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The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains

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ABSTRACT **The DNA binding activity of transcription factor AP-1 is regulated *in vitro* by a posttranslational mechanism involving reduction/oxidation (redox). Redox regulation is mediated by a conserved cysteine residue in the DNA-binding domain of Fos and Jun. Previously, we demonstrated that a DNA repair protein, Ref-1, could stimulate the DNA binding activity of Fos–Jun dimers by reducing this cysteine residue. To examine the relationship between the redox and repair functions of Ref-1, we generated a series of deletion mutants. Analysis of the truncated proteins *in vitro* revealed that the redox and repair activities are encoded by distinct regions of Ref-1. Sequences in the N-terminal domain of Ref-1 that are not present in functionally related proteins from other organisms are required for the redox activity, whereas the DNA repair activity requires conserved C-terminal sequences. Chemical alkylation or oxidation of cysteine sulfhydryls inhibits the redox activity of Ref-1 without affecting its DNA repair activity. Crosslinking studies suggest that a direct cysteine-mediated interaction occurs between Ref-1 and Jun.**

Mutational analysis has shown that a single conserved cysteine residue in the DNA-binding domain of Fos and Jun plays a critical role in regulating the DNA binding activity of AP-1 complexes by a posttranslational mechanism involving reduction/oxidation (redox) (1). A similar mode of regulation has been recently proposed for other nuclear transcription factors (2–6). In the oncogenic v-Jun protein, the critical cysteine residue has been replaced with a serine (7). The same substitution in Fos augments both its DNA binding activity and its transforming potential (8). Chemical oxidation or modification of the cysteine residue inhibits the DNA binding activity of Fos and Jun (1). Oxidation is reversible by thiol compounds or by a cellular redox/DNA repair protein identified originally as Ref-1 (redox factor 1) (9–11). Ref-1 is a ubiquitous nuclear protein that copurifies with HeLa cell AP-1 DNA binding activity during column chromatography (10). Immunodepletion studies using affinity-purified anti-Ref-1 antibodies have shown that Ref-1 constitutes the major component of AP-1 redox activity in HeLa cells (11). Ref-1 is also capable of stimulating the DNA binding activity of other classes of redox-regulated transcription factors, including Myb and NF-κB (11).

Although Ref-1 was purified and cloned on the basis of its AP-1 redox activity, its DNA repair activity was discovered because of sequence identity to a group of DNA repair proteins designated as class II hydrolytic apurinic/apyrimidinic (A/P) endonucleases (12–16). Until recently, Ref-1, like other enzymes in its class, was thought to function mainly in the repair of DNA lesions generated by exposure to reactive oxygen radicals (17). These enzymes act by hydrolyzing DNA at the phosphate bond 5′ to abasic sites. The identification of a DNA repair protein with the ability to also regulate the DNA binding activity of different transcription factors suggested a novel connection between the processes of DNA repair and transcription regulation.

Several examples of transcription–repair coupling have recently been described. In *Escherichia coli*, the product of the mutation frequency decline (*mfd*) gene stimulates repair of the transcribed strand during transcription (18). Mfd displaces RNA polymerase molecules that are stalled at a lesion site in DNA and recruits a nuclease involved in excision repair to the template. The 89-kDa subunit of the human BTF2 basic transcription factor (TFIIH) is a nucleotide-excision repair helicase encoded by the ERCC3 gene (19). A potential role for ERCC3 in melting the DNA helix during transcription initiation has been suggested. In this case, the same helicase activity appears to have been adapted for use during transcription and repair. The situation with Ref-1 is somewhat different in that the same protein catalyzes two apparently distinct functions involved in transcription and repair. Initial studies using recombinant Ref-1 suggested that the redox and A/P endonuclease activities could be distinguished biochemically (11). Here, using a series of truncated proteins, we demonstrate that these activities are encoded by nonoverlapping domains.

MATERIALS AND METHODS

Construction of Ref-1 Deletion Mutants. Ref-1 genes truncated at the N terminus were constructed by polymerase chain reactions using oligonucleotides corresponding to fixed 3′ and variable 5′ end points (62–318ΔN, 116–318ΔN, 162–318ΔN, 205–318ΔN, and 249–318ΔN) of the Ref-1 coding sequence (20).

C-terminal truncations of Ref-1 were generated from the parental Ref-1 expression plasmid pDS56ref-1 (11) by two strategies. This plasmid contains the Ref-1 coding sequence inserted between the *Bam*HI (5′) and *Hin*dIII (3′) sites of the polylinker. The first strategy involved digestion of pDS56ref-1 at the 3′ end with *Hin*dIII together with another restriction enzyme that cut only within the Ref-1 gene, but not in the vector. After double digestion of the plasmid with *Hin*dIII/*Apa* I, *Hin*dIII/*Bgl* II, or *Hin*dIII/*Aha* II, T4 DNA polymerase was used to generate blunt-end DNA fragments. Fragments containing the vector and Ref-1 coding sequences were gel-purified, ligated, and transformed into *E. coli*. The *Apa* I, *Bgl* II, and *Aha* II digests generated truncated proteins with C-terminal endpoints at amino acids 41, 64, and 281, respectively.

In the second strategy, pDS56ref-1 was digested with enzymes that cut both in the gene and in the vector. The resulting fragments were treated with T4 DNA polymerase to generate blunt ends and then digested with *Bam*HI, which cuts 5′ of the Ref-1 gene. Digestion with *Bam*HI/*Rsa* I, *Bam*HI/*Xmn* I, and *Bam*HI/*Sty* I generated fragments with C-terminal endpoints at amino acids 127, 190, and 224, respectively. Fragments containing blunt ends at their 3′ termini and an intact *Bam*HI overhang at the 5′ end were

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Abbreviations: A/P, apurinic/apyrimidinic; DTT, dithiothreitol.

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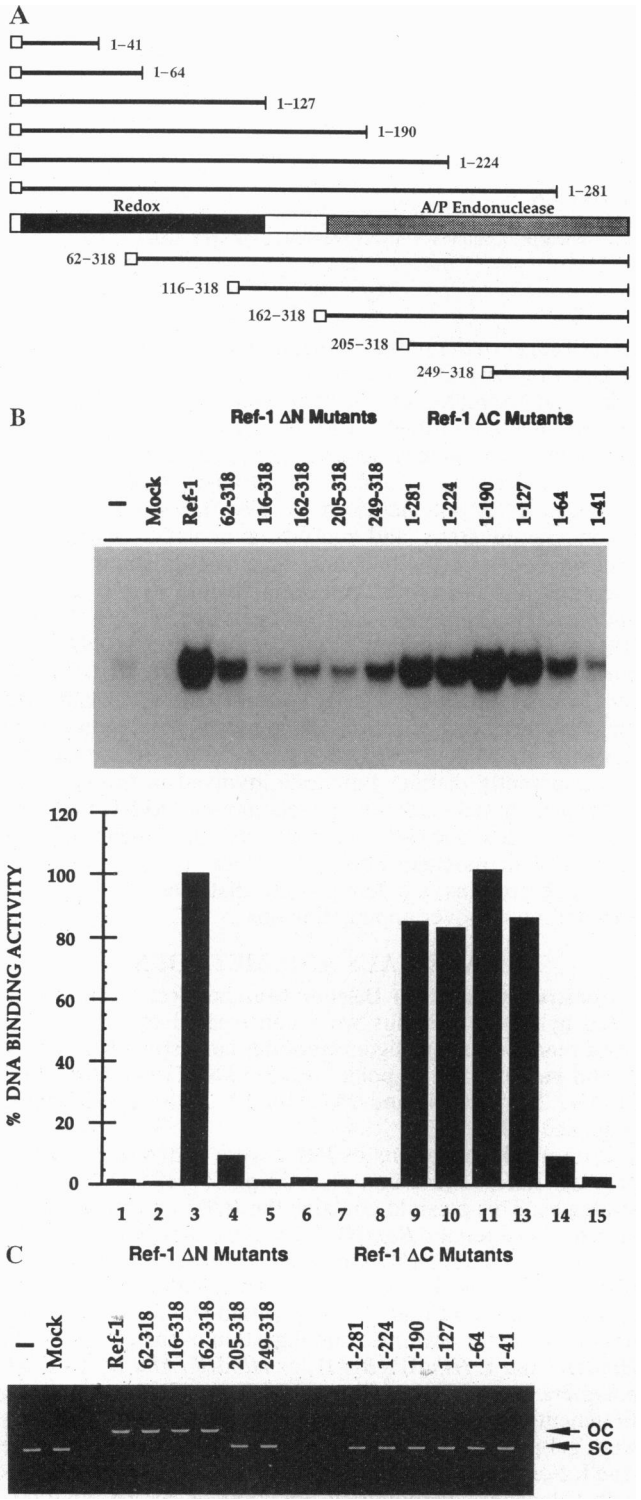

FIG. 1. The redox and A/P endonuclease activities of Ref-1 localize to distinct regions of the protein. N-terminal and C-terminal deletion mutants of Ref-1 were expressed in *E. coli* as histidine fusion proteins and were purified by nickel-chelate chromatography (21). The N- and C-terminal endpoints of each mutant are illustrated (*A*). The open box shown at the N terminus of each protein represents the fused hexahistidine peptide. The redox and A/P endonuclease domains are indicated by the solid and stippled boxes, respectively. An equimolar amount of each truncated Ref-1 protein was assayed for AP-1 redox (*B*) and A/P endonuclease (*C*) activity. OC, open circular DNA; SC, supercoiled DNA. Data expressed as a percentage of activity obtained relative to full-length Ref-1 are summarized in Fig. 3*B*. Background DNA binding activity, from control samples which did not contain Ref-1 (lane 1, buffer; lane 2, mock extract), was not subtracted in the quantitation.

subcloned into the pDS56 vector. The C-terminal deletion constructs were designated 1–281ΔC, 1–224ΔC, 1–190ΔC, 1–127ΔC, 1–64ΔC, and 1–41ΔC.

DNA Binding and A/P Endonuclease Assays. All proteins were expressed in *E. coli* as hexahistidine fusions and purified by nickel-chelate chromatography (1, 11, 21). DNA binding and A/P endonuclease assays were performed with purified Ref-1 or an equimolar amount of the corresponding N- or C-terminal derivative (11).

Chemical Modification of Ref-1 and Crosslinking Assays. Ref-1 (10 μg) was incubated at 25°C for 60 min in the presence of 10 mM diamide or 10 mM *N*-ethylmaleimide. A molar excess of dithiothreitol (DTT, 20 mM) was added to some reaction mixtures (DTT_A) prior to the addition of the modifying agent. The modified Ref-1 protein was dialyzed overnight against consecutive changes of 500 ml of storage buffer [50 mM sodium phosphate, pH 7.3/50 mM NaCl/5 mM $MgCl_2$/1 mM EDTA, 5% (vol/vol) glycerol] containing 1 mM, 10 mM, 1 mM, and 0.5 mM DTT. Dialysis against storage buffer containing 10 mM DTT was performed only for the samples labeled DTT_B, as indicated in Fig. 2. Ref-1 samples (0.1 μg) that were treated with the modifying agent, alone or in conjunction with DTT, were assayed for activity as described above.

In the crosslinking assays Ref-1 (1.0 μg) was incubated for 15 min at 25°C with equimolar amounts of Fos and Jun proteins in the presence of 10 mM diamide as indicated. Complexes were denatured by boiling in the absence or presence of 0.1 M 2-mercaptoethanol and then analyzed by electrophoresis in nonreducing SDS/polyacrylamide gels.

RESULTS AND DISCUSSION

The Redox and DNA Repair Activities of Ref-1 Are Specified by Distinct Domains. Initial biochemical studies of Ref-1 suggested that the redox and A/P endonuclease activities might reside in distinct regions of the protein (11). To understand further the functional relationship between these activities, we generated a series of N- and C-terminal truncations of Ref-1 and assayed equimolar quantities of each mutant for the two activities *in vitro*. The N-terminal and C-terminal endpoints of these mutants corresponded to amino acid positions 62, 116, 162, 205, and 249, and positions 281, 224, 190, 127, 64, and 41, respectively (Fig. 1*A*). Both series of truncations were expressed in *E. coli* and purified as fusion proteins (21). Gel retardation analysis using purified Fos and Jun was used to determine the effect of these deletions on redox activity. Deletion of the N-terminal 62 amino acids of Ref-1 resulted in a 92% loss in redox activity, whereas a deletion to amino acid 116 completely abrogated the activity (Fig. 1*B*). The residual stimulation of Fos–Jun DNA binding activity seen with the 116–318ΔN mutant could be attributed to the carryover of DTT contributed by the buffer in which all the mutant proteins were prepared.

C-terminal deletion analysis was performed to localize the 3′ boundary of the redox domain. Two truncations, 1–281ΔC and 1–224ΔC, displayed a moderate reduction in redox activity (15% and 18%, respectively) relative to full-length Ref-1. Interestingly, truncation of the protein to amino acid 190 resulted in an increase in redox activity restoring it to the level of the wild-type protein. This may reflect removal of negative regulatory sequences that act to modulate the redox function. Deletion of Ref-1 to amino acid 127 had little negative effect on redox activity relative to the more distal truncations, whereas deletion to amino acids 64 and 41 resulted in a marked reduction in activity of 91% and 97%, respectively. These data indicate that sequences proximal to amino acid 127, within the N-terminal third of Ref-1, are required for stimulation of Fos–Jun DNA binding activity *in vitro*. Sequences in the C-terminal region of the protein are

not required for the redox function, although allosteric effects on tertiary structure may influence redox activity indirectly.

The A/P endonuclease activity of the truncated Ref-1 proteins was assayed by monitoring enzyme-mediated conversion of supercoiled, acid-depurinated DNA to open circular forms in an agarose gel. The N-terminal 162 amino acids of Ref-1 could be deleted without significantly affecting the A/P endonuclease activity (Fig. 1*C*). Loss of the endonuclease activity was seen only upon deletion of the protein to amino acid 205. Thus, the 5′ boundary of the A/P endonuclease activity lies in the region encoded between amino acids 162 and 205. The 3′ boundary of the A/P endonuclease activity extends to the C terminus of Ref-1, as even a short truncation of 37 residues from this end completely abolishes the activity. Taken together, these results demonstrate that the redox and A/P endonuclease activities of Ref-1 are encoded by distinct domains and that these activities can function independently.

Cysteine Sulfhydryls Are Essential for Redox Activity of Ref-1. Previously we demonstrated that the redox activity of Ref-1 was inactivated by dialysis against a nonreducing buffer (11). This treatment selectively inhibited the AP-1 redox activity without affecting the capacity of the enzyme to cleave abasic DNA. The redox activity of Ref-1 was restored by incubation with thioredoxin, which suggested that cysteine residues in Ref-1 may be directly involved in reducing Fos and Jun (10, 11). Ref-1 contains a number of cysteine residues distributed throughout its coding sequence. Cysteines at positions 65, 93, and 99 lie in the region encoding the redox activity. To test the functional requirement for reduced cysteines, Ref-1 was treated with diamide and *N*-ethylmaleimide, two sulfhydryl-modifying agents that result in cysteine oxidation and alkylation, respectively. Modified Ref-1 was then assayed for its ability to catalyze the redox and A/P endonuclease reactions (Fig. 2). As a control, a molar excess of the reducing agent DTT was added to the reaction during treatment with the modifying agent to block its effect (DTT_A) or following removal of the modifying agent to reverse its effect (DTT_B). Treatment with either agent abolished the ability of Ref-1 to stimulate binding of Fos–Jun complexes to the AP-1 oligonucleotide (Fig. 2*A*). However, incubation of either modifying agent with Ref-1 in the presence of a molar excess of DTT protected Ref-1 from inactivation. Diamide-mediated inhibition of the redox activity was reversed by subsequent reduction of the protein with 10 mM DTT. This effect was not seen with *N*-ethylmaleimide, which causes irreversible alkylation of cysteine sulfhydryls. In contrast, the A/P endonuclease activity of Ref-1 was unaffected by these treatments as demonstrated by the ability of the enzyme to cleave efficiently abasic plasmid DNA (Fig. 2*B*). Conservation of the A/P endonuclease activity under oxidizing conditions indicates that oxidation did not generate any gross structural alterations in Ref-1. These data confirm that cysteine sulfhydryls are important for the redox activity of Ref-1. Our findings are in accord with those of Walker *et al.* (22), who have recently found that Cys^{65} is important for the redox activity of Ref-1. Replacement of this residue with alanine inhibits the ability of Ref-1 to stimulate binding of Jun homodimers to DNA.

Changes in the redox state of specific cysteines could serve as a mechanism to selectively modulate the redox activity of Ref-1 without altering its endonuclease activity. The endonuclease activity would be required even under normal growth conditions to enable cells to cope with the high rate of spontaneous base loss that occurs at a high frequency in the genome (23). Indeed, Ref-1 mRNA and protein are expressed constitutively in all cells examined (S.X., unpublished data).

The cellular environment contains a number of reducing equivalents (e.g., glutathione, thioredoxin) that may act to

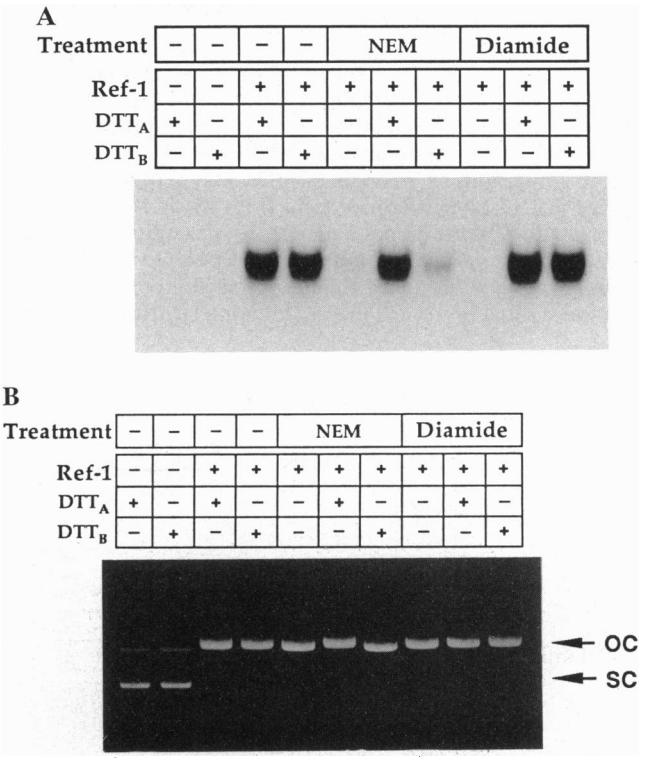

FIG. 2. Treatment of Ref-1 with sulfhydryl-modifying agents selectively inhibits the redox activity. Ref-1 was treated for 60 min at room temperature with 10 mM diamide or 10 mM *N*-ethylmaleimide (NEM). DTT (20 mM) was added to the sample during treatment (DTT_A) or after removal of the modifying agent by dialysis (DTT_B). The protein was then dialyzed extensively to lower the concentration of DTT to 0.5 mM before being assayed for AP-1 redox activity (*A*) and A/P endonuclease activity (*B*). OC, open circle; SC, supercoiled.

regulate the redox activity of Ref-1. For example, stimulation of AP-1 DNA binding activity by Ref-1 could occur following reduction of Ref-1 by thioredoxin or by an analogous activity. Thioredoxin has been implicated in several cellular processes, including transcription regulation (24, 25) and cell proliferation (26), although its role in either process is not well understood. Recent studies suggest that transient expression of thioredoxin in cultured cells stimulates AP-1 (25) and possibly NF-κB (24)-dependent transcription. This effect may be mediated through Ref-1. Thioredoxin fails to activate AP-1 DNA binding activity directly *in vitro* but can act as an intermediate in a redox cascade involving Ref-1 (11). In this system, thioredoxin catalyzes the reduction of oxidized Ref-1, which in turn is able to reduce Fos and Jun and thereby activates AP-1 DNA binding activity. Posttranslational control of Ref-1 by a redox mechanism involving proteins such as thioredoxin would allow rapid responses to the changing redox state of the cellular environment.

Ref-1 Forms Complexes with Jun *in Vitro*. The precise mechanism by which Ref-1 reduces Fos and Jun is not known, although the redox effect is presumably mediated through a direct interaction between the proteins. During purification of Ref-1 from HeLa cells, we noted that several AP-1 proteins copurified through multiple fractionation steps (10). This suggested that Ref-1 might form low-affinity complexes with Fos- and Jun-related proteins. The data presented here indicate that as in other redox systems (27, 28), cysteine sulfhydryls in Ref-1 may participate in the redox reaction by donating the proton required for reduction of Fos and Jun. Therefore we reasoned that it might be possible to trap the interaction by using a crosslinking reagent such as diamide, which oxidizes cysteine sulfhydryls to disulfides. To this end, a series of crosslinking

experiments was performed with purified Ref-1 and several mutated Fos and Jun proteins containing cysteine-to-serine substitutions (1) (Fig. 3). Each of the different combinations of proteins was incubated at equimolar ratios in the presence of diamide. Covalently linked proteins were identified by electrophoresis under nonreducing conditions.

Ref-1 migrated as a single 37-kDa polypeptide in nonreducing SDS polyacrylamide gels (Fig. 3). However, treatment of Ref-1 with diamide generated an additional protein species that migrated just below 37 kDa. On the basis of apparent molecular mass, this more rapidly migrating polypeptide probably represents a monomeric form of Ref-1 containing intramolecular disulfide bonds. No additional protein bands of higher apparent molecular mass were detected, indicating that Ref-1 does not form cysteine-linked multimers under these conditions.

Fos and Jun were incubated with Ref-1 in the presence and absence of diamide and the products were analyzed as outlined above (Fig. 3). Truncated Fos and Jun proteins lacking any cysteine residues (FC154S-C204S, JC272S-C323S) (1) failed to form covalent dimers between themselves or with Ref-1 (Fig. 3*A*). In contrast, Fos and Jun proteins containing a single cysteine-to-serine substitution in the DNA-binding domain (FC154S, JC272S) (1) were capable of

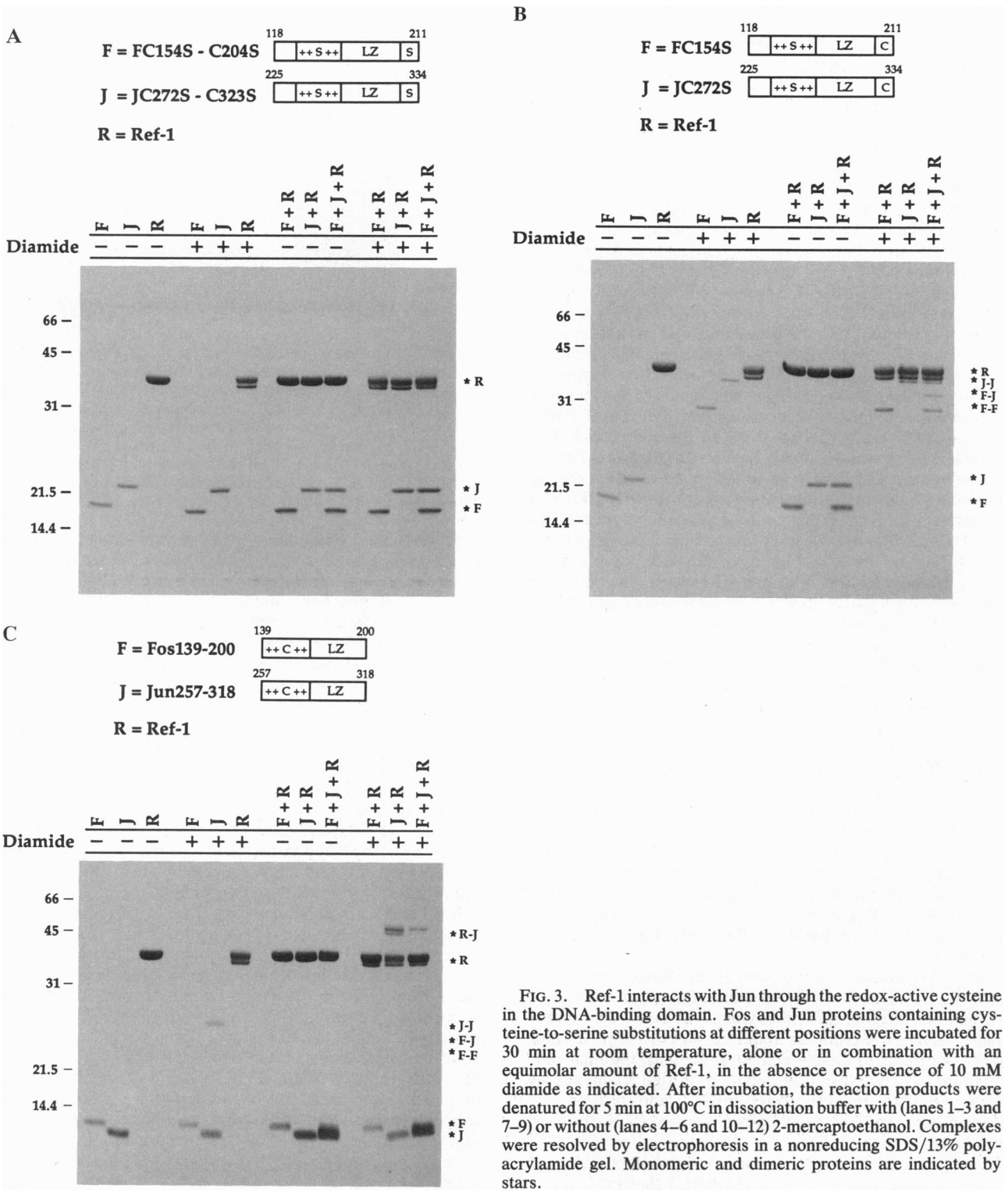

FIG. 3. Ref-1 interacts with Jun through the redox-active cysteine in the DNA-binding domain. Fos and Jun proteins containing cysteine-to-serine substitutions at different positions were incubated for 30 min at room temperature, alone or in combination with an equimolar amount of Ref-1, in the absence or presence of 10 mM diamide as indicated. After incubation, the reaction products were denatured for 5 min at 100°C in dissociation buffer with (lanes 1–3 and 7–9) or without (lanes 4–6 and 10–12) 2-mercaptoethanol. Complexes were resolved by electrophoresis in a nonreducing SDS/13% polyacrylamide gel. Monomeric and dimeric proteins are indicated by stars.

forming covalently linked homodimers as well as heterodimers through a C-terminal cysteine (Fig. 3*B*). However, crosslinking between Ref-1 and Fos or Jun was not detected with this combination of mutants. An interaction was only detected when Ref-1 was incubated with a mutant Jun protein (Jun257–318) that contained a cysteine residue only in its DNA-binding domain (Fig. 3*C*). Surprisingly, the interaction was observed with Jun alone; crosslinking of Ref-1 with Fos139–200 was not detected.

Previously we demonstrated that cysteine residues in the DNA-binding domain of Fos and Jun were involved in the redox regulation of AP-1 DNA binding activity (1). It is not known why covalent Fos–Ref-1 dimers were not detected in these experiments. The lack of an interaction of Ref-1 with Fos may be due to limitations of the assay. Only interactions which involve cysteines that can be oxidized to the disulfide form are detectable by this approach. A likely explanation is that diamide cannot access the Fos–Ref-1 complex. A diminished level of Jun–Ref-1 complex was detected when Ref-1 was incubated with both Fos and Jun as compared to Jun alone. This decrease may reflect the greater stability of Fos–Jun heterodimers relative to Jun homodimers (29). Consistent with this possibility, increased dimerization between Ref-1 and Jun appears to have occurred at the expense of covalent Jun homodimers. In any event, these data suggest that Ref-1 and Jun can interact through the basic region of Jun and that this interaction involves cysteine residues present in each protein.

Conclusions and Implications. This report shows that the different activities catalyzed by Ref-1 localize to distinct domains on the protein. The redox and A/P endonuclease activities reside in the N-terminal 127 and C-terminal 157 amino acids, respectively. These domains can be physically dissociated without significantly disrupting the individual activities. The high degree of sequence similarity shared by the C-terminal half of Ref-1 and other functionally related repair proteins in bacteria (exonuclease III, exonuclease A) (30, 31) and flies (Rrp1) (15) is consistent with the assignment of the A/P endonuclease activity to this region. On the other hand, the redox activity of Ref-1 is localized in a region that is unique to the mammalian enzyme. The N-terminal 62 amino acids of Ref-1 are not present in exonuclease A (30), exonuclease III (31), or Rrp1 (15). Our studies indicate that this short N-terminal region and sequences immediately adjacent to it are critical for Ref-1 redox activity. One of the bacterial proteins, exonuclease III, was recently found to lack any detectable redox activity *in vitro* (22).

It is not clear why a single polypeptide has evolved two apparently distinct activities that function in DNA repair and transcription. One attractive possibility is that the two activities act coordinately to initiate DNA repair and also stimulate the DNA binding activity of latent AP-1 proteins during oxidative stress. The net effect would be to augment the cellular response to adverse oxidative conditions. Rapid activation of AP-1 and other transcription factors by Ref-1, followed by the expression of specific target genes, could act to minimize cellular damage caused by oxidative stress (32). Indeed, transcriptional and posttranslational induction of AP-1 DNA binding activity has been shown to accompany treatment of cells with agents that induce oxidative stress (33–35). Given the ability of Ref-1 to associate with AP-1 (10), another possibility is that Ref-1 may be directed selectively to AP-1 target genes. This would confer an advantage on the cell by increasing the rate of repair to AP-1-responsive genes after oxidative damage. Finally, UV-induced DNA damage is an intermediate in UV-stimulated expression of certain genes, including c-*fos* (35). It will be interesting to determine whether the A/P endonuclease activity of Ref-1 serves a more general role in regulated or basal transcription, as was shown recently for the helicase subunit (ERCC3) of TFIIH/BTF2 (19).

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