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Determinants for Association and Guide RNA-Directed Endonuclease Cleavage by Purified RNA Editing Complexes from *Trypanosoma brucei*

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U-insertion/deletion RNA editing in the single mitochondrion of kinetoplastids, an ancient lineage of eukaryotes, is a unique mRNA maturation process needed for translation. Multisubunit editing complexes recognize many pre-edited mRNA sites and modify them via cycles of three catalytic steps: guide RNA (gRNA)-directed cleavage, insertion or deletion of uridylates at the 3′-terminus of the upstream cleaved piece, and ligation of the two mRNA pieces. While catalytic and many structural protein subunits of these complexes have been identified, the mechanisms and basic determinants of substrate recognition are still poorly understood. This study defined relatively simple single- and double-stranded determinants for association and gRNA-directed cleavage. To this end, we used an electrophoretic mobility shift assay to directly score the association of purified editing complexes with RNA ligands, in parallel with UV photocrosslinking and functional studies. The cleaved strand required a minimal 5′ overhang of 12 nt and an ~15-bp duplex with gRNA to direct the cleavage site. A second protruding element in either the cleaved or the guide strand was required unless longer duplexes were used. Importantly, the single-stranded RNA requirement for association can be upstream or downstream of the duplex, and the binding and cleavage activities of purified editing complexes could be uncoupled. The current observations together with our previous reports in the context of purified native editing complexes show that the determinants for association, cleavage and full-round editing gradually increase in complexity as these stages progress. The native complexes in these studies contained most, if not all, known core subunits in addition to components of the MRP complex. Finally, we found that the endonuclease KREN1 in purified complexes photocrosslinks with a targeted editing site. A model is proposed whereby one or more RNase III-type endonucleases mediate the initial binding and scrutiny of potential ligands and subsequent catalytic selectivity triggers either insertion or deletion editing enzymes.

Keywords: U-insertion/deletion; guide RNA; RNA editing; kinetoplastids; RNP

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Abbreviations used: gRNA, guide RNA; ES, editing site; pre-mRNA, pre-edited mRNA; RNP, ribonucleoprotein complex; EMSA, electrophoretic mobility shift assay; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA; IgG, immunoglobulin G.

Introduction

The majority of primary mRNA transcripts in the single mitochondrion of kinetoplastids, including species of *Trypanosoma* and *Leishmania*, are plagued with frameshifts and stop codons. Protein-encoding sequences are produced via an extraordinary maturation process involving specific insertion and deletion of uridylates often at hundreds of editing sites (ESs) in a single transcript. This process is catalyzed by megadalton multisubunit assemblies known as L-complexes, 20S editosomes, or editing complexes that contain between 16 and 20 known subunits and target ESs specified by the partial complementarity of pre-edited mRNA (pre-mRNA) and guide RNAs (gRNAs). For recent reviews, see Refs. 1,2.

RNA editing has been recreated *in vitro* at single-model ESs in either natural-like[3,4] or completely artificial[5] substrates. Early mechanistic studies indicated that all steps of deletion and insertion editing were catalyzed by distinct enzymatic activities.[6–10] More recently, it was shown that a deletion cycle involves the consecutive action of endonuclease KREN1, 3′-exouridylylase KREX1 and/or KREX2, and ligase KREL1.[9,11–15] Similarly, an insertion cycle involves endonuclease REN2 or REN3, terminal uridylyl transferase KRET2, and, preferentially, ligase KREL2.[9,14,16–18] However, KREL1 may be used in the absence of KREL2 *in vitro* and *in vivo*.[9,14,19,20] Potentially, KREN1 and KREX enzymes could also help proofread misedited insertion ESs bearing extra Us (i.e., misedited insertion sites could be targeted and repaired by deletion editing).[6] Additional observations also suggest that deletion and insertion activities may occur at individual ESs *in vivo*. Namely, RNA interference of KREN1 downregulates editing of CYb and COII pre-mRNAs *in vivo*, which only contain insertion ESs.[11] Also, RET2 was shown to add Us at deletion sites *in vitro*.[21]

Pre-mRNA/gRNA hybrids are proposed to form two helical regions flanking an internal loop. The downstream (relative to the scissile bond) "anchor" duplex directs endonuclease cleavage immediately 5′ to it, whereas the upstream duplex is thought to tether the cleaved 5′ piece during U-specific processing and re-ligation. The mechanisms of substrate recognition in assembled editing complexes are currently being addressed (for a recent review, see Ref. [22]). Previous studies in our laboratory using purified native complexes have shown that secondary structure rather than sequence-specific features are primarily required for full-round insertion editing.[5,23] In a completely artificial 43-nt pre-mRNA/gRNA model substrate with single-helical turns flanking the central loop, simple features of this loop were manipulated to interconvert sites between insertion and deletion editing. Important insights on the specificity of substrate association with purified editing complexes were obtained in competition studies using parallel UV photocrosslinking and full-round catalytic editing assays. Such studies, using a single photoreactive 4-thioU and a ^{32}P atom at targeted ESs, showed a preferential association of complexes with deletion and insertion substrates, particularly with the most efficient model substrate currently available for full-round editing (A6 pre-mRNA/D33 gRNA hybrid).[5,8] The native complexes also exhibited a level of nonspecific binding to unrelated transcripts. Interestingly, ribose 2′-H substitutions on the downstream helix and gRNA side of the central loop significantly inhibited both pre-mRNA cleavage and photocrosslinking activities at a targeted ES. Furthermore, a single 2′-H substitution adjoining the scissile bond obliterated the endonucleolytic activity but had no effect on photocrosslinking, suggesting that the ribose 2′-hydroxyl at this position is relevant for catalysis and not association of editing complexes.[5]

One of the photocrosslinking subunits in assembled editing complexes was proposed to be KREPA2 (MP63),[24] which, as several other subunits, contains conserved domains that predict interaction with nucleic acids.[25,26] Studies of purified recombinant proteins established that KREPA3 (MP42), KREPA4 (MP24), and KREPA6 (MP18) exhibit RNA-binding activity,[27–29] but their precise function in assembled editing complexes remains to be determined. KREPA4 and KREPA6 exhibited preferential binding to poly(U) homopolymers, suggesting a role in the recognition of the natural 3′-poly(U) extension of gRNAs. These recombinant proteins showed low affinity for RNA.

While previous photocrosslinking analyses provided insights on the specificity of the multisubunit editing enzyme/substrate association, absence of crosslinking with certain mutant substrates could not be interpreted with certainty. Furthermore, whether purified editing complexes form transient or stable ribonucleoprotein complexes (RNPs) with cognate substrates is unknown. In this study, we used an electrophoretic mobility shift assay (EMSA) to directly examine, for the first time, RNPs formed by purified editing complexes. We applied EMSA, photocrosslinking, and endonuclease analyses to define substrate determinants for association and endonuclease cleavage, the first catalytic step of RNA editing. Both single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) were required for these two stages of editing, but ssRNA required for association can be satisfied in different ways, whether or not endonuclease cleavage activity is observed. Importantly, the determinants for association and cleavage can be uncoupled, and the determinants for endonuclease cleavage are more complex than those for association but less intricate than those for full-round editing.

Finally, we compared preparations of native and affinity-purified editing complexes in association and catalytic assays and established that one subunit that photocrosslinks at a targeted ES is the essential endonuclease KREN1. The subunit KREPA2 (MP63) was also confirmed to photocrosslink. A model is proposed whereby recognition of basic determinants, including those defined here, leads to a preferential association of editing complexes with

potential substrates. Such initial interactions may precede subsequent specialized contacts that trigger catalysis by either deletion or insertion editing.

Results

Our previous RNA–protein photocrosslinking studies showed that purified native editing complexes preferentially associate with a model A6 substrate for full-round editing (Fig. 1a) via recognition of secondary structure, not sequence-specific, features.[5,24] However, absence of crosslinking due to certain substrate modifications or reaction conditions leaves uncertainties about the editing enzyme/substrate association.

To directly score substrate binding by editing complexes, we established an EMSA. A standard reaction mixture for full-round editing or photocrosslinking studies, using purified editing complexes and an ES1-labeled substrate (Fig. 1a),[24] was briefly incubated and loaded onto native agarose gel. A fraction of radiolabeled substrate exhibited delayed electrophoretic mobility only in the presence of editing complexes (Fig. 1b). This shifted product co-migrated with complexes that were radiolabeled by adenylylation of ligase subunits (Fig. 1c)[30] and was specifically immunodepleted by monoclonal antibodies to editing subunits (Fig. 1d, upper panel). As expected, adenylylatable editing ligases were enriched in the antibody-conjugated immunoglobulin G (IgG) beads but not in beads without antibodies (lower panel).

To further confirm that these ribonucleoprotein assemblies (RNPs) include editing complexes, we examined their substrate specificity using competition analyses as those performed in photocrosslinking and full-round editing studies.[24] Importantly, the competition profiles in photocrosslinking (that we reported[24]) and EMSA were equivalent: the homologous A6 competitor was strongly inhibitory at 5- to 10-fold excess, whereas tRNA and CYb were significantly less inhibitory at 25-fold excess (Fig. 1e and f, respectively; other data not shown). Moreover, a similar competition pattern was observed in assays of gRNA-directed endonuclease cleavage, the first enzymatic step of a full-round editing cycle (Fig. 1g). Together, these data indicate that the EMSA directly scores the editing enzyme/substrate association and specificity of editing complexes. The data using EMSA also mirror the observations in parallel studies of RNA–protein photocrosslinking and editing enzymatic activities. Furthermore, all these activities of editing complexes can be examined using common substrates and reaction conditions.

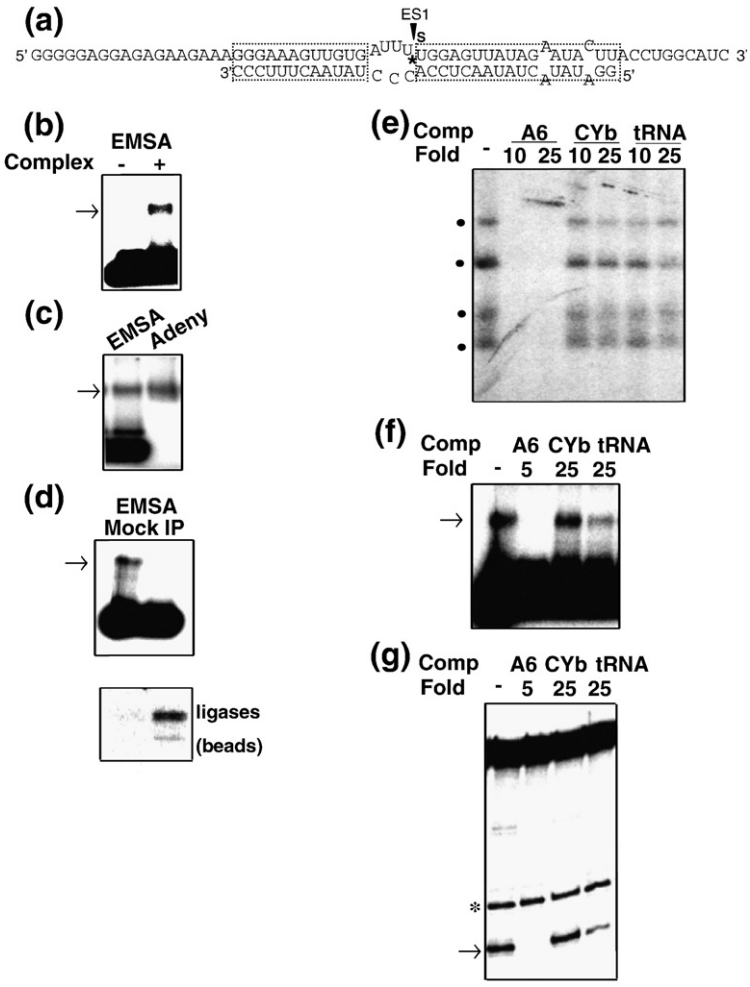

Fig. 1. The association of purified editing complexes with substrates can be directly scored by EMSA, in parallel with UV photocrosslinking and pre-mRNA cleavage assays. (a) Scheme of an ES1-^{32}P labeled (*) and thio-labeled (s) model A6 substrate for EMSA, UV photocrosslinking, and full-round U-deletion editing. (b) EMSA in a native agarose gel showing a shifted band (arrow) only in the presence of editing complexes. (c) Co-migration of the shifted substrate with editing complexes that were radiolabeled by auto-adenylylation (Adeny). (d) Specific depletion of the shifted product by immunoprecipitation (IP; upper) and recovery of self-adenylylatable ligase subunits in the beads (lower). A mock reaction was devoid of antibodies. (e) Preferential association of editing complexes with a substrate (A6) for full-round editing in competition studies using UV photocrosslinking (dots indicate four major crosslinks) or parallel assays of (f) EMSA and (g) endonuclease cleavage (arrow). A spurious cut (*) serves as loading control. In the EMSA, much of the substrate remained unbound. The fold excesses of unlabeled homologous A6 (5- or 10-fold) and heterologous CYb and tRNA competitors are indicated. No competitor is (−).

Based on these observations, we sought to define substrate determinants for association and guide-directed cleavage by editing complexes. We performed competition analyses, as in Fig. 1e–g, to examine the effects of unlabeled derivatives of the homologous (A6) competitor (diagramed in Fig. 2a). Our standard editing mixtures include gRNA at ~120-fold excess over radiolabeled A6 pre-mRNA to ensure quantitative annealing.[5] In these studies, the abundant free gRNA ("guide strand") in the standard mixture was allowed to preanneal with each pre-mRNA derivative ("substrate strand") added at a small excess, 5- to 10-fold, over radiolabeled pre-mRNA (Fig. 2b) in order to form unlabeled competitor duplexes. All constructs in Fig. 2a used the same guide strand, and quantitative annealing was confirmed in native gels[5] (see Methods). Such analysis in binding and catalytic

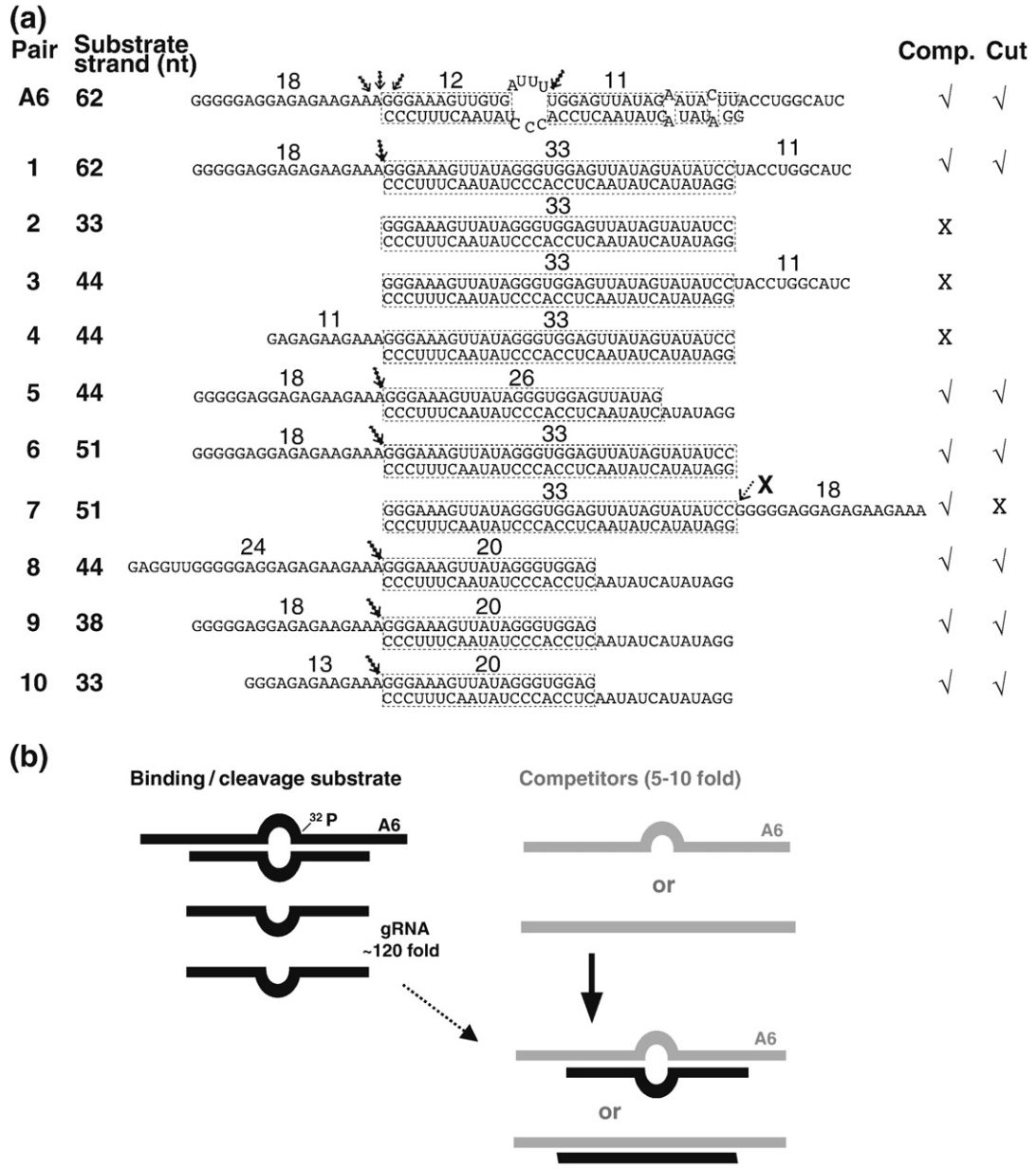

Fig. 2. Constructs tested and scheme for competition assays. (a) Homologous A6 and derivative competitors (top "substrate" strand) paired with gRNA D33 (lower "guide" strand). The assigned number of each competitor RNA pair and size (nt) of the substrate strand are indicated, as well as the size of the predicted helix and overhangs. The demonstrated cleavage sites are noted with an arrow. Evident (✓) or weak-to-undetected (X) competition and cleavage activity for each construct are indicated at the right. Some pairs were not tested for cleavage activity. Pair 7 was tested for cleavage although a negative result was expected (see the text). (b) Cartoon of model RNA construct in standard functional (left) and modified competition (right) assays. ^{32}P-labeled A6 pre-mRNA is usually annealed with complementary gRNA at ~120 fold excess in our standard editing assays. Unlabeled A6 substrate strand or variants (light strand) at 5- to 10-fold excess, over radiolabeled A6, anneal with free gRNA (both as dark strands), forming competitor pairs (light/dark hybrids).

assays performed in parallel is illustrated in Fig. 3. In this example, both the homologous A6 pair and derivative Pair 1 with a fully base paired guide strand (i.e., it forms a continuous 33-bp duplex), were strong competitors in photocrosslinking (Fig. 3a), EMSA (Fig. 3b), and cleavage (Fig. 3c). However, a derivative with the 33-bp duplex but no overhangs (Pair 2) was a poor competitor in all assays. These data suggest that editing complexes associate with Pair 1 but not Pair 2. Thus, the presence or absence of the central loop region in the parental A6 construct does not significantly affect the binding efficiency of editing complexes, although ssRNA seems required for association.

To dissect RNA requirements for association with editing complexes that distinguish Pair 1 from Pair 2, we designed competitors based on Pair 2 that contain substrate-strand upstream or downstream overhangs of various lengths (Fig. 4; diagrammed in Fig. 2a). While 24-nt, 18-nt, and 13-nt extensions

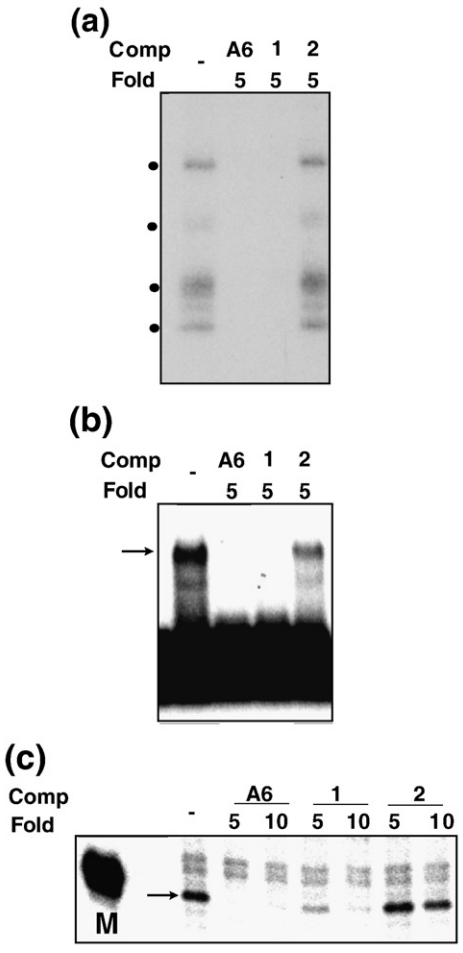

Fig. 3. Parallel competitions in (a) UV photocrosslinking assay, (b) EMSA, and (c) RNA cleavage assay with purified editing complexes, as in Fig. 1. A6 and variant competitors (Comp) diagrammed in Fig. 2a were examined at the indicated fold excess. Our cleavage assays typically included a size marker (M) such as the ^{32}P kinased donor fragment used to prepare the parental A6 substrate (Fig. 1a) or control lanes with and without gRNA.

favored association of editing complexes (Pairs 5–10), 11-nt extensions at either side of the duplex (Pairs 3 and 4) did not. Furthermore, constructs with shorter duplexes, 26 bp (Pair 5) and 20 bp (Pairs 8–10) long, were also effective competitors. Most of these constructs used a 44-nt substrate strand; however, Pair 10 with a 33-nt substrate strand was also a significant competitor. Some competitions are more evident in crosslinking and EMSA than in cleavage studies (Fig. 4a–c). This difference may reflect different dynamics in the assays; that is, the former two assays score RNP complexes that are present at the time of crosslinking and that withstand gel electrophoresis, respectively, whereas the latter assay scores accumulation of cleaved product over time, regardless of the relative stability of RNPs. Together, the competition studies in Figs. 1–4 suggest that association with editing complexes requires recognition of a relatively simple structure bearing discrete ssRNA and dsRNA determinants.

Several constructs examined so far were effective competitors, indicating that they are bound by editing complexes, but it was unclear whether they were also active in enzymatic assays. To directly address this, we tested these constructs for specific gRNA-directed cleavage by editing complexes (Fig. 5). Since the guide strand in these pairs fully complements the substrate strand, we assayed for potential guide-directed cleavage at the phosphodiester bond just 5′ of the duplex.[3] We have reported that this particular bond is cleaved just 5′ of the upstream duplex in the parental construct (Fig. 2a, top construct, and ahead in Fig. 5b).[8] Pairs 1, 5, and 6 generated a predicted 18-nt cleaved product (Fig. 5a) that corresponds to the 5′-end-labeled overhang. This cleavage occurred only in the presence of the guide strand. Furthermore, Pairs 8–10, which form a shorter 20-bp duplex, were also cleaved with comparable efficiencies to the parental A6 construct (Fig. 5b). The expected 24-nt, 18-nt, and 13-nt cleavage products were gRNA dependent. In the parental A6 construct, gRNA-directed cleavages occur 5′ of both downstream (ES1) and upstream duplexes: the 5′-end-labeled substrate strand accumulates a 31-nt product, as a result of consecutive cleavage and removal of 3 Us by U-specific exonuclease activity at ES1[13]; also, multiple cuts 5′ of the upstream duplex probably due to misannealing of this helix are observed. Spurious fragments of the substrate strand often accumulate due to breakage or RNase contamination that preferentially target Us in the absence of guide strand and are more evident with 5′-labeled substrates.

Among constructs found to associate with editing complexes, Pair 7 was not subject to guide-directed endonuclease cleavage as its substrate strand lacks a 5′ overhang and its 3′ ssRNA extension does not undergo cleavage (Fig. 2a; other data not shown). The 18-nt protrusion of Pair 7 rescued the inactive Pairs 2–4 in competition assays by crosslinking (Fig. 2a) and in EMSA (data not shown). In summary, all efficient competitors in EMSA and photocrosslinking assay were also functional for

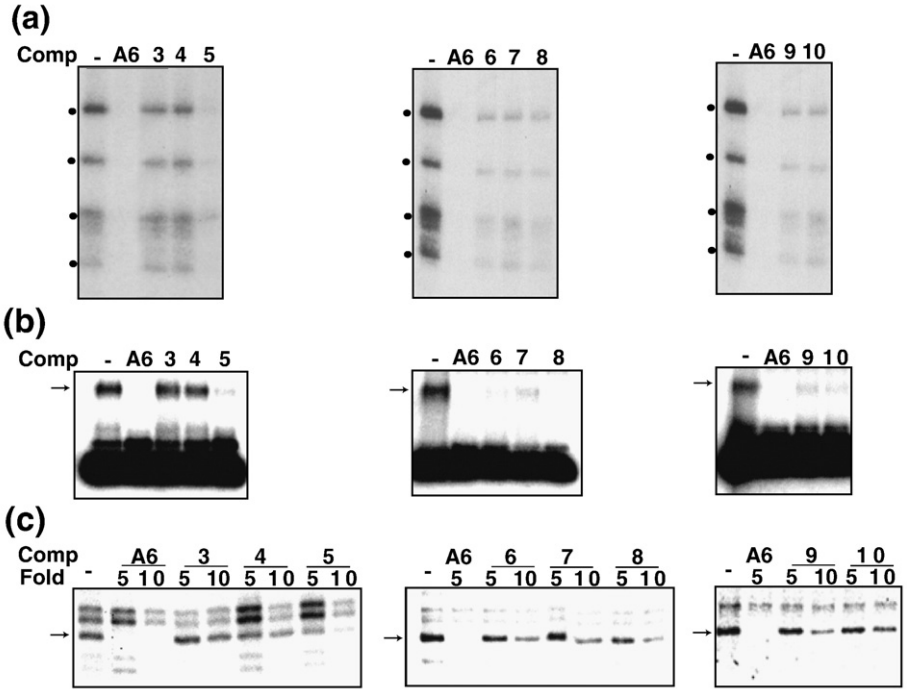

Fig. 4. (a–c) Parallel competitions as in Fig. 3. The homologous A6 and derived competitors are diagrammed in Fig. 2a.

endonuclease cleavage, except for Pair 7. While both association and endonuclease cleavage activities of editing complexes have ssRNA and dsRNA requirements, these can be combined in a way that promotes association but not cleavage. Thus, association and catalysis by editing complexes can be uncoupled.

We decided to further analyze derivatives of Pair 10, the shortest construct tested that supported editing complex association and specific endonuclease cleavage activity. This symmetrical construct with 13-nt overhangs flanking a 20-bp duplex was ideal to dissect determinants involved in selection of the substrate strand. That is, how are the substrate and guide strands distinguished in a duplex? We tested Pair 10 derivatives (Pairs 11–16) bearing progressively shortened 5′ overhangs in the guide strand (Fig. 6a and ahead in d). In these reduced structures, a 3-nt 5′ overhang in the guide strand promoted efficient cleavage of the substrate strand, but 1-nt and 2-nt extensions were strongly inhibitory (Pairs 15 and 16). Also, the latter constructs were not rescued by longer (18 nt) 5′ overhangs in the substrate strand (not shown). This suggests that 5′ overhangs in the substrate and guide strands are not compensatory.

Analysis of constructs bearing shorter 5′ extensions in the substrate strand (Pairs 17–20; Fig. 6b) showed that 12 nt is minimally required for endonuclease cleavage activity (Fig. 6d and e; other data not shown). Constructs with 11-nt 5′ overhangs in the substrate strand were inactive and not rescued by the presence of longer guide-strand overhangs (e.g., Pairs 19 and 20).

To determine whether constructs with duplexes shorter than 20 bp are functional, we examined Pairs 21–26 (Fig. 6c and f). Efficient endonuclease cleavage was supported by Pair 21, which forms a 15-bp duplex, but progressive truncations of the guide-strand 5′ overhang were increasingly inhibitory (Pairs 22–24). Pair 21 also showed that the substrate strand can be shorter than the guide strand and that an ~27-nt substrate strand bearing a 12-nt 5′ overhang supports efficient endonuclease cleavage. In the above constructs, the substrate-strand 5′ extension appears to be separately recognized, as inactivating truncations of this element were not compensated by a longer duplex or extended guide-strand 5′ ssRNA. In contrast, the guide-strand 5′ overhang could be replaced by using either an extended double-stranded terminus (e.g., Pair 6; Fig. 2a) or a 3′ overhang of the substrate strand (Pair 25; Fig. 6c). The latter pair also showed that an 18-nt guide strand, largely annealed with the substrate strand, directs efficient endonuclease cleavage activity. Seiwert *et al.* reported that an 18-nt guide strand directs endonuclease cleavage of a complementary 73-nt A6 mRNA.[3] Pair 25 and Pair 5, both of which generate the same cleaved product, were nearly as efficient as the parental A6 construct (Fig. 6g; see also Fig. 5a). Finally, we found that an 11-bp duplex in Pair 26 failed to direct detectable cleavage of the substrate strand (not shown). Such 11-bp duplex seems relatively stable (−18.4 kcal/mol), and we confirmed efficient annealing with the substrate strand in native gels.[5] Although this simple pair is not cleaved, it binds editing complexes in an EMSA (see the site-specific labeled Pair 27 in Fig. 7a). Importantly, the ssRNA overhang was essential for binding, while pairing with a DNA strand was inhibitory (Pair 28 and Pair 29, respectively). We examined additional constructs for association,

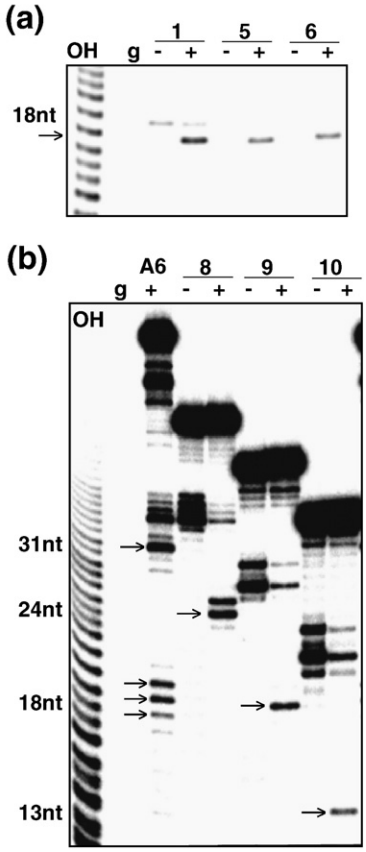

Fig. 5. (a and b) Direct cleavage assays of 5′-end-labeled substrate-strand transcripts paired with the parental gRNA.D33. The homologous A6 and derived competitors are diagrammed in Fig. 2a. Lanes with "+" and without "−" gRNA (g) are shown. Specific cleavage only occurs in the presence of gRNA (marked by an arrow). Spurious fragmentation of these transcripts occurs without gRNA but is inhibited by annealing of gRNA. Partial alkaline RNA hydrolysis "OH" was used as sizing ladder. Guide-directed cleavage of the A6 construct is directed by the downstream duplex (ES1) and by the upstream duplex. The latter occurs at three adjacent positions (~18-nt products) possibly due to alternative pairing. The short upstream duplex may be stabilized by coaxially stacking with the downstream duplex.[8]

whether or not they are cleaved (Fig. 7b). In this case, we prepared derivatives of the thiolated parental A6 (diagrammed in Fig. 1) and tested their ability to photocrosslink with editing complexes. For example, Pair 30 photocrosslinks and is also cleaved (Fig. 7b; other data not shown). Other derivatives with an ssRNA overhang that crosslinked are not cleaved, whereas a blunt helix did not exhibit detectable crosslinking (Pairs 31–33). The parental A6 construct generates more robust signals in association assays than most derivatives tested in our study.

In summary, the construct series in Fig. 6 showed that purified editing complexes only cleave substrate strands bearing a minimal 5′ overhang of 12 nt. The minimal duplex directing specific cleavage was not determined to the nucleotide, but it could be ~15 bp long, if not smaller. In addition to these two features, cleavage activity required the presence of either (a) a substrate 3′ overhang or a guide-strand 5′ overhang when using a 15-bp duplex or (b) a larger duplex without additional ssRNA. Figure 7 confirmed that association and cleavage can be uncoupled although an ssRNA overhang is essential for both stages of the editing reaction. Importantly, association exhibits simpler determinants compared with cleavage.

It is feasible that some, if not all, determinants defined in this study may be recognized by one or more RNA-binding subunits of editing complexes, including RNase III-type, OB-fold, and zinc-finger domains. At least three RNase III-type endonucleases identified in editing complexes are thought to catalyze pre-mRNA cleavage in insertion and deletion editing.[11,12,16,18] However, the composition of the native editing complexes used here, including the presence of reported endonucleases, was unclear. Mass spectrometric analysis of this protein preparation revealed nearly all reported subunits of affinity-purified ~20S editing complexes in *Trypanosoma brucei* and *Leishmania tarentolae*,[11,12,16] in addition to subunits of the MRP (mitochondrial RNA-binding protein) complex that are thought to transiently associate with ~20S editing complexes via an RNA linker (Fig. 8a).[32] Three other proposed editing subunits, KREPA5, KREPA6, and KREH1, were not detected likely because they were sub-stoichiometric, insufficiently ionized, or absent in our preparation. However, KREPA6 was recently reported to be essential[29] and most likely passed undetected in our samples.

Since our previous photocrosslinking studies indicated that at least four subunits of purified ~20S native complexes make intimate contact with model ESs (Fig. 8b),[5,24] we attempted the identification of a crosslinking subunit that migrates at about 100 kDa, where the endonuclease KREN1 was expected. To this end, we made a TAP-KREN1 construct and expressed it in *T. brucei* procyclic cells (see Methods) based on a reported protocol used to generate the same cell line.[31] Tagged editing complexes were purified through IgG and calmodulin-coupled resins and then examined by photocrosslinking. We found that cbp-KREN1 complexes produced a shift of the ~100-kDa crosslink due to the mass added by the tag (~5 kDa; Fig. 8c). These complexes also exhibited the crosslink by endogenous KREN1 and the other major crosslinks observed in native complexes. As far as we know, this is the first evidence that at least two copies of KREN1 are present in editing complexes. Previous characterization of KREL1 affinity-purified complexes showed that endogenous and ectopic copies of this subunit were also present.[14,32] Importantly, the shifted crosslink is specific of our tagged KREN1 cell line and not associated with the cell culture or protein purification conditions, as affinity-purified complexes using a different tagged subunit (TAP-KREPB5; i.e., MP44) exhibited the same crosslinking pattern of native complexes (Fig. 8c), as well as a similar silver staining pattern (Fig. 8d) and full-round insertion and deletion activities (not shown).

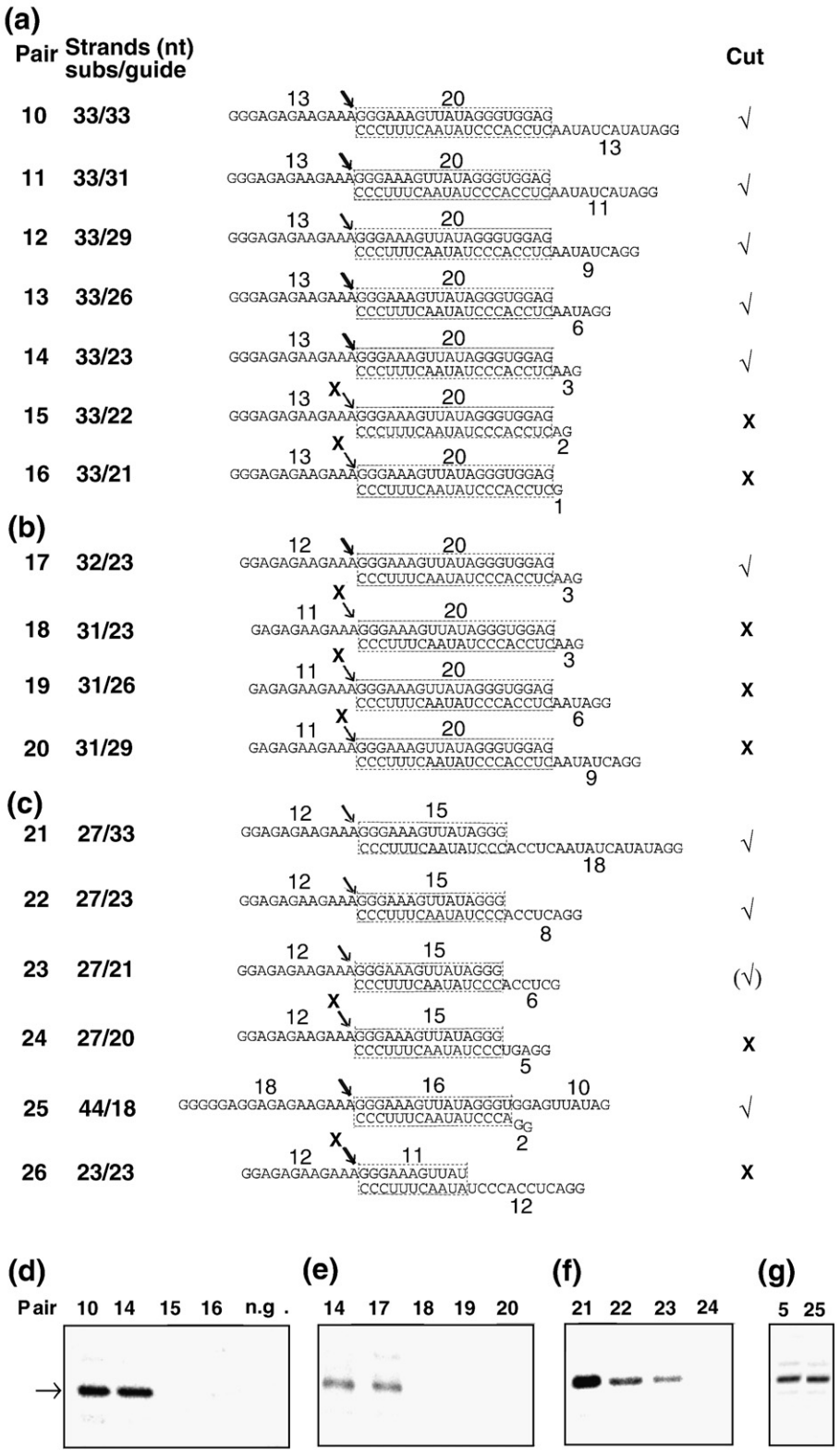

Fig. 6. Diagram of minimized substrates for endonuclease cleavage by purified editing complexes. (a–c) A6 and derivative competitors (substrate strand) paired with parental gRNA.D33 or shorter versions (guide strand). The size of both strands in each pair is indicated. All other labels are as those in Fig. 2a. Detected (↙) or undetected (X) cleavage activity is indicated for each construct. Cleavage activity on Pair 23 was relatively weak. (d–g) Cleavage assays using 3′-end-labeled substrate strand derivatives.

Consistent with the identification of KREN1 in this study, our preliminary crosslinking analysis using aliquots of KREN1 and KREN2 complexes purified and characterized in another study[31] showed that the former but not the latter forms the 100-kDa crosslink (data not shown). The presence of these

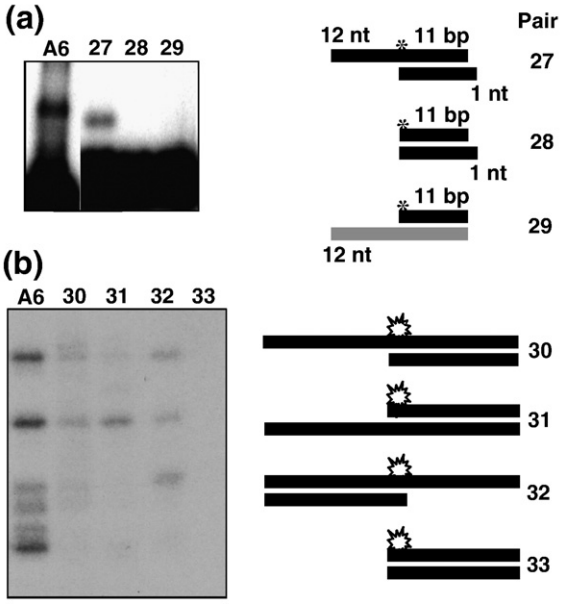

Fig. 7. Additional RNA pairs that associate with purified editing complexes but are not cleaved. (a) EMSA; Pair 27 (derived from Pair 26) forms an RNP but is not cleaved. This RNP exhibits a faster electrophoretic mobility compared with the parental A6, but the reason for this is unclear. Duplexes without the 12-nt overhang or bearing a DNA strand failed to form an RNP (Pairs 28 and 29, respectively). (b) UV photocrosslinking assays of the A6 parental construct in Fig. 1 and derivatives with or without an ssRNA overhang (Pairs 30–33). The site-specific ^{32}P label in (a) and the ^{32}P and thio labels in (b) are depicted by an asterisk and a star, respectively.

KREN proteins was mutually exclusive in the reported purified complexes.[31]

Our previous one-dimensional analyses suggested that the crosslink at ~60 kDa was KREPA2.[24] We confirmed this identification by performing a two-dimensional gel analysis of a partially purified protein preparation exhibiting significant crosslinking activity by editing complexes (Fig. 8d, lower panel). The ~60-kDa crosslink was resolved in a discrete region of the gel, and mass spectrometric analysis of the excised region only contained KREPA2. The crosslinking subunits at about 50 kDa and 40 kDa were more disperse, and mass spectrometric analyses of these gel regions were unsuccessful. Thus, they remain to be identified.

Overall, the native editing complexes used in this study contain most subunits previously observed in purifications by other laboratories, including the RNase III-type endonuclease KREN1, which we showed to directly photocrosslink with model ESs. This subunit may be involved in the recognition of the substrate determinants defined here for association and endonuclease cleavage, but additional work is needed to explore this possibility.

Finally, we compared the substrate specificity of native editing complexes and KREPB5 affinity-purified complexes in parallel EMSA, photocrosslinking assay, and endonuclease cleavage assay (Fig. 9a–c). Native and tagged KREPB5 editing complexes exhibited similar substrate specificity, in the presence of homologous A6 substrate (5-fold excess) and tRNA (25-fold), as positive control and relatively poor competitor, respectively. Thus, the approaches adopted in these studies should be useful in further comparisons of native and affinity-purified editing RNPs that exhibit different protein and functional compositions.

Discussion

The goal of this work was to define substrate requirements for association of purified editing complexes and gRNA-directed cleavage, the first catalytic step of an editing cycle. To this end, we used an EMSA, for the first time, in parallel with UV photocrosslinking and gRNA-directed cleavage assays. Importantly, these assays were performed under comparable reaction conditions and the data obtained were complementary. The RNP assemblies detected by EMSA contained adenylylatable ligases and co-immunoprecipitated with known editing subunits (Fig. 1b–d), and their substrate specificity was conserved in the association and catalytic assays (Fig. 1e–g). Our combined EMSA, photocrosslinking, and enzymatic studies defined ssRNA and dsRNA determinants for association and cleavage, summarized in Fig. 10. Three main combinations of ssRNA and dsRNA determinants that supported endonuclease cleavage are represented by the following pairs: Pair 22 (27-nt substrate strand and 23-nt guide strand) exhibits minimal 5′ overhangs and ~15-bp duplex for cleavage. In this context, a 12-nt 5′ overhang in the substrate strand was minimally required, whereas truncations of the 8-nt 5′ overhang in the guide strand were gradually inhibitory. The size of one overhang did not compensate for the size of the other and thus appears to involve separate recognitions. Pair 25, a long substrate strand annealed to a minimal guide strand of 18 nt (16 nt in a duplex), supports efficient cleavage. This confirms the observation by Seiwert *et al.* that an 18-nt guide strand directed endonuclease cleavage of a complementary 73-nt A6 mRNA.[3] Thus, a substrate 3′ overhang can substitute for a guide 5′ overhang. In Pair 6, a long duplex, overrides a requirement for ssRNA rightward of the duplex. Therefore, neither of these rightward overhangs is essential but an ssRNA extension, abutting a short duplex, may suffice. In this type of construct, the size of the substrate 5′ overhang was also tested. Twelve nucleotides or more supported cleavage (e.g., Pair 6 and other data not shown), but 11 nt was inactivating (i.e., Pair 4 and other data not shown). Additional pairs were bound but not cleaved by editing complexes, demonstrating that these two aspects of editing can be uncoupled. Pair 27 is the simplest construct of this kind. Competition studies or direct association assays by crosslinking or EMSA showed that pairs bearing blunt-ended helices or

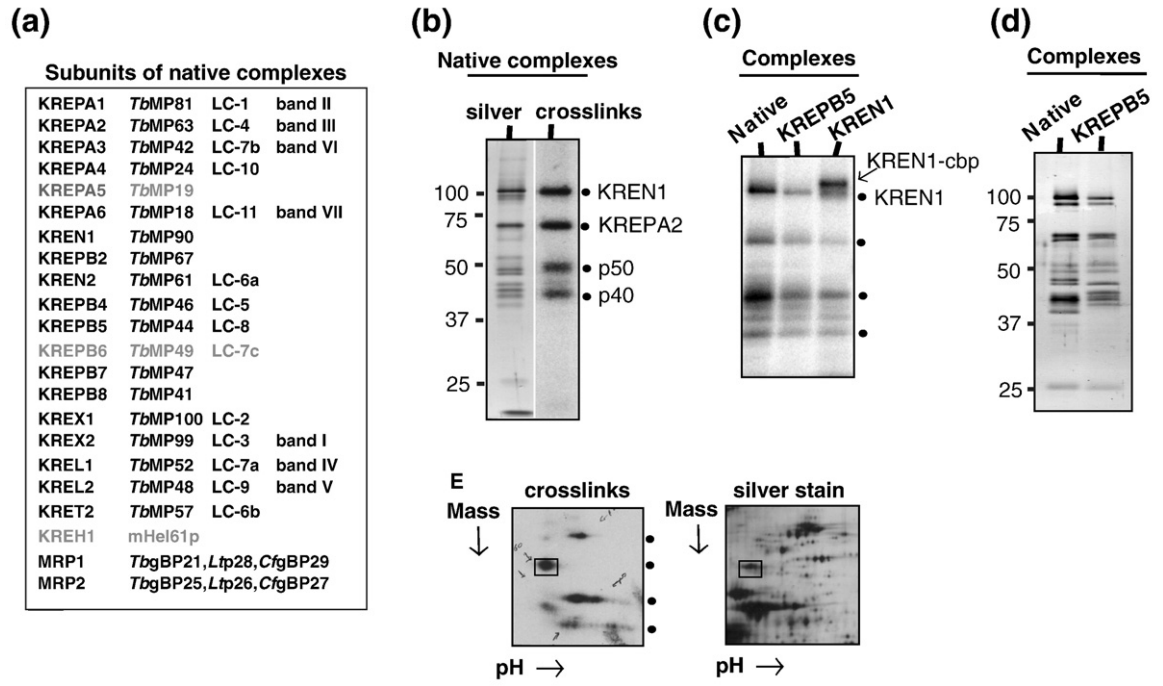

Fig. 8. Composition of native editing complexes and identification of two photocrosslinking subunits: RNase III-type endonuclease KREN1 and structural KREPA2 (MP63). (a) Listing of all subunits detected by mass spectrometry. Alternative nomenclature used in the literature is indicated. Three subunits were not detected (faded). (b) Native editing complexes stained with silver (lane 1) or exposed onto an X-ray film after UV photocrosslinking (lane 2). The crosslinks (dots) by KREN1 and KREPA2 and two more subunits to be identified (p50 and p40) are indicated. (c) Crosslinks by native (lane 1) or affinity-purified KREPB5 (MP44) (lane 2) and KREN1 (lane 3) complexes. Both cbp-tagged (upshift) and endogenous KREN1 are indicted. (d) Silver staining of native and affinity-purified KREPB5 complexes. This panel was prepared using complexes purified during this study (see Methods). Preliminary studies using aliquots from KREN1 and KREN2 complexes characterized in a previous study[31] showed that only the former generate the 100-kDa crosslink (see the text). (e) Two-dimensional gel of partially purified complexes after photocrosslinking (left) or silver staining (right). Crosslinked KREPA2 (boxed) was excised from the gel and identified by mass spectrometry.

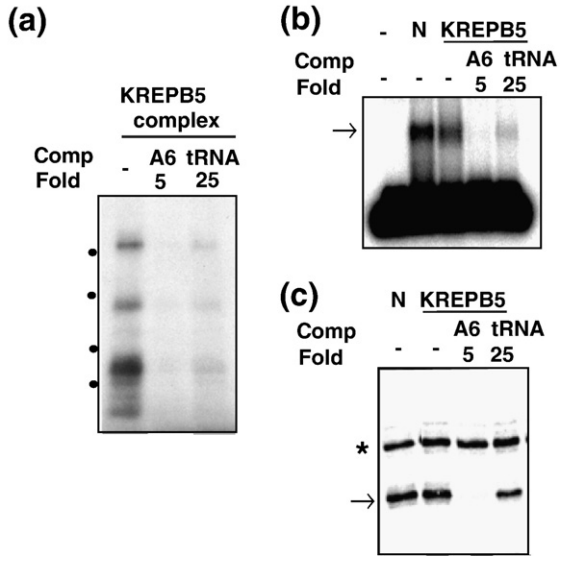

Fig. 9. Association and endonuclease cleavage activity of affinity-purified editing complexes. Parallel (a) photocrosslinking assay, (b) EMSA, and (c) cleavage assay. All labels are as those in Fig. 1. KREPB5-tagged complexes were directly compared with native "N" complexes.

insufficient ssRNA cannot associate with editing complexes. Pair 2 and Pair 28 reproducibly failed to form detectable RNPs, and Pair 3 was significantly less effective than the parental A6 substrate (data not shown). Some constructs that bind but are not cleaved were examined by photocrosslinking or EMSA using 5′-end-labeled rather than more sensitive site-specific labeled RNAs (Fig. 7b and other data not shown. See methods section.).

Together, these constructs, as well as others, examined indicated that an appropriate combination of dsRNA and ssRNA determinants, rather than overall size of the bimolecular structure, is required for both association and endonuclease cleavage by purified editing complexes. The ssRNA requirement (12 nt) 5′ of the scissile bond and the dsRNA/ssRNA combinations 3′ of it seem to involve separate recognitions. The smallest helix tested that directed endonuclease cleavage was 15 bp long (~1.5 helices), but shorter versions similar to Pair 26 may be feasible (Fig. 6c). Although the shortest functional guide strand tested was 18 nt long, the guide strand may be longer than the substrate strand (e.g., Pair 21).

Importantly, the requirements for association and catalysis can be uncoupled. This was shown by Pair 7 (Fig. 2), Pair 27, and A6 thiolated derivatives (Fig. 7) that bind editing complexes but are not cleaved. In

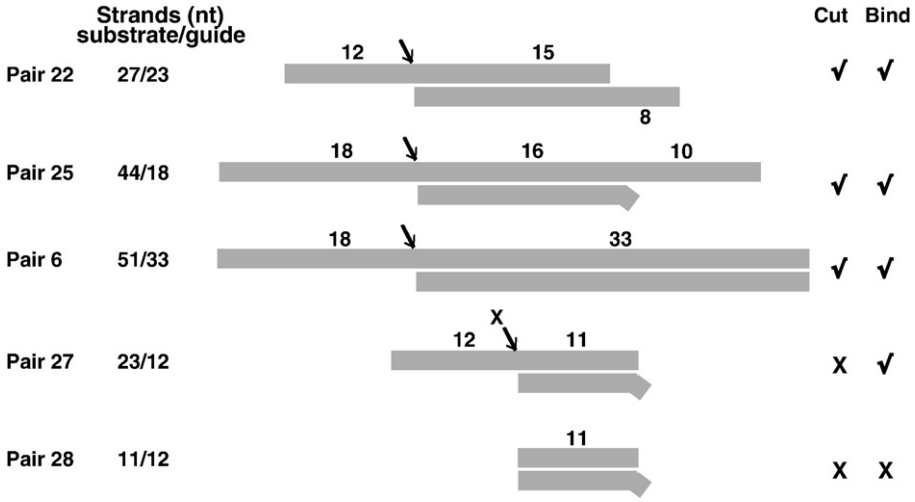

Fig. 10. Summary of defined ssRNA and dsRNA determinants for endonuclease cleavage and association by purified editing complexes. Important variations were observed depending on the secondary structure context. Three main types of cleaved constructs are illustrated by Pair 22: It bears minimal substrate 5′ and guide 3′ overhangs. In this context, further shortening of either element was strongly inhibitory and not rescued by lengthening of the other. Pair 25: Its long substrate strand allowed reducing the guide strand to 18 nt. Thus, a substrate 3′ overhang can substitute for a guide 5′ overhang. Pair 6: Its long duplex can substitute for either substrate 3′ overhang or guide 5′ overhang. Thus, neither of these overhangs is essential but one may suffice in cleaved constructs. Importantly, association can occur without cleavage although it also requires an essential overhang either upstream or downstream of the helix. This is illustrated by Pair 27 and Pair 28. Detected (✓) or undetected (X) binding (bind) and cleavage (cut) are indicated.

Pair 7, the substrate strand forms a 3′ overhang but not a 5′ overhang. Its substrate strand 3′ ssRNA stimulates association (compare with the inactive Pair 2) but, as expected, is not cleaved since editing endonucleases specifically target the phosphodiester bond immediately 5′ of the guiding "anchor" duplex.[3,6] On the other hand, Pair 27 bears the critical 12-nt 5′ overhang, albeit either insufficient duplex or overall length for cleavage. Furthermore, while a substrate-strand 5′ overhang of 12 nt is minimally required for cleavage, whether all residues need to be unpaired or some may partially complement apposing guide-strand residues in a natural-like hybrid was not examined. In full-round editing substrates, single-strandedness of residues near the downstream "anchor" duplex is strongly stimulatory. More distal residues can engage in formation of a proposed upstream "tether" duplex in deletion or insertion *in vitro*.[8,33] Furthermore, the presence and/or the nature of gRNA residues in the internal loop may stimulate full-round editing. Consistent with this idea, the lack or inappropriate number of such residues inhibited full-round deletion and insertion editing[8,33] (and unpublished data), and 2′-deoxy substitutions on the gRNA side of the internal loop inhibited both photocrosslinking and cleavage at the scissile bond.[5]

Previously, our laboratory defined a minimal 43-nt pre-mRNA/gRNA hybrid for efficient full-round editing, which formed 10-bp helices flanking the ES. These nearby helices may be stabilized by coaxial stacking interactions, resembling a continuous helix. Our smallest hybrid identified for endonuclease cleavage activity (including an ~27-nt substrate strand) and even simpler structure for binding imply that editing complexes require gradually increasing RNA contacts from the initial association step to the intermediate cleavage step and to the complete editing cycle. Consistent with this concept, the artificially enhanced A6 parental substrate for full-round editing[8] is more efficient in all EMSA, photocrosslinking, and cleavage studies than most simpler derivatives tested here.

The fact that only one shifted product is reproducibly detected in the EMSA of the constructs examined suggests binding by a single editing complex, whether dimeric or of higher-order composition. A minimal dimeric configuration is consistent with the co-purification of endogenous and ectopically expressed editing subunits—that is, KREN1 in this study (Fig. 8c) and KREL1 in previous studies.[14,32] Mass spectrometric analysis revealed that the native complexes used in this study contain most known subunits of catalytic ~20S editing complexes, as expected from similar biochemical purifications.[34] In addition, we found subunits of the mitochondrial RNA-binding subcomplex as it was reported in purified L-complexes,[25,32] suggesting that at least some of our purified particles represent holoenzyme rather than core complexes.

Several observations lead us to suggest that some, if not all, determinants defined in this study may be recognized by one or more RNase III-type proteins: (a) the shortest duplex tested that directed efficient endonuclease cleavage activity spanned ~1.5 turns (this is also the size of the smallest substrate identified that binds bacterial RNase III[35]); (b) the critical role of 5′ and 3′ overhangs for cleavage at ssRNA–dsRNA junctions by the RNase III family member Drosha[36]; and (c) the fact that KREN1 photocrosslinks with a

site for full-round editing (Fig. 8). This photocrosslink was defined at a deletion site (Fig. 8c) but most likely also corresponds to a co-migrating crosslink at insertion sites.[5] KREN1 endonuclease was proposed to specifically cleave deletion sites[11]; however, since association and cleavage are uncoupled, we propose a model whereby KREN1 and related RNA-binding subunits may help scrutinize potential ligand determinants in the earliest checkpoint of RNA editing. Subsequent to the binding step, catalytic selectivity based on additional specific substrate recognitions may activate either the deletion or insertion enzymes, including proofreading of misedited insertion sites by deletion cycles. A role of REN1 in an early checkpoint of ligand binding may explain why KREN1 downregulation inhibits editing of CYb and COII pre-mRNAs *in vivo*, which only have insertion sites. It is known that bacterial RNase III can undertake a modulatory role as a general dsRNA-binding protein regardless of its catalytic action.[37] Importantly, the crosslinking activities of KREN1, KREPA2 (MP63), and at least two other major crosslinking subunits are conserved in both native and TAP-tagged affinity-purified complexes. Such conservation further suggests that the interactions are relevant and independent of purification protocols and cell lines used. The conserved OB-fold and zinc fingers of KREPA2 may also be involved in recognition of single-stranded determinants defined here.

Finally, while this study shows that RNPs formed by purified editing complexes can be directly visualized, it is currently unclear if the fraction of substrate that remains unbound in association assays reflects the concentration and/or affinity of either total complexes or functional complexes. Also, not all RNPs formed in solution may be stable enough to withstand the forces of gel electrophoresis. These and related questions will be addressed in separate studies.

Methods

Synthesis and labeling of RNA

The ES1-radiolabeled A6 mRNA substrate was prepared by splint ligation as described previously.[24] All other RNAs were synthesized *in vitro* by the Uhlenbeck single-stranded enzymatic method[38] and gel purified.

For the preparation of 5′-end-labeled substrates, gel-purified RNA was dephosphorylated by treatment with alkaline phosphatase at 37 °C for 1 h, followed by addition of SDS, ethylenediaminetetraacetic acid (EDTA), and proteinase K to final concentrations of 1.5%, 5 mM, and 40 μg/mL, respectively, and additional incubation at 50 °C for 30 min. RNA was purified by phenol/chloroform extraction and precipitated with ethanol. A total of 5 pmol of dephosphorylated RNA was incubated at 37 °C for 30 min with [γ-^{32}P]ATP (1:2 ratio of 5′ ends to ATP) and T4 polynucleotide kinase and gel purified. For 3′-end labeling, 5 pmol of gel-purified RNA was incubated at 4 °C for 12 h with an equimolar amount of [5′-^{32}P]cytidine 3′,5′-bis (phosphate) and 15 U of T4 RNA ligase in RNA ligase buffer [50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 μg/mL of bovine serum albumin, 50 μM ATP, 10 mM DTT, 2 U/μL of anti-RNase (Ambion), and 10% DMSO] and gel purified.

Cloning, cell culture, and transfection

Open reading frames were amplified from *T. brucei* genomic DNA, kindly provided by Larry Simpson. The primers for KREN1 were designed as reported previously.[31] For KREPB5, the primers were as follows: forward CCC aagctt ATG AGA CGG GCT GTG GTA CTC CGT AC and reverse CGC ggatcc CCG CCC TCC CAG TGC CAG CGC AAC TA (HindIII and BamHI sites are in small case letters, respectively). The amplified products using Pfu DNA polymerase were treated with HindIII and BamHI and ligated to the pLEW79TAP expression vector, kindly provided by Achim Schnaufer.[31] Constructs were linearized with NotI and used to transfect *T. brucei* strain 29.13 as described previously.[39] Selection of transfectants was applied with 2.5 μg/mL of phleomycin. KREN1 and KREPB5 expression was induced with 100 ng/mL and 1 μg/mL of tetracycline, respectively, and confirmed by immunoblotting with a PAP reagent (Sigma).

Purification and protein composition determination of editing complexes

Chromatographic purification of RNA editing complexes

Mitochondrial extracts were prepared from procyclic *T. brucei* strain TREU667 as described previously.[40,41] Editing complexes were purified from mitochondrial extracts by consecutive anion exchange and DNA affinity chromatography as described previously.[41,42]

Tandem affinity purification of RNA editing complexes

Four liters of culture at a density of $\sim 2.0 \times 10^7$ cells/mL was pelleted and lysed in 25 mL of 10 mM Tris–HCl, pH 8.0, 150 mM KCl, 0.1% NP-40, 1% Triton X-100, and one tablet of EDTA-free complete protease inhibitors (Roche) for 30 min on ice. Lysis was confirmed by microscopy. Lysates were spun at 6000*g* for 15 min, and the clarified extract was purified by sequential IgG and calmodulin affinity chromatography as described previously.[43]

Mass spectrometric analysis of native RNA editing complexes

Proteins in gel bands and complex mixtures were identified by liquid chromatography with tandem mass spectrometry analysis as described previously.[34]

Photocrosslinking, RNA cleavage, EMSA, competition, and adenylylation studies

All assays are variations of the standard editing assay in our laboratory that consists of a mixture of a preannealed mixture of 10 fmol ^{32}P-labeled RNA and 1.25 pmol unlabeled gRNA, completed to 20 μL with MRB [25 mM Tris–HCl, pH 8, 10 mM $Mg(OAc)_2$, 10 mM KCl, 1 mM EDTA, pH 8, 50 μg/mL of hexokinase, and 5% glycerol] and, if applicable, competitor RNA at the indicated molar excess relative to the ^{32}P-labeled substrate. The mixture was preequilibrated for 10 min at 26 °C, and 2 μL of peak editing or TAP fraction was added. Prior to the assays, quantitative annealing of the RNA pairs tested was

confirmed in native gels (as in Ref. 5). The sample was incubated at 26 °C for 10 min and then treated in an assay-specific manner. For crosslinking, samples were irradiated for 10 min under a 365-nm UV lamp, treated with RNase A and RNase T1 (final concentrations of 50 μg/mL and 125 U/mL, respectively) at 37 °C for 15 min, supplemented with SDS loading dye, and loaded onto an SDS polyacrylamide gel. For mRNA cleavage, purified editing complexes were pretreated with 10 mM tetrapotassium pyrophosphate, pH 8, in MRB, for 5 min on ice to inhibit ligase activity[9]; after incubation, the mixture was deproteinized and RNAs were resolved on denaturing polyacrylamide gels. For EMSAs, the reaction mixture was loaded directly (no loading dye) onto 1.5% agarose gel in 0.5× TBE (45 mM Tris–borate and 1 mM EDTA) and run for 2 h at ~5 V/cm at 4 °C. Following electrophoresis, the agarose gel was dried under vacuum. EMSA with site-specific labeled transcripts was significantly more sensitive and reproducible than with end-labeled substrates, since the splint-ligation method used to generate the former (see above) exclusively incorporates phosphorylated fragments. Only the parental A6 substrate and Pair 27 were site-specifically labeled using synthetic donor fragments (e.g., as in Fig. 1),[24] although 5′-end-labeled A6 parental and other constructs were also compared side by side in shift assays. Immunodepletions were carried out as described previously for the immunoprecipitation of RNA crosslinking proteins[24] using a monoclonal antibody against KREPA2 immobilized on goat anti-mouse IgG resin (Dynal). Adenylylation assays were performed as described previously.[30] All assays can be scaled up linearly to enhance signal. The data were reproducible in at least two independent experiments. Each experiment included repeat assays, and those shown are representative. Data were visualized by phosphorimaging and/or autoradiography.

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