. It has been shown that the region between −80 and −8 is necessary for constitutive expression of the gene [26] and the organization of a minimal promoter has been described [27]. As shown in Fig. 1, *cis*-acting elements were identified within the minimal promoter [28]. The role of the activator protein 1 (AP-1) binding site −69/−63 has been shown in the regulation of the *GSTP1-1* gene expression, notably in VCREMS breast cancer cells [29]. In addition, we have demonstrated by promoter deletion analyzes that this site was crucial for 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)-mediated GSTP1 gene transcrip-

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Abbreviations: GST, glutathione *S*-transferase; NF-κB, nuclear factor kappa-B; TNFα, tumor necrosis factor alpha.

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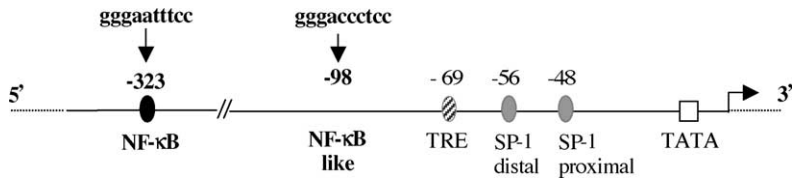

Fig. 1. *GSTP1-1* gene promoter representation. Transcription factor binding sites within the *GSTP1-1* promoter gene are described according to [28,29,31,32,52,66]. Indicated positions along the gene are relative to the transcription initiation site. Putative NF-κB binding sites are represented with their DNA sequences.

tion [30]. Two Sp1 response elements were also identified [31] as well as a NF-κB like sequence [32,33].

As GSH cellular redox status is critical for various biological phenomena including apoptosis and inflammation [34,35] we hypothesized that inflammation could modulate *GSTP1-1* gene expression. Indeed, reactive oxygen species (ROS) are released at inflammation sites and can be eliminated from cells after conjugation to GSH by GSTP1-1. TNFα also known as cachectin or differentiation-inducing factor (DIF) [36] causes inflammation, tumorigenesis, cellular differentiation and proliferation, necrotic and apoptotic cell death, by binding to TNF receptors (TNFR) 1 and 2 [37–39]. However, it is widely admitted that binding of TNFα to TNFR1 initiates the majority of the biological activities of TNFα [40]. This interaction leads to the dissociation of the silencer of the death domain from the intracellular death domain (DD) of TNFR1. The TNF receptor-associated death domain (TRADD) then interacts with TNFR1 through its DD sequence and recruits other adaptor proteins among which the TNF-R-associated receptor 2 (TRAF2). TRAF2 interacts with the NF-κB inducing kinase (NIK) which phosphorylates the enzyme inhibitor of κB kinase (IKK), itself implicated in the activation of the NF-κB transcription factors family by phosphorylation of inhibitor of κB (IκB) leading to its degradation by the proteasome. The mammalian NF-κB transcription factors are involved in immune and inflammatory responses as well as cell growth. They reside in the cytoplasm in an inactive homo- or heterodimer form bound to inhibitory proteins of the IκB family [41,42]. NF-κB activation results in its translocation to the nucleus where it will interact with its target genes, although NF-κB may shuttle between nucleus and cytoplasm in unstimulated cells [43,44]. Redox-sensitive transcription factors such as NF-κB and AP-1 are known to play a key role in proinflammatory processes such as transcription of cytokine genes, and in induction of protective antioxidant genes [45–47]. These transcription factors are modulated by TNFα [48,49] and anticancer drugs which may induce oxidative stress stimulating various signaling pathways including NF-κB [50,51].

Considering the role of the GSTP1-1 enzyme in the cellular response to oxidative stress, we investigated the involvement of both TNFα and NF-κB in the regulation of *GSTP1-1* gene expression in K562 cells. We demonstrated that the *GSTP1-1* promoter is activated by TNFα and NF-κBp65 overexpression in K562 cells and we identified a new TNFα-inducible NF-κB binding site located at −323 within the *GSTP1-1* promoter. The deletion of this site led to a drastic decrease of the TNFα-induced activation of the *GSTP1-1* promoter gene. Altogether, we report here for the first time that the *GSTP1-1* gene expression is inducible by the TNFα signaling cascade leading to NF-κB-activated *GSTP1-1* promoter.

2. Materials and methods

2.1. Cell culture

The human chronic myeloid leukemia (CML) K562 cell line (American Type Culture Collection), was maintained in culture in RPMI 1640 with Ultraglutamine 1 containing 10% fetal calf serum (FCS, BioWhittaker) and supplemented with a penicillin–streptomycin–fungizone mixture 100× (BioWhittaker). The cells in exponential growth phase were cultured in 0.1% FCS containing medium for 48 hr before TNFα treatments.

2.2. Reagents and antibodies

TNFα (Sigma) was resuspended in a 0.5% bovine serum albumin solution in sterile PBS (1×) at 10 μg/mL. Stock solutions of NF-κB inhibitors (all Calbiochem), carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) and (*E*)-3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (BAY11-7082), were resuspended at 20 mM in ethanol and 10 mM in dimethyl sulfoxide, respectively. When used, NF-κB inhibitors were added to the cell cultures 2 hr prior TNFα treatments. The anti-Rel protein antibodies αp50, αp52, αp65, αc-Rel, αrelB, and αBcl3 were purchased from Santa Cruz Biotechnology, Inc.

2.3. DNA sequence analysis

GSTP1-1 promoter sequence was analyzed using Matinspector (Genomatix) [52].

2.4. Nuclear protein extraction and electrophoretic mobility shift assay (EMSA)

Nuclear proteins from 10^7 cells were prepared as previously described [53] in the presence of protease inhibitors pepstatin, leupeptin, aprotinin (Roche), 1,10-phenanthroline

and phenylmethylsulfonyl fluoride. Nuclear protein/DNA binding activity and electrophoretic runs were performed as previously described [54]. Briefly, 10 μg nuclear extracts were incubated for 30 min on ice with [γ-^{32}P]ATP-labeled oligonucleotides in 20 μL reaction mixture containing protease inhibitors, 10 mM Tris–HCl, pH 8.5, 5% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 2.5 mM poly(dI–dC), 0.2 μg/μL BSA and 4 mg/mL spermidine. Reaction mixtures were loaded onto a 5% polyacrylamide gel (29:1 cross-linking ratio) and electrophoresis were performed. In supershift/immunodepletion experiments, the nuclear extracts and labeled probes were incubated in the reaction mixture for 30 min on ice prior a 30 min incubation with 2 μg antibody on ice. In competition experiments nuclear extracts and 50-fold molar excess of unlabeled probes were pre-incubated in the reaction mixture for 20 min on ice. The human recombinant protein NF-κBp50 at 50 gsu (for gel shift units) (Promega) was 30-fold diluted in an anti-proteases containing solution before use and 1 μL was incubated in the presence of oligonucleotidic probes. Sequences of DNA oligonucleotides sense strands (Eurogentec) were as follows: probe −323κB (−328 to −306) 5′-TCTTAGGGAATTTCCCC CCGCGA-3′; probe −98κB (−104 to −82) 5′-GTCCGCGGGACCCTCCA-GAAGAG-3′; probe −323κBM corresponding to the −323κB mutated one 5′-TCTTACTCAATTTCCCCCCG-CGA-3′; probe CκB containing consensus NF-κB binding sequence from the immunoglobulin light κ chain gene 5′-AGTTGAGGGGACTTTCCCAGGC-3′. Sense and antisense strands, 150 μg each, were annealed in 66 mM Tris–HCl, pH 7.5, 13 mM $MgCl_2$, 6.6 mM DTT and 1.3 mM EDTA by the following procedure: 5 min at 90°, 10 min at 65°, 10 min at 37° and 10 min at 20°. The double strand oligonucleotides were ^{32}P-labeled at the 5′-ends by the polynucleotide kinase PNK (Roche, Prophac) following manufacturer's instructions with [γ-^{32}P]ATP 7000 Ci/mmol (ICN) and purified on a spin column QIAquick Nucleotide Removal Kit.

2.5. RNA extraction and Northern blot analysis

Total RNA was isolated from 10^7 treated or untreated cells using NucleoSpin RNAII Kit (Macherey-Nagel) and 10 μg RNA were submitted to electrophoresis in a 1% agarose gel followed by transfer to nylon membrane (Hybond XL, Amersham AP Biotech) using a vacuum blotter (Appligene). Membranes were pre-hybridized for 1 hr in the presence of 200 μg/mL herring sperm DNA and 100 μg/mL yeast tRNA at 42° in 10 mL hybridization buffer (50% (v/v) formamide, 6× SSC, 5 mM EDTA, 0.1% (v/v) SDS and 5× Denhart's). Membranes were then hybridized with 25 ng GSTP1-1 probe and rehybridized with 25 ng GAPDH probe overnight at 42° in 10 mL hybridization buffer containing herring sperm DNA and yeast tRNA. Membranes were washed 5 min with 2× SSC, 0.5% (v/v) SDS at room temperature, three times 5 min with 2× SSC, 0.1% (v/v) SDS at room temperature, and three times 30 min with 1× SSC, 0.1% (v/v) SDS at 55°. cDNA probes were ^{32}P-labeled by random priming using RediprimeII Kit (AP-Biotech). Quantifications were performed by phosphorimaging the membranes on a Cyclone (Perkin-Elmer).

2.6. Plasmid constructs

Human genomic DNA was extracted from human peripheral blood cells following standard procedures. Primers and experimental conditions used for the PCR amplication of a 396 and 1175 bp region from the *GSTP1-1* gene promoter on the basis of sequences deposited in GenBank [26] (accession number: X08058) were previously described [30]. The PCR products were subcloned into a pCRII-Topo plasmid (Invitrogen), excised by *Kpn*I and *Hin*dIII, and then cloned in the dephosphorylated pGL3-Enhancer plasmid (Promega). The final constructs were named −396pGSTP and −1175pGSTP according to the size of promoter region cloned relative to the transcriptional start site. The 5XpκB−323 was prepared by cloning five copies of the −323 to −314 sequence from the *GSTP1-1* gene promoter in the *Hin*dIII/*Xho*I sites of a pMCS vector (Stratagene). The five copies of the consensus NF-κB sequence containing pMCS vector was named 5XpκB-C (Stratagene). TNFR1, TRAF2, p65, NIK, IκB-DN and IKKβ expression vectors were kindly provided by Aggarwal and coworkers [37].

Deletion of the −323/−314 NF-κB site was obtained by directed mutagenesis and led to the pGST-Mut-NF-κB reporter plasmid. Mutagenesis was performed by PCR using the GeneTaylor Kit (Invitrogen), according to manufacturer's instructions. Due to the high G–C content of the promoter sequence, PCRx Enhancer System (Invitrogen) was added to the reaction mixture to allow amplification. Sequences of the primers spanning the NF-κB distal site were as follows: sense, 5′-ATCGCAGCGGTCTTACCCC-GCGATGT-3′; antisense, 5′-TAAGACCGCTGCGATCC-CGG AGCTT-3′. Primers synthesis and sequencing of the mutated plasmids in both orientations were made by Eurogentec.

2.7. Transient transfections

Transfections of K562 cells were performed by electroporation using a BioRad gene Pulser. For each experiment 3.75×10^6 cells at a concentration of 1.5×10^7 cells/mL were electroporated at the following settings: 250 V and 500 μF. Five micrograms luciferase reporter gene construct and 5 μg Renilla plasmid have been used for each pulse. After 48 hr the cells were harvested and resuspended in growth medium (RPMI/FCS 10%) to a final concentration of 10^5 cells/mL in wells of microtitration plates and then treated or not. Following treatments, 75 μL Dual-Glo™ Luciferase Reagent were added to cells for a 10 min-incubation at 22° before luciferase activity assay. Then, 75 μL Dual-Glo™ Stop & Glo® Reagent were added for

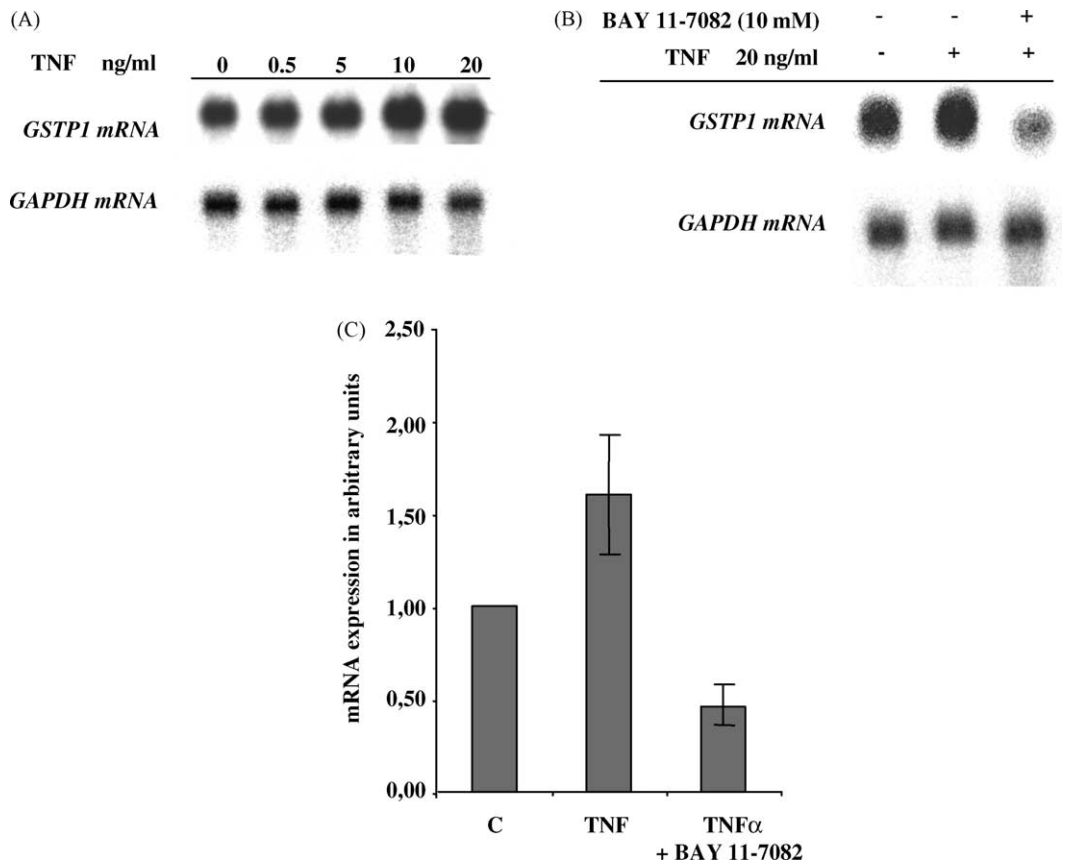

Fig. 2. Effect of TNFα and NF-κB inhibition on the *GSTP1-1* mRNA expression. (A) K562 cells were treated with various TNFα concentrations as indicated for 15 hr. Expression of *GSTP1-1* mRNA was monitored by Northern blot analysis using *GSTP1-1* cDNA as a probe. Human GAPDH mRNA expression was used as an internal control. (B) The effect of 20 ng/mL TNFα alone (T) or with BAY11-7082 (T + B) for 15 hr on the *GSTP1-1* mRNA level was compared to untreated extracts (C) and quantified using a phosphor imager Cyclone (Perkin-Elmer). Results are representative of three independent experiments ± SD ($P < 0.05$). (C) K562 cells were treated for 15 hr with 20 ng/mL TNFα alone or following a 2 hr preincubation with 10 ng/mL BAY11-7082. Expression of *GSTP1-1* mRNA was monitored by Northern blot analysis using *GSTP1-1* cDNA as a probe. Human GAPDH mRNA expression was used as an internal control.

10 min at 22° in order to assay Renilla activity. Luciferase and Renilla activities were measured using a Berthold Orion microplate luminometer (Berthold) by integrating peaks of light emission for 10 s. Data are relative values of firefly luciferase normalized to Renilla luciferase.

3. Results

3.1. Effect of TNFα and BAY11-7082 on the GSTP1-1 mRNA expression

TNFα is an activator of the NF-κB signal transduction pathway and of ROS production, which can be conjugated to GSH. In order to determine the effect of TNFα on *GSTP1-1* expression, K562 cells were treated for 15 hr with various concentrations of TNFα, then total RNA were extracted and analyzed by Northern blot (Fig. 2A). To investigate whether the TNFα-induced *GSTP1-1* mRNA expression could involve NF-κB transcription factors, K562 cells were treated with the inhibitor of TNFα-

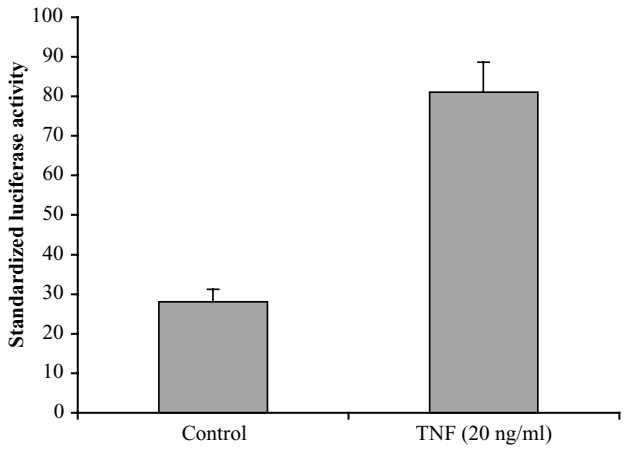

Fig. 3. Effect of TNFα on the *GSTP1-1* promoter activity. K562 cells were transiently co-transfected with 5 μg of the luciferase reporter −396pGSTP plasmid and 5 μg of Renilla plasmid. Transfected cells were treated with 20 ng/mL TNFα for 8 hr. Luciferase activity was measured using Promega's Dual-Glo™ Luciferase Assay system and Berthold Luminometer. Data shown are relative values of firefly luciferase normalized to renilla luciferase. Each bar represents the average of triplicate determinations ± SD.

induced IκBα phosphorylation, BAY11-7082, for 2 hr, prior to a 15-hr TNFα treatment (Fig. 2B). Quantification of *GSTP1-1* mRNA levels showed a significant increase (1.6-fold) in cells treated with 20 ng/mL TNFα as well as a reduction by BAY11-7082 of the TNFα-induced *GSTP1-1* mRNA increase (−72%) (Fig. 2C). Neither TNFα nor BAY11-7082 had an effect on the expression of the human GAPDH mRNA.

3.2. Effect of TNFα on the GSTP1-1 promoter activity

According to the increased expression of the *GSTP1-1* mRNA induced by TNFα we examined the *GSTP1-1* promoter activity in TNFα-treated K562 cells by performing reporter gene transfection assays. K562 cells were transiently transfected with the reporter −1175pGSTP plasmid in which the luciferase gene is driven by a *GSTP1-1* promoter region. Transfected cells were treated with 20 ng/mL TNFα for 8 hr. As shown in Fig. 3, TNFα induced a 2.9-fold activation of the *GSTP1-1* gene promoter suggesting that TNFα signal transduction cascade is able to activate the *GSTP1-1* gene transcription.

3.3. Binding activity of two putative NF-κB sequences within the GSTP1-1 promoter gene

3.3.1. Effect of TNFα on the binding activity of the putative NF-κB sites

As NF-κB inhibition altered the effect of TNFα-induced expression of the *GSTP1-1* mRNA in K562 cells, we investigated the capacity of the *GSTP1-1* promoter to interact with a NF-κB dimer by EMSA. We thus studied the NF-κB like site (−98 to −89) as well as a putative NF-κB sequence in position −323/−314 which was identified by DNA sequence analysis as well as the corresponding mutated sequences. We examined the capacity of TNFα to induce the binding activity on both NF-κB sites. Moreover, the oligonucleotidic probe C-κB, containing the consensus NF-κB binding site, was used as a positive control. With the C-κB probe two binding complexes were increased in TNFα-treated cells, whatever the concentration used (Fig. 4A). Using the −98κB probe, a single binding complex unaffected by TNFα treatments was formed (Fig. 4B). In contrast, the −323κB probe showed a similar pattern to that of the C-κB probe with two binding complexes (C1 and C2) which were strongly induced by TNFα (Fig. 4C). The mutation within the putative NF-κB −323/−314 sequence abolished the TNFα-specific bindings (Fig. 4D). These results suggest that C1 and C2 are composed of nuclear factors which specifically bind to the −323/−314 region.

3.3.2. Specificity of TNFα-induced binding complexes and effect of NF-κB inhibitors

Binding specificity of C1 and C2 was then determined by EMSA using cold competitor oligonucleotides

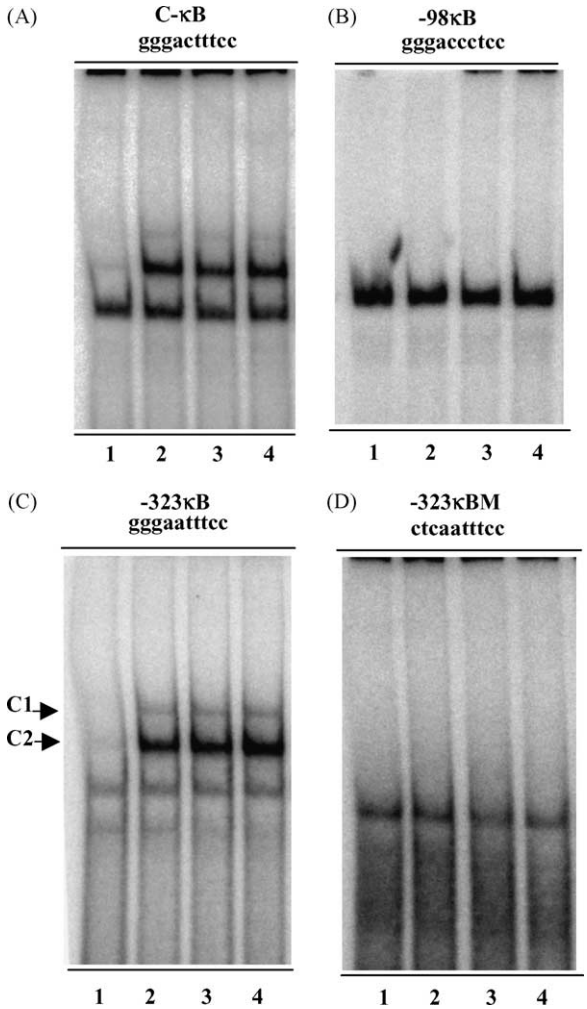

Fig. 4. Binding activity of both putative κB sites. EMSA experiments were performed by incubating 10 μg nuclear extracts from untreated or TNFα-treated K562 cells for 4 hr with oligonucleotide probes containing: (A) the consensus NF-κB binding site C-κB, (B) the NF-κB like site from GSTP1 promoter gene in −98 (−98κB), (C) the putative NF-κB site in −323 from the GSTP1 promoter gene (−323κB). C1 and C2 indicate TNFα-induced binding complexes, (D) the mutated −323κB probe in the putative NF-κB site (−323κBM). Lanes 1: untreated nuclear extracts; lanes 2, 3, 4: TNFα-treated K562 cells with 0.5, 5 and 20 ng/mL, respectively. Results are representative of at least three independent experiments.

(Fig. 5A). TNFα-induced K562 nuclear extracts were incubated in the presence of radiolabeled −323κB probe and with a 50-fold molar excess of the corresponding or the C-κB unlabeled probe. In these conditions C1 and C2 bindings were competed whereas the −323κBM unlabeled probe failed to abolish the binding confirming the specificity of C1 and C2 complexes.

In addition, treatments by BAY11-7082 or the proteasome inhibitor MG132 prior to TNFα exposure suppressed induction of C1 and C2 binding by TNFα (Fig. 5A). Moreover, human recombinant NF-κB p50 subunit bound to the −323κB probe but not to the −93κB probe (Fig. 5B). Altogether, data show that the −323/−314 sequence specifically binds TNFα-inducible NF-κB dimers whereas the

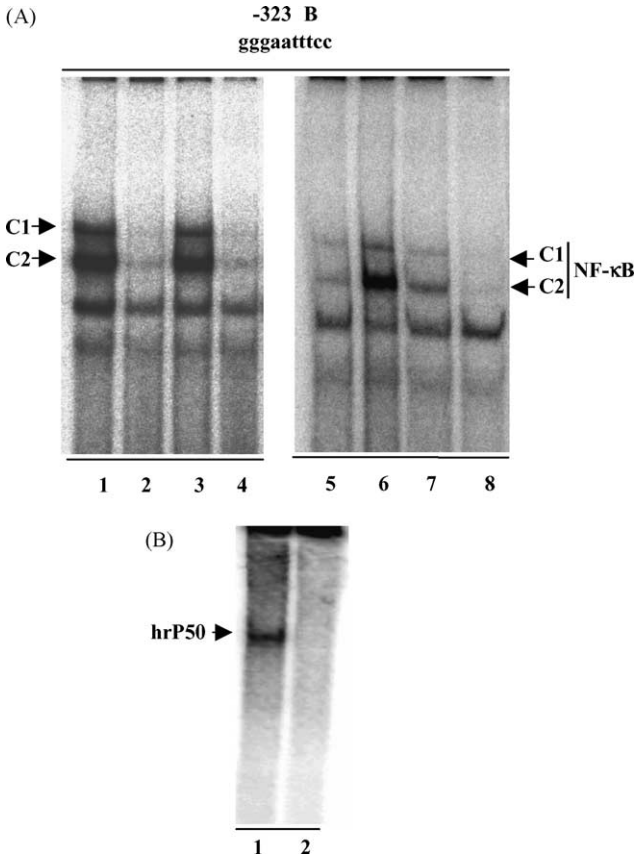

Fig. 5. Specificity of C1 and C2 TNFα induced binding complexes to the −323κB probe. (A) K562 cells were left untreated (lane 5) or were treated with 0.5 ng/mL TNFα (lanes 1–4 and 6–8) during 4 hr alone or in the presence of NF-κB inhibitors MG132 (lane 7) and BAY11-7082 (lane 8). Nuclear extracts (10 μg) were incubated in the presence of the −323κB labeled probe (lane 1) with a 50-fold molar excess of the similar cold probe (lane 2), the −323κBM cold probe (lane 3) or the C-κB cold probe (lane 4). TNFα induced binding complexes are indicated as C1 and C2. (B) Human recombinant NF-κB P50 protein (hrP50) subunit was incubated with the −323κB probe (lane 1) or with the −98κB probe (lane 2).

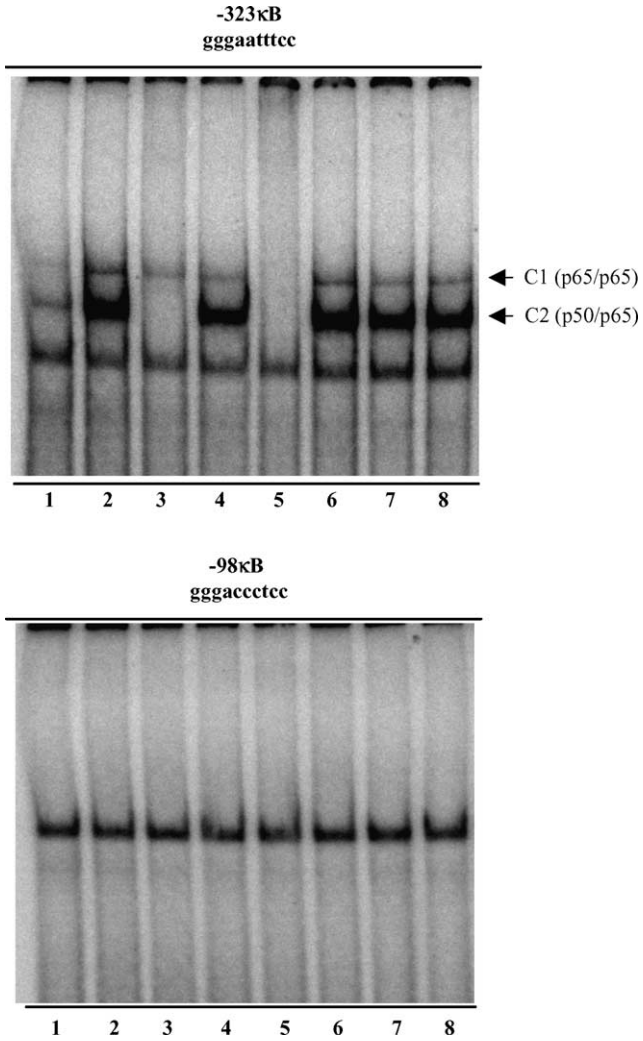

Fig. 6. Identification of TNFα-induced C1 and C2 binding complexes. Nuclear extracts (10 μg) from untreated (lane 1) or TNFα-treated K562 cells (0.5 ng/mL) (lanes 2–10), were incubated in the presence of labeled −323κB or −98κB probes as indicated, with antibodies directed against NF-κB subunits P50 (lane 3), P52 (lane 4), P65 (lane 5), RelB (lane 6), C-Rel (lane 7), and Bcl3 (lane 8). Composition of C1 and C2 binding NF-κB dimers forming complexes on the −323κB probe are indicated.

−98κB site does not act as a NF-κB binding site in our leukemia model.

3.3.3. Composition of the induced binding complexes

To further characterize these complexes, immunodepletion experiments were performed with antibodies directed against p50, p52, p65, c-Rel, RelB and Bcl3. As shown in Fig. 6, TNFα-induced binding complexes C1 and C2 disappeared in presence of αp50 and αp65 antibodies. Thus, complex C1 corresponds to a NF-κBp65/p65 homodimer whereas the main complex C2 is identified as a NF-κB p50/p65 heterodimer. Other antibodies did not affect either the specific or the unspecific bindings. No supershift or immunodepletion was detected in experiments using the −98κB probe. Results led us to conclude that the −323κB site specifically binds the TNFα-induced NF-κB p50/p65 and p65/p65 dimers whereas the −98κB site is not a NF-κB binding site in K562 leukemia cells.

3.4. Activation of GSTP1-1 promoter by TNFα signaling pathway via the −323NF-κB site

Our previous results showed an increased binding to the −323NF-κB site in response to TNFα indicating that this cytokine may be able to regulate *GSTP1-1* gene transcription through this response element. In order to determine whether the −323NF-κB site is sensitive to TNFα treatment, K562 cells were transfected with reporter plasmids containing five copies of the −323/−314 NF-κB site (5XpκB−323) or five repeats of a consensus NF-κB site from (5XpκB-C) (Fig. 7A). TNFα-treatment for 8 hr of cells transfected with 5XpκB−323 or 5XpκB-C resulted in a 14.6- and 6.8-fold stimulation, respectively. Results are consistent with the activation of the promoter and *GSTP1-1*

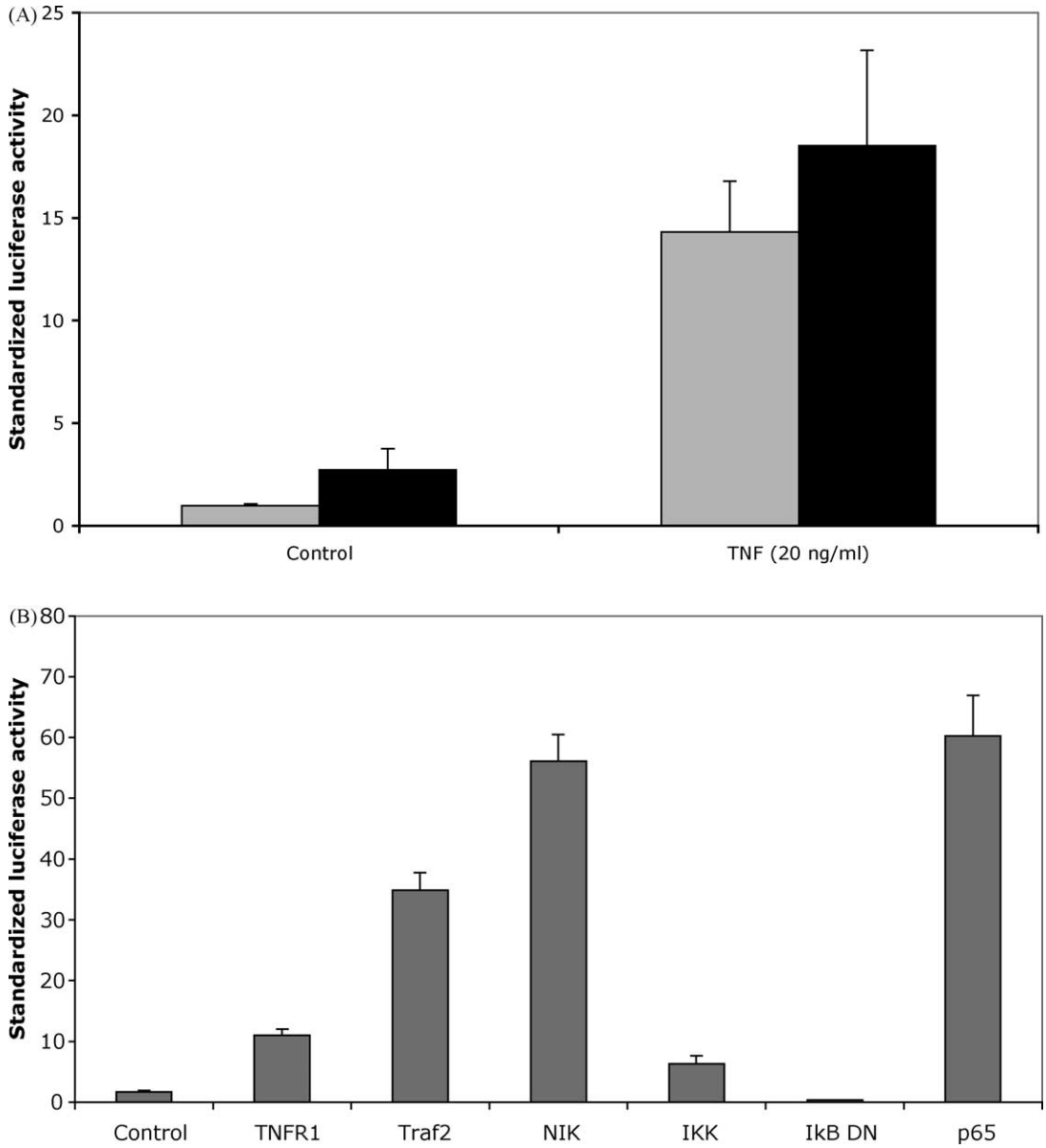

Fig. 7. Effect of TNFα signaling pathway on the 5XpκB−323 reporter plasmid. (A) K562 cells were transfected with 5XpκB−323 (black histogram) or 5XpκB-C (grey histogram) reporter plasmids and then treated with 20 ng/mL TNFα for 8 hr. (B) Expression vectors of the TNFα pathway intermediates (TNFR1, TRAF2, NIK, IKKβ, IκB-DN, NF-κBp65) were co-transfected with the 5XpκB−323 reporter plasmid into K562 cells and promoter activity was determined following 8 hr culture. Luciferase activity was measured using Promega's Dual-Glo™ Luciferase Assay system and Berthold Luminometer. Data shown are relative values of firefly luciferase normalized to renilla luciferase. Each bar represents the average of triplicate determinations ± SD.

mRNA expression by TNFα in K562 cells, *via* the identified NF-κB site.

In addition, expression vectors of the TNFα pathway intermediates (TNFR1, TRAF2, NIK, IKKβ, IκB-DN, NF-κBp65) were co-transfected with the 5XpκB−323 reporter plasmid into K562 cells (Fig. 7B). NF-κBp65 as well as NIK overexpression induced the most effective promoter activation with a 35.6- and 33-fold activation, respectively. TRAF2 and TNFR1 expression vectors induced a 20.6- and 6.5-fold increase, respectively whereas the activation by IKKβ was 3.7-fold increased only. The expression of dominant negative IκBα (IκB-DN) totally inhibited luciferase activity.

Furthermore, co-transfection of −396pGSTP reporter plasmid with the same NF-κB expression vectors led to a significant increase of reporter gene activity (Fig. 8A). NF-κBp65 as well as NIK overexpression induced the most effective promoter activation with a 16.5- and 8-fold activation, respectively. TRAF2 and TNFR1 expression vectors induced a 3- and 1.5-fold increase, respectively, whereas the activation by IKKβ did not induce any increase. The expression of dominant negative IκBα (IκB-DN) again inhibited luciferase activity. In contrast, a construct without a functional NF-κB site at −323 obtained by site-directed mutagenesis (pGST-Mut-NF-κB) significantly reduced the reporter gene activity with

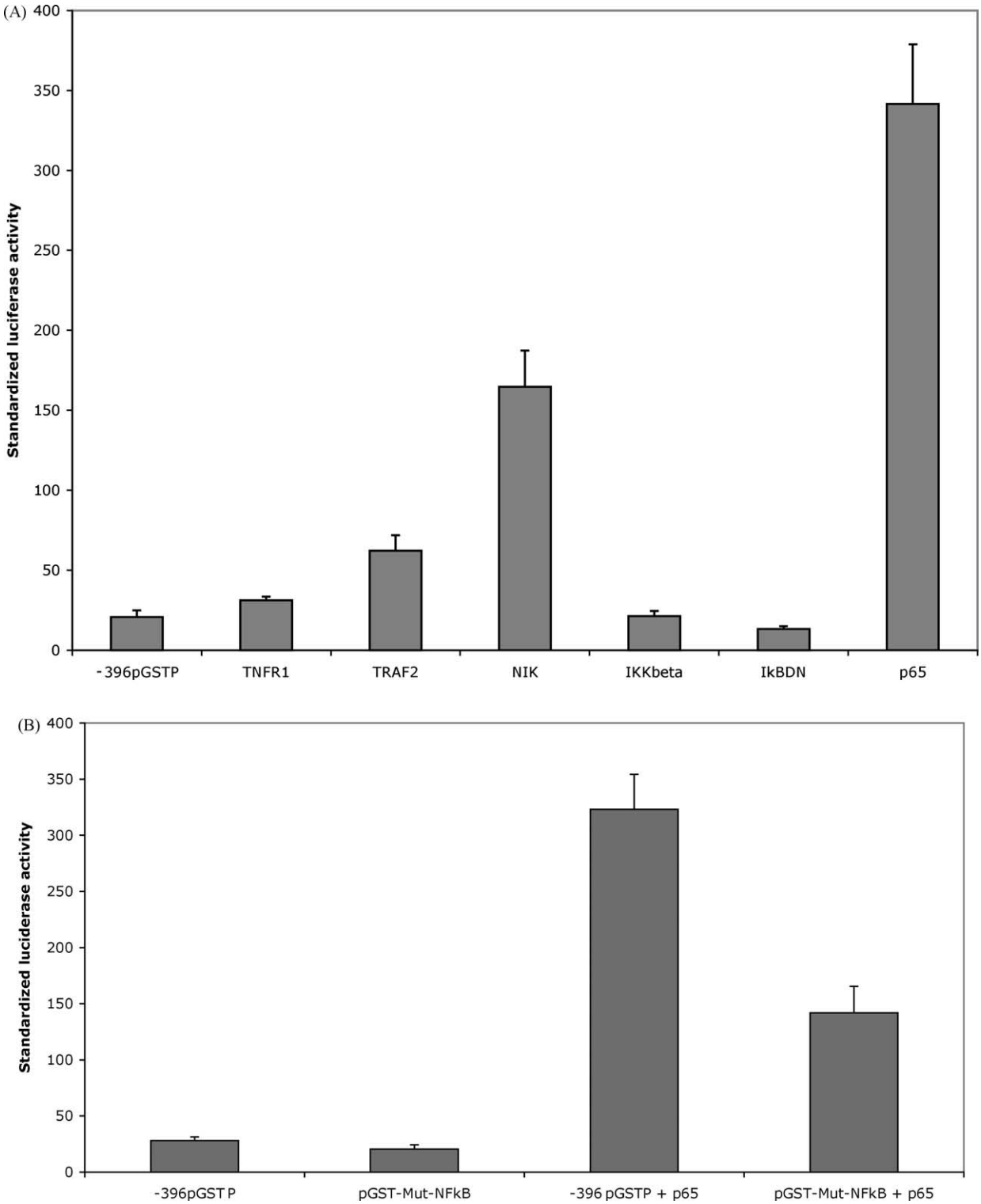

Fig. 8. Effect of TNFα signaling pathway on the mutated −396pGSTP reporter plasmid. K562 cells were co-transfected with expression vectors of the TNFα pathway intermediates (TNFR1, TRAF2, NIK, IKKβ, IκB-DN, NF-κBp65) (A) and −396pGSTP or pGST-Mut-NF-κB (B) reporter plasmids as indicated. Promoter activities were determined following 8 hr culture. Luciferase activity was measured using Promega's Dual-Glo™ Luciferase Assay system and Berthold Luminometer. Data shown are relative values of firefly luciferase normalized to renilla luciferase. Each bar represents the average of triplicate determinations ± SD.

or without a cotransfection with a p65 expression vector (−28 and −56%, respectively) (Fig. 8B). Taken together these results demonstrate that TNFα activates the *GSTP1-1* gene through the −323NF-κB site.

4. Discussion

TNFα regulates various cellular mechanisms, particularly immune responses, by induction of specific early

responsive genes among which transcription factors such as c-*jun* and NF-κB [55,56]. TNFα production is increased in a number of stressful and pathological states and as a proinflammatory cytokine it promotes cell damage through several mechanisms including the overproduction of ROS which is responsible for TNFα toxicity especially in cancer cells [23,57,58]. Besides, it has been reported that TNFα induces several protective genes among which enzymes of the GSH metabolism such as γ-glutamylcysteine synthetase in HepG2 cells [59] and the murine GSTA4 in regenerating liver [60]. Our present study investigates for the first time the potential role of TNFα as well as the NF-κB family of transcription factors in *GSTP1-1* gene expression in human leukemia cells. First of all, an increase in mRNA expression was observed in TNFα-treated K562 cells and transient transfection assays clearly showed an activation of the *GSTP1-1* promoter gene. In addition, inhibition of TNFα-induced mRNA expression using the specific IκBα phosphorylation inhibitor BAY11-7082 [61] supported our hypothesis that NF-κB is involved in the *GSTP1-1* gene activation by TNFα. Results obtained by Mori *et al.* [62] recently demonstrated that BAY11-7082 could be used as a suitable therapeutic agent to treat T cell leukemia since NF-κB pathway is crucial in the development of this pathology and apoptotic resistance. Other detoxifying enzymes including the multidrug resistance P-glycoprotein (MDR1) are inhibited by BAY11-7082 in kidney proximal tubule cells when stimulated with cadmium [63]. Studies by Hideshima *et al.* [64] demonstrate that proteasome inhibitors such as PS1145 and PS341 inhibit TNFα-induced NF-κB activation in a dose and time dependant fashion in multiple myeloma cells. Therefore, proteasome inhibitors are also of interest as therapeutic tools for inhibition of *GSTP1-1*-related drug resistance mechanisms.

In order to determine whether NF-κB could interact with *GSTP1-1* promoter, we performed EMSA. One NF-κB like (−98κB) binding site was previously described in the *GSTP1-1* promoter as a regulator element. However, few data about this site have been published and its role in the *GSTP1-1* gene regulation remains unclear. Indeed, the sequence −98/−89 was shown to act as a repressor binding site in the human mammary carcinoma cell line MCF7 whereas it was not effective in the VCREMS cells, a MCF7 multidrug-resistant derivative cell line [32]. Authors showed that the silencing effect of this NF-κB like site occurred by interacting with the AP-1 site in MCF7 cells but not in the human bladder carcinoma EJ cells [32,33]. In contrast, Zhang *et al.* [65] provided evidence that this NF-κB site is not involved in silencing the TRE/ARE function in keratinocytes. On the other hand, Xia *et al.* [66] showed that response of GSTP1 to oxidants is mediated by a NF-κB like site while response to anti-oxidants is mediated by the AP-1 site. Thus, regulation of GSTP1 can result from cooperation between NF-κB like and AP-1 sites and the function of the −98/−89 sequence might be cell type and/or drug dependent. However, neither the capacity of the −98κB site to bind a NF-κB dimer nor the involvement of this transcription factor in the *GSTP1-1* gene regulation had been demonstrated until now, particularly in leukemic cell lines in which the enzyme is largely expressed [9,67]. By DNA sequence analysis [52] we identified a distal DNA sequence −323/−314 downstream from the minimal promoter as a putative NF-κB binding site. We then studied binding activity of both putative κB sites. EMSA experiments using K562 nuclear extracts and probes containing NF-κB consensus from the immunoglobulin κ light chain (C-κB) or the −323/−314 sequence (−323κB) exhibited similar patterns. Indeed, two binding complexes were induced by TNFα resulting from a specific interaction within the −323/−314 sequence as assessed by competition experiments with wild type (−323κB, C-κB) and mutated (−323κBM) probes. In contrast, we observed a single binding activity on the NF-κB like site (−98/−89) unaffected by TNFα treatments in K562 cells. Moreover, no binding of human recombinant NF-κB p50 protein to this probe was observed suggesting fundamental differences in the potential roles of −98/−89 and −323/−314 sequences. Additional studies are needed to demonstrate the importance of the sequences flanking the two NF-κB sites.

NF-κB activation is regulated *via* a well known signaling pathway [13,15,16]. In order to characterize C1 and C2 bindings, we used MG-132 and BAY11-7082 which inhibit TNFα-induced NF-κB by blocking proteasome activity and phosphorylation of IκBα, respectively [61,68]. The −323κB site was shown to bind TNFα-activated NF-κB dimers since both specific complexes (C1 and C2) were inhibited by MG132 and BAY11-7082. C1 and C2 complexes were then identified by super-shift experiments as p65/p65 and p50/p65 NF-κB dimers, respectively. In contrast, super-shift experiments did not reveal any binding of NF-κB dimers to the −98κB probe.

In order to study the −323κB binding site we performed transfection experiments and so we could establish that its transactivation potential is similar to the one of the consensus site. Previous results clearly demonstrate that anti-inflammatory therapeutic agents block TNF-induced NF-κB activation, IκBα phosphorylation and activation of IKK, JNK, and AP-1, and suppressed TNF-induced apoptosis. Our results obtained by co-transfection of expression constructs coding for TNFR1, NIK, TRAF2, IKKβ, IκBα and p65/NF-κB specifically show that TRAF2, NIK, IκBα and NF-κB/p65 are involved in the regulated activation of the *GSTP1-1* promoter. We show here for the first time that the *GSTP1-1* promoter is regulated by a TNFR1, TRAF2, NIK pathway which leads to activation of NF-κB. The phosphorylation of IκBα is regulated by a large number of kinases, including IKK-α, IKK-β, IKK-γ, NIK, TGF-β-activated kinase-1, AKT, and mitogen-activated protein/extracellular signal-related kinases. However, only IKKβ mediates TNF-induced phosphorylation of IκBα at positions 32 and 36 which explains the strong negative effect of a dominant negative mutant of IκBα in our assays. On the

other hand, a mutated form of the −396pGSTP promoter shows a strongly reduced luciferase activity if co-transfected with p65/NF-κB confirming the importance of NF-κB in the expression of *GSTP1-1* gene. Nevertheless, this mutated construct maintains luciferase reporter gene activity which is potentially due to the presence of a consensus AP-1 site at −73. Our results confirm our previous reports describing the role of the −73 AP-1 site in constitutive and TPA-induced reporter gene activity [30].

The NF-κB family of transcription factors is involved in acquisition of resistance to anticancer drugs as well as to apoptosis. NF-κB-mediated *mdr1* gene upregulation is part of the anti-apoptotic protection mechanism of proximal tubule (PT) cells against cadmium-induced oxidative stress and apoptosis [63]. Bentires-Alj *et al.* [69] have recently shown a new relationship between NF-κB and resistance to chemotherapy through the regulation of human *mdr1* gene expression since the inhibition of NF-κB activity sensitizes resistant colon cancer cells to daunomycin through a decreased *mdr1* gene expression. In addition, NF-κB seems to be an inducer of anti-apoptotic genes [70]. B cell chronic lymphocytic leukemia presents a strong resistance to apoptosis inducing agents due to expression of antiapoptotic genes such as the inhibitor of apoptosis protein (IAP) and TNF receptor associated protein TRAF gene families [71] which are NF-κB target genes. Hodgkin's disease tumor cells constitutively activate NF-κB p50 in Hodgkin/Reed-Sternberg cells preventing apoptosis under stress conditions [72]. Moreover, it is now admitted that most of the anticancer drug families [73–76] have in common the activation of NF-κB [77] which leads to chemoresistance. Thus, inhibition of 5-fluorouracil-activated NF-κB by disulfiram enhances cytotoxicity of the DNA synthesis inhibitor in colorectal cancer cell lines [78] and activation of NF-κB by topoisomerase poisons such as SN38 and doxorubicin protects HeLa cells from the apoptotic effect of the drugs [79].

We show here that *GSTP1-1* gene expression is regulated by NF-κB as a wide range of genes involved in carcinogenesis and in apoptosis inhibition. NF-κB contributes to the age-associated up-regulation of cycloxygenase-2 [80] and controls the over-expression of the 12-lipoxygenase in human erythroleukemia cells [81]. As well, manganese superoxide dismutase (MnSOD) expression is induced by NF-κB in epithelial cancer cells in response to TNFα [82]. In the same way, *GSTP1-1* protein is involved in carcinogenesis and in resistance to apoptosis. Indeed, Gilot *et al.* [83] demonstrated that GSTP1-1 can inhibit Jun N-terminal kinase (JNK), preventing apoptosis in rat hepatocytes and GSTP1/P2$^{(-/-)}$ knock-out mice present increased JNK activity in liver and lung [84]. Furthermore, *GSTP1-1* is up-regulated in chronic lymphoid leukemia [85] and its overexpression has been associated with a severe prognosis in B cell lymphoma [86].

In conclusion, we report for the first time the binding activity of a functional NF-κB site upstream of the minimal promoter of the *GSTP1-1* gene in K562 leukemia cell line whereas the previously described NF-κB like sequence (−98 to −89) in the minimal promoter is not able to bind NF-κB in our experimental conditions and cellular model. Together, our results show the activation of the *GSTP1-1* gene during inflammation and potentially during cancer. Further identification of the mechanisms underlying transcriptional control of *GSTP1-1* gene will be important for the development of novel therapeutic strategies in chemoresistant leukemia.

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