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Translationally controlled tumor protein of *Brugia malayi* functions as an antioxidant protein

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Abstract

Translationally controlled tumor protein (TCTP) is one of the most abundantly expressed proteins in the filarial parasites as well as in the other organisms. Several functions have been suggested for TCTP family of proteins ranging from calcium binding to histamine release function. However, its physiological function is still a mystery. Previous studies showed that the expression of TCTP is increased several-fold during oxidative stress. In the present work, we report the putative antioxidant function of *Brugia malayi* TCTP (BmTCTP). When tested in vitro, rBmTCTP could be reduced by a variety of reducing agents including thioredoxin. Such reduced form of rBmTCTP was able to protect DNA from oxidative damage, suggesting that BmTCTP may have an antioxidant function in the parasite. Sequence analysis of filarial TCTPs revealed that there are three cysteine amino acids located in the central portion of the protein. Subsequent targeted residue modification studies showed that these cysteine residues in rBmTCTP are critical for its antioxidant function. To determine the significance of this finding, rBmTCTP was overexpressed in vivo in *Escherichia coli* and subjected to oxidative stress. These studies showed that rBmTCTP significantly protected cells form oxidative damage. Taken together, these findings suggest that BmTCTP might be functioning as a non-classical antioxidant protein in the filarial parasites.

Introduction

Lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia malayi* is widely prevalent in the tropics, causing significant morbidity in humans throughout the tropics. It is estimated that more than 120 million people are infected, with an additional one billion people at the risk of contracting this dreadful disease (Michael and Bundy 1997). Infection is initiated when infective mosquito bite the susceptible humans living in the endemic areas during the first few years of life followed by the establishment of this infection for many years. From the point of infection to successful establishment, filarial parasites undergo multiple molts beginning with L3 to development of adults in the lymphatic vessels, where adult females liberate millions of mf in the peripheral blood. During this process of infection, all stages of the parasite are constantly exposed to the stressful host environment. It is intriguing how filarial parasites successfully evade or counteract the harmful effects such as oxygen radicals or nitric oxide produced by the host.

In addition to the host oxidative stress, filarial parasites must also counteract its own oxidative stress produced during their aerobic metabolism. Indeed, several lines of studies suggest that filarial parasites have evolved mechanisms to neutralize both the exogenous and endogenous oxidative stress. For example, filarial parasites produce classical antioxidant enzymes such as Cu/Zn superoxide dismutases (James et al. 1994; Dabir et al. 2006), glutathione-*s*-transferase (Rao et al. 2000; Rathaur et al. 2003), and thioredoxin peroxidases (Ghosh et al. 1998) to

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effectively overcome the oxidative stress. In addition to these antioxidant enzymes, parasites possibly utilize non-enzymatic antioxidants which include glutathione, alpha-tocopherol, ascorbic acid, and exogenous albumin for their anti-oxidative defense. Non-enzymatic antioxidants of parasite origin are relatively poorly studied despite their pivotal importance in the defense mechanism. Typically, proteins that fall under this category often operate either in a sacrificial manner or by sequestering transition metals to inhibit the formation of free radicals (Selkirk et al. 1998). Previously, in our laboratory, we have cloned homologues of translationally controlled tumor protein (TCTP) from lymphatic filarial parasites and reported that they have calcium binding and histamine releasing function in vitro (Gnanasekar et al. 2002). A recent study showed that parasite-derived TCTP may be important for the heat stress adaptation (Mak et al. 2001, 2007). Interestingly, expression of TCTP is upregulated by a variety of stress conditions such as oxidative stress, heat shock, and exposure to heavy metals (Sturzenbaum et al. 1998; Bonnet et al. 2000; Mak et al. 2007). For instance, TCTP is upregulated over 12-fold in hypoxic HeLa cells (Rupec et al. 1998) and HepG-2 cells (Wang et al. 2003). Similarly, oxidative stress induced by DTT and hydrogen peroxide (H_2O_2) can induce changes in the expression levels of TCTP in yeast cells (Bonnet et al. 2000) and in human lung epithelial cells (Yoneda et al. 2004). It is worth noting that one of the TCTP genes deposited in the Genbank is designated as a PO_2-related protein cloned from hepatocarcinoma (accession no. AAM51565). Cells, especially embryonic mouse stem cells exposed to dioxin, a potent toxic synthetic environmental pollutants that induces production of reactive oxygen species, cause significant upregulation of the expression and secretion of TCTP, suggesting a potential role for TCTP in oxidative stress (Oikawa et al. 2002). These findings suggest that TCTP may have an antioxidant function. Therefore, in the present study, we have analyzed whether *B. malayi*-derived rTCTP has any antioxidant property.

Experimental procedures

DNA nicking assay

Supercoiled ϕX174 DNA (New England Biolabs, Beverly, MA, USA) was used as a substrate for detecting DNA damage mediated by the metal catalyzed oxidation (MCO) system (Ghosh et al. 1998). The MCO system consisted of 66 mM Fecl3, 6.6 mM DTT, and 2 mM EDTA in a 25 mM Hepes buffer (pH 7). ϕX174 DNA (100 ng) incubated in the MCO system at 37°C for 4−9 h with or without DTT reduced rBmTCTP and rWbTCTP. Before the addition of rBmTCTP or rWbTCTP to the reaction mixture, rTCTP was reduced with 10 mM DTT for 30 min at room temperature. The extent of MCO-mediated nicking was evaluated on ethidium-bromide-stained agarose gels. *Schistosoma mansoni* G box binding factor (rSmGBF; accession number AF102232) expressed in pRSET as a 6-His tagged protein and bovine serum albumin (BSA; Sigma, St. Louis, USA) were used as non-specific control proteins in the MCO nicking assays.

N-ethylmaleimide modification of rBmTCTP

N-ethylmaleimide (NEM) reacts with cysteine amino acids in any given protein and covalently modifies the cystiene residue. Therefore, NEM is widely used to block the function of cysteine residues in proteins (Lim et al. 1993). In the present study, we used NEM to modify the cystiene residues in rBmTCTP. Briefly, rBmTCTP was incubated with NEM in the presence of 10 mM DTT at 30°C and pH 8.0 (100 mM Tris–HCl buffer) for 2 h. The covalent attachment of NEM to rBmTCTP protein was confirmed spectrophotometrically by the decrease in absorbance at 300 nm of the reaction mixture compared to the NEM alone.

Thioredoxin reduction assay

Recombinant BmTCTP was reduced using a Trx/thioredoxin reductase (TR) system as described previously (Redl et al. 1999). *E. coli* Trx and TR were obtained from Sigma

chemicals, St. Louis, USA, and the reduction of rBmTCTP was assayed spectrophotometrically by monitoring the oxidation of NADPH at 340 nm. Briefly, 50 μg/ml of rBmTCTP was added to the reaction mixture (3 μM of Trx and 3 μM of TR) or reference mixture (either 3 μM of Trx alone or TR alone) at room temperature and incubated for a maximum of 10 min. Reactions were performed in Tris–EDTA (TE) buffer containing 400 μM NADPH, and A_{340} nm readings were recorded every 30 s until the completion of experiment. Differences in the A_{340} nm reading of 0.0062 between the reaction (rBmTCTP and Trx/TR system) and reference control (either Trx or TR alone) samples represented a reduction of 1 μM disulfide.

Biotinylation of rBmTCTP

Biotinylation of rBmTCTP was performed using a biotin labeling kit purchased from Pierce Biotechnology. Briefly, 1 mg/ml of rBmTCTP in phosphate-buffered saline (PBS) was mixed with 1 mg of biotin and incubated on ice for 2 h. Unbound biotin was then removed by passing the mixture through a desalting column. Concentration of the biotinylated protein was then estimated by a BCA method (Pierce Biotechnology), and the biotinylated rBmTCTP was stored at 4°C until further use.

Far Western analysis

The interaction of Trx and rBmTCTP were analyzed by an in vitro binding assay. Briefly, 5 μg of *E. coli* thioredoxin (Sigma) was resolved on 15% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was blocked with 3% BSA in Tris-buffered saline (TBS) for 1 h at room temperature. After washing the membrane four times with TBS containing 0.05% Tween-20 (TBST), biotinylated rBmTCTP (5 μg/ml in TBS) was added and incubated for 30 min. After incubation, the membrane was again washed four times with TBST and incubated with streptavidin-conjugated HRP for 15 min at room temperature. After a final wash, the signals in the membranes were developed using a chemoluminescence substrate (ECL, Amersham Biosciences). The interaction of rBmTCTP with Trx was also confirmed by reverse binding. In this case, 10 μg of rBmTCTP was resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane, which was then incubated with 5 μg of *E. coli* Trx and probed with rabbit anti *E. coli* Trx (1:1,000, Sigma), goat anti-rabbit IgG conjugated to HRP (1:5,000, Pierce Biotechnology), and ECL substrate as described above.

Purification of reduced rBmTCTP from Trx/TR system

rBmTCTP was first incubated with Trx/TR system or Trx alone. After this, the mixture was passed through a Cobalt (Co) metal affinity resin (Clontech) column to purify the reduced rBmTCTP as per the manufacturer's instructions. Briefly, the reaction sample was incubated in the Co metal resin for 30 min, followed by washing the unbound samples with wash buffer containing 5 mM imidazole from the resin. The bound-reduced rBmTCTP was eluted with a native buffer containing 50 mM imidazole. The eluted protein was then dialyzed against PBS to remove imidazole. Purity of the reduced rBmTCTP protein recovered was determined by SDS-PAGE analysis (data not shown).

H$_2$O$_2$ tolerance bioassay

In vivo oxidative stress bioassay was performed as described previously by Li et al. 2004. Briefly, rBmTCTP-transformed bacteria [BL21(DE3)] were grown until the OD reading of the culture reached 0.6. Cultures were then induced with isopropyl-beta-ᴅ-thiogalactopyranoside at a final concentration of 1 mM added to Lauria–Bertani (LB) medium containing ampicillin. Induced bacterial cells were then serially diluted, ranging from 1:10 to 1:100, and a 10− μl droplet of each of the dilutions were plated on LB-agar medium containing 1.2 mM H$_2$O$_2$.

Plates were then incubated at 37°C overnight. Bacterial cells expressing the vector protein plated similarly served as controls. Growth of bacterial cells was compared after incubation.

Results

Sequence analysis of TCTP family of proteins

A multiple sequence analysis of the TCTP family of proteins showed that mammalian TCTPs have one N-terminal cysteine residue at aa position 28 and one cysteine residue at the C-terminal at aa position 171 (Fig. 1). However, analysis of the filarial TCTPs showed that they have three cysteine residues (aa63, aa81, aa92) all at the middle region of the protein.

Reduced rBmTCTP protects DNA from metal catalyzed oxidation

Function of intracellular and extracellularly released TCTP is not fully understood. Two previous studies report that TCTP expression is induced during hypoxic or oxidative stress (Rupec et al. 1998; Yoneda et al. 2004). This notion is further supported by the fact that one of the TCTP genes deposited in the GenBank is designated as a PO_2-related protein (GenBank Accession no. AAM51565), meaning a protein that is upregulated during hypoxic conditions of cells. Lymphatic filarial parasite also expresses TCTP; initially, in this study, we wanted to analyze whether rBmTCTP can actually function as an antioxidant protein by protecting the DNA from oxidative damage. To test this, we performed an in vitro DNA nicking assay. This assay employs a metal catalyzed oxidation system that generates hydroxyl (OH^-) and thiol (RS^*) radicals capable of damaging a DNA template such as ϕX174 DNA (Ghosh et al. 1998). The extent of DNA damage could be then assessed by a shift in the DNA in a gel mobility assay. During oxidative damage, the DNA is converted from supercoiled structure to the nicked form. The nicked form migrates faster than the supercoiled form in the agarose gel. Exposure of ϕX174 DNA to the products of MCO system (which are known to nick the DNA) converted the entire DNA into the nicked form (Fig. 2a). When reduced rBmTCTP was added to the DNA before the addition of MCO system, there was substantial inhibition of the nicking reaction, and this inhibition was dose-dependent (from 1 μg to 20 μg/ml; Fig. 2b). Subsequent time kinetic studies showed that rBmTCTP-dependent inhibition of the MCO-induced DNA nicking was evident even up to 4 h after exposure to the MCO system (Fig. 2c). DNA was completely nicked when a non-specific recombinant protein (rSmGBF) or BSA was added to the reaction mixture instead of rBmTCTP (Fig. 2a). These findings clearly suggest that the inhibition of MCO-induced DNA nicking by rBmTCTP is specific.

rBmTCTP reduced by Trx protects DNA from oxidative damage

In the above-described DNA nick assay, we reduced the rBmTCTP with DTT. However, within the cell, TCTP is probably reduced by a variety of reducing agents, among which, Trx is probably a more efficient and abundant reducing agent. Therefore, next, we wanted to test whether rBmTCTP reduced by Trx can function similarly in the DNA nick assay. However, there is a need to first demonstrate that rBmTCTP could be reduced by Trx. Thus, initial studies were set up to determine whether Trx can reduce rBmTCTP (Fig. 3a). These results demonstrated that rBmTCTP could be reduced by Trx. When tested in the DNA nick assay, the Trx-reduced rBmTCTP was able to protect the DNA from oxidative damage similar to the DTT-reduced rBmTCTP (Fig. 2d). These findings further confirmed that the inhibition of MCO-induced DNA nicking by reduced rBmTCTP is specific.

Disulfide bonds in rBmTCTP are the targets of Trx reduction

From the above studies, we know that rBmTCTP could be reduced by Trx. For the reducing function, Trx has to first bind to rBmTCTP. Therefore, to determine whether Trx binds to rBmTCTP, we used a far Western analysis. These studies showed that Trx can bind to

rBmTCTP, and this binding occurs vice versa (Fig. 2f). The next question we asked was how does Trx bring about this reduction. It is well established that the primary target of Trx-induced reduction is the disulfide bond formed between the cysteine residues. rBmTCTP has three disulfide bonds formed by Cys63, Cys81, and Cys92. To determine the role of disulfide bonds, we performed an enzymatic cleavage assay with NADPH consumption as the read out. These studies showed that there was a time-dependent kinetics in the consumption of NADPH during the reaction, suggesting potential cleavage of the disulfide bonds in rBmTCTP by the Trx system (Fig. 3a).

Role of cysteine residues in the antioxidant property of rBmTCTP

Cysteine amino acids present in the sequences of several antioxidant proteins such as glutathione, Trx, and TR are important for scavenging oxygen-free radicals. Figure 1a shows the presence of cysteine residues in both eukaryotic and prokaryotic TCTPs. To determine whether these cysteine residues are critical for the antioxidant function of rBmTCTP, we covalently modified the cystiene residues with NEM. Subsequent DNA nicking assay showed that the cysteine modification significantly interfered with the antioxidant property of rBmTCTP (Fig. 2e).

Overexpression of rBmTCTP conferred resistance to H_2O_2 damage

To further confirm the antioxidant potential of rBmTCTP, an in vivo assay was performed. *E. coli* cells expressing rBmTCTP or the control pRSET A vector alone were cultured in media containing H_2O_2. The concentration of H_2O_2 (1.2 mM) used in this assay was sufficient enough to inhibit the growth of *E. coli* cells transfected with vector alone (Fig. 3b). However, when rBmTCTP was overexpressed in the *E. coli*, they were able to survive in the H_2O_2 environment (Fig. 3b). In fact, rBmTCTP-overexpressed cells were able to survive even at a density of 1:50 dilution. However, growth of *E. coli* containing the vector alone was completely inhibited at a dilution of 1:10.

Discussion

In this report, we describe a potential antioxidant function for *B. malayi* TCTP. Our previous studies showed that BmTCTP is upregulated in the post-infective stages of *B. malayi* (Gnanasekar et al. 2002). While gaining entry into the mammalian host, the infective larval stages are exposed to a variety of hostile factors including oxidative stress. To circumvent the harmful effects of these factors, parasites have evolved several antioxidative defense mechanisms. For example, filarial parasites are known to synthesize antioxidant enzymes such as SOD (Dabir et al. 2006), glutathione peroxidase (Cookson et al. 1992), and thioredoxin peroxidase (Ghosh et al. 1998) to neutralize the oxygen-free radicals generated from exogenous or endogenous sources. In addition to classical antioxidant enzymes, it is suggested that non-enzymatic antioxidants are also produced by filarial parasites. These include glutathione, ascorbic acid, alpha tocopherol, and exogenous albumin (Selkirk et al. 1998). However, the role of these non-enzymatic antioxidants is not fully understood in filarial parasites.

Recent studies by Tuynder et al. 2004 revealed that TCTPs are structurally homologous to methionine sulfoxide reductase (Msr) peptide fragment pilB, which is a potent antioxidant enzyme. Although TCTP is structurally similar to Msr, the amino acids present in the pilB active site are not conserved in TCTP. Typically, antioxidant enzymes perform their function specifically using their active site to scavenge the free radicals generated. Non-enzymatic antioxidants on the other hand function in a non-classical way. Thus, they are either sacrificial or function by chelating transition metals to neutralize the formation of free radicals. As TCTP lacks typical enzymatic active site, it might not be functioning as an antioxidant enzyme.

Therefore, the antioxidant function that we observed in this study suggests that BmTCTP is possibly a non-classical antioxidant protein.

TCTP is a growth-associated protein ubiquitously present in wide variety of organisms from yeast to mammals (Bonnet et al. 2000; MacDonald et al. 2001). In fact, it is one of the 20 most abundantly expressed proteins in the cell. TCTP was initially identified in an Ehrlich ascites tumor cell line, hence, the name (Bohm et al. 1989). Subsequently, TCTP was demonstrated to be present in almost all normal cells (Sanchez et al. 1997). Despite the ubiquitous nature of TCTP, its exact cellular function is not clear, and the true function of TCTP is still being debated. Some of the important cellular functions attributed to TCTP include calcium binding (Gnanasekar et al. 2002; Rao et al. 2002), tubulin binding (Gachet et al. 1999; Yarm 2002), and anti-apoptotic function (Li et al. 2001).

In addition to calcium and tubulin binding, TCTP is shown to be a key target for artemisinin, a drug that is now widely used to treat malaria (Bhisutthibhan et al. 1998). Further, artemisinins are also proven to be effective against schistosome parasites (Utzinger et al. 2007) and for cancer treatment as well (Efferth 2006). Interestingly, TCTP is abundantly expressed in schistosomes (Rao et al. 2002) and in several cancers (Tuynder et al. 2002). Artemisinins are endoperoxide containing drugs that generate toxic free radicals to kill parasites or cancer cells. Hence, presumably, artemisinin might be using a common mode of action by targeting the antioxidant function of TCTP.

Several potential functions have been suggested for TCTP ranging from histamine release to putative anti-apoptotic function. Nevertheless, a critical function for TCTP family of proteins within the cell remains to be described. Some of the recent findings show that the expression of TCTP is increased several-fold during oxidative stress. These findings, coupled with the present observation in this study, suggest that BmTCTP may have a potential antioxidation function. It is well documented that TCTP expression is dysregulated during various stress conditions (Bonnet et al. 2000); especially, the levels of TCTP are upregulated several-fold within minutes under oxidative stress (Rupec et al. 1998; Oikawa et al. 2002; Wang etal. 2003). Recently, it has been reported that TCTP homologue from *Trichinella pseudospiralis* is a heat-inducible protein important for stress adaptation of parasites in the mammalian host. All these reports suggest that TCTP may be a stress-related protein.

A homologue of TCTP has been deposited in the GenBank as PO_2-related protein. Therefore, in the present study, we used a classical MCO-based assay system to determine the antioxidant function of TCTP. This assay is simple, reliable, and has been widely used to determine the antioxidant potential of the target proteins. Recombinant filarial TCTPs that we used in this study exist in oxidized state due to the presence of three cysteine amino acids in the protein sequence (Gnanasekar et al. 2002). This oxidized rTCTP is unable to protect DNA from oxidative damage in MCO assay. On the other hand, when rTCTP was reduced by DTT, significant antioxidant function was exhibited in vitro. However, reducing with DTT is an artificial system.

An MCO-based assay was subsequently used in this study to assess the potential antioxidant function of rBmTCTP. Fe^{3+} ions in the MCO assay catalyze DTT oxidation to form H_2O_2, which subsequently results in the initiation of a free radical chain of reactions and damage the DNA. The reduced form of rBmTCTP, when introduced into this assay, could protect DNA from oxidative damage in a dose-dependent fashion for a prolonged period of time. However, the mechanism of antioxidant protection offered by BmTCTP is unknown at this time. BLAST search analyses of filarial TCTPs did not yield significant similarity hits with any known antioxidant enzymes. Hence, TCTP might not be functioning as an antioxidant enzyme. Other possibility is that BmTCTP may chelate transition metals to inhibit free radical formation or

serve as sacrificial protein as reported for other non-enzymatic antioxidants that would operate in the cell.

Under in vivo conditions, probably, thioredoxin (Trx) system is more physiologically relevant in reducing TCTP, as Trx is the most abundant reducing agent within the eukaryotic cell. Several antioxidant enzymes require Trx as a source of reducing equivalents to remain active. For example, the human tear lipocalin, a member of the lipocalin protein family, interacts with Trx to get reduced for its functional activity (Redl et al. 1999). Thus, as expected, our initial studies confirmed that Trx could bind and reduce rBmTCTP. The next question we asked was how Trx reduces TCTP. The targets of many reducing agents such as thioredoxin are the cysteine amino acids. Critical analysis of the TCTP protein sequences suggested that there are two cysteine residues in the mammalian TCTPs and three cysteine residues in the parasite TCTPs. Covalent modification of these cysteine residues in rBmTCTP with NEM resulted in the failure of TCTP to protect DNA from oxidative damages. Thus, cysteine residues appear to be critical for the antioxidant function of BmTCTP. In addition, the cysteine residues in TCTP also appear to be critical for some of the other functions of TCTP. For example, TCTP is a known target for the antimalarial drug, artemisinin (Bhisutthibhan et al. 1998). Studies by Bhisutthibhan et al. (1998) suggest that the cysteine residues in *Plasmodium falciparum* TCTP are critical for the binding of this drug to TCTP. These studies thus demonstrated that TCTP has significant antioxidant property in vitro.

So then, how does TCTP function as an antioxidant protein? To demonstrate its ability as an antioxidant protein, we overexpressed rBmTCTP in *E. coli* and exposed them to harsh oxidative conditions using H_2O_2. Presence of overexpressed rBmTCTP in the bacterial cell protected these cells from death due to H_2O_2, suggesting that BmTCTP might function as an antioxidant protein within the parasite. Thus, these in vivo studies also demonstrate that BmTCTP can function as an antioxidant protein in the cell. As BmTCTP is an intracellular and a secretory protein (Gnanasekar et al. 2002), it is conceivable that this protein can protect the parasite from both endogenous and exogenous oxidative damages. The present study thus reveals an important but hitherto unknown function for *B. malayi* TCTP. Because of its putative function, BmTCTP appears to be a critical protein for the survival of the parasite in the host. Further studies will determine whether targeting BmTCTP with chemotherapeutic agents or by developing a vaccine can control the infection.

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Human TCTP: N-----------Cys------------------------------------------------------Cys-C
                        (aa28)                                                   (aa172)
Mouse TCTP: N-----------Cys------------------------------------------------------Cys-C
                        (aa28)                                                   (aa172)
Rabbit TCTP:  N-----------Cys------------------------------------------------------Cys-C
                          (aa28)                                                 (aa172)
B.malayi TCTP:N----------------------------Cys------Cys------Cys--------------------------C
                                           (aa63)  (aa81)  (aa92)
W.banc TCTP:  N----------------------------Cys-----Cys-----Cys-------------------------C
                                           (aa63)  (aa81)  (aa92)
O. volv TCTP:  N----------------------------Cys------Cys----Cys--------------------------C
                                            (aa63)  (aa81)  (aa92)
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Fig. 1.
Multiple sequence alignment of the cysteine amino acids of various TCTPs (human, mouse, rabbit, *B. malayi, W. bancrofti*, and *O. volvulus*) was determined by a Clustal W alignment. The analysis showed that cysteine amino acids of mammalian TCTPs and parasite TCTPs are highly conserved
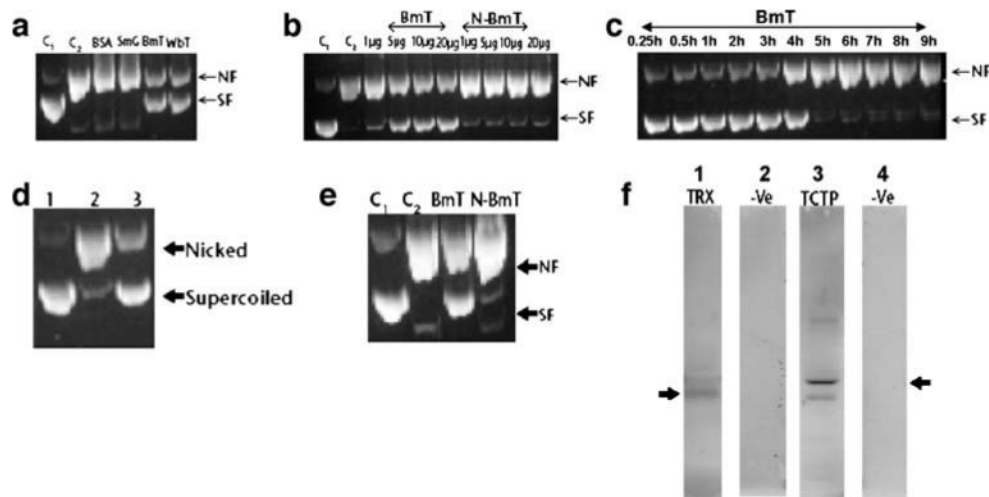
Fig. 2.
DNA nicking assay (**a**–**e**). Approximately 200 ng of variously treated DNA was resolved on a 1% agarose gel followed by staining with Etbr, and bands were visualized using a UV transilluminator. **a** ϕX174 DNA was incubated in the presence of rBmTCTP that was reduced by DTT (reduced BmT) or rWbTCTP that was reduced by DTT (reduced WbT). Results show that the reduced form of BmT and WbT substantially decreased the formation of nicked DNA (*NF*), leaving majority of the DNA in the supercoiled form (*SF*) similar to that of the untreated ϕX174 DNA (C_1). However, incubation of ϕX174 DNA in MFO buffer alone (C_2) or in the presence of BSA or a control recombinant protein, *S. mansoni* G box binding factor (*SmG*), resulted in significant nicking of the DNA. **b** Dose-dependent inhibition of DNA nicking by reduced BmT. Addition of *N*-ethylenemaleimide modified TCTP (*N-BmT*) instead of reduced BmT almost completely reversed its DNA protecting ability. Increasing the concentration of reduced BmT in the reaction mixture substantially decreased the nicked form of DNA. However, addition of N-BmT had no protective effect. **c** Time kinetics studies showed that the DNA protecting effect of reduced rBmTCTP is time-dependent and is present up to 4 h after initiating the incubation. **d** TCTP reduced by Trx system also efficiently protects DNA from oxidative damage compared to unreduced rBmTCTP *Lane 1 Untreated* ϕX174 DNA, *Lane 2* ϕX174 DNA incubated with unreduced rBmTCTP, *Lane 3* ϕX174 DNA incubated with rBmTCTP reduced with Trx. **e** Addition of DTT-reduced rBmTCTP conferred protection from oxidative damage, and this effect was reversed when N-BmT was used instead of reduced BmT. **f** Five micrograms of *E. coli* Trx was resolved on 15% SDS-PAGE, transferred to nitrocellulose membrane, probed with biotinylated rBmTCTP, and developed by ECL. *Arrow* shows that rBmTCTP interacts with Trx (*lane 1*). Similarly, 5 μg of rBmTCTP was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, incubated with Trx, and probed with a horse radish peroxidase labeled-rabbit anti-Trx antibody and color-developed using an ECL kit (*lane 3*). *Arrow* shows the binding of Trx with rBmTCTP. *Lanes 2* and *4* were negative controls using BSA. Data presented are representative of one of three similar experiments
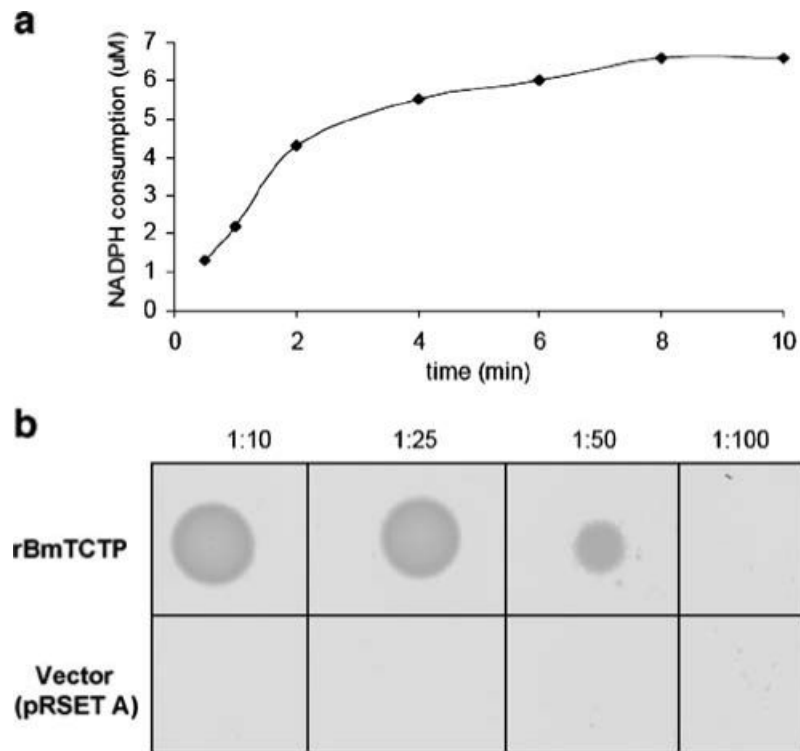
Fig. 3.
a Disulfide reduction of rBmTCTP by Trx system. rBmTCTP was added to a reaction mixture containing 3 μM of TR and 3 μM of Trx in TE buffer containing 400 μM NADPH. Reference controls included reaction mixtures without the addition of Trx or without the addition of TR. Samples were incubated at room temperature, and oxidation of NADPH was monitored spectrophotometrically at 340 nm. Readings were taken every 30 s from 0.5 to 10 min, and the reference readings were subtracted from the test reaction to determine NADPH consumption. Results are representative of three similar experiments. **b** Overexpression of rBmTCTP conferred resistance to H_2O_2 damage in *E. coli* cells. Cells transfected with rBmTCTP or blank vectors were serially diluted from 1:10 to 1:100 and plated on to LB agar plates containing 1.2 mM H_2O_2. After incubation for 12 h at 37°C, growth of bacterial cells was evaluated. Results show that cell transfected with the blank vector failed to grow under these conditions, whereas cells transfected with rBmTCTP showed growth even up to a dilution of 1:50 in the presence of H_2O_2. Data represent results from one of two similar experiments