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Oncogenic conversion of Ets affects redox regulation *in-vivo* and *in-vitro*

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ABSTRACT

The avian acute leukemia virus E26 encodes a fusion protein between viral Gag and the cellular transcription factors cMyb and cEts1(p68). vEts on its own transforms more mature erythroid cells. We have compared the properties of vEts and cEts1(p68). vEts interacts preferentially with an antibody that recognizes the active conformation of the DNA-binding domain. The DNA-binding activity of vEts is particularly sensitive to incubation conditions for band-shift assays, phosphorylation and modification by sulphydryl-specific reagents. Increased sensitivity is due to loss of a protective function of cEts1 C-terminal sequences. cEts2 has a related C-terminal sequence with a similar role. These results suggest that the vEts DNA-binding domain is more accessible to protein-protein interactions and to regulatory mechanisms. Indeed, vEts DNA binding is preferentially inactivated by oxidizing conditions in-vivo. We suggest that the 'open' conformation of the vEts DNA-binding domain favours interactions with other proteins or DNA and facilitates transformation.

INTRODUCTION

Cell growth and differentiation are regulated through the modulation of gene expression by extracellular signals. Proteins of the ets gene-family play key roles in the signal transduction network, like the cellular homologues of other retroviral oncogenes. About 30 Ets-like proteins have been found in species ranging from flies to humans. The conserved 'Ets-domain' is required for DNA-binding to the core-motif 'GGA' and is involved in protein-protein interactions with co-factors. Many of the Ets-related proteins have been shown to be transcription activators, similar to other nuclear oncoproteins and anti-oncoproteins (Jun, Fos, Myb, Myc, Rel, p53, etc., for reviews see 1–4)

The oncogenic counterparts of transcription factors escape normal regulation (5, 6). vEts is a component of the Gag-vMyb-vEts fusion protein encoded by E26 (7, 8; see Fig. 1A). It differs from its progenitor cEts1(p68) by amino acid substitutions in an activation domain [cEts1(p68) position 285, A is changed to V in vEts], in the Ets domain [445 (I to V)], and at the C terminus [473–485 (LHAMLDVKPDADE to HSSASGLTSSMACSSF)]. These changes affect transactivation and DNA-binding. vEts is less efficient than cEts at transactivating through the ets-motif (9, 10). However, vEts retains the capacity to activate the stromelysin and collagenase promoters through their AP1 motifs, possibly by activation of the fos and jun genes (9). There are contradictory reports about DNA binding. Lim et al. (10) found that vEts bound efficiently to DNA, but not cEts1. In contrast, LePrince et al. (11) and Wasylyk and Wasylyk (9) found that only cEts1 bound. We describe properties of vEts that could help to explain these differences.

Sequence alterations in C-terminal sequence of cEts1, such as that found in vEts, induce what can be described as a more 'open conformation', exposing the proteins to antibodies, phosphorylation, a Mg^{2+} sensitive component of BSA and inactivation by chemical modification of sulphydryl groups. Interestingly, this 'open' structure sensitizes DNA-binding to redox regulation in-vivo.

MATERIALS AND METHODS

Construction of recombinants

Standard procedures were used. **vEts, cEts1(p68), cEts2, N70, N70-vt and vN70** expression vectors are published (9, 12, 13). Vector sequences were modified by PCR with appropriate primers and sequenced. **ST/A, MC/A:** C-terminal sequence of N70-vt (HSSASGLTSSMACSSF) mutated to HAAAAGLAAAMACAAF and ASSASGLTSSMAASSF, respectively. ΔA, ΔB, ΔC and ΔD (of either N70 or cEts1): translation stop (TGA) after amino-acids 478, 472, 466 and 459, respectively. **cEts2-vt**: cEts2 C-terminal LHAMLGVQPDTED mutated to HSSASGLTSSMAC= SSF. **cEts2-ΔD**: translation stop after amino-acid 453. ΔD-C/S, -C/A, -C/D: C394 of N70-ΔD mutated to serine (C/S), alanine (C/A) or aspartic acid (C/D). **hERb1-N70, hERb1-N70-vt:** Oligonucleotides coding for hERb1 epitope (RPNSDNRRQGGRERL) in Xho I site of corresponding KOZ1-3 based vectors (13). **pET vectors:** Nco I-Bam HI (-Bgl II for Ets2) fragments from KOZ1-3 based vectors (13) between same sites of pET3d (14).

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Band shift

Proteins were first incubated for 10 min. on ice in 20 μl of 20 mM Hepes pH 7.9, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, 1 μg/20μl poly dIdC, 50 mM KCl, and where appropriate 10 mM $MgCl_2$+ 1 mg/ml BSA (BRL), then for 20 min. at 25°C with excess probe (PEA3* = 5'-TCGAGCCGGAAGTGACGT-CGA-3'). The samples were placed on ice and loaded immediately on pre-run [60 min. at 10 mA (50V)] 6% polyacrylamide (29/1) gels in 0.25×TBE and run for 90 min. at 30 mA (150V) with re-circulating buffer at 20°C.

COS cell transfection and whole-cell extracts

COS cells were transfected by the calcium phosphate procedure (20 μg expression vector per 9 cm. plate) and washed 20 h. later. Cells receiving the same plasmid were trypsinised, pooled and replated (to ensure equal transfection efficiency), incubated for 24 h in medium + 0.5% foetal calf serum and treated for 5 h. with either 30mM N-acetyl cysteine (NAC), 150 mM H_2O_2 or 100 ng/ml TPA. The cells were washed twice with PBS, concentrated by centifugation, resuspended in TGK + protease and phosphatase inhibitors (10 mM Tris−HCl pH 7.9, 10% glycerol, 0.5 M KCl, a cocktail of protease inhibitors, 2 mM PMSF, 10 mM NaF, 2 mM sodium orthovanadate, 4 μM microcystin, 5 mM para-nitrophenyl phosphate), freeze-thawed three times, cleared by centifugation (12,000g 4°C, 15 min.) and stored in aliquots at −80°C. Protein concentrations were determined by the Bradford assay.

Bacterial extracts

BL21(DE3) harbouring pLysS and pET3 based expression vectors were grown to 0.4 OD and induced for 3 h with IPTG. Bacterial pellets were resuspended in 50 mM Tris−HCl pH7.9, 1 mM EDTA with a cocktail of protease inhibitors, freeze-thawed twice and centrifuged to remove debris (14).

Protein synthesis in reticulocyte lysates

RNA was synthesized from linearized pSG5 derived expression vectors with T7 RNA polymerase, quantitated by incorporating radioactive precursors, and verified by gel electrophoresis. Optimised amounts of RNA were used to synthesize proteins in rabbit reticulocyte lysates according to the manufacturers instructions (Amersham or Promega). Efficient synthesis of full-length protein was systematically checked by both measuring incorporation of radioactive precursors ([^{35}S]-methionine) and SDS-PAGE.

Immunoprecipitations

Proteins labelled in reticulocyte lysates ([^{35}S]-methionine) or in gel-shift incubation buffers (γ-[^{32}P]-ATP, 5 μl/40μl reaction, ICN, 7000 Ci/mmole, 22,8 μM) were diluted with 800 μl buffer A (50 mM Tris−HCl pH 7.9, 0.4M NaCl, 180 mg/ml PMSF, 1% Triton X100) at 0°C, incubated with rocking for 90 min. each with antibodies followed by 5 μl Sepharose-A CL (Pharmacia), washed three times with 1 ml buffer A, two times with wash buffer (50 mM Tris−HCl pH 7.9, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 mg/ml PMSF, 1% NP-40, 0.5% sodium deoxycholate) and centrifuged. The pellet was taken up in SDS sample buffer, heated 10 min at 100°C, electrophoresed on 12% polyacrylamide SDS gels and revealed by fluorography. hERb1 tagged N70 and N70-vt were immunoprecipitated in RIPA buffer.

RESULTS

vEts interacts preferentially with an antibody against the Ets-domain

The anti-PA93 antibody was raised against an Ets-domain sequence that is common to vEts and cEts1 (13). [^{35}S]-methionine labelled vEts, Myb-Ets and cEts1(p68) (Fig. 1A) were synthesized in reticulocyte lysates, immunoprecipitated with anti-PA93 and analysed by SDS-PAGE. vEts and Myb-Ets were precipitated more efficiently than cEts1(p68) [Fig. 1B, compare lanes 1−6], showing that the vEts DNA-binding domain is more accessible to the antibody. [The lower minor band of the Myb-Ets doublet (lane 2) could be a degradation product of Myb-Ets resulting from preferential proteolytic digestion of the 'flexible hinge' region between the Ets domain and adjacent N-terminal sequences (Lim et al., 1992; Wasylyk et al., 1992). Digestion would remove the PA93 epitope and prevent immunoprecipitation (see lane 5)].

The altered C-terminus of vEts sensitizes DNA-binding to incubation conditions

There are conflicting data about the relative DNA-binding affinities of vEts and cEts1 (9−11). The vEts DNA-binding domain is more accessible to antibodies and therefore presumably to DNA, in keeping with the results of Lim et al. (10). Whilst trying to resolve this discrepancy we found that the incubation conditions for band-shift had a drastic effect on DNA binding. Equal amounts of cEts1 and vEts synthesised in reticulocyte lysates were incubated in different binding buffers. In the absence of Mg^{2+} and BSA vEts bound specifically and somewhat more efficiently than cEts1 (2 fold) to the PEA3 probe (Fig. 2B, lanes 2, 6). Surprisingly, the addition of both Mg^{2+} and BSA abolished binding by vEts, but had no effect on cEts1 (Fig. 2B,

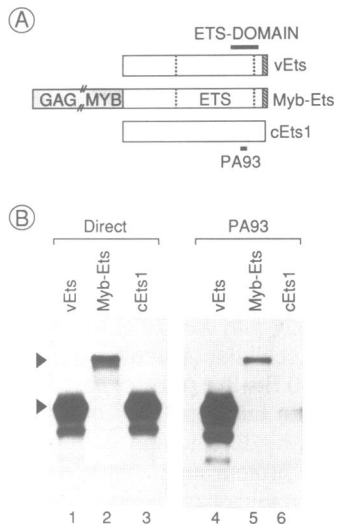

Figure 1. Anti-PA93 antibodies immunoprecipitate Myb-Ets and vEts more efficiently than cEts1(p68). Myb-Ets, vEts and cEts1(p68) (A) were synthesised in reticulocyte lysates with [^{35}S]-methionine and analyzed by SDS-PAGE and autoradiography (B) either before (DIRECT, lanes 1−3) or after (PA93, lanes 4−6) immunoprecipitation with anti-PA93. The PA93 epitope is located in the Ets-domain (A). vEts is that part of the E26 Myb-Ets fusion protein (LePrince et al., 1983, Nunn et al., 1983) that resembles cEts1(p68). It differs by substitutions of two amino-acid (dotted lines) and a C-terminal sequence (shaded box).

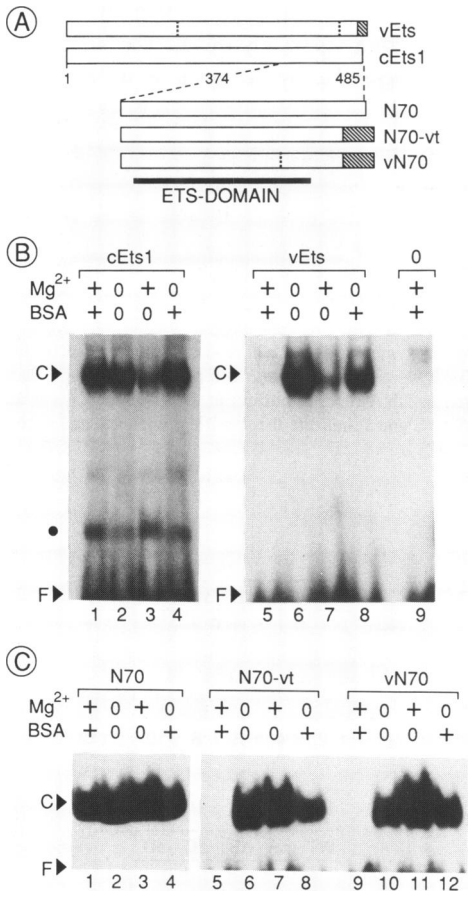

Figure 2. Incubation conditions have a drastic effect on DNA-binding by vEts. vEts, cEts1(p68), N70, N70-vt, and vN70 (part A) were synthesised in reticulocyte lysates and incubated in DNA-binding buffers containing (+) or lacking (0) Mg^{2+} and BSA before gel electrophoresis (parts B & C). C and F indicate specific complexes and excess free probe, respectively. The dot (B, lanes 1–4) indicates a bi-product of protein synthesis that resembles N70 (13). Lane 9 (B) contained lysate primed with non-specific RNA. A). vEts differs from cEts1(p68) by substitutions of two amino-acids and a C-terminal sequence (dotted lines and shaded box, respectively). The position of the Ets-domain is indicated.

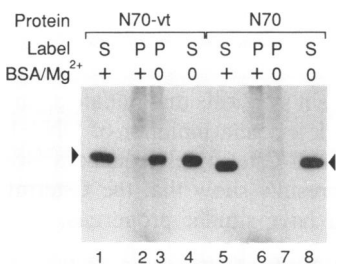

Figure 3. The virus-derived C-terminal sequence confers sensitivity to phosphorylation. N70-vt and N70 (see Fig. 2A for structure) with N-terminal epitope TAGs (hERb1) were synthesised in reticulocyte lysates in the presence of [^{35}S]-methionine (Label S, lanes 1, 4, 5, 8) or unlabelled methionine (other lanes) and incubated in DNA-binding buffers containing (+) or lacking (0) both Mg^{2+} and BSA and with either γ-[^{32}P]-ATP (Label P, lanes 2, 3, 6, 7) or unlabelled ATP (other lanes). Proteins were precipitated with anti-hERb1 monoclonal antibodies and analysed by SDS-PAGE and autoradiography.

lanes 1, 5, 9). Mg^{2+} alone decreased binding by both proteins to some extent (lanes 3, 7), whereas BSA had a small effect on vEts (lanes 4, 8), showing that both Mg^{2+} and BSA are required for the vEts specific effect. A vEts N-terminal deletion mutant, that retains the Ets-domain and C-terminal sequences (vN70, Fig. 2A), was still sensitive to Mg^{2+} + BSA (Fig. 2C, lanes 9, 10), whereas the equivalent cEts1 mutant (N70) bound efficiently (Fig. 2C, lanes 1, 2). The vEts C-terminus was sufficient to confer sensitivity (N70-vt; Fig. 2, A and C lanes 5, 6). N-terminal deletion abolished inhibition by Mg^{2+} alone (Fig. 2C, lanes 2, 3, 6, 7, 10, 11), suggesting that the deleted sequences mediate this common property of vEts and cEts1. [Note that a bi-product of the cEts1 synthesis that retains the DNA-binding domain (13) behaves like N70 (see band labelled with a dot, B lanes 1–4)]. In conclusion, the vEts C-terminal sequence is sufficient to induce sensitivity of specific DNA binding to incubation conditions.

Preferential phosphorylation

We investigated whether phosphorylation by kinases from reticulocyte lysates could account for the above results. N70 and N70-vt (Fig 2A), with N-terminal TAGs for the hERb1 monoclonal antibody (15), were synthesised in reticulocyte lysates, incubated in binding buffers containing γ-[^{32}P]-ATP with or without Mg^{2+} + BSA, immunoprecipitated with the hERb1 monoclonal antibody in RIPA buffer, and analysed by SDS-PAGE and autoradiography. Only N70-vt was phosphorylated, in the absence of Mg^{2+} + BSA (Fig. 3, lanes 2, 3, 6, 7). This was not due to specific degradation or preferential immunoprecipitation, as shown with [^{35}S] labelled proteins (lanes 1, 4, 5, 8). The TAG did not affect DNA binding (data not shown). These results show that the Ets DNA binding domain with vEts C-terminal sequences is phosphorylated in particular incubation conditions.

cEts1 and cEts2 C-terminal sequences protect against loss of DNA-binding activity

Phosphorylation of the vEts C-terminus on serines and threonine (HSSASGLTSSMACSSF) could introduce negative charges similar to those of the cEts1 C-terminus (LHAMLDVKPDADE) and stimulate DNA-binding. However, mutation to non-phosphorylatable alanines (ST/A, Figure 4A1) did not affect either DNA binding or sensitivity to Mg^{2+} + BSA (Fig 4A2, lanes 2, 5, 1, 4). Treating N70 and N70-vt with various phosphatases, or kinase and phosphatase inhibitors, did not affect either DNA-binding activity or sensitivity to the incubation conditions (results not illustrated), showing that differences in phosphorylation were not involved.

We observed that Zn^{2+} had an effect similar to Mg^{2+} (results not shown). vEts has a repetition of His and Cys somewhat reminiscent of zinc fingers (His-12X-Cys-12X-His-11X-Cys, 447–485), that is incomplete in cEts1 (His-12X-Cys-13X-His, 447–474). Mutation of His and Cys in the C-terminal sequence to Ala (MC/A, Fig. 4A1) did not alter the DNA-binding properties (Fig. 4A2, lanes 3, 6, 1, 4). Three different C-terminal sequences allow inhibition, suggesting that they all abrogate a specific function of the cEts1 C-terminal sequence.

Partial deletion of the cEts1 sequence that is replaced in vEts conferred some sensitivity to the incubation conditions (N70-ΔA; Fig 4, B1, B2 lanes 1–4), whereas total deletion gave a complete effect (N70-ΔB; B1, B2 lanes 5, 6). More extensive deletions

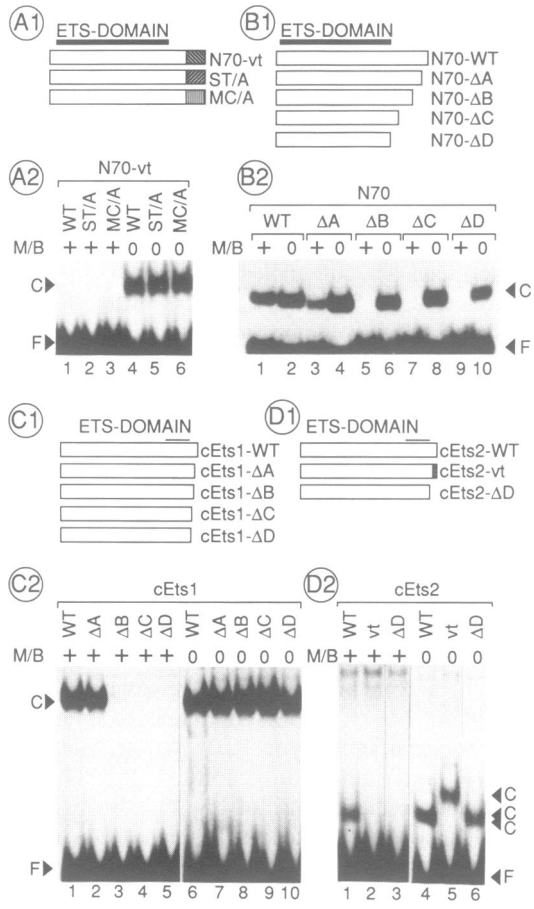

Figure 4. C-terminal sequences of vEts and cEts1(p68) confer sensitivity to incubation conditions. Mutated Ets proteins (A1−D1) were synthesised in reticulocyte lysates, incubated in DNA-binding buffers containing (+) or lacking (0) Mg^{2+} and BSA (M/B) and analysed by band shift (parts A2−D2). C and F indicate the specific complexes and excess free probe, respectively. ST/A and MC/A have alanines in the place of serines and threonine, or methionine and cysteine, respectively, in the C-terminal vEts specific sequence (see shaded sequences, A1). ΔA−D have progressive C- terminal deletions up to the Ets domains of N70 (B1) or cEts1(p68) (C1). cEts2-vt (D1) has a C-terminal substitution by vEts sequences. cEts2-ΔD (D1) is deleted from the C-terminus to the Ets-domain.

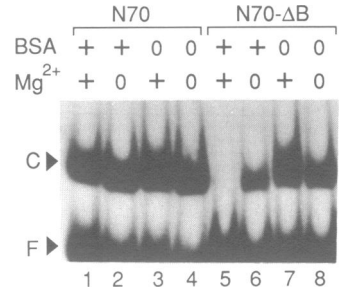

Figure 5. Proteins synthesised in *E.coli* retain sensitivity to incubation conditions. N70 and N70-ΔB were synthesised in *E.coli* using pET vectors. Bacterial extracts were incubated in DNA-binding buffers in the presence (+) or absence (0) of Mg^{2+} and BSA. C and F indicate the specific complexes and excess free probe, respectively.

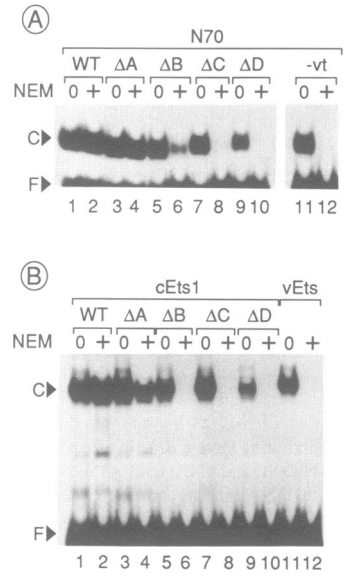

Figure 6. NEM preferentially inactivates C-terminal mutants of cEts1(p68). Proteins were synthesised in reticulocyte lysates and incubated with (+) or without (0) 10mM NEM for 30 min at 25°C in DNA-binding buffer lacking Mg^{2+} and BSA, before adding the probe. C and F indicate the specific complexes and excess free probe, respectively. For structures of recombinants see Figs. 2A, 4B1 and 4C1.

up to the Ets-domain had no further effect (N70-ΔC, N70-ΔD; B1, B2 lanes 7−10). Similar results were obtained with C-terminal deletions in the context of the whole protein (Fig. 4, C1, C2 lanes 1−10). These results show that the Ets-domain is sufficient for specific DNA binding to the PEA3(ets)-motif. Alterations of the C-terminal sequence beyond the Ets-domain, most notably by oncogenic conversion, alters the sensitivity to binding conditions.

21 of the 26 C-terminal amino-acids are identical in cEts1 and cEts2. Derivatives of cEts2 with either a substitution of C-terminal sequences equivalent to that in vEts (cEts2-vt, Fig. 4D1), or a C-terminal deletion up to the Ets-domain (cEts2-ΔD, Fig. 4D1) gave protein products in reticulocyte lysates that bound only in the absence of Mg^{2+} + BSA (Fig. 4D2, lanes 2, 3, 5, 6). In contrast, cEts2 bound in both conditions (lanes 1, 4). Deletion of amino acids equivalent to those replaced by viral sequences (similar to ΔB above) was sufficient to confer sensitivity to Mg^{2+} + BSA (results not shown). Note that the cEts2 proteins that bind in these assays are specific secondary products of protein synthesis that initiate at an internal initiation codon. They lack a potent inhibitor of DNA binding but retain the Ets-domain and C-terminal sequences (see 13 and data not shown). These results show that the C-terminal sequences of cEts2 and cEts1 have similar properties.

Mg^{2+} + BSA inhibit binding of proteins produced in *E.coli*

Mg^{2+} + BSA could affect the activity of components of reticulocyte lysates. However, proteins synthesised in *E.coli* were also sensitive to incubation conditions (compare N70 and N70-ΔB, Fig. 5 lanes 1−8, and results not shown for other recombinants), suggesting that Mg^{2+} + BSA were the direct cause of inhibition. Inhibition was variable, depending on the source and batch of BSA. The ovalbumin and gelatin that we tested did not inhibit (results not shown). We found that excluding BSA and Mg^{2+} gave the best results for vEts.

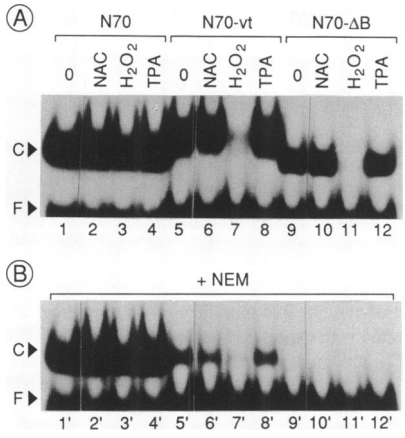

Figure 7. Redox regulation in-vivo. COS cells were transfected with expression vectors for N70, N70-vt and N70-ΔB. 24 h later the cells from all the plates receiving the same expression vector were trypsinised, mixed and replated to homogenise the transfection efficiency. The cells were incubated in medium + 0.5% foetal calf serum for 24h and then mock treated (lanes 1, 1′, 5, 5′, 10, 10′), or treated for 5 h with either 30 mM NAC (lanes 2, 2′, 6, 6′, 10, 10′), 150 μM H_2O_2 (lanes 3, 3′, 7, 7′, 11, 11′) or 100 ng/ml TPA (lanes 4, 4′, 8, 8′, 12, 12′). Cell extracts were incubated in DNA-binding buffer lacking Mg^{2+} and BSA. In B (+NEM) cell extracts were pre-incubated for 30 min. at 25°C with 10mM NEM. C and F indicate the specific complexes and excess free probe, respectively.

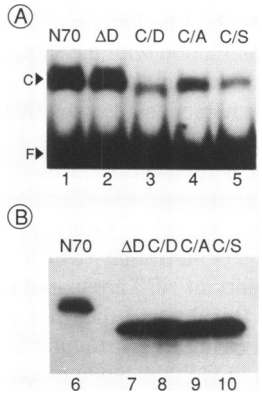

Figure 8. Inhibition of DNA binding by mutation of cysteine 394. N70, N70-ΔD, ΔD-C/D, ΔD-C/A and ΔD-C/S were labelled with [^{35}S]-methionine during synthesis in rabbit reticulocyte lysates and analysed by band-shift for DNA binding (A) and SDS-polyacrylamide gel electrophoresis for protein (B). C and F indicate specific complexes and excess free probe, respectively.

NEM preferentially inhibits binding by Ets1 proteins with altered C-terminal sequences

The results with antibodies, phosphorylation and Mg^{2+} + BSA suggest that modifying C-terminal sequences induces an 'open conformation,' exposing the Ets-domain to antibodies and enzymes. The 'open conformation' could confer sensitivity to regulation by phosphorylation or redox. N-ethyl maleimide (NEM) forms an adduct with cysteine and inhibits DNA binding by several transcription factors that are regulated by redox. NEM inactivated DNA binding by Ets1 proteins with modified C-terminal sequences, in the context of N70 or the whole protein (Fig. 6, A and B lanes 3−12). Wild type cEts1 or N70 were not inactivated (Fig. 6, A and B lanes 1, 2). However, longer

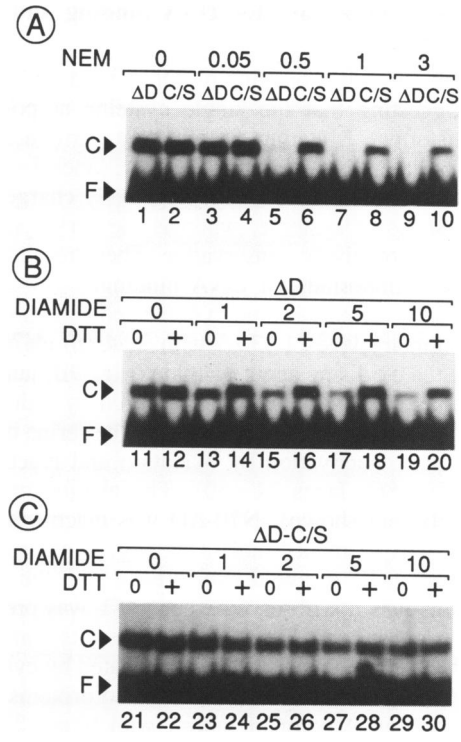

Figure 9. Cysteine 394 mediates inactivation by NEM and diamide in-vitro. N70-ΔD and ΔD-C/S were incubated in binding buffer lacking DTT at 25°C with up to 3 mM NEM for 20 min and 10 mM diamide for 15 min. Where indicated, 25 mM DTT was then added and incubation continued for 15 min at 25°C. Probe was then added and complex formation analysed by band-shift. C and F indicate specific complexes and excess free probe, respectively.

incubations with cysteine modifying agents inhibited the wild-type proteins (results not shown), suggesting that they were more resistant to modification, rather than there being a fundamental difference in the way they bind to DNA. Similar results were obtained with bacterially synthesized proteins (not shown). The reversible cysteine modifying agent diamide gave similar results to NEM, except that the proteins were re-activated by reducing agents such as β-mercaptoethanol and dithiothreitol (results not shown and see below). Reducing agents did not affect DNA binding by the Ets derivatives (results not shown). These data show that Ets proteins with an altered C-terminus are redox-regulated in-vitro, and suggest that they could become sensitive to redox regulation in-vivo.

Redox regulation of Ets proteins *in-vivo*

COS cells expressing N70, N70-vt and N70-ΔB were treated with N-acetyl cysteine (NAC), H_2O_2 and TPA. Oxidising conditions (H_2O_2) severely inhibited DNA binding by proteins with C terminal viral sequences or an equivalent deletion (Fig. 7A, lanes 5, 7, 9, 11). In contrast, the wild-type protein was not affected (Fig. 7A, lanes 1, 3). H_2O_2 had no effect when added directly to the incubation mix (results not shown). TPA and NAC (a reducing agent) had no effect (Fig. 7A, lanes 2, 4, 6, 8, 10, 12). Adding NEM to the extracts inhibited only N70-vt and N70-ΔB (Fig. 7B, lanes 1·-12·), showing that their sensitivity was not due to synthesis in-vitro or bacteria (see above). We could not detect DNA-binding by full length proteins expressed in COS cells, precluding their analysis. These results show that C-terminal modifications, such as that found in vEts, alter the sensitivity of the DNA-binding domain to redox-regulation in-vivo.

Cysteine 394 is important for DNA binding and mediates redox regulation *in-vitro*

Sulphydryl modifying agents inhibit DNA binding by N70-ΔD in vitro, suggesting that the single cysteine at position 394 mediates this effect. Mutating cysteine to aspartic acid, alanine or serine inhibited DNA-binding (Fig. 8A, lanes 1−5, and 8B for protein levels). Aspartic acid is negatively charged, similar to oxidised states of cysteine, whereas the serine and alanine substitutions are relatively conservative. These results show that cysteine 394 is important for DNA binding.

The cysteine to serine substitution prevented inhibition by NEM (Fig. 9A, compare lanes 1, 3, 5 with 2, 4, 6). N70-ΔD was inhibited by the oxidising agent diamide (Fig. 9B, lanes 11, 13, 15, 17, 19), and DNA binding was restored by reduction with DTT (Fig. 9B, lanes 12, 14, 16, 18, 20). The serine mutant was resistant to both inactivation by oxidation and reactivation by reduction (Fig. 8C, lanes 21−30). The alanine mutant gave similar results (not shown). N70-ΔD was inactivated to some extent in these experiments (lanes 1, 2, Fig. 9) since the buffers did not contain DTT to avoid interference with the sulphydryl reagents. This was not observed when DTT was present in all buffers (see lanes 2, 5, Fig. 8). These results show that Ets sensitivity to oxidative inactivation is mediated by cysteine 394. However, with excess reagents, the mutated proteins could still be inactivated (Fig. 9, compare lanes 2 with 10 and 22 with 30), and the wild type could not be fully reactivated with DTT (lanes 12, 20). Amino-acids other than cysteine become sensitive to modification due to local envoironmental effects. These results raise the possibility that oxidative inactivation may be mediated by amino-acids other than cysteine in-vitro, and possibly also in-vivo.

Cysteine 394 mutants are sensitve to redox regulation *in-vivo*

Treating COS cells expressing N70-vt, N70-ΔD and ΔD-C/S with H_2O_2 inhibited DNA binding by ΔD-C/S (Fig. 10, lanes 5, 6), to a similar extent as N70-vt and ΔD (lanes 1−4). Similar results were observed with the C/A mutant (not shown).These results suggest that amino-acids other than cysteine 394 mediate the sensitivity to oxidation in-vivo. Further experiments are required to identify these residues.

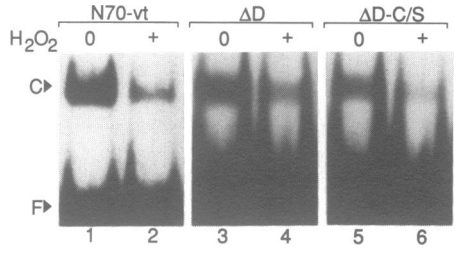

Figure 10. Effect of cysteine 394 modification on redox regulation in-vivo. COS cells were transfected with expression vectors for N70-vt, N70-ΔD and ΔD-C/S, treated with H_2O_2 and analysed as described above (Figure 7). ±C and F indicate specific complexes and excess free probe, respectively.

DISCUSSION

DNA binding by vEts can be rapidly lost in a variety of experimental conditions, due to an 'open' conformation that exposes reactive amino acid side-chains to fortuitous modification. In one series of experiments we found that buffer components had a drastic effect on DNA binding. Mg^{2+} + BSA in the band shift binding buffer abolished binding by vEts without affecting cEts1.The inactivating component most probably originates from BSA, since inhibition is observed with proteins synthesised in reticulocyte lysates, *E.coli* and COS cells, and depends on the batch of BSA. Mg^{2+} might stimulate an enzymatic contaminant of BSA. It appears unlikely that it induces the open conformation, since vEts is also preferentially inactivated by chemical modification in the absence of Mg^{2+}.

The C-terminal sequences of cEts1 and cEts2 confer resistance to the Ets-domain to a variety of inactivating agents. These sequence are more similar than most of the rest of the protein (81% identity in 26 amino-acids), in keeping with their having an important function. Only the Ets-domains are more conserved (one difference in 84 amino-acids). Erg and Fli1, two other members of the Ets-family, resemble each other more than the rest of the family. Again, sequence immediately C-terminal to the Ets-domain are particularly well conserved, suggesting that they may also have an important function. Altering the C-terminal sequence of cEts1 in a number of ways leads to loss of protection. In particular, the critical sequences appear to be precisely replaced in vEts (cf. ΔA-D, Results). How do C-terminal sequences affect the structure of the Ets-domain?

vEts is preferentially recognised by antibodies raised against the sequence DEVARRWGKRKNKPK in the Ets-domain. This region is positively charged and predicted to be exposed to solvent. It could adopt either a helix-loop-helix (16) or a β-turn-α-helix (17) structure and interact directly with DNA. A sequence located N-terminal to the Ets-domain of Ets1 and 2 inhibits both DNA-binding and interaction with the same antibodies (13). Both the N-terminal inhibitors and the C-terminal sequences of cEts1 and 2 are negatively charged, suggesting that they interact directly with the positively charged DNA-binding domain. The negatively charged C-terminus is replaced by a neutral sequence in vEts, which would weaken the interaction and expose the Ets-domain to antibodies. DNA binding by cEts may displace and expose the negatively charged C-terminus, which would account for the unusually high mobility of N70 compared to C-terminal mutants in band shifts (see Results and data not shown). The vEts C-terminal sequence does not appear to have a role, beside replacing cEts sequences. The vEts DNA binding domain is phosphorylated by kinases in reticulocyte lysates. Phosphorylation in-vivo could introducing negative charges that affect interactions between the C-terminus and the Ets-domain, or perhaps with other proteins.

Alterations of C-terminal sequences potentiate redox regulation in-vitro. Similar results are obtained in the context of the entire protein, or just the DNA binding domain. The Ets-domain alone (N70-ΔD) is sensitive to both oxidation by diamide and alkylation by NEM, principally due to the single cysteine at position 394. A cysteine is found in an equivalent position in Ets2, Elf1 and E74, and in the same region (7 amino-acids) in most of the rest of the Ets-family, suggesting that may also be important for DNA binding. Basic amino acids are thought to enhance sensitivity to redox. However, the cysteines in the Ets proteins are not surrounded by basic amino acids, in contrast to the redox-sensitive cysteines of Jun (18), NF-κB (19−21) and Myb (22, 23). Modification of cysteine might alter either the structure of the Ets-domain or interactions with DNA.

vEts is sensitive to redox regulation in-vivo. The oxidant H_2O_2 inhibits Ets in cells by an indirect mechanism, since it has no effect when added directly in-vitro (our unpublished results). Furthermore, adding reducing agents to COS cell extracts does

not reactivate DNA binding (unpublished data). Cysteine 394 in the Ets domain does not appear to mediate this effect, suggesting that the mechanisms of inactivation are different in-vitro and in-vivo. Little is known about the signalling pathways induced by oxidants. TPA induces in cells an oxidative state that appears to be important for activation of NF-$\varkappa$B (eg. 24). However, TPA does not inhibit Ets DNA binding in COS cells, perhaps because they lack certain pathways, or that Ets regulation differs from NF-$\varkappa$B. Hydrogen peroxide and oxygen radicals are commonly produced in inflammatory processes and induce endogenous gene expression (20). Redox regulation of Fos-Jun is mediated by a DNA repair enzyme, Ref-1 (25). In conditions of oxidative stress the cell probably inactivates proteins that stimulate growth, and, once DNA repair is underway, reactivates them with proteins such as Ref-1. Ets-like proteins might mediate redox regulation. cEts1 is less sensitive than vEts to redox regulation. It is possible that cEts1 can adopt a sensitive conformation, perhaps through post-translational modification or protein-protein interactions. vEts might be fixed in an 'open' conformation, to facilitate interactions with other proteins or DNA, or to increase susceptibility to redox regulation.

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