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Nitric oxide (NO) disrupts specific DNA binding of the transcription factor c-Myb in vitro

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Abstract In an attempt to elucidate signal transduction pathways which may modulate DNA binding of the transcription factor c-Myb, we investigated whether c-Myb could be a target for the signaling molecule nitric oxide (NO) in vitro. NO-generating agents severely inhibited specific DNA binding of the c-Myb minimal DNA-binding domain R_2R_3. This inhibition was readily reversible upon treatment with excess DTT. A redox-sensitive cysteine (C130) was required for this NO sensitivity. Moreover, a DNA-binding domain carrying two of the avian myeloblastosis virus (AMV)-specific mutations (L106H, V117D) appeared to be less sensitive to *S*-nitrosylation than the wild-type c-Myb. This difference in NO sensitivity may influence the regulation of wild type versus AMV v-Myb protein function.

Key words: Myb; DNA binding; Avian myeloblastosis virus; Nitric oxide; Redox

1. Introduction

The activity of many transcription factors is regulated by signal transduction-dependent mechanisms, ranging from the regulation of transport into the nucleus to modifications that modulate DNA binding or interaction with other proteins, which result in altered transactivation of target genes [1]. Modification and regulation by phosphorylation is well established, and has been studied in detail for several transcription factors. More recently, ayyention has also been focused on the regulation of transcription factors by redox mechanisms and nitric oxide. Nitric oxide (NO) is involved in a wide range of biological processes, including blood vessel and macrophage function, neurotransmission, growth control and regulation of transcription (reviewed in [2–5]). NO exerts its effect on downstream protein targets either by generating *S*-nitroso modifications of specific protein thiols or by reacting with metal-containing proteins [2]. Nitric oxide bound to proteins (e.g. albumin in plasma) or to peptides (e.g. glutathione in cells) serves to prolong the biological activity of nitric oxide by acting as a slow-releasing reservoir [3,6]. In *Escherichia coli*, the transcriptional activity of OxyR (inducer of hydroperoxidase I) is activated by *S*-nitrosylation [7], whereas the SoxR transcription factor (an activator of antioxidant genes) is activated by NO reacting with an iron-sulfur cluster [8]. In higher eukaryotes, central signal-responsive transcription factors such as NF-κB and AP-1 have been reported to be stimulated or inhibited by NO depending on the cell system and signaling pathway [9–15].

The c-*myb* proto-oncogene has been extensively characterized (reviewed in [16,17]), but little information is available regarding regulation of c-Myb function by signal transduction mechanisms. One report has shown that CK2 phosphorylation of serines 11 and 12 inhibits specific DNA binding, whereas MAPK-dependent phosphorylation of serine 532 in the negative regulatory domain increased c-Myb's transcriptional capacity on some promoters (see references in [17]). The c-Myb DNA-binding domain consists of three tryptophan-rich repeats, R_1, R_2 and R_3. A highly conserved cysteine residue (C130) is located in a flexible region of the R_2 repeat, and thought to line a cavity in the structure [18,19]. This residue is essential for DNA binding, transformation and transcriptional transactivation [20,21]. The strong conservation of this cysteine cannot be explained simply by being required for DNA binding, as several hydrophobic replacements did not weaken the sequence-specific DNA-binding activity compared to the wild type protein [22]. This cysteine residue is also highly redox-sensitive. We have previously proposed a model where the C130 residue may function as a redox-sensitive molecular switch, which by induction of a conformational change in the R_2 repeat affects specific DNA binding of c-Myb [22]. Regulation of transcription factor DNA binding by redox modification of cysteines has been reported for an increasing number of transcription factors, including AP-1, Sp-1, NF-κB and p53 [23]. Cysteine residues in both AP-1 and NF-κB are targeted by both redox and NO-generating agents/pathways. We therefore tested whether c-Myb could be a target for *S*-nitrosylation and whether this modification could alter the DNA-binding activity of the protein.

In this report we show that the NO donors SNP (sodium nitroprusside) and SNOG (*S*-nitrosoglutathione) severely reduce the DNA-binding activity of c-Myb and that the C130 residue is required for this inhibitory effect. *S*-Nitrosylated c-Myb could be readily reactivated by treatment with an excess of dithiothreitol (DTT). We also found that the DNA-binding domain of avian myeloblastosis virus (AMV) v-Myb was less sensitive towards *S*-nitrosylation than the wild type, and thereby possessed significant DNA-binding activity under conditions where the wild type protein was severely inhibited.

2. Materials and methods

2.1. Expression and purification of Myb proteins

The minimal DNA-binding domain of chicken c-Myb, R_2R_3, and mutant derivatives R_2R_3[C130V] and R_2R_3[AMV] (mutations L106H and V117D in R_2, described in [24]) were expressed in *E. coli* using the T7 system [25]. Recombinant proteins were purified as described previously [26] and diluted in HGE_{500} (20 mM HEPES, 1 mM EDTA, 10% v/v glycerol, 500 mM NaCl, 0.05% v/v Triton X-100, pH 8.0).

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2.2. Electrophoretic mobility shift assay

DNA binding was monitored by electrophoretic mobility shift assay (EMSA) [24] using the double-stranded oligonucleotide probe (5′-GCATTATAACGGTTTTTTAGCGC-3′) (mim-1 site A) [17]. SNP, glutathione (GSH) (both from Sigma), and SNOG (Calbiochem) were made fresh from powder dissolved in N_2-flushed MilliQ-purified distilled water.

All binding reactions were performed in an oxygen-free atmosphere (N_2 gas) with 20 fmol protein in a total volume of 15 μl in 24 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 12% v/v glycerol, pH 8.0. Samples treated with SNP and/or with DTT were incubated at 25°C for 5 min. Samples treated with SNOG or with GSH were incubated for 30 min at 25°C. After treatments, 5′-^{32}P-labelled DNA oligo probe (10 fmol) was added and the binding mixture incubated for an additional 2 min at 25°C before electrophoresis.

For investigation of the stability of nitrosylated c-Myb R_2R_3[wt] in normal atmosphere, the samples were prepared as follows: R_2R_3[wt] protein (400 fmol) was treated with 1 mM DTT and with or without 6 mM SNP in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.1 mg/ml BSA for 5 min at 25°C in an oxygen-free atmosphere. Excess SNP was removed by centrifugation through a Sephacryl S-200 HR gel filtration column (Pharmacia Biotech) equilibrated in TE buffer. The eluate was incubated in normal atmosphere at 25°C. Aliquots were removed at various timepoints and added to the binding buffer in a total volume of 15 μl (final conditions as above) containing 10 fmol probe. Binding reactions were incubated for 2 min at 25°C before immediate loading on the running EMSA gel.

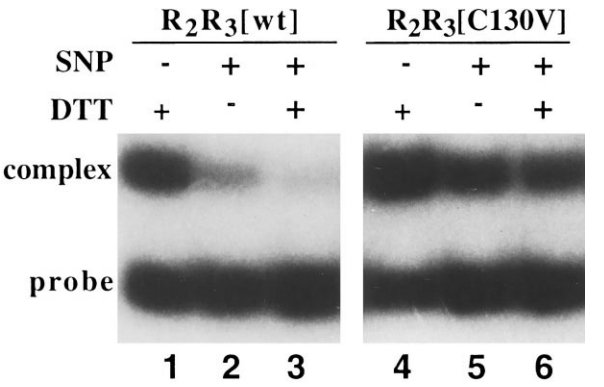

Fig. 1. Inhibition of the DNA-binding activity of the c-Myb R_2R_3[wt] protein by the NO donor SNP. Wild type and mutant forms of the minimal DNA-binding domain of c-Myb, R_2R_3, were expressed in *E. coli* and purified to near homogeneity. The effect of NO on DNA binding was analyzed as follows: wild type c-Myb R_2R_3[wt] (lanes 1–3) and mutant R_2R_3 [C130V] (lanes 4–6) proteins (20 fmol) were treated without (−) or with 5 mM SNP (+) in the presence (+) or absence (−) of 1 mM DTT for 5 min before binding to a mim-1 A site oligo probe. The protein-DNA complexes were analyzed by electrophoretic mobility shift assay (EMSA) as described in Section 2. Positions of protein-DNA complexes and free probe are indicated.

3. Results

3.1. Inactivation of c-Myb R_2R_3 [wt] DNA binding by the NO donors SNP and SNOG

We first investigated the effects of NO-generating agents on the DNA-binding activity of chicken c-Myb wild type minimal DNA-binding domain R_2R_3[wt]. Recombinant c-Myb R_2R_3[wt] was incubated with the NO donor SNP in an oxygen-free atmosphere in the presence or absence of DTT before analysis by EMSA. The DNA-binding activity of R_2R_3[wt] was greatly reduced by treatment with SNP (Fig. 1, lanes 1–3).

We have previously shown that the cysteine residue C130 in the R_2 subdomain is readily oxidized, and that oxidation of this residue disrupts DNA binding of the R_2R_3[wt] protein [22]. Since cysteines are potential targets for *S*-nitrosylation [2], we tested whether the C130 residue of c-Myb was involved in the NO-mediated inhibitory effects. The R_2R_3[C130V] mutant protein, where cysteine-130 is replaced by valine, was similarly treated with SNP and DTT. EMSA analysis showed only a slight reduction in the DNA binding of the C130V mutant protein (Fig. 1, lanes 4–6). In other experiments, this reduction was hardly apparent (see Fig. 4, lanes 13 and 14). Accordingly, the C130 residue must be the target for the major SNP effect. The latter result also excludes the possibility that SNP or SNP-derived products act independently of cysteine modification and mediate significant inhibition of c-Myb R_2R_3[wt] DNA-binding activity.

Treatment with SNP and DTT together inhibited DNA binding of R_2R_3[wt] to a greater extent than SNP alone (Fig. 1, lanes 2 and 3). This effect was not due to inhibition of DNA binding by DTT itself. Previous reports have shown that DTT stimulated DNA-binding activity of oxidized c-Myb R_2R_3[wt] [20,24]. Furthermore, the enhanced negative effect of DTT on DNA binding was not observed with the [C130V] mutant (lanes 4 and 6). Tabuchi and colleagues also noted the apparent DTT enhancement of the SNP effects on AP-1 DNA binding, and suggested that this may be due to the formation of *S*-nitroso-DTT which subsequently reacts with the target protein [14].

To confirm and extend these findings, we tested the more physiological NO donor SNOG. C-Myb R_2R_3[wt] and [C130V] mutant proteins were incubated with increasing amounts of SNOG followed by EMSA analysis of DNA-binding activity. As shown in Fig. 2, treatment with increasing concentrations of SNOG rapidly abolished c-Myb R_2R_3[wt] DNA-binding activity (lanes 1–5) whereas the c-Myb R_2R_3[C130V] mutant was unaffected (lanes 7–11), again demonstrating the specificity of the nitrosylation. Treatment with glutathione alone (lanes 6 and 12) had no such effect on either wild type or mutant protein, which excluded the glutathione moiety as an inhibitor of DNA binding. The NO donor SNOG was much more efficient at inhibiting DNA binding than SNP.

3.2. The stability of S-nitrosylated c-Myb R_2R_3[wt] and reactivation of the protein by DTT

NO radicals released from NO donors such as SNP are short-lived reactive molecules with a half-life in the order of seconds depending on the concentration [27]. Compounds such as *S*-nitroso proteins have a much longer life-time. The half-life of *S*-nitroso-BSA is ∼24 h in phosphate buffer (pH 7.4; 25°C) and ∼40 min in plasma [28]. Data suggest that particularly thiol nitrosylation serves to prolong the biological activity of nitric oxide by acting as a slow-releasing reservoir [28,29]. A regulatory role for nitrosylation of c-Myb would necessitate the modification being readily reversible and sufficiently stable for effects on downstream targets. We therefore investigated the stability of *S*-nitrosylated c-Myb R_2R_3[wt] in a normal aerobic atmosphere, and whether the *S*-nitrosylation of C130 was reversible.

Purified c-Myb R_2R_3[wt] was treated with SNP in the presence of DTT in an oxygen-free atmosphere and centrifuged through a gel filtration column to remove excess SNP. The column eluate was then exposed to air at 25°C for up to

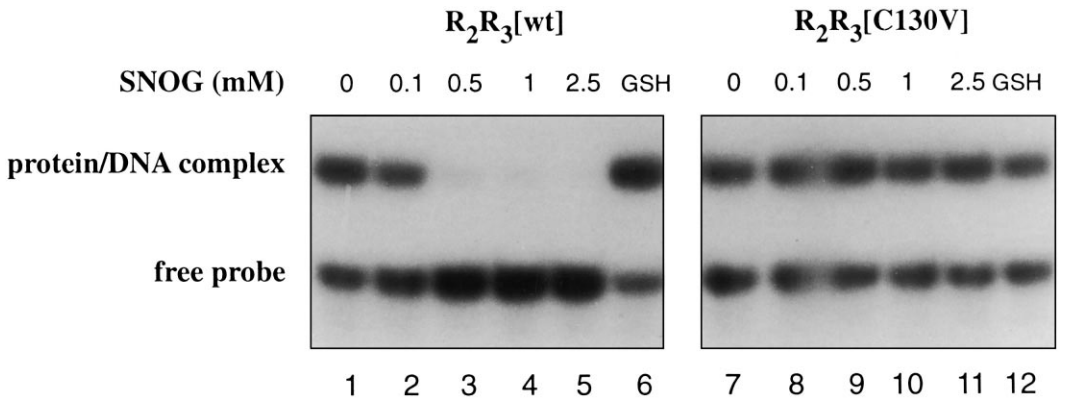

Fig. 2. Inhibition of the DNA-binding activity of the c-Myb R_2R_3[wt] protein by the NO donor SNOG. Purified c-Myb R_2R_3[wt] or R_2R_3[C130V] proteins (20 fmol) were treated with 0, 0.1, 0.5, 1.0 or 2.5 mM SNOG (lanes 1–5 and 7–11, respectively) or with 2.5 mM GSH alone (lanes 6 and 12) for 30 min before binding to the oligo probe and EMSA analysis. Positions of protein-DNA complexes and free probe are indicated.

20 min before initiating the DNA-binding reaction. As shown in Fig. 3, there was no reactivation of DNA binding of c-Myb R_2R_3[wt] during this 20 min incubation period (lanes 2–7). In contrast, addition of excess DTT to the binding buffer (lane 1) readily reactivated DNA binding. Control EMSA reactions with c-Myb R_2R_3[wt] protein treated as above except for SNP demonstrated that active protein was eluted in a high yield from the column and was not inactivated by the column matrix, nor by incubation under aerobic conditions (lanes 8–10). We conclude that *S*-nitrosylated c-Myb R_2R_3[wt] is stable for at least 20 min in normal atmosphere, and that the *S*-nitrosylation is readily reversible.

3.3. The AMV-specific mutations in R_2 alter the sensitivity of the DNA-binding domain towards S-nitrosylation

The oncogenic AMV version of c-Myb carries three amino acid substitutions in the R_2 subdomain. Two mutations have been mapped by transformation assays to be essential for the AMV-transformed phenotype [16]. We have recently shown that a mutant protein carrying these two mutations (L106H, V117D) exhibits a more compact conformation in R_2 and reduced sensitivity towards cysteine-specific alkylation and oxidation [24]. We asked whether these two AMV-specific mutations also could influence nitrosylation of the C130 residue. C-Myb R_2R_3[wt] and R_2R_3[AMV] recombinant proteins were treated with increasing concentrations of SNP in the presence of DTT and analyzed by EMSA. As shown in Fig. 4, the R_2R_3[AMV] protein (lanes 7–12) was more resistant than R_2R_3[wt] (lanes 1–6) to SNP-induced loss of DNA-binding activity. The R_2R_3[C130V] mutant was unaffected by the SNP treatment (lanes 13 and 14), demonstrating the cysteine specificity of the nitrosylation. This experiment shows that the AMV-specific mutations in R_2 render the protein less sensitive towards nitrosylation, possibly as a result of a more compact structure induced by the point mutations in R_2.

4. Discussion

We have presented data showing that the second messenger molecule NO is able to disrupt the specific DNA-binding activity of the transcription factor c-Myb by interaction with a specific cysteine residue (C130) located in the R_2 repeat of the DNA-binding domain. This inhibitory effect is fully reversible by treatment with excess DTT.

We have previously shown that when the C130 residue in c-Myb is oxidized, alkylated or mutated to a polar residue, the protein adopts a more open structure and has a severely impaired specific DNA-binding activity [22] (note that C130 was designated C43 in this reference). The critical importance of the size and polarity of the residue at this position may be explained by the cysteine lining a hydrophobic cavity in the R_2 domain [19]. This cavity may confer the necessary degree of flexibility needed for specific DNA interaction. The basic region encompassing the C130 residue resembles the basic DNA-binding motif (containing a highly conserved redox-sensitive cysteine residue) of the Fos/Jun and other leucine zipper family transcription factors [30]. As pointed out recently by

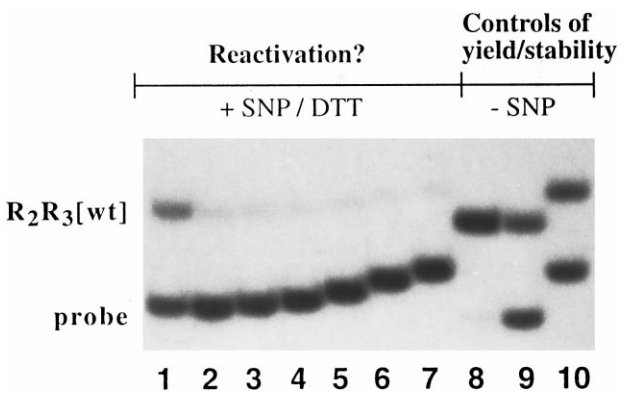

Fig. 3. Stability of nitrosylated c-Myb R_2R_3[wt] protein in normal atmosphere. Purified c-Myb R_2R_3[wt] (400 fmol) was treated with 1 mM DTT and with or without 6 mM SNP in an oxygen-free atmosphere before removal of excess SNP by centrifugation through a gel filtration column (described in Section 2). Column eluates were incubated in normal atmosphere at 25°C. Aliquots from SNP-treated protein were removed at 0, 2, 5, 10, 15 and 20 min (lanes 2–7). Aliquots from protein treated identically, but omitting SNP, were removed after 0 and 20 min (lanes 9, 10) and added to the binding reactions. Lane 1: 50 mM DTT was included in the binding reaction. Lane 8: precolumn aliquot of protein treated without SNP. The complexes were analyzed as previously described. The difference in migration of the protein-DNA complexes and free probe is due to samples being loaded on a running gel. Positions of protein/DNA complexes and free probe are indicated.

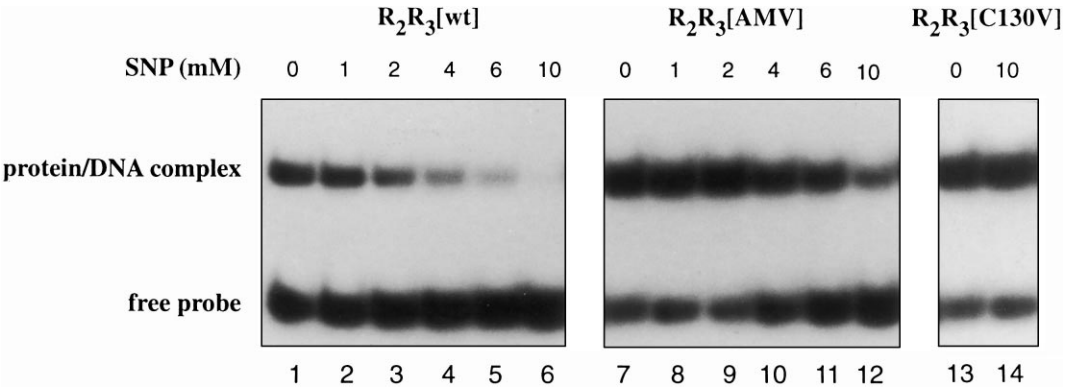

Fig. 4. Comparison of NO sensitivity between c-Myb R_2R_3 wild type and AMV R_2R_3 domains. To analyze whether oncogenic mutations affected NO sensitivity, mutations found in AMV v-Myb were introduced and recombinant R_2R_3[AMV] was compared to the wild type protein. Purified c-Myb R_2R_3[wt] and R_2R_3[AMV] proteins (20 fmol) were treated with 0, 1, 2, 4, 6 and 10 mM SNP and 1 mM DTT (lanes 1–6 and 7–12, respectively) for 5 min before addition of the oligo probe. Purified mutant c-Myb R_2R_3[C130V] was similarly treated with 0 or 10 mM SNP (lanes 13 and 14) before the addition of the oligo probe and EMSA analysis. Positions of protein-DNA complexes and free probe are indicated.

Stamler et al. [4], relevant cysteine targets for *S*-nitrosylation seem to be located close to basic and acidic residues probably because such residues allow base-catalyzed nitrosylation and acid-catalyzed denitrosylation. The c-Myb C130 seems to fit this requirement since it is located in a stretch of six residues of which three are basic and one acidic. Taken together, these particular structural features make C130 a very sensitive target for modification. We predict that *S*-nitrosylation disrupts DNA binding by a structural effect similar to the one previously proposed for redox modification of C130 in c-Myb [22].

Nitric oxide mediates inhibition of DNA binding of NF-κB both in vivo and in vitro [9–12]. The p50 cysteine-62 residue in the DNA-binding domain is specifically *S*-nitrosylated, which leads to a four-fold reduction in DNA-binding activity. The DNA-binding activity of AP-1 is also inhibited both in vitro and in vivo [14,15]. A highly conserved cysteine in the basic DNA-binding domain is the target of *S*-nitrosylation, but the exact molecular mechanism for inhibition of DNA binding has not yet been determined. We speculate that a common theme for c-Myb, NF-κB and AP-1 may be the targeting of specific cysteine residues by NO signaling pathways, and loss of DNA-binding activity by *S*-nitrosylation-induced conformational changes in the DNA-binding domains.

This biochemical study has not addressed directly the question of whether *S*-nitrosylation plays a role in the regulation of c-Myb activity in vivo. In general, the intracellular environment is strongly reducing, with intracellular concentrations of GSH and GSSG of approximately 10 mM and 0.5 mM, respectively [31]. One would therefore expect regulatory mechanisms to be highly specific and readily reversible. One requirement would be that c-Myb is sufficiently sensitive towards *S*-nitrosylation at physiological concentrations of NO. Free NO in the brain is in the range 10 nM–2 μM, although this would be expected to vary with cell type and intracellular conditions [32]. The concentration of free NO in EMSA binding buffer containing 1–5 mM SNP has been measured to be in the range 20–100 nM [11], well within physiologically relevant concentrations of NO. The DNA binding of c-Myb was strongly inhibited by 5 mM SNP and 0.5 mM SNOG.

Nitric oxide has been reported to act as an antiproliferative agent, both during *Drosophila* development [33] and in neuronal cells after NGF stimulation [5]. Expression of c-Myb, on the other hand, has been tightly associated with stimulation of proliferation. A crucial role for c-Myb in hematopoiesis seems to be to maintain the proliferative state of immature cells (reviewed in [16,17]). Likewise, antisense oligonucleotides directed against c-*myb* have antiproliferative effects [17]. The targets for the antiproliferative effects of NO have yet to be identified. Based on our results presented in this paper, we suggest that c-Myb may be one such target for NO action.

We have previously shown that the introduction of two AMV mutations (L106H, V117D) into the c-Myb minimal DNA-binding domain resulted in less stable protein-DNA complexes in vitro, and reduced transactivation in vivo compared to the wild type protein [24]. In the present work we found that an AMV v-Myb DNA-binding domain with the L106H and V117D mutations was less sensitive towards *S*-nitrosylation than the wild type protein. These are intriguing properties for a product of a potent nuclear oncogene. One possibility may be that oncogenic AMV v-Myb may be active in the cell under circumstances where normal c-Myb should be inactive. A better understanding of NO signaling in c-Myb expressing cells will be required to finally evaluate the importance of the reduced NO and redox sensitivity in v-Myb caused by the AMV-specific point mutations.

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