Biochemical and Biophysical Research Communications 373 (2008) 169–173

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Glutathionylation regulates IκB

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ARTICLE INFO

Article history:
Received 31 May 2008
Available online 13 June 2008

Keywords:
NFκB
IκB
Glutathionylation
Phosphorylation
Ubiquitination

ABSTRACT

Although there has been considerable interest in the regulation of NFκB activation by glutathionylation, the possibility of IκB as a target for glutathionylation has not been investigated. We now report that Cys^{189} of IκBα is a target for *S*-glutathionylation. This modification is reversed by thiols such as dithiothreitol and GSH. The glutathionylated IκBα appears to be significantly less susceptible than is native protein to phosphorylation by IκB kinase and casein kinase II, as well as to *in vitro* ubiquitination. This finding suggests that glutathionylation plays a regulatory role, presumably through structural alterations. HeLa cells treated with oxidant inducing GSH oxidation such as diamide showed the accumulation of glutathionylated IκBα. This mechanism suggests an alternative modification to the redox regulation of cysteine in IκBα and a possible mechanism in the regulation of NFκB activation.

The initial cellular response to oxidative stress is often a reduction in the levels of GSH, which represents the major low molecular weight antioxidant in mammalian cells, and a corresponding increase of GSSG, the oxidized form of GSH [1,2]. It is well established that GSH plays a central role in the cellular defense against oxidative damage [3]. Thus, the oxidation of a limited amount of GSH to GSSG can dramatically change this ratio and affect the redox status within the cell. In these conditions of moderate oxidative stress, thiol groups of intracellular proteins can be modified by the reversible formation of mixed disulfides between protein thiols and low molecular mass thiols, such as GSH, in a process known as *S*-glutathionylation [4]. Glutathionylation, which is reversible by the actions of the enzyme glutaredoxin (thioltransferase) [5,6], may serve as a means of protection by preventing the irreversible oxidation of cysteine to cysteine sulfinic and sulfonic acid.

One proposed mechanism leading to protein glutathionylation *in vivo* is the thiol/disulfide exchange mechanism [7], which occurs when an oxidative insult changes the GSSG/GSH ratio and induces GSSG to bind to protein thiols. The GSSG/GSH ratio is an indicator of the redox status of the cell, and the extent of protein glutathionylation will vary accordingly: a higher ratio will promote glutathionylation, and a lower ratio will result in deglutathionylation of glutathione [8]. Therefore, the regulated formation of mixed disulfides between protein thiols and glutathione redox changes has the potential to act as a reversible switch in much the same way as phosphorylation [9]. A growing list of enzymes, including glyceraldehyde-3-phosphate dehydrogenase [10], protein kinase C [11], guanylate cyclase [12], mitochondrial $NADP^+$-dependent isocitrate dehydrogenase [13], and glucocorticoid receptors [14], are potentially influenced by the formation of protein adducts with glutathione. Transcription factors such as c-Jun appear to be redox-regulated by mechanisms that include protein *S*-thiolation [9,15], and ubiquitin-activating enzymes become glutathionylated, with a concomitant decrease in the ubiquitinylation pathway, when cells are exposed to oxidants [16].

Nuclear factor κB (NFκB) is a major transcription factor that regulates the expression of a large number of genes that code for cytokines, cytokine receptors, adhesion molecules, and antiapoptotic proteins [17,18]. In unstimulated cells, NFκB is retained in the cytoplasm as a complex with inhibitory proteins known as IκB (inhibitor of NFκB) [19]. Upon stimulation, IκBα is phosphorylated, ubiquitinated, and degraded by the proteasome, and it allows the translocation of NFκB to the nucleus [18].

In this study, we report that IκBα is modified by the formation of a mixed disulfide at Cys^{189}, and that this modification is reversed by thiols such as dithiothreitol (DTT) and GSH. This mechanism suggests an alternative modification to the redox regulation of cysteine in IκBα and a possible mechanism in the regulation of NFκB activation.

Materials and methods

Materials. GSH, GSSG, *N*-acetylcysteine (NAC), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), diamide, cysteine, *N*-acetyl-DL-penicillamine, ubiquitin, and 8-anilino-1-naphthalene sulfonic acid (ANSA) were purchased from Sigma Chemical Co. (St. Louis,

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doi:10.1016/j.bbrc.2008.06.007

MO). Electrophoresis reagents and Bio-Rad protein assay kits were purchased from Bio-Rad. In order to prepare recombinant human IκBα, *Escherichia* coli transformed with expression construct pGEX-IκBα (a kind gift of Dr. Gary D. Bren, Mayo Clinic) were grown and lysed, and the glutathione-*S*-transferase protein was purified on GSH-agarose, as described elsewhere [20]. Anti-IκBα, anti-ubiquitin, and anti-IκB kinase β (IKKβ) antibodies were purchased from Santa Cruz (Santa Cruz, CA) and anti-GSH antibody was purchased from ViroGen (Watertown, MA). *S*-Nitrosothiols were prepared as described previously [21].

Cell culture. HeLa, a human cervical cancer cell line, was purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate, respectively. The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Immunoblot analysis. The proteins were separated on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose membranes, and subsequently, subjected to immunoblot analysis using appropriate antibodies. The immunoreactive antigen was then recognized by using horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). For glutathionylation detection by immunoblot, IκBα was mixed with a 5× SDS sample buffer, without reducing reagents and with a supplement with 5 mM NEM to block unreacted thiol groups, and then subjected to SDS–PAGE followed by electroblotting onto nitrocellulose membranes.

Structural analysis. Steady-state fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer with the sample compartment maintained at 22 °C. A 150 W xenon source was used. The slit-width was fixed at 5 nm for excitation and emission. Unless otherwise stated, samples were excited at 278 nm, and the emission was monitored between 300 and 400 nm. ANSA (100 μM) was incubated with the various forms of IκBα in 25 mM potassium phosphate buffer, pH 7.0/50 mM KCl. The fluorescence emission spectra (excitation, 370 nm) of the different mixtures were monitored on a spectrofluorometer. The binding of ANSA to the protein was evidenced by subtracting the emission spectrum of ANSA from that of ANSA in the presence of the protein.

Site-directed mutagenesis and preparation of recombinant proteins. The site-directed mutagenesis was performed using the Quick-change™ site-directed mutagenesis kit (Stratagene). In order to prepare recombinant proteins, *E. coli* transformed with pGEX containing the cDNA insert for human IκBα or mutant IκBα construct was grown and lysed, and GST-tagged proteins were purified by affinity chromatography on GSH–agarose as described elsewhere [20].

In vitro phosphorylation. The IKK complex was immunoprecipitated from 200 μg of lipopolysaccharide-treated cell extract with IKKβ IgG. Precipitates were washed once with a lysis buffer and twice with a kinase buffer (20 mM Hepes/20 mM β-glycerophosphate/1 mM $MnCl_2$/5 mM $MgCl_2$/2 mM NaF/250 μM DTT). Kinase reactions were performed with 1 μg of IκBα or GSSG-treated IκBα as a substrate and 5 μCi of [γ-^{32}P]ATP at 30 °C for 30 min. Phosphorylation of IκBα by casein kinase II (CKII) was performed in a reaction mixture containing 20 mM Tris/HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 5 μCi of [γ-^{32}P]ATP, and 1 μg of IκBα or GSSG-treated IκBα. The reactions were started by the addition of CKII and incubated for 15 min at 30 °C. After terminating the phosphorylation reactions by the addition of 10 μl of 4× SDS sample buffer, the samples were subjected to SDS/PAGE using a 10% running gel. The labeled proteins were visualized by autoradiography of dried gels.

In vitro ubiquitination assay. Reactions were performed in a 100 μl mixture containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, 0.2 U/ml creatine phosphokinase, 2 mg/ml ubiquitin, and recombinant IκBα that had been treated with various concentrations of GSSG for 1 h. The reactions were carried out in darkness and devoid of any reducing agents such as DTT at 37 °C. After 1 h, the reactions were terminated with an equal volume of a 2× SDS sample buffer, and the products were subjected to Western blot analysis using anti-ubiquitin antibody.

IκBα glutathionylation in HeLa cells. HeLa cells in confluent 10-cm dishes that were exposed to diamide and incubations were terminated by removing the medium and washing them twice with ice-cold PBS, and lysed in a buffer containing 50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 1% NP-40, and 1× protease inhibitor cocktail. The insoluble material was removed by centrifugation 15,000g for 30 min. The proteins were pre-cleared with protein A–Sepharose (Amersham Biosciences) for 1 h at 4 °C. The supernatants were then incubated with rabbit polyclonal anti-IκBα (5 μg) for 12 h at 4 °C followed by protein A–Sepharose incubation for 1 h at 4 °C. The immunoprecipitated proteins were washed, separated by SDS–PAGE, and visualized by Western blotting with anti-GSH antibody.

Replicates. Unless otherwise indicated, each result described in the paper is representative of at least three separate experiments.

Results and discussion

Glutathionylation of IκBα in vitro

Regulation of biological activity by the reversible modification of protein thiol is a growing concept in cellular defense, such as glutathionylation [22]. Cysteine-containing proteins are susceptible to protein *S*-glutathionylation, the reversible covalent addition of glutathione to cysteine residues on target proteins. In this study, we present evidence indicating that IκBα can be modified by reversible glutathionylation. It has been shown that a polyclonal anti-GSH antibody is very useful for detecting glutathionylation [13]. When IκBα was incubated with various concentrations of GSSG and subjected to Western blot analysis with a polyclonal anti-GSH antibody, the intensity of the immunoreactive band was increased in a concentration- and time-dependent manner (Fig. 1A and B). As shown in Fig. 1C, the addition of 10 mM DTT or 5 mM GSH reversed glutathionylation, suggesting that GSSG is modifying susceptible cysteine(s) on the protein through the formation of a mixed disulfide. In order to determine whether glutathionylated cystein(s) in IκBα are susceptible to sulfhydryl modifying agents, IκBα was allowed to react simultaneously with 5 mM GSSG and various concentrations of NEM, NAC, and *S*-nitrosothiols for 1 h. As shown in Fig. 1D, the dose-dependent decrease of glutathionylated IκBα was observed. To further confirm which cysteine residue is a target for glutathionylation, eight single C → A mutant IκBα proteins (C135A, C152A, C156A, C167A, C186A, C215A, C239A, and C308A) were prepared. When mutant proteins were exposed to 5 mM GSSG, only the C186A mutant was not glutathionylated among the eight mutant IκBα proteins, confirming that Cys^{186} is a target of glutathionylation of IκBα (Fig. 1E).

Glutathionylation of IDPm in intact cells

Because GSSG readily glutathionylates IκBα *in vitro*, we examined IκBα glutathionylation in HeLa cells after treatment with diamide. It has been reported that chemical oxidants such as H_2O_2 or diamide can serve as a catalyst in promoting the formation of protein-mixed disulfides with glutathione [23]. Cell lysates from both control and diamide-treated cells were subjected to immunoprecipitation with anti-IκBα antibody followed by separation by SDS–PAGE. Western blot analysis of purified IκBα with anti-GSH IgG revealed a concentration- and time-dependent increase of

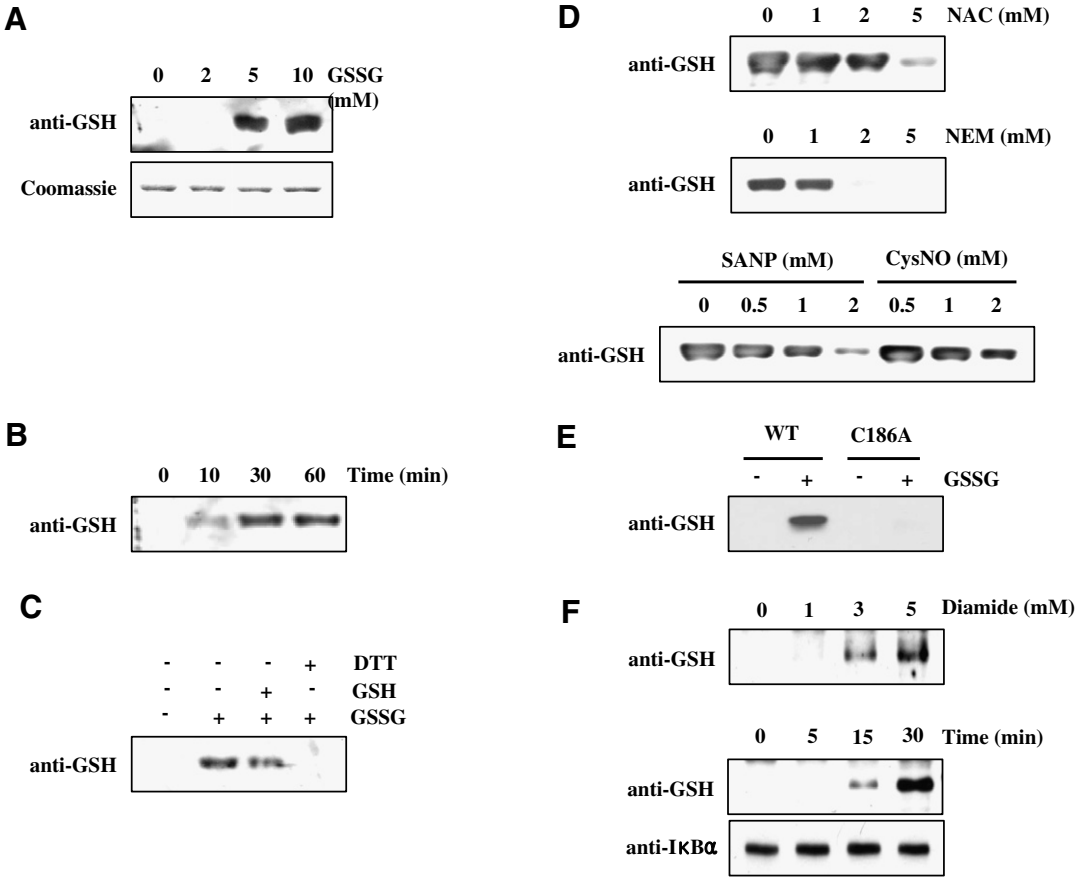

Fig. 1. Glutathionylation of IκBα. (A) Immunochemical analysis of GSS-IκBα. After incubation with various concentrations of GSSG for 1 h at 37 °C, samples were subjected to SDS–PAGE for immunoblotting with anti-GSH IgG. (B) After incubation with 5 mM GSSG for various lengths of time at 37 °C, samples were subjected to SDS–PAGE for immunoblotting with anti-GSH IgG. (C) Reversal of glutathionylated IκBα by thiols. IκBα was incubated with 5 mM GSSG for 1 h, and subsequently treated with 10 mM DTT or 5 mM GSH for 1 h at 37 °C. Samples were characterized by SDS–PAGE followed by immunoblotting with anti-GSH IgG. (D) Effect of sulfhydryl modifying agents on the glutathionylation of IDPm. After incubation of IκBα with 5 mM GSSG in the presence and absence of NAC, NEM or *S*-nitrosothiols for 1 h at 37 °C, samples were characterized by SDS–PAGE followed by immunoblotting with anti-GSH IgG. SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; CysNO, *S*-nitrosocysteine. (E) Identification of GSS-Cys on IκBα with a site-directed mutagenesis. After incubation with 5 mM GSSG for 1 h at 37 °C, wild-type and C189A mutant IκBα were characterized by SDS–PAGE followed by immunoblotting with anti-GSH IgG. (F) Accumulation of glutathionylated IκBα in HeLa cells by diamide. HeLa cells were incubated with diamide at 37 °C and disrupted by sonication. IκBα was purified from the control and from the oxidant-treated HeLa cells using immunoprecipitation with anti-IκBα antibody as described, and then characterized by SDS–PAGE followed by immunoblotting. Purified IκBα was probed with anti-GSH IgG.

immunoreactive bands in diamide-treated cells, while no immunoreactive bands were found in the control cells (Fig. 1F).

Structural changes in modified IκBα

There are several lines of evidence obtained from the present study indicating that glutathionylation of IDPm results in structural alterations. These findings are reflected in the changes in intrinsic tryptophan fluorescence and in the binding of ANSA. Native IκBα exhibits a fluorescence emission spectrum typical for tryptophan residues in proteins. Upon excitation of native IκBα at 278 nm, an emission spectrum with a maximum at 337 nm was observed. The fluorescence spectra of native and GSSG-treated IκBα, normalized to the protein content, show that modified IκBα displays a dose-dependent increase in quantum yield of the emission spectra and a red shift of the maximum emission wavelength (Fig. 2A). Among the techniques aimed at following the conformational changes of proteins, binding of the fluorescent probe ANSA has been used to detect the accessibility of the hydrophobic regions on protein upon increases in flexibility or partial unfolding. Binding can be easily monitored since it is accompanied by an increase in fluorescence associated with the transfer of the ANSA from a hydrophilic to a hydrophobic environment [24]. When IκBα was exposed to various concentrations of GSSG for 1 h, it bound the hydrophobic probe ANSA more efficiently than does the native protein. The representative result with GSSG is shown in Fig. 2B. A change in the ANSA fluorescence in IκBα modified by glutathionylation indicates conformational changes of protein.

Glutathionylation of IκBα regulates phosphorylation and ubiquitination

The proteasome pathway of IκBα proteolysis has been induced by phosphorylation of IκBα on serine 32 and 36 by the IKK [25]. This phosphorylation serves as a signal for subsequent ubiquitination, which promotes IκBα degradation by the 26 S proteasome [26]. Recently, additional kinases have been implicated in the regulation of IκBα activity or stability [27]. Protein kinase CKII phosphorylates IκBα on serines and threonines in the proline–glutamic acid–serine–threonine (PEST) sequence domain, which affects the intrinsic stability of this inhibitory protein [28]. It has been demonstrated that the CKII phosphorylation of serine/threonine residues in the PEST domain promotes the calpain-mediated degradation of IκBα [29]. A number of previous studies have reported the conformation-dependent phosphorylation of proteins. It has been demonstrated that the phosphorylation of p53 and

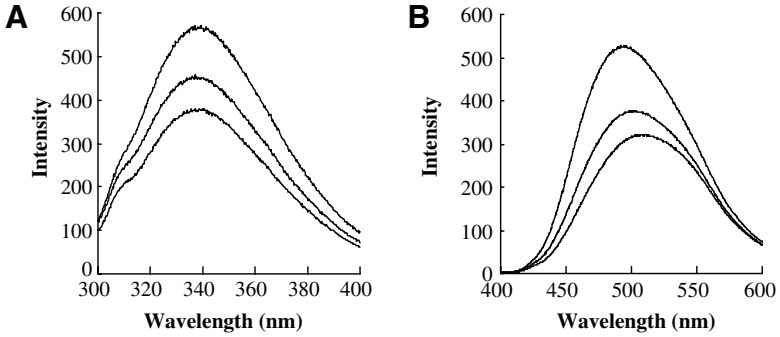

Fig. 2. Structural changes in glutathionylated IκBα. (A) Steady-state emission spectra of intrinsic fluorescence of native (*lower trace*) and IκBα treated with 2 and 5 mM GSSG for 1 h at 37 °C (*middle and upper traces*, respectively) were analyzed in a spectrofluorimeter. Spectra were obtained using an excitation wavelength of 278 nm and excitation and emission slits of 5 nm. (B) Spectrofluorometric analysis of ANSA binding to the glutathionylated IκBα. Emission spectra from 400 to 600 nm (excitation, 370 nm) of ANSA (100 μM) bound to native IκBα (*lower trace*) and IκBα treated with 2 and 5 mM GSSG for 1 h at 37 °C (*middle and upper traces*, respectively) were obtained.

p47 of NADPH oxidase by CKII is modulated by conformational alteration of substrate proteins [30,31]. To study if the glutathionylation affected its phosphorylation through a slight conformational change, recombinant IκBα was treated with various concentrations of GSSG for 1 h, and then phosphorylated it with IKKβ. Without glutathionylation, IκBα was phosphorylated by IKKβ, however, the phosphorylation occurred less efficiently when IκBα was glutathionylated, and this effect depended on the dose of GSSG (Fig. 3A). Besides inhibiting the phosphorylation of IκBα by IKKβ, the glutathionylation of IκBα also suppressed its phosphorylation by CKII (Fig. 3B). In addition, the *in vitro* ubiquitination of IκBα was significantly inhibited by glutathionylation in a dose-dependent manner, as shown in Fig. 3C. Accordingly, we propose the following model seen in Fig. 4. With an increased level of GSSG, IκBα is glutathionylated and *S*-glutathionylation inhibits the degradation of IκBα by decreasing its susceptibility to kinases, which is responsible for two degradation pathways: proteasome and calpain. Furthermore, the glutathionylation of IκBα suppresses the ubiquitination process. The regulatory role of glutathionylation on IKKβ and NFκB has been proposed [32–34]. It has been demonstrated that the p50 subunit of NFκB undergoes *S*-glutathionylation in the Cys62 residue of its DNA-binding domain and that this modification can reversibly inhibit its DNA-binding activity

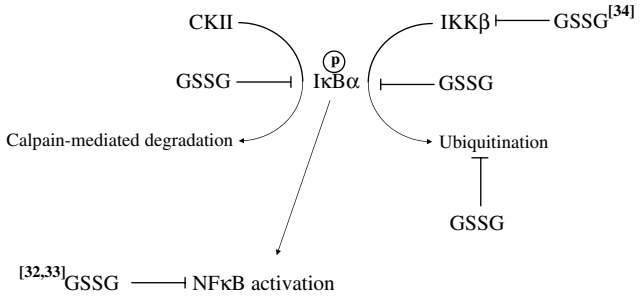

Fig. 4. Proposed model of inhibition of the NFκB pathway by *S*-glutathionylation of IκBα. See text for details.

[32]. In addition, hypoxia and NAC treatment led to the inactivation of the p65 subunit of NFκB, and glutaredoxin was shown to restore the p65 transcriptional activity, which is indicative of p65-SSG formation *in situ* [33]. NFκB activity was recently shown to be regulated by the *S*-glutathionylation of IKKβ in lung epithelial cells [34]. Collectively, it can be proposed that glutathionylation is a mechanism for controlling the activation of the NFκB pathway at multiple levels.

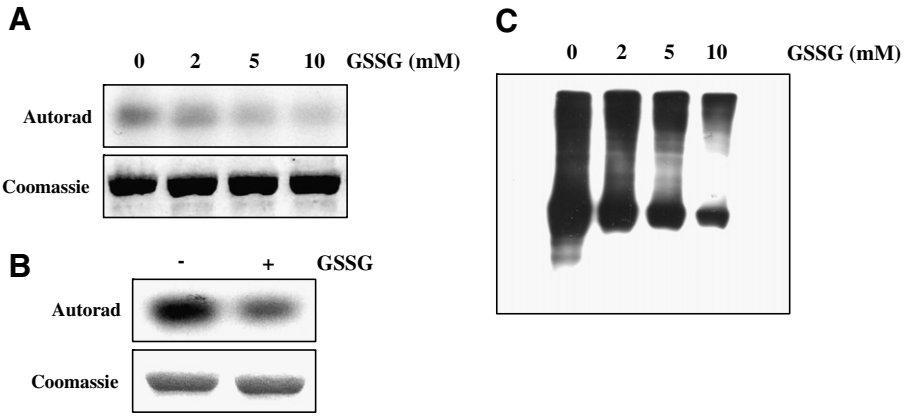

Fig. 3. Effect of glutathionylation on the phosphorylation and ubiquitination of IκBα. (A) Effect of glutathionylation on the phosphorylation of IκBα by IKKβ. IκBα treated with various concentrations of GSSG for 1 h at 37 °C and glutathionylated IκBα were phosphorylated with IKKβ for 30 min. The labeled proteins were separated by SDS–PAGE and the labeled bands were located by autoradiography. (B) Effect of glutathionylation on the phosphorylation of IκBα by CKII. IκBα treated with 5 mM GSSG for 1 h at 37 °C and glutathionylated IκBα were phosphorylated with CKII for 15 min. The labeled proteins were separated by SDS–PAGE and the labeled bands were located by autoradiography. (C) Effect of glutathionylation on the ubiquitination of IκBα. IκBα treated with various concentrations of GSSG for 1 h and glutathionylated IκBα were ubiquitinated *in vitro*. Samples were subjected to Western blot analysis and anti-ubiquitin antibody.

Acknowledgments

This work was supported by the National R&D Program for Cancer Control, Ministry of Health & Welfare, Republic of Korea (0720250) and a Nuclear Research Program from the Korea Science and Engineering Foundation Grant M2-0513-000102-05-B09-00-102-10.

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