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A chromosomal SIR2 homologue with both histone NAD-dependent ADP-ribosyltransferase and deacetylase activities is involved in DNA repair in *Trypanosoma brucei*

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SIR2-like proteins have been implicated in a wide range of cellular events including chromosome silencing, chromosome segregation, DNA recombination and the determination of life span. We report here the molecular and functional characterization of a SIR2-related protein from the protozoan parasite *Trypanosoma brucei*, which we termed TbSIR2RP1. This protein is a chromosome-associated NAD-dependent enzyme which, in contrast to other known proteins of this family, catalyses both ADP-ribosylation and deacetylation of histones, particulary H2A and H2B. Under- or overexpression of TbSIR2RP1 decreased or increased, respectively, cellular resistance to DNA damage. Treatment of trypanosomal nuclei with a DNA alkylating agent resulted in a significant increase in the level of histone ADP-ribosylation and a concomitant increase in chromatin sensitivity to micrococcal nuclease. Both of these responses correlated with the level of TbSIR2RP1 expression. We propose that histone modification by TbSIR2RP1 is involved in DNA repair.

Keywords: DNA damage/histone deacetylase/histone ribosyltransferase/SIR2 homologue/*Trypanosoma*

Introduction

The yeast SIR2 is the paradigmatic member of a large family of proteins present in many organisms from bacteria to metazoan eukaryotes (Brachmann *et al*., 1995; Frye, 2000). Members of this family share a core domain of ~250 amino acids that exhibits 25–60% sequence identity between different organisms. SIR2-like proteins have been implicated in a wide range of chromosome-associated phenomena, such as gene silencing, cell cycle progression, chromosome segregation, DNA repair and life span (Brachmann *et al*., 1995; Tsukamoto *et al*., 1997; Freeman-Cook *et al*., 1999; Martin *et al*., 1999; Xie *et al*., 1999; Lin *et al*., 2000; Tissenbaum and Guarente, 2001). The first indication that these proteins might be enzymes came from studies on cobalamine biosynthesis in *Salmonella typhimurium*, which revealed that CobB, the *Salmonella* SIR2 homologue protein, was able to compensate in part for the loss of CobT, a protein that functions in the cobalamine biosynthesis pathway as a nicotinate mononucleotide: 5,6-dimethylbenzimidazole phosphoribosyltransferase (Tsang and Escalante-Semerena, 1998). This complementation suggested that SIR2-like proteins might be ribosyltransferases. Initially, biochemical studies on SIR2 from yeast seemed to support this view since the recombinant protein appeared to ADP-ribosylate bovine serum albumin (BSA) and histones in an *in vitro* assay (Frye, 1999; Tanny *et al*., 1999). However, it became clear from further studies that the nuclear SIR2 was an NAD-dependent deacetylase and that cleavage of the β-glycosidic linkage between the nicotinamide ring and the ribose sugar of NAD was coupled to the deacetylation reaction liberating free nicotinamide and a novel product, *O*-acetyl-ADP-ribose (Imai *et al*., 2000; Landry *et al*., 2000a,b; Smith *et al*., 2000; Tanner *et al*., 2000; Sauve *et al*., 2001; Tanny and Moazed, 2001; Borra *et al*., 2002). The consensus view at present is that SIR2-like proteins constitute a new family of NAD-dependent deacetylases, termed class III, but do not possess genuine ADP-ribosyltransferase activity.

The observation that histones at silenced loci in yeast are hypoacetylated (Braunstein *et al*., 1993, 1996) provided an obvious link between the deacetylase activity of SIR2 and heterochromatin structure. However, the wide phylogenetic distribution of SIR2-like proteins plus their presence in different subcellular compartments within eukaryotes suggests that SIR2-mediated deacetylation might not be restricted to regulation of chromatin structure (Zemzoumi *et al*., 1998; Afshar and Murnane, 1999; Perrod *et al*., 2001). For example, it was demonstrated recently that the human SIR2 orthologue hSIRT1 negatively regulates the tumour suppressor p53 by deacetylation of a known control site (Luo *et al*., 2001; Vaziri *et al*., 2001; Langley *et al*., 2002), and that hSIRT2 deacetylates tubulin *in vivo* (North *et al*., 2003), while the murine mSIR2a represses Pol I transcription by deacetylation of the transcription factor TAF_I68 (Muth *et al*., 2001). Futhermore, in bacteria, CobB inactivates acetyl-CoA synthetase by deacetylation of an active lysine (Starai *et al*., 2002) and ssSIR2 from *Sulfolobus solfataricus* mediates transcriptional repression by deacetylating the major archaeal chromatin protein (Bell *et al*., 2002). In summary, the evidence emerging from the literature is that the SIR2 proteins regulate the structure/function of multiple targets by deacetylation of specific residues.

African trypanosomes, e.g. *Trypanosoma brucei*, are protozoan flagellates that grow in the mammalian vasculature and cause sleeping sickness in man and Nagana in cattle. These parasites are transmitted between hosts by the tsetse fly, where they develop as procyclic forms in the insect midgut (Vickerman, 1985). In the bloodstream, they evade the immune response of the host by continually changing their variant surface glycoprotein

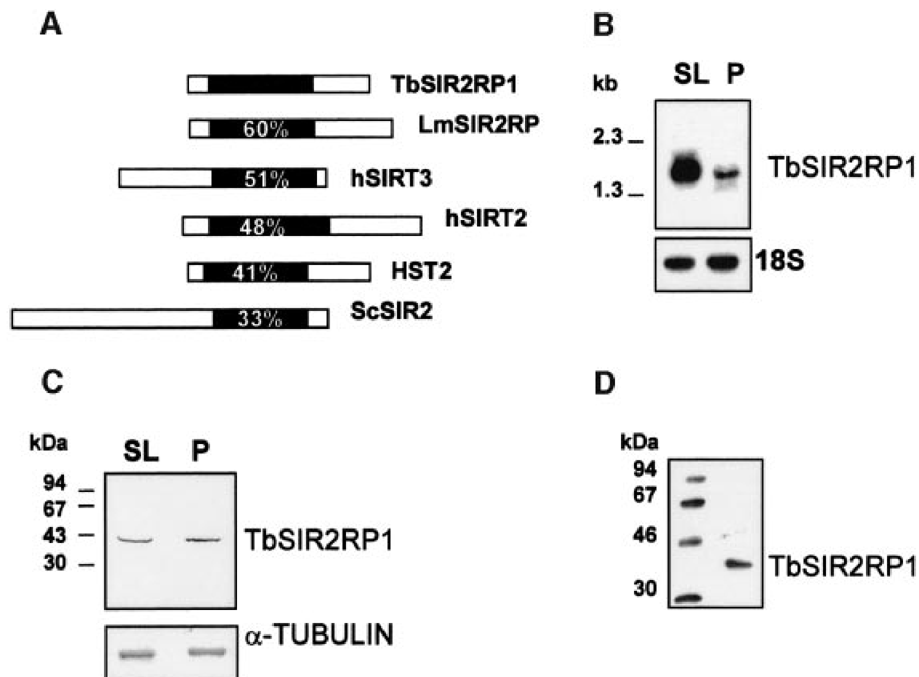

Fig. 1. Characterization of TbSIR2RP1. (**A**) Sequence comparison between TbSIR2RP1 and homologous proteins. The position of the conserved core domain is shown in black, and numbers represent the percentage amino acid identity with this region of TbSIR2RP1. The SIR2-like proteins compared are: LmSIR2RP from *L.major* (Yahiaoui *et al*., 1996), hSIRT2 and hSIRT3 from man (Frye, 1999) and HST2 and SIR2 from *S.cerevisiae* (Brachmann *et al*., 1995). (**B**) Northern blot analysis of *TbSIR2RP1* mRNA. A 10 µg aliquot of poly(A)$^+$ RNA from *T.brucei* AnTat 1.1 bloodstream long slender (SL) and procyclic forms (P) was separated on a formaldehyde agarose gel, transferred to nitrocellulose and probed with ^{32}P-labelled *TbSIR2RP1* cDNA. The filter was subsequently rehybridized with a ribosomal 18S probe, as control for RNA loading. (**C**) Immunoblot analysis of TbSIR2RP1. Total bloodstream slender (SL) and procyclic (P) cell lysates in SDS–PAGE sample buffer were examined by western blot analysis with affinity-purified anti-TbSIR2RP1. A control analysis was performed on a similar filter with an anti-α-tubulin monoclonal antibody. (**D**) Immunoprecipitation of *in vitro* translated TbSIR2RP1 with affinity-purified anti-TbSIR2RP1 antibodies.

(VSG) (reviewed in Cross *et al*., 1998; Pays and Nolan, 1998; Borst and Ulbert, 2001). Individual bloodstream forms express a single VSG at a time, and the expressed VSG gene is located at the end of a telomeric expression site harbouring a polycistronic transcription unit. Variation of the VSG occurs either by gene conversion or by the transcriptional activation of a new expression site. The genome of *T.brucei* contains 11 diploid pairs of megabase chromosomes (1–6 Mbp), which carry the essential genes (Melville *et al*., 1998), and ~100 minichromosomes (50–150 kb) which consist predominantly of a tandemly repeated 177 bp element (Weiden *et al*., 1991). Both megabase chromomes and minichromosomes have canonical telomeres at both ends containing long TTAGGG repeats. As in yeast, the trypanosome chromosomes never condense and the nuclear envelope remains intact during mitosis (Solari, 1980, 1983). In order to characterize factors that influence chromatin structure and the regulation of gene expression in *T.brucei*, we initiated a search for SIR2 homologues in this parasite. Here we show that a homologue of SIR2 from *T.brucei*, termed TbSIR2RP1, is a chromosome-associated protein that catalyses the NAD-dependent ADP ribosylation and deacetylation of histones. TbSIR2RP1 appears to have a role in the mechanism of DNA repair probably by converting target regions of chromatin into a more relaxed or open state. Significantly, there was a marked increase in the level of histone ribosylation and a concomitant increase in chromatin sensitivity to micrococcal nuclease (MNase) in response to the oxidizing DNA damage agent methyl methanosulfonate (MMS). Both of these responses correlated with the level of TbSIR2RP1 expression. We propose that histone modification by TbSIR2RP1 is critical for DNA repair.

Results

Identification of a gene coding for a homologue to SIR2 in T.brucei

We employed degenerate primers based on two highly conserved motifs of the SIR2 sequence (Brachmann *et al*., 1995) to amplify a 250 bp fragment from *T.brucei* genomic DNA. This amplification product subsequently was used to isolate a full-length cDNA that we have named *TbSIR2RP1*, for *T.brucei* SIR2-related protein 1. During the course of these studies, the sequence of *TbSIR2RP1* was deposited in the data bank with the accession No. AF102869 (M.Hoek and G.A.M.Cross, unpublished). In addition, a search of the current *T.brucei* genome database allowed the identification of two other SIR2-related genes that we termed TbSIR2RP2 and TbSIR2RP3 (accession Nos AC119406 and AF102869). These genes shared 45.7 and 43.2% nucleotide sequence identity, respectively, with TbSI2RP1over the entire open reading frames (ORFs).

TbSIR2RP1 encodes a protein of 351 amino acids that, like all members of this family, contains a core of 250 amino acids including the GAG and NID motifs and a four-Cys-zinc finger as well as the HG residues adjacent to the zinc finger that have been shown to be essential for its

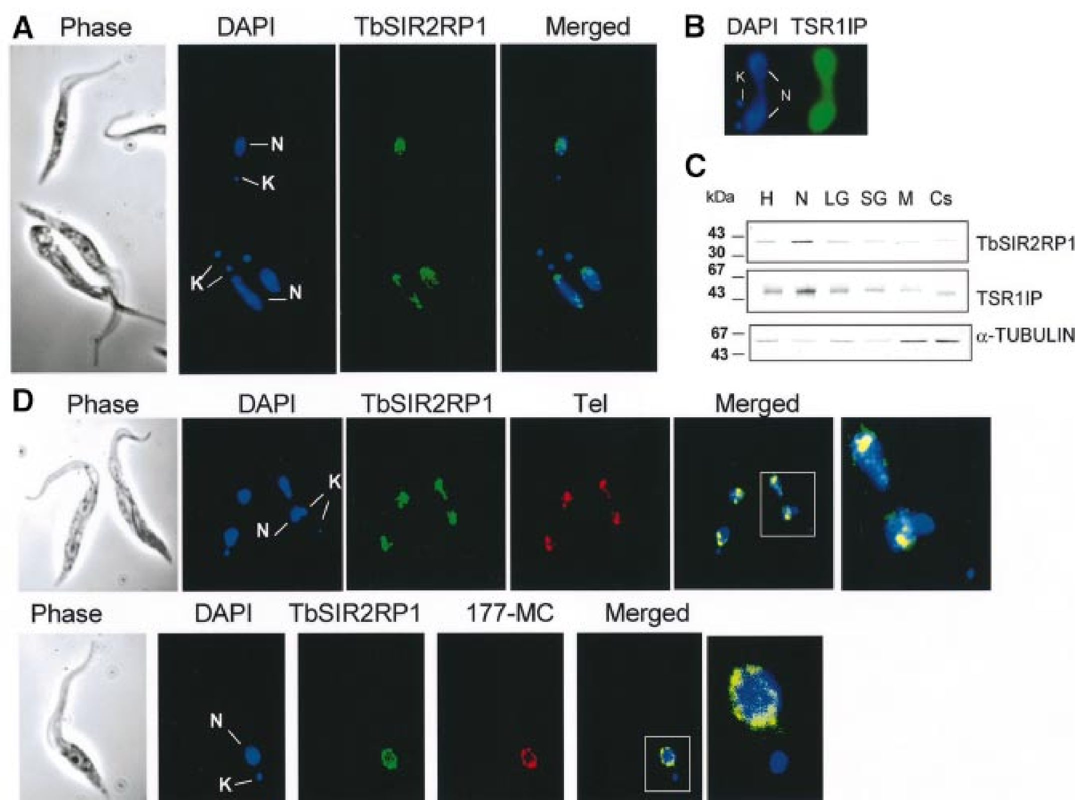

Fig. 2. Cellular localization of TbSIR2RP1. (**A**) Procyclic forms were stained by immunofluorescence with anti-TbSIR2RP1 antibodies and counter-stained with DAPI. The DAPI staining reveals the location of the nucleus (N) and kinetoplast (mitochondrial DNA, K). (**B**) Immunolocalization of TSR1IP nuclear protein. (**C**) Distribution of TbSIR2RP1 in subcellular fractions from procyclic forms of *T.brucei*. Subcellular fractions were separated by SDS–PAGE, transferred to nitrocellulose and probed with anti-TbSIR2RP1, anti-α-tubulin and anti-TSR1IP antibodies. Subcellular fraction abbreviations are as follows: H, homogenate; N, nuclear; LG, large granular (mitochondrial); SG, small granular (glycosomal); M, endosomal; Cs, cytoplasmic (soluble). (**D**) TbSIR2RP1 co-localizes with telomeres and minichromosomes. Procyclic forms were fixed and subjected to IF and FISH using anti-TbSIR2RP1 antibodies and probes for telomeric repeats (Tel) and minichromosome (177-MC). Overlap of the two signals is yellow/orange.

enzymatic activities (Frye, 1999; Tanny *et al*., 1999; Imai *et al*., 2000). The primary sequence of TbSIR2RP1 predicted a mol. wt of ~40 kDa and a pI of 5.7. Comparison of the sequence of TbSIR2RP1 with SIR2-like proteins from other organisms revealed that sequence homology was restricted to the 250 amino acid core domain (Figure 1A). Within this conserved domain, TbSIR2RP1 had the highest sequence homology (between 41 and 60%) with LmSIR2RP (Yahiaoui *et al*., 1996), human SIRT3 and SIRT2 (Frye, 1999), and the yeast HST2 (Brachmann *et al*., 1995) (Figure 1A). Together, this group of proteins represents a branch of the SIR2 family tree that has been designated class Ib (Frye, 2000) or HST2-like proteins (Perrod *et al*., 2001).

Expression of TbSIR2RP1

A Southern blot analysis demonstrated that *TbSIR2RP1* is present as a single copy gene (data not shown). A northern blot analysis of poly(A)$^+$ RNA prepared from different stages of the parasite life cycle revealed that the *TbSIR2RP1* mRNA was more abundant in bloodstream long slender than in procyclic forms (Figure 1B). However, a western blot analysis using affinity-purified antibodies raised against recombinant TbSIR2RP1 demonstrated that, in contrast to the northern blot data, TbSIR2RP1 was present at the same relative abundance in bloodstream slender and procyclic forms (Figure 1C). *In vitro* translation followed by immunoprecipitation confirmed the predicted size of TbSIR2RP1 (40 kDa) and demonstrated the specificity of the anti-TbSIR2RP1 antibodies (Figure 1D).

TbSIR2RP1 is a nuclear protein that co-localizes with telomeric sequences and minichromosomes

In order to localize TbSIR2RP1, immunofluorescence (IF) microscopy was performed on fixed procyclic cells with anti-TbSIR2RP1 antibodies. TbSIR2RP1 was found in the nucleus where it exhibited a punctate distribution (Figure 2A). In contrast, the transcription factor TbSR1IP used as an internal control showed the expected more diffuse distribution over the entire nucleus (Figure 2B) (Ismaili *et al*., 2000). Since this was the first example of a member of the HST2 subfamily located in the nucleus, an alternative approach was employed to confirm this location. Western blot analysis of subcellular factions prepared from procyclic forms confirmed that TbSIR2RP1 was recovered mainly in the nuclear fraction (Figure 2C). The distribution of TbSIR2RP1 in these fractions was very similar to that observed for the nuclear TbSR1IP but clearly different from that of α-tubulin. Interestingly, the distribution of TbSIR2RP1 in the nucleus varied depending on the stage of the cell cycle. The cell cycle is easy to

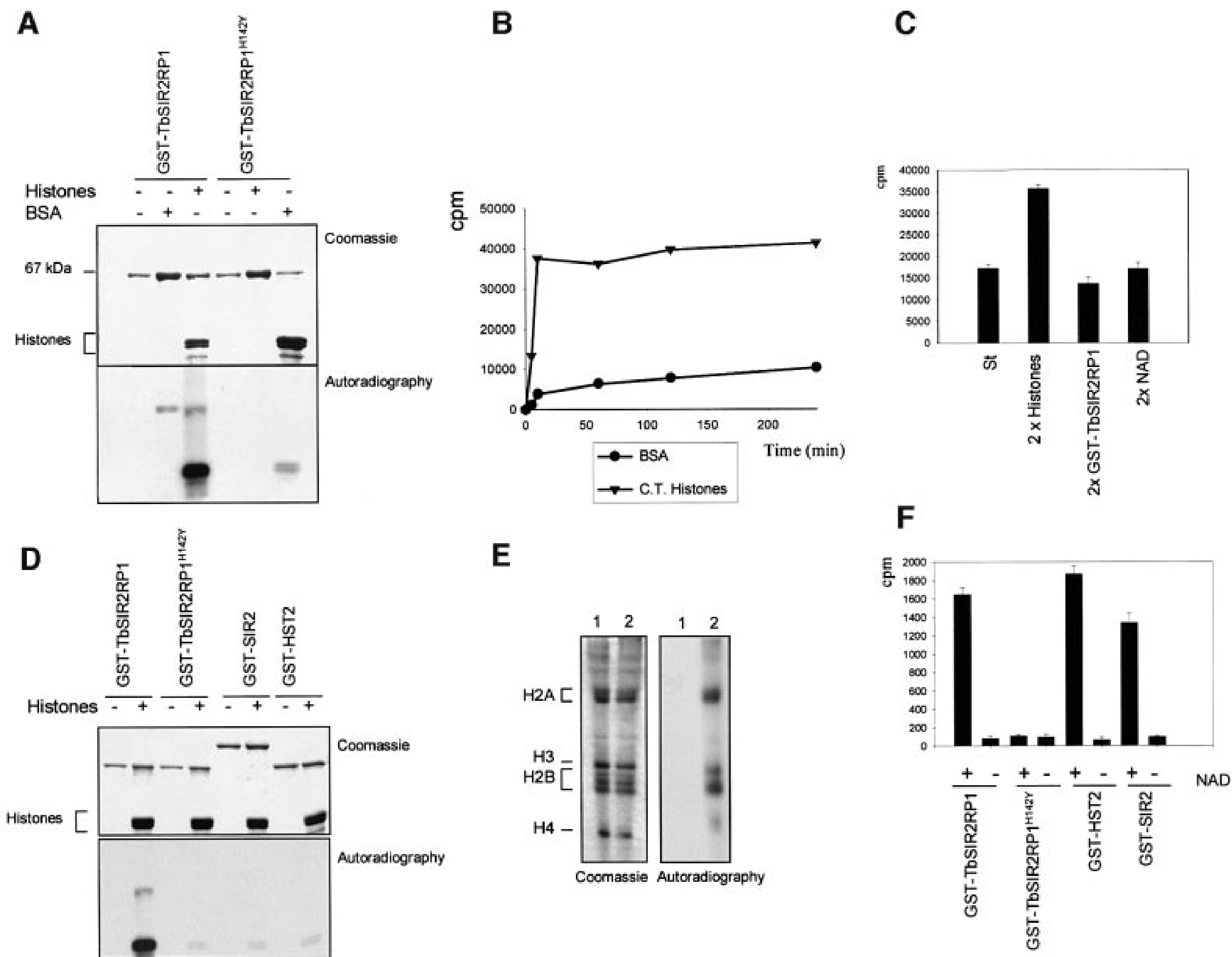

Fig. 3. NAD-dependent ribosylation and deacetylation of proteins by TbSIR2RP1. (**A**) *In vitro* ribosylation reactions were performed with 1 μg of GST–TbSIR2RP1 or GST–TbSIR2RP1^{H142Y}, plus 5 μg of BSA and 5 μg of histones using [^{32}P]NAD as donor. The top panel illustrates a Coomassie-stained gel of reaction products resolved by 10% SDS–PAGE, whereas the bottom panel shows the autoradiograph of the gel. (**B**) Time curves of histone and BSA ribosylation by GST–TbSIR2RP1 at 37°C. Each point represents the ribosylation products of 5 μg of substrate by 0.5 μg of GST–TbSIR2RP1 using 5 μCi of [^{32}P]NAD at the indicated time. Reaction products were precipitated with 20% TCA (w/v), collected and washed on a GF/C glass fibre filter (Whatman), and then counted after addition of liquid scintillation fluid. (**C**) Ribosylation reactions were performed with 5 μg of histones, 0.5 μg of GST–TbSIR2RP1 and 5 μCi of [^{32}P]NAD (standard reaction, column St) or a double concentration of histone (10 μg, column 2× histones), GST–TbSIR2RP1 (1 μg, column 2× GST–TbSIR2RP1) and [^{32}P]NAD (10 μCi, column 2× NAD). (**D**) Comparison of TbSIR2RP1 ribosylation activity with that of other members of the SIR2-like family. Ribosylation reactions with 1 μg of GST–TbSIR2RP1, GST–TbSIR2RP1^{H142Y}, GST–SIR2 or GST–HST2 were performed with and without 5 μg of histones using [^{32}P]NAD as donor. The top panel illustrates a Coomassie-stained gel of the reaction products, and the bottom panel shows the autoradiograph of the gel. (**E**) Triton–acid–urea (TAU) gel of *T.brucei* histones treated with GST–TbSIR2RP1 or GST–TbHST1^{H142Y} and [^{32}P]NAD, respectively stained with Coomassie blue and autoradiographed. The histone types are indicated on the left. (**F**) Analysis of the NAD-dependent histone deacetylase activity. Histones were acetylated with [^{3}H]acetyl-CoA by HAT1 and then treated with GST–TbSIR2RP1, GST– TbSIR2RP1^{H142Y}, GST–HST2 and GST–SIR2. The amount of acetate released was measured in the presence or absence of NAD.

follow in trypanosomes by monitoring both nuclear elongation and the segregation of the mitochondrial (kinetoplast) DNA (Sherwin and Gull, 1989). In the G_1 phase, trypanosomes have a single kinetoplast and nucleus. After kinetoplast segregation, G_2 phase cells contain two kinetoplasts and one nucleus, while after mitosis but before cell division two kinetoplasts and two nuclei are present. In G_1 phase cells, the TbSIR2RP1 immunofluorescent signal was always in tight clusters spread throughout the entire nucleus or near the periphery (Figure 2A, top). However, in dividing cells (two kinetoplasts and one or two nuclei), the signal was located at the poles of the dividing nucleus (Figure 2A, bottom, and D, top). This behaviour was very similar to that reported for telomeres (Chung *et al*., 1990; Ersfeld and Gull, 1997; Perez-Morga *et al*., 2001) and raised the possibility that TbSIR2RP1 is associated with telomeres. In order to test this possibility, IF was combined with fluorescence *in situ* hybridization (FISH) using fixed procyclic cells and a fluorescent probe for telomeric repeats (Figure 2D). A punctate staining throughout the nucleus was observed with both TbSIR2RP1 antibodies (green) and the probe for the telomeric repeats (red). The merged image of the two probes showed significant overlap (orange/yellow), suggesting that TbSIR2RP1 is associated with telomeres. Overlapping signals were also obtained with a specific probe for minichromosomes (a 177 bp repeat, 177-MC in Figure 2D). The latter are

known to be concentrated in a few foci and to be >10-fold more numerous than large chromosomes (Weiden *et al.*, 1991). Therefore, both telomeric and minichromosome probes essentially recognize spots where chromosomes are concentrated. The minimal conclusion from these data is that TbSIR2RP1 is associated with chromosomes, at least minichromosomes.

TbSIR2RP1 is an NAD-dependent ADP-ribosyltransferase

Two reports have suggested that members of the SIR2 family can mediate the transfer of ribose 5′-phosphate from nicotinic acid mononucleotide to amino acid residues of BSA, histones or SIR2 proteins themselves (Frye, 1999; Tanny *et al.*, 1999). To assess whether TbSIR2RP1 also possessed ADP-ribosylase activity, a GST fusion protein containing the entire ORF fused C-terminal to GST was expressed in *Escherichia coli* and purified to homogeneity by glutathione affinity chromatography. The purified protein was assayed for ADP-ribosyltransferase using [^{32}P]NAD as the donor and BSA or calf thymus histones as acceptor substrates. As a negative control, an essential histidine (H142) required for enzyme activity within the core domain of the protein (Frye, 1999; Tanny *et al.*, 1999; Imai *et al.*, 2000) was mutated by site-directed mutagenesis and converted into tyrosine to generate TbSIR2RP1^{H142Y}. Analysis of the products of these assays by SDS–PAGE/autoradiography revealed the presence of a strongly labelled band corresponding to histones, together with a much weaker band that migrated with the same apparent molecular mass as BSA (67 kDa), but was also present with histones (Figure 3A). Although GST–TbSIR2RP1 migrated with a similar molecular mass to BSA, no signal was detected when the fusion protein was incubated in the absence of substrate. As detailed in the Discussion, at least in the case of histones, this weakly labelled band may reflect transient auto-ADP-ribosylation of TbSIR2RP1 during the course of its activity. Significantly, when the inactive TbSIR2RP1^{H142Y} was employed in the assay, only a very minor incorporation of the ^{32}P label into histones was observed (Figure 3A). This result clearly demonstrated that active TbSIR2RP1 was required to stably and covalently modify the target protein and the modification was not due to some non-specific non-enzymatic reaction. Taken together, these data indicated that TbSIR2RP1 is a genuine ADP-ribosyltransferase with at least two specific substrates, histones and BSA, the affinity for histones being higher than for BSA.

In order to compare the rate of ribosylation of histones or BSA by TbSIR2RP1, the assay was stopped at various times and the level of the ^{32}P incorporation was measured after precipitation of the proteins (Figure 3B). The time curves confirmed that TbSIR2RP1 has a far higher activity using histones than BSA (Figure 3B). When histones were the substrate, the reaction progressed much more rapidly and reached a plateau after 10 min. To investigate this plateau further, ribosylation reactions were performed using a double concentration of substrate, enzyme and NAD, respectively. Only a doubling of histone concentration yielded a doubling of ^{32}P incorporation into the substrate (Figure 3C). We conclude that histones are the limiting factor in the ribosylation reaction by TbSIR2RP1.

Comparison of TbSIR2RP1 ribosylation activity with that of other members of the SIR2-like family

It has been proposed that the weak ribosyltransferase activity of other SIR2 proteins is not genuine, but reflects an intermediate step during NAD hydrolysis (Tanner *et al.*, 2000). This suggestion prompted us to compare the ribosyltranferase activity of TbSIR2RP1 with that of other SIR2-like proteins. Two other members of the family, the yeast SIR2 and HST2, were expressed as C-terminal GST fusion proteins exactly as for TbSIR2RP1, and subjected to the identical ADP-ribosylase assay. Consistent with the previous results, a strong ribosylation activity was observed with TbSIR2RP1 (Figure 3D), but not with its H142Y mutant. In contrast, very little ADP-ribosyltransferase activity was observed in the two other cases (Figure 3D), as expected (Landry *et al.*, 2000b).

Histones H2A and H2B are major targets for in vitro TbSIR2RP1 ribosylation

We subsequently analysed whether all of the histones are equally effective as acceptors of ADP-ribosyl groups. Trypanosome histones were incubated with GST–TbSIR2RP1 in the presence of [^{32}P]NAD and the reaction products were separated on a Triton–acid–urea (TAU) gel. These gels allow the separation of the various classes of histones as well as their modified species. Figure 3E demonstrates that two types of histone were ribosylated by TbSIR2RP1 and these appeared to correspond to histones H2A and H2B, respectively (Burri *et al.*, 1994). Protein digestion and mass spectrometry (MALDI-TOF) of the ribosylated bands confirmed the assignment of histones H2A and H2B as the major ribosylation products, whereas histone H4 was ribosylated to a lesser extent. These results indicate that GST–TbSIR2RP1 is an ADP-ribosyltransferase with high affinity for histones H2A and H2B.

TbSIR2RP1 is an NAD-dependent histone deacetylase

It is well established that SIR2-like proteins constitute a new class of protein deacetylases. To analyse whether TbSIR2RP1 also possesses NAD-dependent deacetylase activity, trypanosome histones were first acetylated with [^{3}H]acetyl-CoA using the enzyme HAT1, a yeast histone H4 acetyl transferase (Kleff *et al.*, 1995). Radiolabelled histones were then incubated with GST–TbSIR2RP1, GST–TbSIR2RP1^{H142Y}, GST–SIR2 and GST–HST2 with and without NAD, and the release of [^{3}H]acetyl groups was monitored. As shown in Figure 3F, similar levels of deacetylation were observed with GST–TbSIR2RP1, GST–SIR2 and GST–HST2, and in all cases this activity was absolutely dependent on the presence of NAD. As expected, the H142Y mutant was inactive.

TbSIR2RP1 confers resistance to DNA damage

In yeast, SIR proteins have been reported to play a role in DNA repair, although the mechanism remains unclear (Tsukamoto *et al.*, 1997; Lee *et al.*, 1999; Martin *et al.*, 1999; Mills *et al.*, 1999). The human homologue, hSIRT1, is involved in cell survival by inactivating the tumour suppressor p53, the primary mediator of the cellular DNA damage response (Luo *et al.*, 2001; Vaziri *et al.*, 2001; Langley *et al.*, 2002). In mammalian cells, histones are

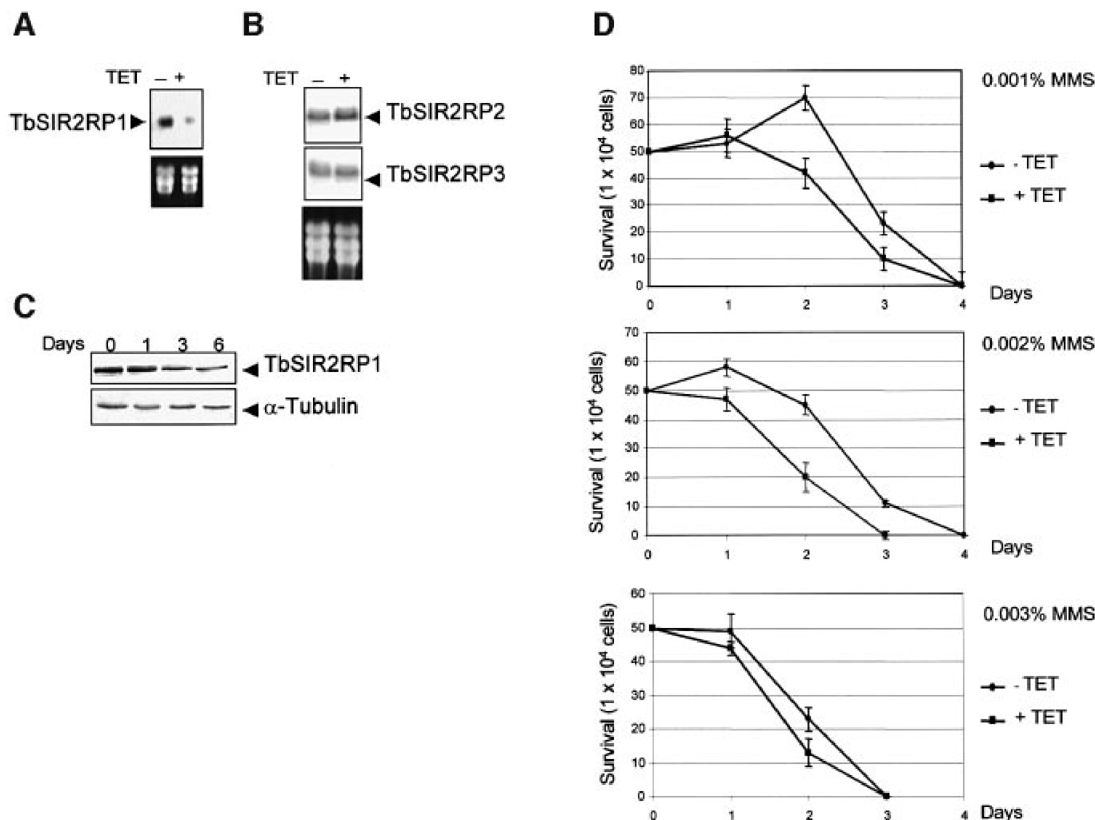

Fig. 4. Effect of TbSIR2RP1 RNAi on cell sensitivity to DNA damage. (**A**) Northern blot analysis of mRNA levels from cells incubated for 24 h without (–) or with (+) tetracycline. rRNAs stained with ethidium bromide are shown as loading controls. (**B**) A similar northern blot was hybridized with probes corresponding to the entire ORF of two other trypanosomal homologues of SIR2 (TbSIR2RP2, accession No. AC119406; TbSIR2RP3, accession No. AF102869). (**C**) Western blot analysis of whole-cell lysate (1×10^6 cell equivalents/lane) from uninduced (day 0) and tetracycline-induced cells (days 1–6) using anti-TbSIR2RP1 and anti-α-tubulin antibodies. (**D**) Cell survival curves are shown for uninduced (TET–) and tetracycline-induced cells (TET+) treated with different concentrations of MMS.

mono- and polyribosylated by a poly(ADP)ribose polymerase in response to DNA damage and oxidative stress. These findings prompted us to consider whether the trypanosomal SIR2 homologue is involved in the response to DNA damage. This question was addressed by specific ablation of the TbSIR2RP1 mRNA using RNA interference (RNAi) (Ngo *et al*., 1998; Wang *et al*., 2000). Therefore, we generated a trypanosome cell line that expressed a double-stranded RNA corresponding to the first 600 nucleotides of *TbSIR2RP1* under the control of the tetracycline-inducible promoter. The effect of *TbSIR2RP1* RNAi was assessed by northern and western blot analysis. *TbSIR2RP1* transcripts were reduced to ~75% after 24 h in the presence of tetracycline (Figure 4A). This RNAi specifically affected *TbSIR2RP1* mRNA since no change was observed in the expression of the other two SIR2-related genes found in the *T.brucei* genome database, which share 47.7 and 46.9% nucleotide sequence identity, respectively, over the region covered in the RNAi construct (Figure 4B). The level of TbSIR2RP1 decreased to approximately one-third of the control level after 3 days of induction (Figure 4C). This decrease did not lead to any detectable effect on trypanosome growth (not shown), but it clearly affected the cell resistance to DNA damage. As shown by the growth curves in Figure 4D, the tetracycline-induced loss of TbSIR2RP1 led to an increased sensitivity to the DNA alkylating agent MMS. This effect was more obvious at lower than at higher concentrations of the drug.

Since a decrease of TbSIR2RP1 levels correlated with an increase of sensitivity to MMS, we investigated whether overexpression of TbSIR2RP1 could increase resistance to DNA damage. TbSIR2RP1 and TbSIR2RP1^{H142Y} were overexpressed in procyclic forms of *T.brucei* by targeting both genes into the tubulin locus (Sommer *et al*., 1992). As shown in Figure 5A, an increase of ~2- and 3-fold was observed for TbSIR2RP1 and TbSIR2RP1^{H142Y}, respectively. These increased levels of expression did not affect the localization of TbSIR2RP1 (Figure 5B) or cell growth (not shown). However, overexpression of TbSIR2RP1, but not TbSIR2RP1^{H142Y}, conferred resistance to MMS since cells overexpressing TbSIR2RP1 clearly resisted better than the controls in the presence of MMS (Figure 5C). In 0.001% MMS, the cells overexpressing TbSIR2RP1 grew at a rate similar to the parental cell line in the absence of MMS. Altogether, these data support the view that the level of expression of TbSIR2RP1 correlates with cellular tolerance to agents that damage DNA.

TbSIR2RP1 enzymatic activities play a role in DNA damage response

Since TbSIR2RP1 possesses an ADP-ribosyltransferase activity, we investigated whether ribosylation of histones was involved in the response to DNA damage caused by MMS. First, we assessed whether DNA damage resulted in ribosylation of endogenous histones *in vitro*. Nuclei

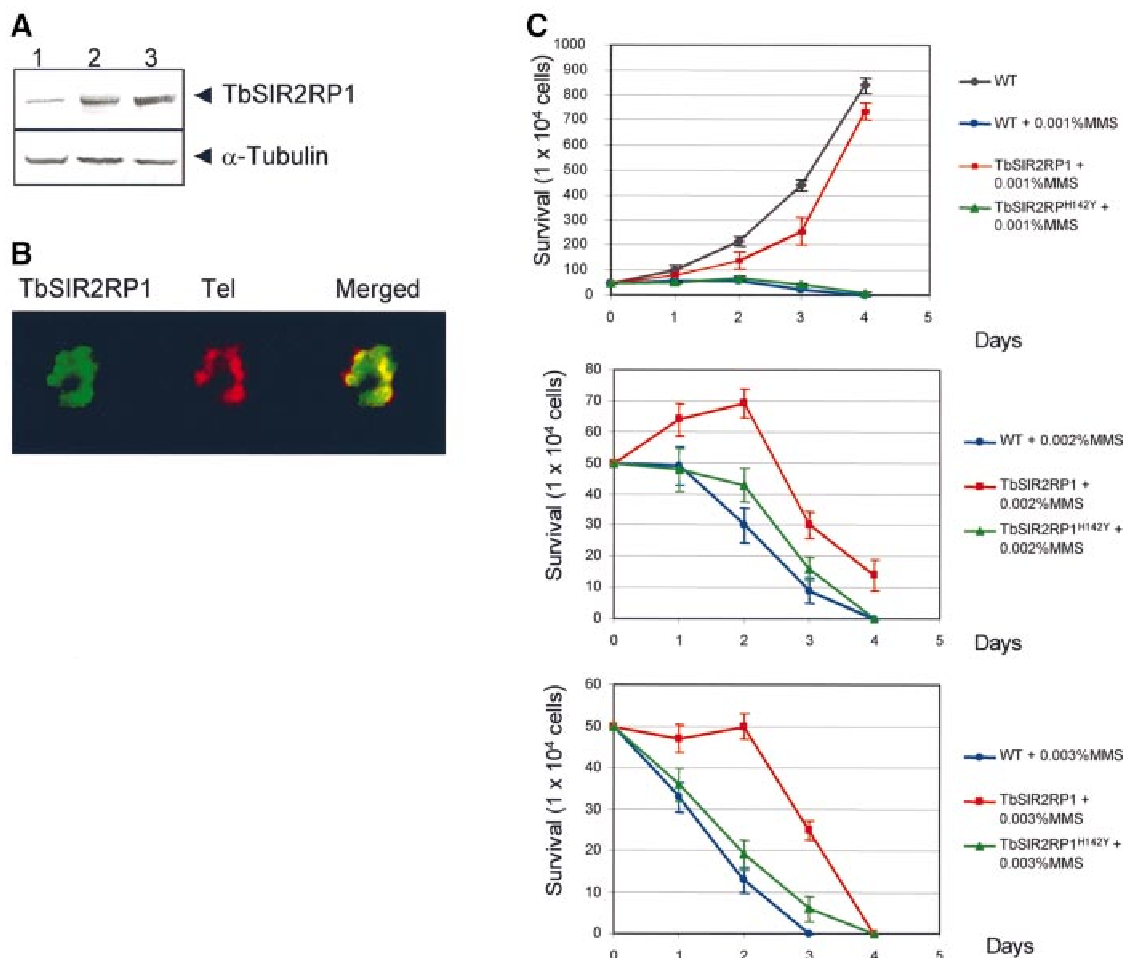

Fig. 5. Effect of ectopic expression of TbSIR2RP1 and TbSIR2RP1^{H142Y} on cell sensitivity to MMS. (**A**) Western blot analysis of control cells (lane 1) or cells overexpressing TbSIR2RP1 (lane 2) and TbSIR2RP1^{H142Y} (lane 3) using anti-TbSIR2RP1 and anti-α-tubulin antibodies. (**B**) Subcellular localization of TbSIR2RP1 in cells overexpressing the protein. Procyclic forms overexpressing TbSIR2RP1 were subjected to IF and FISH using anti-TbSIR2RP1 antibodies and probes for telomeric repeats (Tel). Overlap of the two signals is yellow/orange. (**C**) Cell survival in the presence of different concentrations of MMS, comparing wild-type and cells overexpressing TbSIR2RP1 and TbSIR2RP1^{H142Y}.

isolated from trypanosomes were incubated for 1 h at 37°C with MMS and radioactive NAD in the presence of 3-aminobenzamide, a specific inhibitor of poly(ADP)ribose polymerase (Farzaneh *et al*., 1985). TAU gel analysis of histones purified from these nuclei revealed a significant increase in histone ribosylation in response to MMS (Figure 6A). Moreover, the extent of histone ribosylation was higher in nuclei isolated from cells overexpressing TbSIR2RP1 compared with wild-type and mutant overexpressor cells (Figure 6A). In contrast, the bulk of histone ribosylation in TbSIR2RP1 knocked-down cells was lower compared with control cells (Figure 6B). Interestingly, the profile of histone ribosylation under these conditions indicated that histones H2A and H2B were the major products, with histone H4 being modified to a lesser extent. This pattern of modification was similar to that obtained when recombinant TbSIR2RP1 was incubated with a mixture of histones *in vitro*. Together, these data support the view that histones are ribosylated by TbSIR2RP1 in response to oxidative stress. Finally, we analysed whether like in yeast, TbSIR2RP was mislocalized in response to DNA damage. As illustrated in Figure 6C, most of the signal remained in clusters co-localizing with telomeres.

Histone modification by TbSIR2RP1 enhances sensitivity to DNases

In order to understand how TbSIR2RP1-mediated ribosylation might protect cells from DNA damage, we investigated the effects of this modification on the chromatin structure using MNase. Chromatin was isolated from nuclei treated as described previously but with cold NAD, and then digested with increasing concentrations of MNase. Although the same nucleosome spacing was observed in intact and oxidized (MMS-treated) chromatin, the sensitivity to MNase was significantly greater in the latter case, i.e. damaged DNA was easier to digest (Figure 7A). These results indicated that the majority of damaged chromatin is organized in arrays of regularly spaced nucleosomes but in a more open or relaxed state than in intact chromatin. Similar results were obtained with chromatin extracted from procyclic cells overexpressing TbSIR2RP1 or TbSIR2RP1^{H142Y} but, in the presence of MMS, cells overexpressing TbSIR2RP1 were more

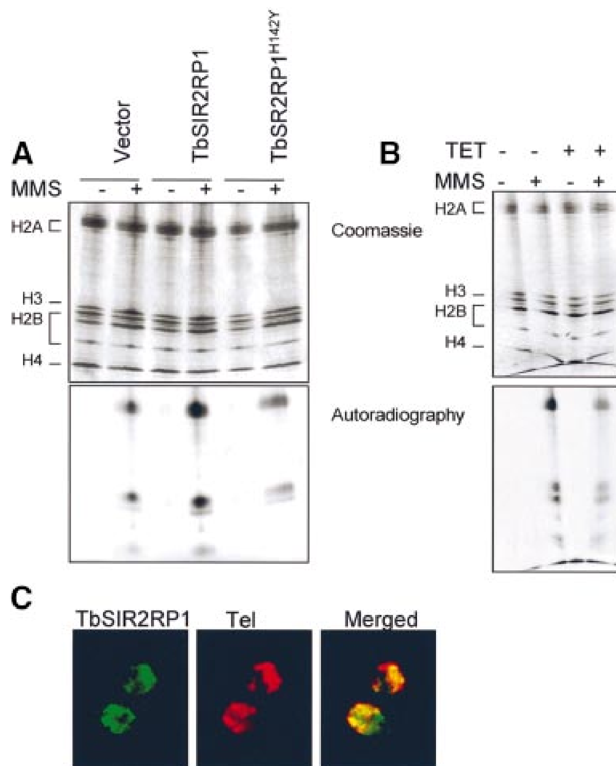

Fig. 6. Extent of histone ribosylation in response to DNA damage correlates with the cellular concentration of TbSIR2RT1. The results of TAU gel analysis of histone ribosylation in nuclei from trypanosomes overexpressing TbSIR2RP1 and TbSIR2RP1^{H142Y} (**A**) and from TbSIR2RP1 knocked-down cells (**B**). Histones were purified from nuclei treated for 1 h with MMS and [^{32}P]NAD in the presence of 5 mM 3-aminobenzamide. The bottom panel shows the autoradiograph of the gel. (**C**) The localization of TbSIR2RP1 in cells with damaged DNA. Procyclic forms incubated in 0.001% MMS for 12 h were subjected to IF and FISH with anti-TbSIR2RP1 antibodies and telomeric probes, respectively.

sensitive to MNase compared with wild-type and TbSIR2RP1^{H142Y} (Figure 7B and C). In contrast, no significant differences in MNase pattern were observed in cells where TbSIR2RP1 was depleted by RNAi induction (Figure 7D).

Thus, chromatin appears to be more open in response to DNA damage, and when cells overexpress TbSIR2RP1.

Discussion

Two NAD-dependent enzymatic activities in TbSIR2RP1

TbSIR2RP1 appears to be an unusual member of the SIR2 family since this homologue possesses NAD-dependent histone ADP-ribosyltransferase as well as deacetylase activities. Repeated *in vitro* assays using [α-^{32}P]NAD demonstrated that TbSIR2RP1 catalyses the stable covalent incorporation of ^{32}P label, consistent with ADP-ribosyl transfer, into histones and to a lesser extent BSA. However, this activity was not detected with GST-tagged yeast SIR2 or HST2 homologues under the same assay conditions. This latter result was in agreement with those of other workers who employed histidine-tagged versions of the proteins and concluded that these proteins are not ADP-ribosyltransferases (Landry *et al*., 2000b; Tanner *et al*., 2000). ADP-ribosylation in our assay was not due to non-specific chemical modification since it was not observed when a mutant form of the protein (TbSIR2RP1^{H142Y}) was employed. The activity was also detected using low amounts of TbSIR2RP1 (~100 ng). Moreover, the reaction was relatively specific since histones H2A and H2B were the major ribosylation products and the labelling of histones was ~10-fold higher than that of BSA. The final level of histone ADP-ribosylation was reached rapidly and appeared to depend solely on the amount of histones present in the assay, which suggested that only a limited number of specific sites in the substrate are available for modification by TbSIR2RP1. These assays also demonstrated an apparent auto-ADP-ribosylation of TbSIR2RP1 during the course of the assay. This observation is consistent with the generation of an ADP-ribosyl-TbSIR2RP1 intermediate during the course of the reaction. These intermediates are predicted to form part of the catalytic mechanism of ADP-ribosyltransferases due to nucleophilic attack on the C-1 of ribose. The nucleophilic group from the ADP-ribosyltransferase carries the ADP-ribosyl group that can then be transferred by a second displacement onto a suitable nucleophilic acceptor group (Koch-Nolte *et al*., 1996). The observation that the TbSIR2RP1 ADP-ribosyl intermediate was relatively stable, i.e. resistant to boiling in SDS–PAGE sample buffer, suggests that direct transfer to water to give free ADP-ribose does not occur but that transfer of the ADP-ribose group requires a specific nucleophilic acceptor group in the histone substrate. Presumably the build up of this intermediate during the reaction occurred because of the limiting amount of specific nucleophilic acceptor group in the histone substrate.

We also demonstrated that TbSIR2RP1 possesses the expected deacetylase activity associated with the SIR2 family and readily catalysed the release of ^{3}H-labelled acetyl groups from histones. In common with other members of the family, the activity required NAD and was not observed using the TbSIR2RP1^{H142Y} mutant. This finding agrees with the current view that the deacetylation step is coupled to the cleavage of the glycosidic bond and suggets that histone deacetylation and ADP-ribosylation activities are also coupled in the case of TbSIR2RP1. At present, it is unclear whether the same residues or even the same histones are undergoing coupled deacetylation/ADP-ribosylation reactions. For example, it was notable that histones H2A and H2B were the major ribosylation products, whereas histone H4 was the substrate for deacetylation since the enzyme used to acetylate histones, HAT1, mainly acetylates histone H4 (Kleff *et al*., 1995). However, other workers have noted that the deacetylase activity of SIR2 proteins may be relatively relaxed in these assays (Landry *et al*., 2000a).

The identity between TbSIR2RP1 and eukaryotic homologues within the core catalytic domain is between 30 and 50% with no significant homology outside this region. This degree of homology is typical of the conservation observed within the catalytic core of other enzymes, which raises the question of why is the ADP-ribosylation activity apparently unique to TbSIR2RP1. The simple answer might be that the ADP-ribosyltransferase activity is contained within the non-homologous

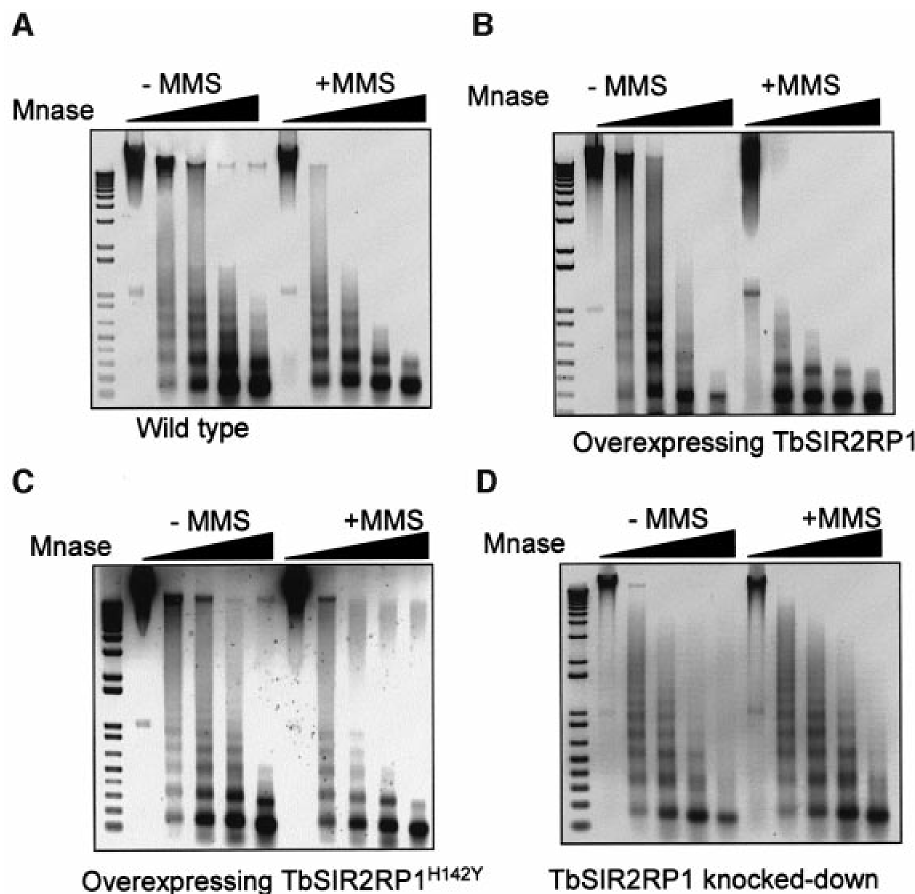

Fig. 7. Alkylated chromatin is more sensitive to MNase. Untreated and MMS-treated chromatin from wild-type procyclic cells (**A**) and from cell overexpressing TbSIR2RP1 (**B**) or TbSIR2RP1^{H142Y} (**C**), or from TbSIR2RP1 knocked-down cells (**D**) were incubated with MNase. All digestion reactions contained 4 μg of DNA. Chromatin was digested with 0.00025, 0.0005, 0.0015 and 0.005 U of MNase for 10 min at 30°C. A negative print of the ethidium-stained DNA is shown. A 1 kb PLUS DNA ladder (Invitrogen) is on the left of each panel.

region rather than the conserved core domain. It is difficult to assess this possibility given the lack of a well established consensus motif for ADP-ribosyltransferases (Takada *et al*., 1993), but it is clear that a mutation within the conserved core region (TbSIR2RP1^{H142Y}) also abolished ADP-ribosyl transfer activity.

A role for TbSIR2RP1 in DNA repair

There are indications in the literature that SIR2-like proteins are involved in cellular responses to DNA damage and oxidative stress, but clear evidence as to their precise role has remained elusive (Tsukamoto *et al*., 1997; Lee *et al*., 1999; Martin *et al*., 1999; Mills *et al*., 1999; Luo *et al*., 2001; Langley *et al*., 2002). Our data suggest a correlation between the activity of TbSIR2RP1 and extent of chromatin ADP-ribosylation and the sensitivity of trypanosomes to agents that damage DNA, i.e. the ability to mount an effective repair response. For example, a decrease in the level of TbSIR2RP1 resulted in an increased sensitivity to the DNA alkylating agent MMS, while overexpression of TbSIR2RP1 resulted in an increased resistance to the agent. Moreover, both effects correlated with the extent of ADP-ribosylation of histones. In contrast, overexpression of an inactive mutant form of the protein did not increase resistance to MMS or the ribosylation status of histones. Incubation with MMS resulted in changes in the organization of trypanosomal chromatin, which appeared to adopt a more relaxed or less condensed state as it became more sensitive to MNase. Although the repair of damaged DNA is obviously a complex process, a key requirement appears to be a relaxation or reduction in the condensation state of chromatin in the region of the damage. Indeed, the efficiency of nucleotide excision repair (NER) mechanisms of UV-induced DNA lesions is reduced in chromatin substrates (Smerdon and Conconi, 1999; Green and Almouzni, 2002), nucleosome core particles (Hara *et al*., 2000; Liu and Smerdon, 2000; Kosmoski *et al*., 2001) and within SV40 minichromosomes compared with naked DNA (Wang *et al*., 1991; Sugasawa *et al*., 1993). In view of these considerations, we propose that TbSIR2RP1 is a component of the chromatin remodelling machinery that disassembles nucleosomes, by affecting the acetylation and ribosylation status of specific residues, to create enough space for subsequent binding of other NER factors.

There is evidence for a functional link between deacetylation and ADP-ribosylation in other cells. Repair of DNA damage is associated with increased mono-ADP-ribosylation of core histones as well other nuclear proteins (Bredehorst *et al*., 1981; Wielckens *et al*., 1982; Kreimeyer *et al*., 1984; Boulikas, 1988, 1989). Interestingly, histone H2B served as the major acceptor of

ADP-ribosyl groups in mammalian nuclei *in vivo* (Burzio *et al*., 1979; Ogata *et al*., 1980; Adamietz and Rudolph, 1984). There is also evidence for a link between deacetylation and ribosylation since deacetylated and ribosylated histones appear to be associated with silent regions of the mammalian genome (Tikoo and Ali, 1997). In *Physarum polycephalum*, inhibition of histone deacetylases by *n*-butyrate was accompanied by an increase in ADP-ribose incorporation into highly acetylated histone H4 subspecies (Golderer and Grobner, 1991). In addition, *n*-butyrate also increased the utilization of NAD and ADP-ribosylation of proteins in HeLa, V79, mouse B16, mouse Fib/T and human T1 kidney cells in culture (Bohm *et al*., 1997). All of these findings support the view that connection of ADP-ribosylation and deacetylation of proteins is a feature of all cells. It is tempting to speculate that one member of the SIR2 family, among the seven identified so far in human, may possess the same activities as TbSIR2RP1.

Finally, the histone code hypothesis predicts that modification marks on the histone tails provide binding sites for effector proteins (Jenuwein and Allis, 2001). Hence, histone mono- and polyribosylation may act as markers for damaged chromatin.

Materials and methods

Trypanosomes

Procyclic *T.brucei* from strains EATRO 1125 and 29-13 were grown in SDM-79 medium (Brun and Schonenberger, 1979) supplemented with 15% fetal bovine serum. Strain 29-13, which harbours integrated genes for T7 polymerase and tetracycline repressor (Wirtz *et al*., 1999), was used for RNAi. Transfection of procyclic cells was as described previously (García-Salcedo *et al*., 2002). The transfectants were selected with 2.5 μg/ml phleomycin for RNAi analysis (Wang *et al*., 2000) or 50 μg/ml hygromycin for overexpression studies.

Cloning a SIR2 homologue from T.brucei

A partial *TbSIR2RP1* sequence was isolated by PCR amplification of *T.brucei* genomic DNA using degenerate oligonucleotide primers based on two highly conserved regions within the catalytic core domain of the SIR2-like proteins family (Brachmann *et al*., 1995). The PCR amplification was performed with 100 ng of genomic DNA as template and 30 pmol of primers 5′-GGNATYCCNGAYTTYMG-3′ and 5′-CKR-AARTCNGGRATNCC-3′ with an initial 2 min of denaturation at 94°C followed by 35 cycles of 1 min at 90°C, 1 min at 5°C, 1 min at 72°C and a final elongation step of 10 min at 72°C. The amplification products were subcloned into pCR2.1 vector (Invitrogen). A 250 bp fragment amplified using this approach was used to screen a λgt10 cDNA library from *T.brucei* procyclic forms, allowing the isolation of a full-length cDNA clone.

Plasmid constructions and expression of recombinant proteins

Plasmids pGEX-TbSIR2RP1 and pMAL-TbSIR2RP1 were generated by cloning the entire TbSIR2RP1 coding sequence in-frame with the C-terminus of GST into the *Bam*HI–*Xho*I site of pGEX5. *Saccharomyces cerivisieae SIR2* and *HST2* genes were PCR amplified from yeast genomic DNA as template and cloned into the *Eco*RI–*Xho*I sites of pGEX5 to yield pGEX-HST2 and pGEX-SIR2, respectively. Site-directed mutations were generated in the plasmid pGEX-TbSIR2RP1 using the Gene Editor system (Promega) according to the manufacturer's instructions and verified by sequencing. TbSIR2RP1 and the mutated gene were cloned in pTSA-HYGRO for overexpression in trypanosomes (Sommer *et al*., 1992). The HAT1 *E.coli* expression plasmid, pSTT21, was a gift from Dr R.Sternglanz (Kleff *et al*., 1995).

Expression of the recombinant proteins was induced in mid-logarithmic phase DH5α cells, by incubation with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 5 h at 37°C. The bacteria were lysed by sonication and the fusion proteins purified on glutathione–agarose (Pharmacia) as described by the manufacturer.

Immunofluorescence and in situ hybridization

Trypanosoma brucei cells were harvested by centrifugation. The pellets were resuspended in phosphate-buffered saline (PBS; 1.7 mM NaH_2PO_4, 9.1 mM Na_2HPO_4, 150 mM NaCl pH 7.5) and settled onto poly-L-lysine-coated slides. Trypanosomes were fixed in 100% methanol for 4 min at –20°C, rehydrated for 20 min in PBS and processed for IF using a 1:500 dilution of anti-TbSIR2RP1 antibodies. Rabbit anti-TbSIR2RP1 was raised against recombinant maltose-binding peptide fused to TbSIR2RP1 expressed in *E.coli*. FISH was performed according to instructions provided by the manufacturer (Dako, Glodstrup Demmark) using a PNA (peptide nucleic acid) fluorescein isothiocyanate (FITC)-labelled probe against the human telomeric repeats, which are identical to those in *T.brucei*. When IF and FISH were combined, samples were first processed as described for IF and then for FISH. Images were taken on a Zeiss Axioskop 2 microscope coupled to a CCD camera and processed by ISIS 3.

Enzyme activity assays

Protein ADP-ribosylation assays were performed in a volume of 20 μl containing 0.1–1 μg of each GST fusion protein, 5 μCi of [^{32}P]NAD and 5 μg of calf thymus (Sigma) or *T.brucei* histones and BSA as indicated. The reaction buffer contained 150 mM NaCl, 10 mM dithiothreitol (DTT), 50 mM Tris–HCl pH 8.8 (Tanny *et al*., 1999). Samples were incubated for the indicated period of time at 37°C and reactions were stopped by the additon of Laemmli gel loading buffer. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, dried on Whatman paper and exposed to film for 4–12 h at –80°C.

For deacetylation assays, calf thymus and *T.brucei* histones were first acetylated *in vitro* with [^{3}H]acetyl-CoA and the yeast histone acetyltransferase HAT 1 (Kleff *et al*., 1995) as described elsewhere (Landry *et al*., 2000b). Reactions were incubated at 37°C for 30 min and then heated at 55°C for 30 min to inactivate the histone acetyltransferase. Labelled histones were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 20% (w/v) and incubating on ice for 1 h. Precipitates were collected by centrifugation, washed with 10% TCA (w/v) and dissolved in 100 mM Tris pH 8.0. Approximately 10 μg of labelled histones (~30 000 c.p.m.) were assayed for deacetylase activity in 50 μl reactions including 0.5 mM NAD, 0.5 mM DTT, 50 mM sodium phosphate pH 7.2 and 0.5 μg of enzyme to be tested. Reactions were stoped by addition of 18 μl of 0.1 M HCl/0.4 M acetic acid. Released acetyl groups were extracted by adding 400 μl of ethyl acetate. Afer 5 min centrifugation in a microfuge, 300 μl was counted in 4 ml of scintillation fluid (Insta-gel II, Packard).

Nuclear extraction and histone and chromatin purification

Trypanosome nuclei were prepared as described previously (Murphy *et al*., 1987) and then lysed in 100 mM NaCl, 0.1% Triton X-100, 1% CHAPS, 50 mM Tris pH 7.8 at 4°C for 1 h. Histones were extracted with 0.4 M H_2SO_4 for 2 h at 4°C. After centrifugation at 10 000 *g* for 10 min at 4°C, histones were recovered from the supernatant by precipitation in 20% (w/v) TCA. Precipitated histones were washed successively with acidic acetone (1% HCl in acetone) and acetone, desiccated, and dialysed against 10 mM Tris pH 8. A cocktail of aminoethylbenzenesulfonyl fluoride (AEBSF), E64 and pepstatin protease inhibitors was used during the purification process.

For ribosylation reactions, nuclei from 1×10^8 cells were incubated with 0.3% MMS and 5 μCi of [^{32}P]NAD in 20 μl of ribosylation buffer for 1 h at 37°C. Nuclei were then treated with 10 U of DNase I for 1 h at 37°C. Proteins were precipitated with 20% TCA (w/v), washed with 10% TCA (w/v), 10% ethanol (v/v) and resuspended in TAU loading buffer. Afer electrophoresis, TAU gels were stained with Coomassie brilliant blue, dried on Whatman paper and exposed to Hyperfilm film (Amersham Pharmacia) for 1–3 weeks at –80°C.

Microccal muclease digestion of chromatin

A 4 μg aliquot of chromatin was digested in 20 μl of 250 mM sucrose, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 50 mM Tris pH 7.5 with 0.00025, 0.0005, 0.0015 and 0.005 U of MNase at 30°C for 10 min. The reaction was stopped by addition of 5 mM EDTA. DNA was extracted, precipitated, washed, dried and resolved on a 1% agarose–TBE gel. The gel was stained with ethidium bromide and digitally scanned.

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