Roles for ligases in the RNA editing complex of *Trypanosoma brucei*: band IV is needed for U-deletion and RNA repair

Catherine E. Huang, Jorge Cruz-Reyes, Alevtina G. Zhelonkina, Sean O'Hearn, Elizabeth Wirtz¹ and Barbara Sollner-Webb²

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205 and ¹Laboratory of Molecular Parasitology, The Rockefeller University, New York, NY 10021, USA

²Corresponding author
e-mail: bsw@jhmi.edu

Trypanosome RNA editing utilizes a seven polypeptide complex that includes two RNA ligases, band IV and band V. We now find that band IV protein contributes to the structural stability of the editing complex, so its lethal genetic knock-out could reflect structural or catalytic requirements. To assess the catalytic role in editing, we generated cell lines which inducibly replaced band IV protein with an enzymatically inactive but structurally conserved version. This induction halts cell growth, showing that catalytic activity is essential. These induced cells have impaired *in vivo* editing, specifically of RNAs requiring uridylylate (U) deletion; unligated RNAs cleaved at U-deletion sites accumulated. Additionally, mitochondrial extracts of cells with reduced band IV activity were deficient in catalyzing U-deletion, specifically at its ligation step, but were not deficient in U-insertion. Thus band IV ligase is needed to seal RNAs in U-deletion. U-insertion does not appear to require band IV, so it might use the other ligase of the editing complex. Furthermore, band IV ligase was also found to serve an RNA repair function, both *in vitro* and *in vivo*.

Keywords: RNA editing/RNA ligase/trypanosome/U-deletion/U-insertion

Introduction

RNA editing in trypanosomes is an unusual form of post-transcriptional RNA processing in which uridylylate (U) residues are inserted into and deleted from pre-mRNAs to create start codons, stop codons, and much of the coding regions in many mitochondrial transcripts (reviewed in Arts and Benne, 1996; Alfonzo *et al.*, 1997; Stuart *et al.*, 1997; Gott and Emeson, 2000). The information for editing is provided by short (~70 nt), trans-acting guide RNAs (gRNAs) that are complementary to blocks of edited sequence (Blum *et al.*, 1990). The first gRNA overlaps the 3' end of the editing domain, so could anchor to the unedited mRNA forming a partial duplex. The editing site can be identified as the mismatch abutting the anchor duplex: a mismatched purine in the gRNA specifies U insertion, while a mismatched U in the pre-mRNA specifies U-deletion.

RNA editing occurs through a series of protein catalyzed reactions (Figure 1; Cruz-Reyes and Sollner-Webb, 1996; Kable *et al.*, 1996; Seiwert *et al.*, 1996), much as initially proposed (Blum *et al.*, 1990), and individual editing cycles can be reproduced *in vitro* (Seiwert and Stuart, 1994; Kable *et al.*, 1996). Each editing cycle begins with endonuclease cleavage of the pre-mRNA at the first mismatch adjacent to the anchor duplex. Then U residues are removed by 3'-U-exonuclease or added by terminal-U-transferase (TUTase) at the 3' end of the upstream cleavage fragment. Finally, RNA ligase seals the pre-mRNA. This allows the gRNA:pre-mRNA duplex to zip up to the next mismatch where the next cycle of editing occurs.

In our mitochondrial extracts, the activities described above for the editing cycles (Figure 1) were found to copurify, revealing a complex of only seven detectable polypeptides, potentially in equimolar ratios (Rusché *et al.*, 1997). This complex catalyzes all the component reactions plus full round U-deletion and full round U-insertion cycles, and it appears to be a single major kind (Rusché *et al.*, 1997; Cruz-Reyes *et al.*, 1998b). Two of these seven proteins are RNA ligases, band IV and band V (Rusché *et al.*, 1997). Their purification as part of the editing complex can be followed readily by radioactive labeling, since ligation begins with adenylation, covalently binding an AMP from ATP while releasing pyrophosphate (PPi).

The simplest model for enzymatic catalysis of U-insertion and U-deletion envisaged these two kinds of editing cycles occurring by a common pathway in which the same endonuclease activity performs the cleavages, a common enzyme adds Us or acts in the reverse direction to remove Us, and a single RNA ligase reseals the mRNA (Hajduk, 1997; Stuart *et al.*, 1997). However, our finding that the endonuclease cleavage steps and the U addition/removal steps of U-insertion and U-deletion exhibit very different biochemical properties has instead suggested that these parallel reactions use distinct catalytic activities (Cruz-Reyes and Sollner-Webb, 1996; Rusché *et al.*, 1997; Cruz-Reyes *et al.*, 1998a,b; Sollner-Webb *et al.*, 2001). Nonetheless, distinct activities could reside in the same protein, since it is not known which proteins catalyze these reactions. For the ligation step, the presence of two distinct RNA ligases in the editing complex (Rusché *et al.*, 1997) raises the possibility that one could function in U-insertion and the other in U-deletion, but there are several other possibilities. For instance, one ligase could function in proper RNA editing while the other serves to repair incorrect cleavages (see Discussion; Sturm and Simpson, 1990; Koslowsky *et al.*, 1991); or one ligase could serve both U-insertion and U-deletion in the procyclic (insect host) stage trypanosomes while the other ligase serves both in the bloodstream (mammalian host) stage; or the

ligases could have partially redundant catalytic functions. Notably, the *in vivo* stability of the band IV ligase protein evidently depends on it being associated with other components of the editing complex (Rusché *et al.*, 2001), so the assembly or stability of other editing proteins could similarly depend on the presence of band IV protein. Thus, this protein could appear to be essential in a particular life-stage because its physical presence rather than its catalytic activity is critical for the function of the editing complex.

The genes for both the band IV and band V RNA ligases have recently been cloned (McManus *et al.*, 2001; Panigrahi *et al.*, 2001; Rusché *et al.*, 2001; Schnauffer *et al.*, 2001; S.O'Hearn, C.E.Huang and B.Sollner-Webb, in preparation). They are quite similar to each other, but much more distantly related to other known ligases. These ligases have also been called TbMP52 and TbMP48 (Panigrahi *et al.*, 2001; Schnauffer *et al.*, 2001) and p52 and p48 (McManus *et al.*, 2001); they are named for the size of the primary translation product before it is shortened upon mitochondrial import. These proteins were previously called 57 and 52 kDa (Sabatini and Hajduk, 1995; Rusché *et al.*, 1997) or 54 and 47 kDa (Corell *et al.*, 1996). Genetic knock-out studies of the band IV ligase have shown that this protein is essential in the procyclic (Rusché *et al.*, 2001) and bloodstream (Schnauffer *et al.*, 2001) form of *Trypanosoma brucei*. However, these studies did not address whether band IV is essential for its catalytic or structural role, and if catalytic, whether this ligase is needed for U-deletion, U-insertion, both forms of editing or neither form of editing. The current work was undertaken to determine the precise role of the band IV RNA ligase in editing cycles of procyclic cells.

Results

Band IV protein favors stability of the editing complex

Procyclic trypanosomes in which one of the two band IV genes is knocked out grow at approximately normal rates (Rusché *et al.*, 2001). However, closer examination revealed that these growing cells could appear like 'couch potatoes', settling to the bottom of the culture flask, while parallel transfection controls and normal trypanosomes swam more homogeneously throughout the media (data not shown). Since phenotypic effects are only rarely reported in trypanosomes bearing single allele knock-outs of other genes, we wanted to confirm that this propensity to sink indeed correlates with reduced band IV protein levels. Assessing protein levels by adenylation of mitochondrial extracts, multiple lines of single allele band IV knock-out cells and control cells showed that band IV ligase protein was reproducibly ~2-fold reduced in the single allele knock-outs relative to total protein, while the band V ligase protein remained at nearly control levels (Figure 2A). Thus, a single gene copy appears insufficient to produce normal levels of band IV protein.

Because the simple editing complex normally contains approximately stoichiometric amounts of the seven component polypeptides including the band IV and band V ligase proteins (Rusché *et al.*, 1997), we examined whether reduction of the band IV protein could affect the stability of the residual complex. When mitochondrial

Fig. 1. Mechanism of trypanosome RNA editing. This RNA editing involves the enzymatic steps indicated as described in Introduction.

extracts of control cells were resolved by glycerol gradient sedimentation, the vast majority of the editing complexes, as well as the component band IV and band V RNA ligases, co-sedimented at ~20S (Figure 2B; Sabatini and Hajduk, 1995; Cruz-Reyes and Sollner-Webb, 1996; Rusché *et al.*, 1997). Notably, when repeated using extracts of the single allele knock-out cells, which contain half the usual amount of band IV and approximately normal amounts of band V protein, there was a peak at the usual ~20S position which contained almost all the band IV protein and about half the total band V protein at a fairly normal ratio. However, the remaining half of the band V protein sedimented much more slowly at ~7S (Figure 2B). The finding of these considerably smaller associations, which contain band V without corresponding band IV protein, indicates that in the absence of band IV protein the remaining components of the editing complex are less stably associated and at least *in vitro* can form partial or broken complexes. It follows that band IV protein does indeed serve a structural role in maintaining the editing complex. Since integrity of the editing complex is assuredly important for the concerted action of its component editing activities, the finding that band IV protein is essential *in vivo* at a particular life-stage (Rusché *et al.*, 2001; Schnauffer *et al.*, 2001) does not demonstrate its catalytic importance.

Band IV's catalytic activity is critical *in vivo*

To determine if band IV's catalytic activity is indeed essential, we wanted to generate trypanosomes bearing a structurally conserved but catalytically inactive version. Construction of the inactive band IV protein utilized a

Fig. 2. Analysis of single allele band IV knock-out cell lines. (A) Adenylation assay using small-scale mitochondrial extracts from three different single allele band IV knock-out cell lines (single K.O.) and two control cell lines: WT 427 (lane 2) and 427 transfected with pLew13 vector (lane 4). (B) Adenylation assay following glycerol gradient fractionation of mitochondrial extracts from wild-type cells and band IV single allele knock-out cells. Fractions 5 and 6 of the 15 fractions (numbered from the bottom) represent ~20S (determined by 19S thyroglobulin marker). Slightly more protein, and therefore slightly more band V adenylation activity, was loaded onto the gradient of the single allele knock-out cells.

KXXG motif, conserved in all known RNA and DNA ligases and known to be necessary for their activation by adenylation (Heaphy *et al.*, 1987; Tomkinson *et al.*, 1991; Shuman and Ru, 1995; Shuman and Schwer, 1995). Band IV has two such KXXG motifs, so we introduced separately the charge-conservative lysine to arginine mutations K86R and K109R. When expressed in *Escherichia coli*, the K86R band IV protein exhibits no adenylation or ligation activity, while the K109R protein retained activity (Figure 3A; data not shown). This indicates that lysine 86 is the active site, adenylylatable residue of this ligase.

We then generated trypanosomes which inducibly replace the majority of their endogenous band IV protein with this K86R catalytically inactive version. This was feasible because the amount of band IV protein in the trypanosome is regulated so as not to exceed its normal level, evidently because any excess that is not incorporated in the editing complex is short-lived (Rusché *et al.*, 2001). Specifically, when a band IV gene with an active tetracycline (tet) regulatable T7 promoter is ectopically introduced into the rDNA spacer, its protein product replaces ~2/3 of the endogenous band IV protein (Rusché *et al.*, 2001). We repeated the protocol using an identical construct except that the band IV gene bears the K86R mutation (Figure 3B). This gene should be transcribed and translated as before, and since the encoded protein bears only one charge-conservative amino acid replacement, it should have the wild-type structure and similarly replace much of the endogenously encoded band IV protein (Rusché *et al.*, 2001). Therefore, this catalytically inactive

protein should fulfill any structural requirement for editing complex integrity, allowing the catalytic role of band IV to be assessed.

When the integrated K86R band IV gene was induced with tet for 48 h and equal amounts of total protein were compared, these 'induced K86R cells' contained only about a third as much adenylylatable band IV as the parental 29-13 trypanosomes, but normal amounts of band V (Figure 3C; lanes 1 and 2). This indicates that the K86R mutant protein is so similar to wild type that the trypanosomes' regulatory mechanism does not distinguish the two forms; the ~2/3 decrease in adenylylatable protein indicates that ~2/3 of the band IV protein incorporated into the editing complexes is the catalytically inactive K86R version, and that excess band IV protein is degraded. Thus, the editing complex should contain a full complement of protein with band IV's structure, but only ~1/3 with band IV ligation activity.

To verify that the K86R protein is indeed incorporated into the ~20S complex and fulfills band IV's stabilizing role, extracts from induced mutant cells were fractionated on glycerol gradients. If the K86R protein was excluded from editing complexes or did not serve to stabilize them, the gradient-isolated ~20S complexes should exhibit the wild-type ratio of band IV to band V adenylation. Yet the ~20S fraction contained only ~1/3 the normal amount of adenylylatable band IV protein but normal levels of adenylylatable band V (Figure 3C; lanes 3 and 4), the same as in the whole extract (Figure 3C; lanes 1 and 2). These data demonstrate that ~20S complexes containing the catalytically inactive band IV protein assemble and are stable, underscoring the value of the K86R mutant approach.

Induction of the K86R band IV protein has profound effects on trypanosome growth and morphology (Figure 3D and E). These effects become apparent at ~24 h and are maximal 48–72 h post-induction (Figure 3D), paralleling but lagging ~12 h behind the time course for replacing the majority of the endogenously encoded band IV protein (Rusché *et al.*, 2001). Normal cell growth ceases (Figure 3D), debris from dead cells becomes abundant, and most of the remaining live cells exhibit various abnormal morphologies (Figure 3E; right three panels; compare to left panel). These include cells which are unnaturally round and unusually large or small, with 4'-6-diamidine-2-phenylindole (DAPI) staining patterns indicative of abnormal nuclear or nucleolar structures. Thus, the enzymatic activity of the band IV RNA ligase, and not only its structural presence, is crucial for normal trypanosome growth.

Induction of the K86R band IV protein disrupts *in vivo* editing

To assess RNA editing upon K86R band IV induction, we analyzed several *in vivo* mRNAs by primer extension poisoned with ddGTP (which terminates extension at their infrequent C residues). Using steady state RNA from four K86R band IV cell lines and parental 29-13 cells, both non-induced and following 48 h of tet treatment, and transcript-specific primers just 3' of their editing domains, we scored their edited and unedited mRNAs as well as accumulated cleaved species. The never-edited cytochrome oxidase subunit I (COI) mRNA provided a control

Fig. 3. Effects of K86R mutant band IV protein expression. **(A)** Adenylation assay of extracts from *E. coli* expressing wild-type and mutant band IV proteins (arrow); some degradation products are also seen. An adenylylatable *E. coli* protein (asterisk) serves as an internal control. **(B)** piLigIV-K86R drives expression in trypanosomes of the K86R mutant band IV protein and a linked phleomycin-resistance gene (Phleo^R, the *ble* gene from *Streptothalotrichus hindustanus*) from a T7 promoter with a tet operator and uses trypanosome 5' and 3' processing signals. It is transfected into 29-13 procyclic trypanosomes, which express T7 RNA polymerase and the tet repressor. **(C)** Adenylation assay of mitochondrial extract made from non-induced (lanes 1 and 3) and induced (lanes 2 and 4) K86R cells. The assays in lanes 1 and 2 used unfractionated extract and those in lanes 3 and 4 used the ~20S peak from glycerol gradient fractionated extract. **(D)** Growth of two lines of K86R cells and control cells (the 29-13 parental line) with none or 1 μg/ml tet added at 0 h. Media was added to maintain cell densities supporting log phase growth. This semi-log plot is based on total cell numbers (cells/ml × volume), scaled to equalize minor differences in starting cell numbers. Error bars denote standard deviation for K86R-1 cells and are similar for the other cell lines. After much longer periods of induction (selection), the cultures can be overtaken by normal looking cells, possibly due to loss or inactivation of the ectopic gene. **(E)** Representative morphologies of K86R cells non-induced (first panel) and induced with tet (subsequent three panels), DAPI-stained and photographed 66 h post-induction at 100× magnification.

(Figure 4A; data not shown). The relatively constant level of its extension product indicates that no global changes in mitochondrial RNA abundance or stability occur upon tet administration or K86R mutant protein induction.

The 5' domain of NADH dehydrogenase subunit 7 (ND7) RNA is the smallest of the common kind of *T. brucei* editing domain that contains multiple U deletions and numerous U insertions extending over a considerable length (a pan-edited domain). Upon K86R mutant ligase induction, the extension products corresponding to ND7 mRNA fully edited across the assessed region were dramatically reduced, although products corresponding to fully unedited ND7 mRNA remained at a fairly constant level (Figure 4B). This major effect on ND7 editing due to partial replacement of the active band IV ligase by the K86R mutant form thus indicates that the catalytic activity of the band IV ligase protein is critical for RNA editing.

If partial replacement of active band IV ligase with the inactive version compromised ligation at specific editing sites of the 24 assessed in this ND7 segment and the cleaved RNA accumulates *in vivo*, extension products

corresponding to these unligated mRNAs could be more apparent. Indeed, products seven nucleotides larger than the primer were significantly more abundant with RNA from induced K86R cells in repeated experiments (Figure 4B; inset). This size corresponds to mRNA remaining cleaved at the second editing site, which is the first U-deletion site. These extension products are indeed due to RNA that remained cleaved, for they are similarly obtained in a primer extension assay performed without dideoxy nucleotide addition (data not shown), unlike the extension products representing the edited and unedited species. In contrast, mRNAs cleaved at the first editing site, a U-insertional site four nucleotides beyond the primer, or at the many subsequent U-insertion sites, do not appear more abundant with the induced K86R mutant (Figure 4B, inset). This suggests that the band IV RNA ligase may be needed in ND7 RNA editing, specifically at U-deletion sites.

We next examined other edited RNAs to determine whether band IV ligase was generally important in editing at U-deletion sites. Another pan-edited RNA,

Fig. 4. Primer extension analysis of *in vivo* RNA editing. Representative assays are shown; all four K86R cell lines gave comparable results. For each transcript, poison primer extension assays used ddGTP and RNA from parental (29-13) and K86R cells grown in the absence or presence of tet for 48 h. 'ed' denotes product from RNA fully edited through the region analyzed; 'uned' denotes product from fully unedited RNA. (A) Analysis for COI, a never edited transcript, shown by the arrow. (B) Analysis for ND7, 5' domain, a small pan-edited region, covering 5 U-deletion and 19 U-insertion sites. The inset (from another gel) shows the position corresponding to RNAs cleaved but not ligated at the first U-deletion site (*del) or at the first U-insertion site (-ins). (C) Analysis for MURF2, covering 1 U-deletion and 9 U-insertion sites. This editing domain extends into the 5'-UTR, and fully edited product forms a doublet (Carrillo *et al.*, 2001). The position corresponding to RNAs cleaved but not ligated at the U-deletion site (*del) or at the two preceding U-insertion sites (-ins) are shown. Bands in this region corresponding to RNA cleaved at positions that are not normal U-deletion or U-insertion sites are significantly more intense from the induced K86R cell lines (see Discussion). (D and E) Analysis for CYb and COII, covering 10 and 3 U-insertion sites, respectively, and no U-deletion sites.

ATPase subunit 6 (A6), yielded consistent results and showed an increase in RNA remaining unligated specifically at U-deletional sites upon K86R induction (data not shown). We then examined the three RNAs with small editing domains, mitochondrial unidentified reading frame 2 (MURF2) which has one U-deletion site plus 10 U-insertion sites as well as apocytochrome b (Cyb) and cytochrome oxidase subunit 2 (COII) which have only U-insertion sites, 13 and 3, respectively. Primer extensions of these RNAs showed no significant diminution of the fully edited species upon K86R induction in any of the multiple K86R band IV cell lines tested (Figure 4C–E; data not shown), probably because these RNAs have insufficient numbers of affected sites. However, one extension product corresponding to mRNA cleaved but unligated at an editing site accumulated on K86R induction (marked by the asterisk in Figure 4C; data not shown). This product is from MURF2 mRNA and is 19 nt larger than the primer, corresponding to the only U-deletion site in these three mRNAs. No such accumulation was seen for products diagnostic of RNA cleaved at any of the U-insertion sites of these three mRNAs, including the previous two editing sites in MURF2, which would direct products 9 and 15 nt larger than the primer (Figure 4C–E; data not shown). These data provide further evidence that ligation by band IV is required for mRNA joining specifically in U-deletion.

The *in vivo* RNAs also show extension stops corresponding to cleavages at positions that are not normal U-insertion or U-deletion sites, and they are more abundant when using RNA from cells with reduced band IV functional ligase. This is most striking for MURF2 RNA and is seen with all examined induced K86R cell lines (Figure 4C; data not shown), but is also the case for other edited mRNAs. Such accumulation of cleaved molecules suggests that, *in vivo*, the band IV ligase can also act to reseat mRNAs cleaved at incorrect sites (see Discussion).

Band IV RNA ligase is responsible for resealing mRNAs during *in vitro* U-deletion

To verify that band IV ligase acts preferentially in U-deletion by assessing fully edited cellular RNA levels, one would need to examine pan-edited RNAs bearing only U-insertion sites (to compare with ND7) or RNAs with only U-deletion sites (to compare with Cyb or COII), but natural *T.brucei* RNAs have neither of these editing patterns. We therefore turned to *in vitro* editing, using mitochondrial extracts prepared in parallel from these induced and non-induced K86R mutant cell lines, with active gRNAs D32a for U-deletion (Cruz-Reyes *et al.*, 2001) and I47G for U-insertion (J.Cruz-Reyes, A.G.Zhelonkina and B.Sollner-Webb, in preparation). In this way, we can readily score individual U-deletion

Fig. 5. *In vitro* U-deletion using extract from non-induced and induced K86R cells. (A) Full round U-deletion reactions (lanes 1 and 2) and parallel reactions with PPI to assess cleavage (lanes 3 and 4). The inset is a blow-up of lanes 1 and 2 to better resolve the partially edited (-2) and fully edited (-3) products from the input 3'-end-labeled mRNA. (B) Reactions using 5'-end-labeled mRNA and PPI to assess 3'-U-exonuclease activity; positions corresponding to the cleaved fragment lacking 0, 1, 2, or all 3 of the terminal Us are indicated. These assays used whole mitochondrial extract, but extract that was fractionated on a glycerol gradient and used in Figure 6 and extracts from a different K86R mutant cell line gave similar results.

and U-insertion cycles as well as their three component reaction steps.

Assessing U-deletion, we found that extracts from cells with reduced band IV ligase activity generate only about half as much of the -3 U-deletion product as extracts of non-induced cells, using equal amounts of extract protein (Figure 5A; data not shown). The finding that induced K86R cell extracts are deficient in catalyzing full round U-deletion is consistent with the evidence from *in vivo* RNA analysis (Figure 4).

This decreased U-deletion is due to a deficiency in the ligation step. This step was implicated because significantly more cleaved mRNA remains unligated in the U-deletion reaction using induced K86R cell extracts (Figure 5A; lanes 1 and 2). Indeed, the cleaved RNA increases by about the same amount as the U-deletional product decreases. In contrast, the first two steps in the U-deletion cycle, mRNA cleavage and U removal, are not inhibited in induced K86R cell extracts. The cleavage step was analyzed in a U-deletion assay inhibited for ligation by supplementing with PPI and omitting ATP (Cruz-Reyes *et al.*, 1998a). Non-induced and induced extracts generated the same amount of cleavage product (Figure 5A; lanes 3 and 4). The 3'-U-exonuclease step was assayed by comparing the amount of completely U-deleted versus partially U-deleted product in the complete editing reaction (A.G.Zhelonkina, J.Cruz-Reyes and B.Sollner-Webb, in preparation). It yielded the same ratio of the -3 and -2

Fig. 6. *In vitro* U-insertion using fractions from non-induced and induced K86R cells. (A) Full round U-insertion reactions. (B) Parallel reactions with PPI to assess cleavage. The inset is a blow-up to better resolve the fully edited (+2) product from the input 3'-end-labeled mRNA. Full round U-insertion was analyzed on a 1 m gel, while all other reactions were analyzed on 0.4 m gels. These assays used the same mitochondrial extract as in Figure 5, but purified additionally by glycerol gradient fractionation; unfractionated mitochondrial extract gave a qualitatively similar result with lower overall U-insertion, and extracts from a different K86R mutant cell line gave similar results. U-insertion and U-deletion assays were routinely performed in parallel.

bands with the two kinds of extracts (Figure 5A; lanes 1' and 2'). The 3'-U-exonuclease step can also be assessed by examining U-removal from the 5' cleavage product in reactions using 5' labeled mRNA, added PPI, no ATP, and a gRNA that prevents release of the cleaved 5' mRNA (like the D32a used here) (Cruz-Reyes *et al.*, 2001; A.G.Zhelonkina, J.Cruz-Reyes and B.Sollner-Webb, in preparation). This analysis (Figure 5B) confirmed that the two kinds of extract catalyze the same extensive level of U-removal. Repeating these U-deletion experiments using a more natural gRNA (Anc+U₁₆; Cruz-Reyes *et al.*, 2001) yielded similar results (data not shown). Together, these data demonstrate that the band IV RNA ligase acts in U-deletion, serving to join the mRNA fragments following cleavage and U-removal, and that band V ligase activity does not substitute for this band IV activity.

Band V RNA ligase appears sufficient for resealing mRNA during *in vitro* U-insertion

Using similar reaction conditions, we analyzed the same extracts of non-induced and induced K86R cells for U-insertion. Unlike in U-deletion, these extracts exhibit no significant differences in the amount of the +2 U-insertion product they generate (Figure 6A). We conclude that the extracts with diminished band IV ligase activity show no deficiency in catalyzing U-insertion. Therefore, the decrease in functional band IV ligase specifically impairs U-deletion. It follows that U-insertion does not have the same requirement for band IV activity as U-deletion does.

The simplest explanation is that the other RNA ligase of the editing complex, band V, serves to join the mRNA in U-insertion (see Discussion).

Finally, in the full round U-insertion reaction, appreciably more cleaved mRNA remains when using the induced rather than the non-induced K86R cell extracts (Figure 6A). This occurs even though both extracts support the same amount of cleavage (Figure 6B) and full round U-insertion (Figure 6A), and no unidentified ligation products are observed (Figure 6A; data not shown). These data imply that the band IV ligase seals some mRNAs cleaved at the U-insertion site, forming molecules which we did not detect, likely regenerated input mRNA (see Discussion). We conclude that, at least *in vitro*, mRNAs cleaved at U-insertion sites have an additional, previously unappreciated potential fate—they become rejoined without U-addition by band IV ligase.

Discussion

Roles of band IV and band V in RNA editing

The studies reported in this paper address the functional roles of the two distinct RNA ligases, band IV and band V, present in the seven polypeptide RNA editing complex from *T.brucei* (Rusché *et al.*, 1997). Because a double gene knock-out is inviable, the band IV RNA ligase protein appeared essential in procyclic (Rusché *et al.*, 2001) and bloodstream (Schnauffer *et al.*, 2001) trypanosomes, but it remained unclear what its specific role might be. Using extracts prepared from single allele band IV knock-out cell lines, we now find that editing complexes depleted for band IV protein tend to disassociate (Figure 2B). Since enzymes of the editing complex normally appear to act in concert, deficient editing in band IV knock-out cells could be due to loss of the ligation activity, to the destabilized complex causing altered effectiveness of other components, or to both effects. To address the catalytic role of the band IV ligase independently of any structural role the protein may serve, we constructed a K86R mutant where the charge-conservative amino acid replacement at the active site abolished adenylation and ligation activity (Figure 3A). Upon its inducible expression in trypanosomes (Figure 3B), the regulation that prevents excess band IV protein from accumulating (Rusché *et al.*, 2001) causes this inactive protein to replace ~2/3 of wild-type, active band IV protein (Figure 3C), so all editing complexes contain protein with the structure of band IV, even though only ~1/3 have catalytic activity. This reduced band IV activity causes arrest of cell growth (Figure 3D), with the remaining cells exhibiting aberrant morphologies (Figure 3E). Notably, *in vivo* RNA editing is severely restricted (Figure 4). Accumulation of cleaved species indicates that this is due to deficient ligation in U-deletion and not in U-insertion (Figure 4). Furthermore, extracts from these induced mutant lines are defective at catalyzing U-deletion (Figure 5). Indeed, they are defective specifically at its ligation step (Figure 5), while the cleavage and 3'-U-exonuclease steps of U-deletion and overall U-insertion proceed unimpaired (Figures 5 and 6). These data demonstrate that: (i) band IV's catalytic activity is critical for RNA editing, (ii) it serves to ligate the mRNA in U-deletion, and (iii) band V ligase is not capable of

sustaining U-deletion. Our data additionally show that (iv) band IV ligase also acts to rejoin cleaved mRNA *in vitro* and apparently also *in vivo*, potentially serving a repair function, and they suggest that (v) band V protein may serve to ligate the mRNA in U-insertion. These results also reinforce the value of analyzing the function of an enzyme that is a key constituent of a complex by substituting a structurally homologous but inactive protein.

The first four conclusions are mandated by our data, while the fifth statement is an inference based on two controlled but negative results. Specifically, induced K86R cells abnormally accumulated mRNAs cleaved at U-deletion sites but apparently not mRNAs cleaved at U-insertion sites (Figure 4) and extracts of induced K86R cells are deficient at *in vitro* U-deletion but apparently not at U-insertion (Figure 6). These results do not exclude more complicated possible explanations. For example, U-insertion could use a novel third ligase of the editing complex that does not obviously adenylylate, while band V ligase serves an unidentified function, or U-insertion could use band IV ligase so efficiently that its joining step is not perceptibly impaired *in vivo* or *in vitro*, even when most editing complexes contain the inactive band IV. Nonetheless, the inference that band V acts in U-insertion is the simplest explanation of these results. Furthermore, this inference, as well as our demonstration that band IV serves in U-deletion, is supported by combining several previously published findings, as described in the following two paragraphs.

First, a potential 'ligation bridge' in which base pairing with the gRNA could hold the two mRNA ends in precise register for ligation (see Seiwert *et al.*, 1996) appears not relevant in U-deletion (Cruz-Reyes, 2001) but quite important in U-insertion (Igo *et al.*, 2000; J.Cruz-Reyes, A.G.Zhelonkina and B.Sollner-Webb, in preparation), suggesting that the relevant ligation steps may have different specificities and therefore could use different ligases. Furthermore, our finding that recombinant band IV protein directs active ligation in the absence of such a bridging RNA (Rusché *et al.*, 2001) is supportive of band IV ligase serving in U-deletion, which Figures 4–6 show to be the case.

Secondly, Igo *et al.* (2000) report an efficient pre-cleaved U-insertion system that uses substantially purified editing complex and two RNA fragments perfectly aligned for U-addition by a complementary bridging RNA. Without added ATP, specifically RNAs that acquired the guided number of U residues became ligated, mimicking faithful U-insertion. This result implies that faithful U-insertion uses a ligase which is pre-adenylylated in their purified complex. Although Igo *et al.* (2000) do not suggest that their results can help define the ligase that functions in U-insertion, earlier work showed that specifically band V RNA ligase becomes adenylylated upon similar purification of the trypanosome editing complex (Rusché *et al.*, 1997). From these combined results, we deduce that band V may act in U-insertion, as our data also imply. Moreover, parallel biochemical analyses based on the different adenylation properties of band IV and band V ligases (J.Cruz-Reyes, A.G.Zhelonkina and B.Sollner-Webb, in preparation) also support our designation of which of the ligases function principally in U-deletion and in U-insertion.

It is also conceivable that the band IV and band V RNA ligases could have partially redundant functions in RNA editing. However, two results suggest that band V cannot function efficiently in U-deletion. First, if band V could efficiently replace the activity of band IV *in vivo*, RNA from the induced K86R cells should not show the joining deficiencies that it does at U-deletion sites (Figure 4). Secondly, the induced K86R extracts show diminished U-deletion when using a gRNA that has the potential to form a ligation bridge and therefore may favor ligation by band V, as well as when using a gRNA unable to form this structure (Anc+U₁₆ and D32a, respectively; Cruz-Reyes *et al.*, 2001). Thus, the band V ligase appears unable to join RNAs efficiently in U-deletion, either *in vitro* or *in vivo*. To address the converse possibility, whether band IV RNA ligase can also serve in U-insertion, will require assessing editing when band V protein is present but catalytically inactive.

A function for band IV in RNA repair

It is also possible that band IV and band V RNA ligases may serve additional, different functions. Notably, an additional ligation function has been observed in editing reactions using all but the most efficient artificial gRNAs. There is appreciably more cleavage (assayed with PPI) than is inferred in the complete editing reaction by summing the edited plus remaining cleaved plus any chimeric RNA; this suggests that cleaved molecules are also re-ligated back to input RNA (A.G.Zhelonkina, J.Cruz-Reyes and B.Sollner-Webb, in preparation). In the current *in vitro* U-insertion studies, considerably more cleaved RNA remains when using extract with reduced band IV ligase activity than when using control extract (Figure 6A). This occurs even though both extracts have equal amounts of band V ligase activity and support equal levels of cleavage, full round U-insertion and chimera formation (Figure 6A and B; data not shown). These results imply that the band IV ligase serves to rejoin unedited mRNA cleaved at this U-insertion site. In fact, it makes sense that such a rejoining of unedited mRNA, which does not have a precise ligation bridge, would use band IV since this ligase functions without a bridge (see above). These results indicate that *in vitro* the band IV ligase can perform a repair-like joining at U-insertion sites.

The long-standing observation that mis-editing appears astonishingly frequent, accounting for >90% of pan-edited COIII RNA (Feagin *et al.*, 1988; Decker and Sollner-Webb, 1990), actually suggests that a repair-like joining may occur in normal growing trypanosomes. This mis-editing arises when a wrong gRNA fortuitously anchors and directs several cycles of editing (Sturm and Simpson, 1990; Koslowsky *et al.*, 1991), and it frequently terminates at positions that are not edited when using the correct gRNA (Decker and Sollner-Webb, 1990). Once every ~10 nt, such mis-editing should encounter an adenine (A) in the mRNA opposite a cytosine (C) in the gRNA, which should direct cleavage but not subsequent editing steps. Yet these cleaved mRNAs do not accumulate (Feagin *et al.*, 1988; Decker and Sollner-Webb, 1990; Sturm and Simpson, 1990), even though cleaved mRNAs can accumulate (Figure 4C; lane 4). This implies that they become re-ligated *in vivo*. In fact, band IV ligase appears a good

candidate to perform this function, since it can rejoin cleaved but unedited mRNAs *in vitro* (Figure 6; see above). Notably, when band IV activity is diminished, multiple K86R cell lines show mRNAs that are cleaved at other than normal editing sites (Figure 4). This is most striking for MURF2 (Figure 4C; data not shown) but is also seen for ND7 (Figure 4B) and A6 (data not shown). These RNAs provide experimental evidence that, *in vivo*, certain transcripts become fortuitously cleaved and that band IV ligase functions to repair these breaks. Furthermore, should such rejoined molecules be correctly re-edited, this RNA repair could augment the overall editing process.

The two different forms of editing

Previous data from this laboratory have shown that the U-deletion and U-insertion cycles use different activities for their cleavage steps (Cruz-Reyes *et al.*, 1998a) and different activities for their U-addition/removal step (Cruz-Reyes and Sollner-Webb, 1996; Rusché *et al.*, 1997). The *in vivo* and *in vitro* studies reported in this paper (Figures 4–6) show that band IV ligase is used in U-deletion and suggest that band V ligase is used in U-insertion. These conclusions are also supported by combining results from other lines of study (see above). These aggregate data suggest that catalysis of U-deletion and U-insertion could involve entirely different enzymatic activities. Thus, having the activities reside in a common editing complex (Rusché *et al.*, 1997) may not be critical for the individual editing cycle but rather reflect some other selective force. One advantage of a common location for catalyzing U-deletion and U-insertion could relate to these two kinds of editing occurring alternately along an mRNA: a single complex would allow the editing cycles to be processive without the mRNA needing to bind different U-insertion and U-deletion complexes as editing progresses.

Materials and methods

Plasmid constructs and prokaryotic expression

Cloning of the *T.brucei rhodesiense* band IV RNA ligase gene, its subcloning into vector pTrc99A for prokaryotic expression (generating pTrc-IV), into pLew 82 (Wirtz *et al.*, 1998) for expression in trypanosomes under a tet regulatable T7 promoter (generating pT7LigIV), and into pLew13 (Wirtz *et al.*, 1999) for knock-out of a band IV allele [generating pLew13-IV-k/o (G418)] were as described in Rusché *et al.* (2001). Lysine to arginine mutation of either of the two KXXG motifs in band IV was performed using a Transformer Site-Directed Mutagenesis Kit (Clontech) according to the manufacturer's directions. Using oligonucleotides K86R (5'-GTTGCATGTG AAAGAGTGCA TGGGAC) or K109R (5'-GTGA-GGTTTG CAAGGCGTAG TGGCATC), and selection/*Hind*III (5'-GCGTCACAAG CGTGGCTGTT TTG), plasmids pTrc-IV-K86R and pTrc-IV-K109R were generated. They were transfected into *E.coli* HR171pr⁺, induced, and extracts were prepared as previously described (Rusché *et al.*, 2001). These mutated positions correspond to K87 and K110 of the *T.brucei brucei* sequence. [Previous suggestions that *E.coli*-expressed band IV protein is associated with poly(A) polymerase activity were not reproduced using other expression systems.] For overexpression in trypanosomes, an 828 bp *Nsi*I-*Bam*HI fragment from pTrc-IV-K86R containing the mutated site was exchanged with the same fragment in pT7LigIV, yielding pLigIV-K86R.

Trypanosome propagation and transfection

Trypanosoma brucei brucei procyclic strain 427 was grown in SDM-79 media with 10% fetal bovine serum (Gibco-BRL). These cells were transfected with the knock-out cassette released from pLew13-IV-k/o

(G418) as previously described (Rusché *et al.*, 2001). Cell line 29-13, a 427 derivative that expresses the T7 RNA polymerase and the tetracycline repressor protein (Wirtz *et al.*, 1999) was grown in this medium with 15 µg/ml G418 (Gibco-BRL) and 50 µg/ml hygromycin (Sigma), as were transfectants bearing pLigIV-K86R. To assess the effects of induction, 10% tetracycline system-approved fetal bovine serum (Clontech) was used.

pLigIV-K86R linearized with *NotI* within the rDNA segment was transfected in 29-13 cells, as described by Wirtz *et al.* (1998) using 2.5 µg/ml phleomycin (Sigma) for selection and 10 ng/ml tet for low-level induction of the resistance gene and the linked gene of interest. Four of the clones obtained at ~2–3 weeks post-transfection were selected randomly and propagated non-induced (without phleomycin or tet). When desired, maximal ectopic expression was induced with 1–2 µg/ml tet. Cell densities were determined using a hemacytometer. To visualize cells, ~10⁷ were gently pelleted, washed repeatedly in phosphate buffered saline (PBS), and allowed to adhere to poly-L-lysine coated slides. After fixing with 2% paraformaldehyde in PBS for 10 min, the slides were washed for 5 min each in PBS, PBS with 0.1% saponin, PBS, and PBS containing 0.5 µg/ml DAPI (Sigma), then twice for 10 min in PBS. Coverslips were mounted using Vectashield (Vector laboratories, Inc.) and the cells were visualized using a Zeiss Axioskop microscope (Carl Zeiss, Inc.) and a 100×, 1.3 NA plan-NEOFLUAR oil immersion objective.

Extract preparation and adenylation

Mitochondrial extract was prepared as described by Sabatini *et al.* (1998) except that the mitochondrial vesicles were suspended at 2.5×10^{10} cell equivalents/ml in MRB [25 mM Tris–HCl pH 8.0, 10 mM Mg(OAc)₂, 10 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol (DTT) and 5% glycerol] and lysed by the addition of 5% Triton X-100. Extracts compared for *in vitro* editing were prepared in parallel. Small-scale extract preparations were as described by Rusché *et al.* (2001). Extracts were standardized by protein concentration. Mitochondrial extract (500 µl) was also fractionated on 10–30% glycerol gradients in MRB for 12 h, similarly to Rusché *et al.* (1997).

Adenylation assays were carried out on ice in MRB plus 5 mM DTT, using ~4 µg crude *E. coli* extract, 0.3 µg trypanosome mitochondrial extract, or 4 µl glycerol gradient fraction; these components were pre-incubated for 5 min with 10 mM tetra potassium PPi to de-adenylate any pre-adenylated ligases before 1 U inorganic pyrophosphatase (Sigma) and 33 nM [α -³²P]-ATP (ICN) were added for a 10 min incubation.

In vitro editing assays

PCR amplification of template DNA, RNA production, and its 3' and 5' end-labeling were as described previously (Cruz-Reyes and Sollner-Webb, 1996). Natural A6 pre-mRNA m[0,4] contains four Us at editing site 1 (ES1) and none at ES2 (Cruz-Reyes and Sollner-Webb, 1996). gRNA D32a directs removal of three Us at ES1, is ~30 times more efficient than the natural A6 gRNA, and forms no chimeras (Cruz-Reyes *et al.*, 2001). gRNA I47G directs insertion of two Us at ES2 and is a few fold more efficient than natural A6 gRNA (J.Cruz-Reyes, A.G.Zhelonkina and B.Sollner-Webb, in preparation). U-deletional editing reactions (20 µl) were in MRB supplemented with 0.15 mM ATP, 3 mM ADP, 5 mM CaCl₂, 0.045 ng/ml torula RNA, 10 mM DTT and 0.8 U/ml RNase inhibitor (Promega), plus 25–50 fmol of end-labeled m[0,4] and 1.25 pmol of D32a (Cruz-Reyes *et al.*, 2001). U-insertional editing reactions were the same except that the gRNA was I47G, ATP was 3 µM, ADP was omitted, and 0.15 mM UTP was used (Cruz-Reyes *et al.*, 1998b). mRNAs and gRNAs were pre-annealed in TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA) at 42°C for 5 min followed by cooling to 22°C over ~15 min. Reactions were incubated 5 min on ice and then 40 min at 22°C. Deletion and insertion reactions were performed in parallel. Total cleavage was assayed in editing reactions supplemented with 1.5 mM PPi to inhibit ligase, and no ATP or CaCl₂ (Cruz-Reyes *et al.*, 1998a). RNAs were resolved on 9% polyacrylamide/7.5 M urea gels and visualized by autoradiography. Quantitation used a Fuji phosphorimager (Fujix BAS 1000) with Image Gauge version 1.7 software.

Primer extension analysis of in vivo editing

Total RNA from ~3 × 10⁹ trypanosomes grown to 0.5–1.0 × 10⁷ cells/ml (Carrillo *et al.*, 2001) was extracted using Trizol Reagent (Gibco-BRL) according to the manufacturer's instructions and suspended in 50 µl TE at ~5 µg/µl. Extension reactions used 10 U of AMV reverse transcriptase (Promega) in the manufacturer's buffer plus 150 µM each dATP, dCTP, dTTP and 100 µM ddGTP, 20 µg of the above RNA and 0.25–0.5 pmol of

5'-[³²P]-labeled primer (below) pre-annealed in TE at 70°C for 10 min and then cooled to ~37°C over ~30 min. After 1 h at 47°C, and then 20 min at 37°C with 200 µg/ml RNase A, the DNA was extracted, precipitated and analyzed on polyacrylamide gels (as described above). The primers were: COI-RT (5'-GTAATGAGTA CGTTGTAAAA CTG-3'); ND7-RT (5'-CACATAACTT TTCTGTACCA CGATGC-3'); MURF2-RT (5'-CAAAAACACG ACTACAATCA AAG); CYb-RT (5'-CAACCTGACA TTAAGAC-3'); COII-RT (5'-ATTCATTAC ACCTACCAGG-3') (Lambert *et al.*, 1999). The amount of editing for different mRNAs evidently varies in different strains of trypanosome (compare Figure 4 with Lambert *et al.*, 1999). However, controls show that the amount of ND7 editing is minimally affected when the cells were harvested at very late log rather than in mid-log (data not shown; see also Carrillo *et al.*, 2001).

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