Human thioredoxin/adult T cell leukemia-derived factor activates the enhancer binding protein of human immunodeficiency virus type 1 by thiol redox control mechanism

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Abstract

Transcription from the human immunodeficiency virus type 1 (HIV-1) provirus is activated by a cellular factor, NFkB, recognizing the tandemly repeated 10-base-pair sequences, termed the xB sequence, present in the enhancer region within the viral long terminal repeat (LTR). Using electrophoretic mobility shift assay (EMSA), which demonstrates specific DNA–protein interaction in vitro, we could demonstrate that reducto-oxidative modulation of NFkB dramatically changes its DNA binding activity and that a cellular physiological reducing catalyst, thioredoxin (TRX) also known as adult T cell leukemia derived factor (ADF), fully restored the DNA-binding activity of the oxidized NFkB. We also observed that purified TRX/ADF protein could augment gene expression from HIV LTR as demonstrated by transient chloramphenicol acetyltransferase (CAT) assay. These observations confirmed the previous notion that ADF might be an inducing factor of cellular interleukin-2 receptor α subunit (IL-2Rα) through the xB sequence that is a common central cis-regulatory element in both IL-2Rα and HIV gene expression. These observations indicate that reducto-oxidative regulation (or redox regulation) of a cysteine residue(s) on the NFkB molecule might play an important role in its specific DNA interaction and that it might provide a clue to the understanding of a pathway of cellular signal transduction to NFkB that is independent from the known pathways involving protein phosphorylation.

Introduction

NFkB is a pleiotropic mediator of transcription for various promoters (1–8). It activates a wide variety of viral and cellular genes including human immunodeficiency virus-1 (HIV-1) (9–13), interleukin-2 receptor α (IL-2Rα) (4,14,15), tumor necrosis factor-α (TNF-α) (16) and GM-CSF (17). NFkB is initially located in the cytoplasm as an inactive form associated with an inhibitory protein, IκB (18). The finding that phorbol esters induce nuclear translocation of NFkB, and that NFkB is released in vitro by treatment of the cytosol fraction with purified protein kinase C (PKC) (19), have suggested that IκB may be inactivated by phosphorylation. However, one of the few known physiological inducers of NFkB, TNF-α, was found to activate NFkB even in the presence of protein kinase inhibitors, implying that a kinase-independent mechanism was involved (20).

Within the HIV-1 long terminal repeat (LTR), two tandemly repeated xB sites have been identified in the region initially
identified as enhancer. This cis-regulatory element has been shown to regulate the transcriptional inducibility of HIV in activated T cells and in TNF-treated cells (2,9,12,20,21). Thus, NFκB may serve as an intracellular mediator in activation of the latent HIV-1 provirus and thus participate in a critical step preceding the clinical manifestation of the acquired immunodeficiency syndrome (AIDS).

In T cells transformed by human T lymphotropic virus type 1 (HTLV-1), NFκB is constitutively activated causing overexpression of IL-2Rα on the cell surface (15,22) and release of TNF-α (16) and GM-CSF (17). Moreover, replication of HIV-1 is greatly augmented in these cells (23,24) and its augmentation is dependent on the κB sites within LTR (9,12,21). Interestingly, Tagaya et al. (25) demonstrated that a cellular factor, known as adult T cell leukemia-derived factor (ADF), which is released from cells transformed by HTLV-1 (26,27) or Epstein – Barr virus (28) and has activity to stimulate IL-2Rα expression, is the human homolog of thioredoxin (TRX) (see ref. 29 for review). Human TRX is a 13 kDa thiol protein that is a strong catalyst of dithiol-disulfide exchange reactions (A. Mitsui, T. Hirakawa, and J. Yodoi, submitted). TRX participates in redox reactions through reversible oxidation of its active center dithiol to disulfide. The actual role of TRX in inducing IL-2Rα is to be clarified, but there is much evidence to suggest that the reducto-oxidative conditions of sulfhydryl (SH) groups of some nucleic acid-binding proteins reversibly modulate their binding capabilities (30–33). Recently, several reports have indicated that reducto-oxidative conditions modulated the DNA binding activity of NFκB by using non-physiological inorganic reagents (34–36). These findings prompted us to investigate whether redox regulation by the TRX system participates in the regulation of HIV gene expression.

**Methods**

**Nucleic acids**

Oligodeoxynucleotides were synthesized in an Applied Biosystems 381 DNA synthesizer (Applied Biosystems, USA).

**Preparation of nuclear and cytosolic extracts**

Nuclear and cytosolic (S100) extracts were prepared from MT2 cells (37) in the logarithmic growth phase by the method of Dignam et al. (38). NFκB activity in the crude MT2 nuclear extract was partially purified through heparin–agarose and DEAE–Sepharose columns as reported previously (39,40). All operations after cell harvest were performed at 4°C. All buffers contained protease inhibitors, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.01 U/ml apotinin. Briefly, the crude nuclear extract after dialysis against a buffer D (containing 20 mM Tris – HCl (pH 7.9), 0.1 M KCl, 0.2 mM EDTA, 1 mM DTT and 20% glycerol) was loaded onto a heparin–agarose column, washed with five column volumes of buffer D, and the bound proteins were eluted with buffer D with 0.4 M KCl. This 0.4 M KCl eluate was dialysed against buffer D and loaded onto a DEAE – Sepharose column. The NFκB activity recovered in the 0.225 M KCl eluate (called ‘DEO.225’) was concentrated by ultrafiltration (using Centriprep; Amicon, MA, USA) and dialysed against buffer containing 20 mM HEPES – KOH (pH 7.9), 0.1 M KCI, 0.2 mM EDTA, 0.5 mM PMSF, and 20% (v/v) glycerol (no DTT was added) and stored in liquid nitrogen until use. The endogenous TRX, examined by Western blotting with specific antiserum, and its activity, measured by the ability to convert NADPH to NADP+, was not detected in the MT2 DEO.225 fraction.

**DNA binding assay**

Kinase reactions of oligonucleotide DNA were performed with [γ-32P]ATP and T4 polynucleotide kinase. Binding reactions were performed at 30°C for 10 min in a total volume of 10 µl of buffer containing 20 mM HEPES – KOH (pH 7.9), 60 mM KCl, 1 mM MgCl2, 5% (v/v) glycerol, 0.1% NP40, 1.0 µg of poly(dI-dC), 0.1 ng (20 000 – 30 000 c.p.m.) of labeled probe, and DEO.225 containing NFκB, DTT was not added in the experiments using sulphhydryl-modifying reagents. After addition of loading buffer, the mixture was analyzed by electrophoresis in 6% non-denaturing polyacrylamide gel with 0.5 × TBE buffer (4.5 mM Tris, 4.5 mM boric acid, 0.1 mM EDTA, pH 8.0). For competition assays, the competitor DNA (5 ng: i.e. 50-fold excess over the probe) together with poly(dI-dC) was preincubated with NFκB on ice for 5 min before addition of the rest of the reaction mixture. NFκB usually gave two major retarded bands on electrophoretic mobility shift assay (EMSA), as reported previously (3,7,8,14,21,41). The additions of the wild-type κB sequences reduced the intensities of both bands equally.

**Modification of sulphydryls**

N-ethylmaleimide (NEM) and diamide (diazenedicarboxylic acid bis(N,N-dimethylamide)) were purchased from Sigma (St Louis, USA). NEM alkylates and irreversibly blocks SH groups by the following reaction:

$$R-SH + NEM \rightarrow R-S-NEM$$

On the other hand, diamide reversibly converts thiols to disulfides (42) and the diamide-oxidized protein could be recovered by appropriate reducing agents such as DTT, 2-mercaptoethanol (2-ME) and the reduced form of TRX.

Recombinant human TRX, or ADF, and its mutant were purified to near homogeneity, reduced by incubation with excess DTT (usually 10 mM DTT was used for reducing 1 mM TRX) for 1 h at room temperature and dialyzed overnight against buffer saturated with nitrogen gas. Purified and reduced TRX was stored in liquid nitrogen until use.

**Transfection and chloramphenicol acetyltransferase (CAT) enzyme assay**

For transfection of plasmid DNA into COS-1 cells, the calcium phosphate precipitation protocol was used as previously reported (9,24). Construction of HIV LTR – CAT, CD12, and its deletion mutant, CD52, lacking the upstream sequence from nucleotide position – 65, was reported in the previous papers (13,40). Cells were harvested after 48 h of transfection and the CAT enzyme activity in 100 µg of cell lysate was measured by the standard method (9,24). Purified recombinant TRX/ADF was added to the cell culture media 24 h before cell harvest at the concentrations indicated. Protein concentration of each lysate was determined using Bradford’s method (BioRad, USA).
Results

Preparation of 'activated' NF\(\times\)B and its specificity

NF\(\times\)B was partially purified from one of the HTLV-1-transformed cell lines, MT2 (37). For this, a crude nuclear extract was prepared from MT2 cells in the logarithmic growth phase by the method of Dignam et al. (38) and NF\(\times\)B in the extract was fractionated using heparin-agarose and DEAE-Sepharose (Fig. 1). Since in MT2 cells genes such as HTLV-1-immortalized sequences of the mutants were synthesized as described under Methods. (a) The xB DNA-binding activities of fractions after each stage of purification by EMSA. 32P-labeled double-stranded HIV \(\times\)B probe (see Fig. 2a for the sequence) was incubated with each fraction. The binding reactions and electrophoresis were as described under Methods. One microliter (about \(10^6\) cell equivalent) of each fraction was added to the binding reaction (lanes 2 through 5). Two retarded bands are indicated by close arrow heads, in lane 1, no protein was added (c, control). In lanes 8 and 10, 2 \(\mu\)l of DEAE 0.225 nuclear fraction or cytosolic fraction of MT2 cells (S100) were incubated with 0.8% deoxycholate and 1.2% NP40 (final concentrations) before addition of the probe. The attenuation of the upper band and the slight downward shift of the lower band with the DEAE 0.225 fraction was noted upon treatment with detergents, probably due to the dissociation of the protein complexes or the conformational changes. Positions of DNA–protein complexes and free unbound probe are shown by arrow heads and an arrow, respectively.

Thiol redox control of the HIV-1 enhancer-binding protein

The requirement of free sulphydryl groups of NF\(\times\)B in DNA recognition was investigated using a moderate sulphydryl-specific alkylating agent, NEM, to block the sulphydryls on the molecular
Thiol redox control of the HIV-1 enhancer-binding protein

(a) HIVwt: GATCTAGGAGCTTTCGGAGCATCTTCAG
HIVmut: GATCTAGGAGCTTTCGGAGCATCTTCAG
IL2Rwt: GATCTAGGAGCTTTCGGAGCATCTTCAG
IL2Rmut: GATCTAGGAGCTTTCGGAGCATCTTCAG
H2TF1wt: GATCTAGGAGCTTTCGGAGCATCTTCAG
H2TF1mut: GATCTAGGAGCTTTCGGAGCATCTTCAG

(b) DNA binding assays

Fig. 2. (a) DNA sequences of synthetic oligonucleotides for NF-κB binding sites used for EMSA. Indicated are the HIV-1 enhancer elements located between -104 and -78, containing the wild-type NF-κB site ('HIV wt'), the IL2-Rα enhancer region containing a single NF-κB site (nucleotide position from -289 to -235) ('IL2R wt'), and their mutants, 'HIV mut' and 'IL2R mut', respectively. Mutations are indicated by lower-case letters with underlines. The linker sequences (GATCT in the 5' end and G in the 3' end) were added for cloning purposes. (b) Analysis of binding to the wild-type and mutant NF-κB binding sites by EMSA. 32 P-labeled HIV wt double-stranded oligonucleotide was incubated with partially purified NF-κB prepared from MT2 (DE0.225 nuclear fraction). Dose-dependent binding was observed in lanes 2 through 4. The specificity of the protein-DNA interaction is demonstrated in lanes 5 through 11. In lanes 6 through 8, increasing amounts of cold HIV wt DNA were added (molar ratio of the probe to the competitor are indicated). In lanes 9 to 11, the unlabeled competitors, IL-2R wt, HIV mut, or IL-2R mut, were added at a molar ratio of 1:50.

Redox regulation of NF-κB activity in vitro

Next we examined the effect of diamide, which catalyzes chemical oxidation of free sulfhydryls, and of purified recombinant human TRX, a physiological reducing catalyst, on DNA recognition by NF-κB. In the following experiments, DTT was not added to most of the reactions because of its possible intervention with the oxidative reagents to be examined, although the clarity of the retarded bands deteriorated (which was probably due to partial oxidation of the protein). As demonstrated in Fig. 4b, oxidation of NF-κB by diamide inhibited the binding dose-dependently (lanes 1 through 6). The aberrant band obtained on treatment with 1 mM diamide (lane 5) did not appear to be specific for the NF-κB sequence judging from the results of competition experiments (compare lanes 5, 7, and 8), and so was probably due either to modification of the NF-κB protein by oxidation, such as by a conformational alteration or dissociation of the multimer structure, or to the formation on oxidation of a non-
Fig. 4. Reducto-oxidative regulation of the DNA-binding activity of NFkB. (a) Amino acid sequences of human TRX/ADF (25). The active site, which is completely conserved in human and E. coli, is underlined. A mutant in which cysteine 31 was replaced by serine had no ability to catalyze the dithiol-disulfide exchange reaction, as shown by insulin reduction assay (43) (Mitsui et al., in preparation). (b) Reversible inactivation of NFkB activity by oxidation and reactivation catalyzed by TRX. EMSA reactions were performed as for Figs 1 and 2 except that 1 mM (final concentration) OTT was not added to the reaction mixture. Incubation of NFkB with diamide was performed on ice for 5 min and the subsequent reduction with TRX at the final concentrations indicated was carried out at 30°C for 5 min. For lanes 3 - 6, increasing concentrations of diamide were added. The aberrant band (indicated by an open arrow head) observed in lane 5 (with 1 mM diamide) appeared not to be specific for the xB sequence judging from the results of competition experiments with cold DNA (lanes 7 and 8). Diamide-pretreated NFkB was subsequently incubated with wild-type human TRX (TRX wt) (lanes 9 - 11) or the mutant (TRX mut) (lanes 12 - 14). In lane 15, NFkB oxidized with diamide was treated with 2-mercaptoethanol (2-ME) (80 mM final concentration).

Fig. 5. Effects of oxidation and reduction of CCAAT binding protein (CBP) on its DNA-binding activity. The 0.1 M KCl eluate from the DEAE-Sepharose column (Fig. 1) was used as a source of CBP. The probe for CBP was the synthetic oligonucleotide, 5'-GATCCAAACGCAATGAGACTGCTCCA-3' (44).
TRX at 4°C (not shown). This temperature dependence of reduction was in contrast with that by the inorganic reducing agent 2-ME, which restored the binding activity equally at both temperatures although the reducing activity of 2-ME in this assay system was ~1000 x less than that of human TRX. Mutant TRX could not restore the NFkB activity at either temperature, confirming that cysteine 31 was crucial for the reducing activity (lanes 4 and 8). These observations demonstrated that TRX reduced the oxidized NFkB and restored its DNA-binding activity through its active thiol on cysteine 31 by the enzymatic mechanism. Preincubation of TRX with diamide before incubation with NFkB was less efficient than addition of diamide after preincubation of TRX with NFkB in restoring the NFkB activity (Fig. 6b), suggesting that TRX-responsive sulfhydryls on the NFkB molecule require a highly reduced form of TRX (in which the active sulfhydryls are probably most prone to be oxidized) or that TRX in the presence of its target protein is more resistant to oxidation as in the case of the TRX-phage T7 DNA polymerase complex (45).

**Effect of TRX/ADF on HIV gene expression in vivo**

To examine further the effect of TRX/ADF on HIV gene expression in vivo, a transient CAT assay was performed with COS-1, a monkey kidney-derived cell line, and CAT gene expressing plasmids under the control of HIV-1 LTR, CD12, or its derivative, CD52, with the deleted upstream sequence from -65, therefore not containing the xB sequences (13,40). Plasmids expressing CAT under the control of chicken β-actin promoter were used as control. After transfection, purified recombinant TRX/ADF was added 24 h prior to cell harvest and CAT enzyme activities were measured according to the standard protocol (9,24). Representative results are shown in Fig. 7. When cells were treated with TRX/ADF, CAT enzyme activity from the wild-type HIV LTR was stimulated in a dose-dependent manner. However, the HIV LTR mutant lacking the upstream sequence including xB sites, as well as the control promoter, did not respond to the effect of TRX/ADF. These observations with the cell culture experiment confirmed the in vitro effect of TRX/ADF on the DNA-binding activity of NFkB.

**Fig. 6.** (a) Temperature dependence of the action of TRX. For lanes 6-8, protein reduction by TRX was performed at 30°C for 5 min, while for lanes 2 and 3, the reaction was carried out at 4°C for 5 min. Lanes 3 and 7, mutant TRX; lanes 4 and 8, wild-type TRX. Temperature dependence of TRX activity has also been observed by insulin disulfide-reducing assay (according to the method of Saby and Holmgren (45)); also our unpublished results at 12°C and 30°C). (b) Effect of preincubation of TRX with NFkB on its susceptibility to oxidation by diamide. For lanes 1-4, the reaction protocol was as for Fig. 3b. For lane 5, the same amount of TRX as for lane 4 was preincubated with diamide on ice for 5 min before its incubation with NFkB. Autoradiographs of 5-day exposure are shown to demonstrate the differences of DNA binding.

**Fig. 7.** Effects of purified TRX/ADF on HIV gene expression in vivo. Results of transient CAT gene expression assays were demonstrated. (a) Dose-dependent stimulation of HIV gene expression by purified recombinant TRX/ADF. A CAT-expressing plasmid under the control of HIV LTR, CD12, was transfected into COS cells. Purified TRX/ADF was added to the cell culture medium to the indicated concentrations 24 h before cell harvest. (b) Effect of deletion of the upstream element containing the NFkB binding site from the HIV LTR. A deletion mutant lacking the upstream sequence (up to the nucleotide position -65 from the CAP site), CD52, was similarly tested for its responsiveness to the exogenous TRX/ADF. A CAT-expressing plasmid under the control of chicken β-actin promoter (46) was also used as a control.
Discussion

Here we present evidence which suggests that TRX/ADF might activate HIV gene expression by a dithio-disulfide exchange reaction of the cysteine residues on the NF\(x\)B molecule. First, the present study has shown that free SH groups of NF\(x\)B required for its specific DNA-binding activity. Oxidation of already ‘activated’ NF\(x\)B prepared from MT2 cell nuclei inactivated its specific DNA-binding activity and subsequent reduction of the oxidized protein by TRX/ADF efficiently restored its activity. Moreover, the transient CAT gene expression assay has demonstrated that gene expression from HIV LTR was augmented by the exogenously added TRX/ADF. Since this activity was abolished when the upstream cis-regulatory region containing the NF\(x\)B-binding sites was deleted from the HIV LTR, this in vivo effect of TRX/ADF might also be mediated through NF\(x\)B.

This kind of control mechanism involving sulfhydryls of cysteine residues is collectively called ‘redox regulation’ (29). Similar phenomena have been observed with other nucleic acid-binding proteins. For example, oxidation of the iron-responsive element binding protein (IRE-BP), which specifically interacts with the 5' untranslated region of the ferritin heavy-chain mRNA, increased its binding affinity to its RNA target (IRE) by a factor of over 100 (31). On the other hand, in the case of the bacterial transcriptional regulatory protein OxyR, which regulates gene expression in response to oxidative stress, oxidation of a single essential cysteine changed its DNA-binding specificity and activated gene expression of a set of genes responsible for the resistance to oxidative environment (32). Furthermore, the DNA-binding activity of the Fox – Jun heterodimer has been shown to be modulated by the redox status of a single conserved cysteine residue in the DNA-binding domain of each of the two proteins (33). However, similar experiments with other transcription factors, namely CBP, Sp-1 and NF-1 ([46] and our unpublished results) did not show any effects of oxidation or reduction of sulfhydryls on their DNA-binding activities. Thus, one of the physiological roles of the cellular redox regulation system might be that it serves as a novel modality in selective activation of a certain set of genes by modulating the activity of particular nucleic acid-binding proteins.

Besides the TRX system, there are other cellular reducing systems such as the glutaredoxin (GSH) system (29,48). The TRX and GSH systems are considered to have specific, but overlapping functions in many reduction systems. Since reduced glutathione, even in the presence of glutathione reductase, did not restore the activity of the oxidized NF\(x\)B in vitro (T. Kawabe, T. Okamoto, and Y. Yodoi, submitted), TRX is more likely to be important in the reduction of NF\(x\)B. Recently, Staal et al. (49) reported that N-acetyl-L-cysteine (NAC), acting both as a scavenger for oxygen radicals and as a precursor of GSH, blocked activation of HIV transcription by TNF-\(\alpha\) through NF\(x\)B when added at a very high concentration. It is possible that there are cross talks within the cells among different reducing systems. However, the in vivo effects of reducing agents, especially in the presence of oxidative stress, such as that evoked by TNF-\(\alpha\), need to be evaluated carefully. For example, the effect of TNF-\(\alpha\) in inducing endogenous TRX may have been blocked in the presence of excess intracellular GSH because of the radical-scavenger activity of GSH. Thus, cellular reducing systems, such as those of TRX and GSH, might have distinct roles in the signal transduction pathway involving NF\(x\)B. In T cells infected with HTLV-1, where TRX (or ADF) (25–27) as well as TNF (16) is overexpressed, NF\(x\)B is known to be constitutively activated (15,22) and replication of HIV is greatly augmented (23,24). It is interesting to see if excessive amounts of the extracellular reducing agent such as NAC could inactivate NF\(x\)B even in cells infected with HTLV-1.

There are many cysteine residues in the putative DNA-binding domain of the NF\(x\)B molecule, but the cysteine residue(s) required for DNA recognition has not yet been identified. Nevertheless, our results, together with others (34–36), suggest that a novel molecular mechanism involving the sulfhydryls has an important role in specific DNA recognition by NF\(x\)B. The concept of ‘redox regulation’ may not only provide an insight into a novel mechanism of DNA-binding by NF\(x\)B but also may explain, at least partly, why gene expression of HIV and IL-2Rx is augmented in the cells transformed by HTLV-1. Further investigation will hopefully lead to development of a new strategy which can efficiently decelerate the pathological process of AIDS.

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Abbreviations

ADF: adult T cell leukemia-derived factor
CAT: chloramphenicol acetyltransferase
CBP: CCAAT-binding protein
EMSA: electrophoretic mobility shift assay
GSH: glutaredoxin
HIV: human immunodeficiency virus
HTLV-1: human T-lymphotropic virus type 1
IL-2Rx: interleukin-2 receptor \(\alpha\)
IRE: iron responsive element
LHR: long terminal repeat
NAC: N-acetyl-L-cysteine
NEM: N-ethylmaleimide
PCK: protein kinase C
PMSF: phenylmethylsulfonyl fluoride
SH: sulfhydryl
TNF: tumor necrosis factor
TRX: thioredoxin

References

Thiol redox control of the HIV-1 enhancer-binding protein