The conserved redox-sensitive cysteine residue of the DNA-binding region in the c-Rel protein is involved in the regulation of the phosphorylation of the protein

Corine GLINEUR^{*1}, Elisabeth DAVIOUD-CHARVET[†] and Bernard VANDENBUNDER^{*}

^{*}EP560-CNRS-Université Lille II-Institut Pasteur de Lille, Institut de Biologie de Lille, 1 rue du Professeur Calmette, 59021 Lille, France, and

[†]UMR8525-CNRS-Université Lille II-Institut Pasteur de Lille, Institut de Biologie de Lille, 1 rue du Professeur Calmette, 59021 Lille, France

The DNA-binding activity of the transcription nuclear factor κ B (NF- κ B) is regulated by a redox-control mechanism involving the reduction of a disulphide bond from a specific cysteine residue conserved in all members of the NF- κ B family. Thio-redoxin is involved in this redox control. DNA binding and transactivating capacity of NF- κ B are up-regulated by inducible phosphorylation. Here we demonstrate that the conserved redox cysteine in the c-Rel protein is involved in the phosphorylation regulation of the protein. When this cysteine residue is mutated to an aspartic acid residue, the mutant protein loses its capacity to be phosphorylated and its DNA-binding activity. In addition, our results suggest that, when the conserved redox cysteine is chemically modified by *N*-ethylmaleimide and 2-chloro-1,3-

dinitrobenzene, the protein c-Rel cannot be phosphorylated. In contrast, the protein in which the cysteine residue was replaced by a serine residue, creating a potential phosphorylation site, is highly phosphorylated and binds κ B sequences. The protein could lose the redox regulation of the phosphorylation when the residue replacing the cysteine can be itself phosphorylated. We also show that specific inhibitors of thioredoxin reductases impair the phosphorylation of the c-Rel protein, suggesting that the redox regulation of the protein controls its phosphorylation.

Key words: NF- κ B, oxidative stress, thioredoxin, thioredoxin reductase.

INTRODUCTION

A growing body of evidence has demonstrated that the DNA-binding properties of numerous eukaryotic transcription factors are thiol-redox regulated [1–9]. The best-studied examples include NF- κ B, c-Jun, c-Fos, Myb, p53, TFIIC and BZLF1. The transcription nuclear factor κ B (NF- κ B) plays important roles in immune function, inflammation response, cell adhesion and growth control [10]. The NF- κ B family includes structurally related transcription factors RelA, RelB, c-Rel, NF- κ B1 p50 and NF- κ B2 p52 which associate to form transcriptionally competent homo- and hetero-dimers [11].

NF- κ B is stored as an inactive cytoplasmic complex in association with inhibitory proteins called I κ Bs (inhibitory κ Bs). Treatment of cells with inducers of NF- κ B activity leads to phosphorylation, ubiquitination and degradation of the I κ B proteins, thus allowing NF- κ B to translocate into the nucleus [12]. NF- κ B is activated by a plethora of distinct stimuli, including mitogens, cytokines, bacterial lipopolysaccharide, viruses, UV radiation, γ -radiation and oxidants (reviewed in [13]). Many of these stimuli cause oxidative stress in cells due to an increased production of reactive oxygen intermediates (ROIs) such as superoxide, hydrogen peroxide, hydroxyl radicals and secondary reactive intermediates [14]. Several lines of evidence suggest that ROIs, in particular H₂O₂, serve as common second-messenger-

like molecules in the various pathways leading to NF- κ B activation. Thus ROIs act as mediators of NF- κ B activation, although NF- κ B must be in a reduced state to bind DNA *in vitro* [4]. This effect is mediated by the redox state of a cysteine residue conserved in all members of NF- κ B family, corresponding to Cys⁶² in the p50 subunit [1,15,16]. It is difficult to envisage that ROIs act directly on NF- κ B, since the direct action of ROIs on NF- κ B would result in the oxidation of NF- κ B and consequently the impairment of DNA-binding activity. However, the incubation of different cell types with H₂O₂ induces increased levels of thioredoxin (Trx) and H₂O₂ induces in HeLa cells the translocation of Trx in the nucleus. Trx seems to play dual and opposing roles in the regulation of NF- κ B [17]. In the cytoplasm it interferes with signals to I κ B kinases and blocks the degradation of I κ B. In the nucleus, Trx enhances NF- κ B transcriptional activities by enhancing its ability to bind DNA.

Trx is a ubiquitous disulphide reductase which catalyses reduction of exposed S–S bridges in a variety of proteins through an NADPH-dependent thioredoxin reductase (TrxR) system [18]. The TrxRs are homodimeric flavoproteins that catalyse the reduction of Trx by NADPH [19]. The Trx–TrxR system plays an important role in many aspects of cell function, including cell growth, protection against oxidant damage and apoptosis [20]. Human tumours have been shown to have increased levels of TrxR activity, and inhibition of TrxR in cultured cells is

Abbreviations used: NEM, *N*-ethylmaleimide; CDNB, 2-chloro-1,3-dinitrobenzene; ROI, reactive oxygen intermediate; Trx, thioredoxin; hTrx, human thioredoxin; TrxR, thioredoxin reductase; hGR, human glutathione reductase; DCIP, 2,6-dichloroindophenol; NZ, naphthazarine (5,8-dihydroxy-1,4-naphthoquinone); OA, okadaic acid; NF- κ B, nuclear factor κ B; I κ B, inhibitory κ B; CEF, chicken embryo fibroblast(s); PEI, polyethyleneimine; wt, wild-type; EMSA, electrophoretic-mobility-shift assay; CIP, calf intestine phosphatase; C26D, Cys²⁶ → Asp; C26S, Cys²⁶ → Ser; LTR, long terminal repeat; SNV, spleen necrosis virus; TNF α , tumour necrosis factor α .

¹ To whom correspondence should be addressed (e-mail Corine.Glineur@pasteur-lille.fr).

associated with decreased cell growth and apoptosis. Structural-biological approaches have shed new light on the redox regulation of NF- κ B by Trx. The solution structure of a complex between human Trx (hTrx) and a 13-residue peptide comprising residues 56–68 of the p50 subunit has been determined [21]. The structure reveals that, in addition to the intramolecular disulphide bridge between Cys³² of hTrx and Cys⁶² of the NF- κ B peptide, the complex is stabilized by numerous hydrogen-bonding, electrostatic and van der Waals interactions. A site on the surface of Trx containing the redox-active cysteine residues can recognize the DNA-binding loop of NF- κ B and is likely to reduce the oxidized cysteine residue by donating reducing equivalents in a structure-dependent way. Several studies have shown that TrxR is inhibited by some quinones, an important class of redox-active compounds that are known for their anticancerous or anti-parasitic properties [22]. The thiol alkylating reagent 2-chloro-1,3-dinitrobenzene (CDNB) is an irreversible TrxR inhibitor, acting by alkylation of both the redox-active selenocysteine residue and its neighbouring cysteine residue of the active site [23]. This irreversible inactivation, which is faster with hTrxR than other thiols such as GSH or the homologous flavoprotein human glutathione reductase (hGR), is accompanied by a large increase of the NADPH oxidase activity, producing superoxide anion radicals. 2,6-Dichloroindophenol (DCIP) is also an irreversible hTrxR inhibitor and is reduced via a single-electron mechanism, leading to the production of DCIP semiquinone imine free radical and superoxide anion radicals [24]. Naphthazarine (5,8-dihydroxy-1,4-naphthoquinone, NZ) is a reversible TrxR inhibitor which also leads to production of superoxide anion radicals. Several studies suggest that the activation of NF- κ B is triggered through a cascade of membrane-bound and cytoplasmic protein kinases. Several of these kinases were found to be activated by hydrogen peroxide and by the thiol oxidizing agent diamide [25,26]. Using a variety of experimental conditions and stimuli, it has been demonstrated that the DNA binding of NF- κ B [27,28] and NF- κ B-dependent transcription [29,30] are positively regulated by phosphorylation. Different inducers can activate different kinases to phosphorylate NF- κ B at distinct sites to modulate its transcriptional activity. Zhong et al. [30] observed that, upon lipopolysaccharide stimulation, the transcriptional activity of RelA was increased after phosphorylation on Ser²⁷⁶ in the consensus protein kinase A phosphorylation site. However, a mutant RelA protein with an alanine-to-serine substitution at position 276 can still be phosphorylated upon tumour-necrosis-factor- α (TNF- α) treatment. The TNF- α -induced phosphorylation of RelA occurs on Ser⁵²⁹, which is in the C-terminal transactivation domain. It was also reported that casein kinase II can phosphorylate the RelA subunit and that casein kinase II is associated with RelA *in vivo* [31]. The c-Rel protein is phosphorylated on serine and threonine residues and, to a lower extent, on tyrosine residues [32,33]. However, under standard labelling conditions, very limited amounts of ³²P are incorporated into these proteins *in vivo*, probably due to the constitutive action of a c-Rel-specific phosphatase. A previous report [34] has shown that a protein phosphatase called PPX specifically interacts with c-Rel. In an effort to increase the efficiency of ³²P labelling of Rel proteins, the cells were labelled and lysed in the presence of okadaic acid (OA), a potent inhibitor of phosphatases 1 and 2A [35]. OA has been reported to be a potent activator of NF- κ B [36]. Hyperphosphorylation of the c-Rel proteins following the treatment with OA caused a significant retardation in the electrophoretic mobilities on SDS/polyacrylamide gels [32].

In the present paper we show that the redox regulation of the c-Rel protein controls its phosphorylation. We have tested the

effect of inactivation of TrxR by different inhibitors on the phosphorylation of the c-Rel protein. Interestingly, three of the compounds, DCIP, NZ and CDNB used in the present study interfere with the phosphorylation of the c-Rel protein. In agreement with these results, the replacement of the redox cysteine residue by an aspartic acid residue in the c-Rel protein corresponding to Cys⁶² in the p50 subunit impairs the phosphorylation of the protein *in vitro* and *in vivo*. This is the first demonstration of a relationship between the redox regulation and the phosphorylation of a transcription factor.

MATERIALS AND METHODS

Cells

Chicken embryonic fibroblasts (CEF) were prepared from 6-day-old chick embryos and used for transient transfection experiments after four to eight passages. The canine osteosarcoma-derived D17.2G cell line, kindly provided by Dr. Anthony M. C. Brown (Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 New York Avenue, NY 10021, U.S.A.) and Dr Thierry Jaffredo (UPR9064-CNRS, Institut d'embryologie cellulaire et moléculaire, Collège de France, 94736 Nogent-sur-Marne cedex, France), was cultured in Dulbecco's medium supplemented with 7% fetal-bovine serum and methothrexate at 20 μ M.

Transfections

The CEF primary cells were transiently transfected using the cationic polymer polyethyleneimine (PEI; Exgen 500 from Euro-medex) as carrier for delivering plasmids in cells. Cells were seeded at 300 000 cells/well in six-well dishes 18 h before transfection. A 1 μ g portion of the plasmid DNA and 3 μ l of PEI stock solution were diluted into 50 μ l of 150 mM NaCl. The two solutions were mixed and, after 15 min, the transfection mixture was added to the cells. After 5 h incubation, fresh medium was added to the cells. At 48 h post-transfection, the cells were rinsed and lysed in RIPA buffer [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM EDTA/1% (v/v) Triton X-100/0.5% sodium deoxycholate/0.1% SDS/1% aprotinin/1 mM PMSF/1 mM orthovanadate and 10 mM NaF]. The D17.2G cells were seeded the day before the transfection in six-well plates at 5×10^5 cells/well. The plasmids (1 μ g) were introduced into the cells using PEI reagent (3 μ l/ μ g of DNA). Clonal cell populations were selected in the presence of 12.5 μ g/ml phleomycin (CAYLA ZI Montaudran, Toulouse, France) and harvested at day 15 post-transfection.

Reagents

NZ was purchased from Acros (Noisy Le Grand, France) and DCIP sodium salt was purchased from Aldrich. *N*-Ethylmaleimide (NEM) and CDNB were purchased from Sigma. Carbobenzoyloxy-L-leucyl-L-leucyl-L-leucinal (MG-132) was purchased from Calbiochem.

Plasmids

The c-*rel* cDNA was cloned in the eukaryotic TA cloning plasmid pCR3 (Invitrogen). The vector pcDNA3.1/Myc-His (Invitrogen) was used to express the Myc-His-tagged proteins. A *Bgl*III site at the codon stop of c-*rel* cDNA was created by site-directed mutagenesis using the QuikChange Kit (Stratagene). The *Bam*HI-*Bgl*III fragment of the c-*rel* sequence was inserted in the pcDNA3.1/Myc-His, creating an expression vector. The

Myc-His-tagged c-Rel proteins were detected using a monoclonal antibody directed against the Myc epitope (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The *EcoRI* fragment containing the cDNA isolated from a construct provided by Dr Paula Enrietto was cloned in the eukaryotic TA cloning plasmid pCR3. For stable transfection in D17.2G, the *c-rel* cDNA was cloned in the construct spleen necrosis virus (SNV)-bleomycin::alcohol dehydrogenase which contains the phleomycin-resistance gene and was provided by Dr Thierry Jaffredo [37].

Antibodies

The polyclonal anti-c-Rel antibodies, called 'α-c-Rel 1 antiserum', were generated by immunization of rabbits with a peptide corresponding to amino acids 428–442 of the chicken c-Rel protein. This peptide was synthesized by Hélène Gras and Marc Bossus (UMR8525-CNRS-Université Lille II-Institut Pasteur de Lille, Institut de Biologie de Lille, 59021 Lille, France) and was coupled to BSA with 6-maleimidohexanoic acid. The mouse monoclonal phosphotyrosine antibody was purchased from Santa Cruz Biotechnology.

Oligonucleotide-directed mutagenesis

The cDNA encoding the c-Rel mutant C26D (Cys²⁶ → Asp) and C26S (Cys²⁶ → Ser) proteins were generated using the QuikChange Site-Directed Mutagenesis Kit (from Stratagene). Point mutations were introduced in the *c-rel* DNA using *Pfu* DNA polymerase, double-stranded DNA plasmid and two synthetic complementary oligonucleotide primers containing the desired mutation. The mutated cDNAs have been completely sequenced.

In vitro translation of proteins

Chicken c-Rel wild-type (wt) and mutant proteins, and chicken RelA proteins were produced in 25 µl of TNT wheat-germ extract (from Promega) containing 0.5 µg of DNA template in accordance with the manufacturer's recommendations. A 5 µl portion of ³⁵S-labelled proteins were loaded on a 10% (w/v) polyacrylamide gel. The gel was fixed, treated with Amplify, dried and autoradiographed using an intensifying screen.

The effect of ATP and OA on the phosphorylation extent of the c-Rel proteins translated in a wheat-germ extract was analysed by mixing 5 µl of the c-Rel proteins synthesized *in vitro* in the presence of [³⁵S]methionine to 45 µl of a TMg buffer [50 mM Tris/HCl (pH 7.6)/5 mM MgCl₂], containing 2 mM ATP, an ATP-regenerating system (10 mM creatine phosphate, 3.5 units/ml creatine kinase and 0.6 unit/ml inorganic pyrophosphatase) and/or 1.25 µM OA. After 1 h incubation at 37 °C, 50 µl of the reaction mixture was analysed on a 10% polyacrylamide gel. As a control, 45 µl of TMg buffer was added to 5 µl of ³⁵S-labelled proteins. For ³²P-labelling experiments, 5 µl of c-Rel proteins synthesized in wheat-germ extract were added to the TMg buffer containing [γ-³²P]ATP (10 µCi, 3000 Ci/mmol) and 1.25 µM OA and further incubated for 10 min. The 50 µl mixture was diluted 10-fold in RIPA buffer containing protease and phosphatase inhibitors [50 mM Tris/HCl (pH 7.4)/150 mM NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/10 mM NaF/1% aprotinin/0.1 mg/ml PMSF/1 µg/ml leupeptin], mixed with 5 µl of α-c-Rel 1 antiserum and incubated overnight at 0 °C. The ³²P-labelled c-Rel immunocomplexes purified on Protein A-Sepharose were boiled in 1% SDS buffer and the c-Rel proteins were immunoprecipitated again after adjusting the concentration of SDS to that used in the RIPA buffer.

Oligonucleotides and labelling procedure

Oligonucleotide probes were prepared by annealing of a primer 5'-CTCGAGCCTCTC-3' for HIV probe to oligonucleotide templates followed by primer extension with the Klenow fragment of DNA polymerase I (Boehringer-Mannheim) in the presence of [α-³²P]dCTP. The following template was used: 5'-AGCTTCAACAGAGGGGACTTTCCGAGAGGCTCGAG-3' containing the HIV type 1 long terminal repeat (LTR) κB site.

Electrophoretic mobility-shift assays (EMSAs)

In vitro-translated proteins (4 µl of extracts) were incubated in the presence of 0.5 µg of poly(dI-dC) and incubation buffer [10 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM dithiothreitol/1 mM EDTA/5% glycerol], followed by addition of 50 000–100 000 c.p.m. of ³²P-labelled oligonucleotide and incubation at room temperature for 20 min. The reactions were performed in a final volume of 18 µl and samples were run on native 5% polyacrylamide gels at 200 V in TBE (25 mM Tris base/22 mM boric acid/0.25 mM EDTA/0.1% Triton, pH 8.0) at 4 °C. For supershift experiments, 1 µl of α-c-Rel 1 antiserum was added to 4 µl of extract and incubated for 15 min at 0 °C before addition of the incubation buffer, poly(dI-dC) and probe. The incubation was continued at room temperature for 20 min.

Immunoblotting and immunoprecipitation analyses

Expression of transfected *c-rel* cDNA was monitored using the specific antipeptide antibody c-Rel 1 antiserum. Cells were rinsed in ice-cold PBS and lysed in RIPA buffer. After centrifugation at 100 000 g, equal amounts of total proteins were analysed by SDS/PAGE. The proteins were transferred to nitrocellulose and probed with the α-c-Rel 1 antiserum as the primary antibody and detected using the ECL[®] (enhanced chemiluminescence) Western blotting analysis system (Amersham Pharmacia Biotech). The D17.2G-c-*rel* cells that overexpress the c-Rel protein were treated for 2 h with the TrxR inhibitors and 1 h before harvesting, OA was added to the cells at 600 nM. The cells were lysed directly in Laemmli buffer [62.5 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 700 mM 2-mercaptoethanol, 10% (v/v) glycerol and 0.1% Bromophenol Blue] and the lysates were analysed by Western blotting using the α-c-Rel 1 antiserum. The blots were stripped and analysed with anti-phosphotyrosine antibody.

Glutaraldehyde cross-linking

³⁵S-labelled *in vitro*-translated proteins (5 µl of extracts) were incubated with 0.004% glutaraldehyde at room temperature in a final volume of 25 µl. To avoid aggregates, the glutaraldehyde was added step by step over 5 h. Cross-linked products and untreated proteins were electrophoresed on an SDS/gelatin 6–12% PAGE gel.

RESULTS

Mutagenesis in the RXXRXXXC Rel/κB DNA-binding motif of chicken c-Rel

All the members of the NF-κB family contain in their DNA-binding domain a motif RXXRXXXC. It has been demonstrated that the cysteine residue contained in this motif plays a critical role in the DNA-binding properties of NF-κB, since chemical modifications of this residue impairs the DNA-binding properties of the protein [15]. In addition, this cysteine residue needs to be in a reduced state for maximal DNA-binding activity

Figure 1 *In vitro* translation of c-Rel wt, C26D and C26S proteins in wheat-germ extract

The proteins were translated from pCR3-*c-rel* in the presence of [35 S]methionine. The arrowhead indicates the position of c-Rel. The positions of retarded forms of c-Rel are also indicated. In order to test the nature of the slower-migrating forms of c-Rel, 5 μ l of Rel translated proteins were incubated with 1 unit of CIP for 1 h at 37 °C.

Figure 2 Phosphorylation of c-Rel wt, C26D and C26S proteins *in vitro*

(A) The c-Rel proteins were translated in wheat-germ extract and incubated with OA and [γ - 32 P]ATP for 10 min. The c-Rel proteins were immunoprecipitated using the α -c-Rel 1 antiserum. The immunoprecipitated proteins were boiled in 1% SDS to dissociate c-Rel-associated proteins and immunoprecipitated again using the same c-Rel antiserum. (B) Effect of OA and ATP on the phosphorylation extent of the c-Rel wt, C26D and C26S proteins in wheat-germ extract. *In vitro*-translated c-Rel proteins synthesized in the presence of [35 S]methionine were incubated with ATP and an ATP-regenerating system and/or OA for the indicated time periods. 'ctrl' (control) corresponds to incubation with the buffer only.

[4]. In order to better understand the role of this conserved cysteine in the function of NF- κ B protein, we have replaced the cysteine residue in c-Rel (Cys²⁶) with a serine residue to get the mutant c-Rel protein C26S and by an aspartic acid residue for the mutant c-Rel C26D protein. It has previously shown that the conservative substitution of Cys⁶² by a serine residue in the p50 subunit did not affect the DNA-binding activity of the protein [3]. In contrast, the substitution of the Cys⁶² in p50 with an aspartic acid reduced its DNA-binding activity.

The cDNAs encoding the wt avian c-Rel protein and mutant C26D and C26S proteins, were cloned in the pCR3 vector to allow expression of the c-Rel proteins *in vitro* or *in vivo*. As shown in Figure 1, these different c-Rel wt, C26D and C26S proteins were expressed in wheat-germ extract. The upper bands above c-Rel disappeared after treatment with calf intestine phosphatase (CIP), suggesting that they correspond to

Figure 3 Phosphorylation of c-Rel wt and C26D and C26S proteins *in vivo*

(A) Phosphorylation of the c-Rel wt protein in stably *c-rel*-overexpressing D17.2G cells. The D17.2G cells encoding the c-Rel wt protein and control D17.2G cells were labelled with [32 P]P_i for 2 h in the presence (+) or absence (–) of OA. For OA-treated cells, OA was added to cells at 1 μ M 1 h before lysis. The proteins from the different cell lysates were immunoprecipitated using the α -c-Rel 1 antiserum. (B) Phosphorylation of c-Rel wt, C26D and C26S proteins in transiently transfected CEF cells. Whole-cell lysates from CEF cells transiently transfected with the pcDNA3-*myc-his-c-rel* constructs encoding the tagged c-Rel wt, C26D or C26S proteins were analysed by Western blotting using the α -c-Rel 1 antiserum. The cells were lysed 48 h post-transfection. For OA-treated cells, OA was added to cells at 1 μ M 1 h before lysis. Equal amounts of total proteins were loaded on a SDS/10%-polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with Myc epitope antiserum as the primary antibody. The position of tagged Myc-His c-Rel proteins is designated by an arrow.

phosphorylated forms. According to this hypothesis, the *in-vitro*-translated c-Rel C26D protein is not phosphorylated.

The c-Rel wt and mutant C26S proteins are phosphorylated in wheat-germ extract and the c-Rel C26D protein is not phosphorylated

It has been previously shown that c-Rel is a phosphoprotein [32,33]. We have shown that the slower-migrating forms of c-Rel in wheat-germ extracts corresponded to phosphorylated forms. The c-Rel proteins synthesized in wheat-germ extracts were incubated with [γ - 32 P]ATP and OA, an inhibitor of the serine/threonine phosphatases PP2A and PP1A, and the c-Rel proteins were double-immunoprecipitated with the α -c-Rel antiserum 1 as indicated in the Materials and methods section. The double immunoprecipitation permits the elimination of c-Rel-associated proteins. As expected, the c-Rel wt and C26S proteins incorporated [32 P]P_i (Figure 2A). On the other hand, in accordance to previous results, [32 P]P_i is not incorporated in the c-Rel C26D protein (Figure 2A). As a control, we double-immunoprecipitated the *in-vitro*-translated 35 S-labelled c-Rel wt and mutant proteins with the α -c-Rel 1 antiserum. Our results indicate that the absence of bands for C26D in Figure 2(A) is due to the inability of the protein to be phosphorylated. We thus concluded that the mutant c-Rel C26D protein is not phosphorylated *in vitro*.

In vitro-translated 35 S-labelled c-Rel wt, C26D and C26S proteins were incubated with ATP and an ATP-regenerating system and/or OA at 37 °C for different times (Figure 2B). The incubation of the protein with the buffer or ATP with an ATP-regenerating system for 60 min leads to the dephosphorylation of the c-Rel wt and C26S proteins. The addition of OA prevents this dephosphorylation. The incubation of extracts with ATP, ATP-regenerating system and OA leads to complete phosphorylation of the proteins within less than 30 min. The results also indicate that the phosphorylation of the c-Rel wt and C26S proteins in wheat-germ extracts increases sequentially

Figure 4 Importance of phosphorylation on the DNA-binding properties of the c-Rel protein

(A) EMSA analysis of the c-Rel wt, C26D and C26S proteins. *In-vitro*-translated c-Rel proteins in wheat-germ extract were mixed with ³²P-labelled HIV oligonucleotide probe. After 20 min of incubation at room temperature, the reaction mixture was loaded on to a 5%-polyacrylamide non-denaturing gel. (B) EMSA analysis of the c-Rel wt protein treated with CIP compared with EMSA analysis of the c-Rel C26D protein. A 4 μ l portion of c-Rel wt proteins produced in an unlabelled wheat-germ system were incubated with 1 unit of CIP for 1 h at 37 °C or 0 °C and mixed with 1 ng of ³²P-labelled HIV DNA probe. A 4 μ l portion of untreated wt or C26D c-Rel protein was also mixed with the HIV probe DNA. Lanes called 'c' correspond to EMSA analysis with wheat-germ extract programmed with the empty vector pCR3. (C) Analysis by Western blotting of the c-Rel proteins used in the EMSA analysis. A 4 μ l portion of *in-vitro*-translated c-Rel proteins untreated or treated with CIP were allowed to migrate on a SDS/10%-PAGE gel, then transferred to a PVDF membrane. The membrane was incubated with α -c-Rel 1 antiserum and proteins were revealed with the ECL[®] system.

over time. In this experiment we showed that the c-Rel C26D protein does not undergo a significant mobility shift after addition of ATP, ATP-regenerating system and OA.

The c-Rel mutant protein C26D is not phosphorylated *in vivo*

OA has been reported to be a potent activator of NF- κ B, supporting a role of protein phosphorylation in the NF- κ B activation process [36], since OA is a potent inhibitor of protein phosphatases. We have tested the effect of OA on the phosphorylation of the c-Rel wt protein in *c-rel*-overexpressing cells. We have stably transfected the D17.2G cell line with a SNV-derived construct encoding the c-Rel protein and individual clones were tested for expression of the c-Rel protein. We have chosen a clone with significant amount of c-Rel protein and labelled the cells with [³²P]P_i in the presence of OA. As shown in Figure 3(A), the immunoprecipitated protein is hyperphosphorylated when cells are treated with OA, and this hyperphosphorylation caused a significant retardation in the electrophoretic mobilities of c-Rel on SDS/polyacrylamide gels as observed in wheat-germ extracts.

Since we did not get stable transfectants overexpressing the mutant C26D c-Rel protein, we tested the effect of OA on the phosphorylation status of the c-Rel wt, C26D and C26S

proteins transiently expressed in CEF primary cells. We have transfected these cells with the different pcDNA3-myc-his-c-rel constructs. These expression vectors encode tagged c-Rel proteins which can be detected by Western blotting using a Myc epitope antiserum (Figure 3B). In the CEF cells, the mutant Myc-His-c-Rel C26D protein is more expressed than the wt and the mutant Myc-His-c-Rel C26S protein (Figure 3B). A plausible explanation could be that the mutant C26D c-Rel is more stable than the wt and mutant C26S c-Rel proteins. The Myc-His-c-Rel wt or C26S proteins transiently expressed in cells treated with OA appear as diffuse bands scarcely visible in relation to phosphorylated forms. In contrast, the OA treatment of the cells transiently expressing the Myc-His-c-Rel C26D does not change the amount and the migration profile of the protein. These results suggest that the c-Rel protein C26D is not phosphorylated *in vivo*.

The replacement of the Cys²⁶ in the c-Rel protein affects its DNA-binding properties

wt and mutant forms of c-Rel proteins translated in wheat-germ extract were tested for their ability to bind the κ B site from the LTR of HIV in EMSAs. The results show that the substitution of Cys²⁶ with a serine residue did not impair the DNA-binding properties of c-Rel (Figure 4A). A visible increase in the

Figure 5 Interaction of the c-Rel wt, C26D and C26S proteins with I κ B α in a wheat-germ extract

³⁵S-labelled *in-vitro*-translated c-Rel wt, C26D or C26S proteins were either directly analysed by SDS/PAGE (lanes —) or analysed after treatment with 0.004% glutaraldehyde (lanes +). *In-vitro*-translated I κ B α was added to ³⁵S-labelled c-Rel wt, C26D or C26S proteins and the complexes were treated with 0.004% glutaraldehyde. The autoradiogram shows the homodimers and the complexes containing I κ B α . The negative control, 'c', corresponds to a c-Rel protein mutated in the dimerization region.

binding could be observed. A PhosphorImager quantification indicated that the difference in intensity between the c-Rel wt and C26S complexes was twofold. In contrast, the replacement of the cysteine with an aspartic acid residue abolished the DNA-binding activity of the protein. The retarded forms observed when the c-Rel proteins C26D were mixed with the HIV κ B sequence should correspond to the binding of endogenous proteins from the wheat-germ extract, since an extract programmed with the empty vector pCR3 presents the same complexes to the same extent. In conclusion, the c-Rel C26D protein would seem to have lost its capacity to interact efficiently with κ B site. To determine if the binding of the c-Rel protein to DNA is correlated with its phosphorylation status, the wt protein synthesized in wheat-germ extract was treated with CIP and its binding to the HIV κ B probe was analysed by EMSA. We have tested two conditions of CIP treatment: 1 h at 37 °C, which should give the best conditions for dephosphorylation, and 1 h at 0 °C, conditions that should result in partial dephosphorylation (lanes 4 and 5 in Figure 4B). The results indicate that the more the c-Rel protein is dephosphorylated, the less it binds the HIV κ B probe. The supershift with the α -c-Rel 1 antiserum indicates the position of retarded c-Rel complexes. These results show clearly the absence of c-Rel complexes bound to κ B site for the C26D protein (lanes 3 and 6 in Figure 4B). The preimmune serum was used as a control in the supershift study (lane 7 in Figure 4B). Figure 4(C) shows the Western blotting of the c-Rel wt, c-Rel C26D and the gradually dephosphorylated c-Rel wt proteins used for the EMSA.

We have demonstrated, by using glutaraldehyde as cross-linking agent, that the change in the DNA-binding properties of the c-Rel protein C26D is not the result of its inability to form homodimers, since, as shown in Figure 5, the c-Rel wt, C26D and C26S proteins are able to form homodimers with the same efficiency. The migration of homodimers were retarded compared with the expected size of the dimers, probably due to cross-linking with glutaraldehyde. A c-Rel protein mutated in the dimerization domain did not form these complexes, suggesting that the high-molecular-mass bands observed for the c-Rel wt, C26D and C26S proteins cross-linked with glutaraldehyde corresponded to homodimers. In the same series of experiments, we have found that the non-phosphorylated protein C26D c-Rel interacts with the inhibitor I κ B α , suggesting that this mutant protein did not undergo an important conformational change that could have modified its DNA-binding specificity.

Figure 6 NEM and CDNB inhibit the phosphorylation of the c-Rel wt protein *in vivo*

The c-*rel*-overexpressing D17.2G cells were treated with the indicated concentrations of NEM or CDNB for 2 h. OA was added to culture medium 1 h before harvesting the cells. The effect of dithiothreitol (DTT) was tested by adding it at the same time as NEM or CDNB. Whole-cell lysates were prepared and analysed by Western blotting using the α -c-Rel antiserum 1. The phosphorylated forms of the c-Rel protein are indicated as c-Rel-P.

Thiol modifiers inhibit the phosphorylation of the c-Rel protein *in vivo*, and this effect can be prevented by reducing agents

Different thiol modifiers, such as NEM and CDNB, inhibit NF- κ B activity in TNF α and interleukin-1-activated cells as determined by EMSA analysis [38]. Both of these compounds were also shown to react directly with NF- κ B *in vitro*, and it has been suggested that they can modify Cys⁶² in the p50 subunit [1]. We have tested the effect of NEM and CDNB *in vivo* on the phosphorylation of the c-Rel protein. The c-*rel*-overexpressing cells D17.2G were treated with different concentrations of NEM and CDNB for 2 h. In order to amplify the phosphorylation of c-Rel *in vivo*, the cells were incubated with the phosphatase inhibitor OA 1 h before harvesting the cells. The results of Western blotting in Figure 6 indicate a strong inhibition by NEM of the phosphorylation of the c-Rel protein. Dithiothreitol counteracts this action. CDNB also modifies the phosphorylation pattern of the protein, although to a lesser extent, and dithiothreitol prevents this. These results suggest that the phosphorylation of c-Rel is triggered *in vivo* only when the cysteine residues involving the conserved cysteine residue of the DNA-binding domain are in a reduced form.

TrxR inhibitors impair the phosphorylation of the protein

It has been shown that NF- κ B DNA-binding activity is enhanced by thiols, including dithiothreitol, cysteine, GSH and reduced Trx [1,39,40]. The latter is by far the most effective, and likely the only, physiologically relevant thiol compound performing this function. Trx was found to restore the DNA-binding activity of oxidized NF- κ B *in vitro* and to increase gene expression from HIV-LTR in intact cells [1,40]. Trx acts as an efficient protein disulphide reductase, and the major Trx form has been detected in the reduced state inside the cell [41]. The reduced state of Trx is maintained by homodimeric TrxR, which uses NADPH as hydrogen donor [19]. We have tested the effect of two TrxR inhibitors, NZ and DCIP, on the phosphorylation of the c-Rel protein. The results presented in Figure 7(A) indicate that the two agents decrease the OA-induced phosphorylation of the c-Rel protein in a dose-dependent manner. In Figure 7(B) we have analysed the effect of TrxR inhibitors on overall tyrosine phosphorylation by stripping the blots of the Figure 7(A) and incubating them with an anti-phosphotyrosine antibody. Our results indicate that NZ and DCIP increase tyrosine-specific phosphorylation. Thus the reduction in c-Rel phosphorylation in

Figure 7 Two TrxR inhibitors with different action modes, NZ and DCIP, impair the OA-induced phosphorylation of c-Rel in D17.2G-c-rel cells

The c-rel-overexpressing D17.2G cells were treated with the indicated concentrations of NZ and DCIP for 2 h. In lanes OA, 1 h before the harvesting, OA was added to the cells at 600 nM and the cell extract analysed by Western blotting (A) with α-c-Rel 1 antiserum or (B) with an anti-phosphotyrosine antibody. In control, the cells were treated with DMSO ('v'). The lanes labelled 'MG' correspond to lysates of cells treated with MG-132 for 2 h.

cells treated with NZ and DCIP is unlikely due to an effect of TrxR inhibitors on kinase activities in the cells. In Figure 7(A) we noticed the presence of high-molecular-mass bands recognized by the c-Rel antiserum in cells treated with the different inhibitors. The treatment of the cells with an inhibitor of proteasome, MG-132, at 10 μM for 2 h, does not increase the intensity of these bands, suggesting that they do not correspond to ubiquitinated forms. In these conditions, MG132 does not induce apoptosis of the treated cells. We suggest that these bands originate from aggregation of oxidized forms of c-Rel. Formation of protein aggregates occurs during free-radical reactions [42].

DISCUSSION

In the present study we have shown that redox regulation and phosphorylation of the c-Rel protein are not separate events, but rather the redox status of the conserved cysteine residue of the DNA-binding domain is important for the phosphorylation of the protein. It was proposed that all the agents that activate NF-κB stimulate it by causing oxidative stress. This hypothesis is based on several lines of evidence. First, direct addition of H₂O₂ to culture medium activates NF-κB in some cell lines [43]. Antioxidants inhibit pathways to NF-κB activation [14]. Finally, inhibition or overexpression of enzymes that affect the level of intracellular ROIs have been shown to modulate the activation of NF-κB by some agents [29,44,45]. Like c-Jun, c-Fos, Myb, p53, PEBP2 and nuclear receptors such as glucocorticoid receptor or oestrogen receptor, NF-κB has a well-conserved cysteine residue in its DNA-binding loop. The requirement of free thiol groups in NF-κB in the DNA recognition was investigated using a moderate thiol-group-specific alkylating agent, NEM, which blocks the thiol groups irreversibly, or with diamide, which catalyses chemical oxidation of free thiol groups. Alkylation of NF-κB by NEM or oxidation of the factor by diamide inhibited the DNA binding [40].

We have replaced the redox-conserved cysteine residue in the c-Rel protein by an aspartic acid residue or a serine residue to create the mutant C26D or C26S proteins respectively. The choice of these two substitutions was based on results obtained with the subunit p50 of NF-κB. Whereas the conservative substitution of the conserved Cys⁶² with a serine residue did not affect the DNA-binding activity of p50, the replacement of the same cysteine residue by an aspartic residue reduced DNA-binding activity of the p50 subunit [3]. Other studies have demonstrated that DNA binding by NF-κB can be inhibited by the modification of the sensitive thiol of Cys⁶² in the p50 subunit [1]. This thiol must be maintained in a reduced state for DNA binding to occur [4]. These results therefore suggest that the DNA-binding activity of the Rel homology domain could be regulated by cellular redox factors and the cysteine residue conserved in this region is a target for this regulation. Redox regulation of DNA binding via a conserved cysteine residue has also been proposed for the AP-1 subunits Jun and Fos [5]. We have found that Cys²⁶ of the c-Rel protein, corresponding to Cys⁶² in p50, is implicated in the control of its DNA-binding activity, since the mutant c-Rel C26D protein is unable to bind the κB site. Cys²⁶ in the c-Rel protein resembles the redox-responsive cysteine residue identified in Jun and Fos, which is also flanked by basic amino acids and gave increased DNA binding when it was substituted with serine. In agreement with these results, the mutant c-Rel C26S protein shows an enhanced DNA-binding activity.

It was previously shown that the oxidant hydrogen peroxide activated NF-κB in Jurkat T cells and HeLa cells in a post-translational fashion [43]. We have found that the substitution of the redox-sensitive cysteine residue with an aspartic acid residue modified the phosphorylation pattern of the protein *in vitro* and *in vivo*. The wild-type c-Rel protein is phosphorylated in wheat-germ extract, and this post-translational modification leads to the appearance of slower-migrating bands. A similar electro-

phoretic pattern has been observed in cells overexpressing the c-Rel protein and treated with OA, a serine/threonine phosphatases PP2A and PP1A inhibitor. The mutant c-Rel C26D protein, in contrast with the c-Rel wt and C26S proteins, is not phosphorylated *in vitro* and *in vivo*, and this absence of phosphorylation correlates with the absence of DNA binding. Kinetic experiments indicate that, in wheat-germ extracts, the phosphorylation events occur in stages over 30 min and need ATP and an inhibitor of phosphatase to be completed. The substitution of cysteine with aspartic acid in the c-Rel protein could impede the first phosphorylation event on a residue located near Cys²⁶. A report by Neumann et al. [33] showed that the activation of Jurkat cells by phorbol methyl ester and phytohaemagglutinin results in the increase of c-Rel phosphorylation on tyrosine residues. In addition, the tyrosine kinase inhibitor herbimycin A was found to block NF- κ B stimulation in response to interleukin-1 and PMA in Jurkat cells [46]. Tyr²⁴ near Cys²⁶ in the c-Rel protein could be a target of an inducible phosphorylation, and this phosphorylation would occur only if the nearby cysteine residue is in the reduced state. On the other hand, the substitution of Cys²⁶ by a serine residue in the c-Rel protein creates a potential RXXS phosphorylation site with a consensus sequence for kinases like protein kinases A and C. The protein seems to have a better affinity for the DNA target than the wt, and we show that the DNA binding is dependent on the phosphorylation of the protein. We suggest that Ser²⁶ in the mutant c-Rel C26S protein could be phosphorylated independently of the redox environment and this phosphorylation could induce a conformational change, making the phosphorylation sites accessible to other kinases. Interestingly, it has been shown that the replacement of Cys¹⁵⁴ in Fos and Cys²⁷² in Jun with a serine residue increased DNA-binding activity and loss of redox control [5]. The conformational change necessary for activation of the c-Rel protein could occur naturally when the protein is reduced on the conserved redox cysteine. In the case of the mutant C26D c-Rel protein, no conformational change could occur, preventing the phosphorylation of the protein. It has been previously shown that the c-Rel protein contains a negative regulatory domain in its N-terminal part [47]. In the full-length protein, the negative regulatory domain is dominant, as judged by poor transcriptional activation. When the N-terminal half of the protein is removed, the C-terminal half of the protein functions as a potent transcriptional activator. We hypothesized that the reduction of the conserved cysteine of the DNA-binding domain located in the N-terminus could induce a conformational change and allow the phosphorylation of the protein. The case of v-Rel is also very interesting. In contrast to c-Rel, v-Rel transforms haematopoietic cells. The oncogenic activity of v-Rel results from deletions and single-amino-acid substitutions. Nehyba et al. [48] have shown that the replacement of Met²⁰ in c-Rel by a threonine residue in v-Rel alters DNA-binding specificities of v-Rel. Since this replacement introduces a potential phosphorylation site in v-Rel, we believe that the v-Rel protein could have lost its redox regulation, as suggested for the mutant C26S c-Rel protein, and could, in consequence, be constitutively active.

It has been previously reported that the DNA-binding and the transcriptional activities of NF- κ B are regulated by the cellular reducing catalyst Trx through a redox-control mechanism. In order to test the correlation between the redox state of the c-Rel protein and its phosphorylation, we analysed the effect of different TrxR inhibitors on the OA-induced phosphorylation of the c-Rel protein *in vivo*. Our results showed a clear reduction in phosphorylation of the c-Rel protein with the three different compounds tested (NZ, DCIP and CDNB) in the treated cells. We have tested the action of these inhibitors on the overall

phosphorylation in the cells. NZ and DCIP increase tyrosine-specific phosphorylation, and this effect is probably associated with the oxidative stress caused by the production of superoxide radical after the treatment with the different inhibitors. A free-radical-mediated mechanism was suggested for the quinone stimulation of protein phosphorylation [49]. We hypothesized that the effect of the different inhibitors observed on the phosphorylation of the c-Rel proteins is due to the inactivation of TrxR and consequently to the absence of reduced Trx rather than to a secondary inactivation of protein kinases. All these results indicate a dissociation between the effect of the different inhibitors on the phosphorylation of the c-Rel protein and on the tyrosine kinase activity. These data could explain the apoptotic effect of the TrxR inhibitors, since it has been reported that NF- κ B is involved in the regulation of some anti-apoptotic genes [50].

In conclusion, we show that a link exists between the redox regulation of the c-Rel protein and its phosphorylation. Although the mechanism is not fully understood, the phosphorylation of a key residue could depend on the oxidation–reduction status of NF- κ B. A specific kinase could recognize the target residue only when a neighbouring cysteine residue is in a reduced form. On the other hand, the reduction of the c-Rel protein could induce a conformational change and consequently render the protein competent for specific DNA binding. In this form the protein could be phosphorylated to interact with different co-activators. At this time we do not know whether the other members of the NF- κ B family are similarly regulated, but the conservation of the cysteine involved suggests that the mechanism could be conserved. These results suggest that the phosphorylation of I κ B by the I κ B kinase complex is not sufficient for NF- κ B activation. The phosphorylation necessary for the activity of the NF- κ B protein depends on the redox regulation.

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