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Structures of a key interaction protein from the *Trypanosoma brucei* editosome in complex with single domain antibodies

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ABSTRACT

Several major global diseases are caused by single-cell parasites called trypanosomatids. These organisms exhibit many unusual features including a unique and essential U-insertion/deletion RNA editing process in their single mitochondrion. Many key RNA editing steps occur in ~20S editosomes, which have a core of 12 proteins. Among these, the "interaction protein" KREPA6 performs a central role in maintaining the integrity of the editosome core and also binds to ssRNA. The use of llama single domain antibodies (VHH domains) accelerated crystal growth of KREPA6 from *Trypanosoma brucei* dramatically. All three structures obtained are heterotetramers with a KREPA6 dimer in the center, and one VHH domain bound to each KREPA6 subunit. Two of the resultant heterotetramers use complementarity determining region 2 (CDR2) and framework residues to form a parallel pair of beta strands with KREPA6 – a mode of interaction not seen before in VHH domain–protein antigen complexes. The third type of VHH domain binds in a totally different manner to KREPA6. Intriguingly, while KREPA6 forms tetramers in solution adding either one of the three VHH domains results in the formation of a heterotetramer in solution, in perfect agreement with the crystal structures. Biochemical solution studies indicate that the C-terminal tail of KREPA6 is involved in the dimerization of KREPA6 dimers to form tetramers. The implications of these crystallographic and solution studies for possible modes of interaction of KREPA6 with its many binding partners in the editosome are discussed.

1. Introduction

Trypanosomatids are a group of unicellular eukaryotic organisms of which several members are the causative agents of major human diseases. These include: (i) *Trypanosoma brucei*, causing human trypanosomiasis, also called sleeping sickness, with ~60 million people at risk for acute infections in sub-Saharan Africa; *Trypanosoma cruzi*, responsible for Chagas disease, also called American trypanosomiasis, with ~25 million people at risk in Latin America, and (iii) various *Leishmania* species, which cause cutaneous and disseminated leishmaniasis in the tropics and subtropics with ~350 million people in danger of infection (http://www.who.int/health-topics/idindex.htm, http://www.cdc.gov/chagas/factsheet.html). The available drugs for these tropical parasitic diseases are limited by poor efficacy, toxicity, and/or increasing resistance (Croft et al., 2006; Fairlamb, 2003; Hotez et al., 2007; Tarleton et al., 2007). Vaccines have been difficult to develop for these parasites due to their ability to undergo antigenic variation and thereby evade the immune response (Smith and Parsons, 1996). Hence, there is a tremendous need for new therapeutic agents to treat the diseases caused by these pathogenic protozoa.

Trypanosomatids contain a substantial number of unusual and essential biochemical characteristics, several of which are currently being explored as targets for new chemotherapeutics (De Souza, 2002; Hammarton, 2007; Moyersoen et al., 2004). One of the most remarkable features of these organisms is a very elaborate U-insertion/deletion RNA editing process of most messenger

Abbreviations: CDR, complementarity determining region; gRNA, guide RNA; KREL, kinetoplastid RNA-editing ligase; KREPA6, kinetoplastid RNA-editing interaction protein A6; KRET2, kinetoplastid RNA-editing TUTase 2; KREX2, kinetoplastid RNA-editing 3′ → 5′-exonuclease 2; OB, oligonucleotide binding ; TEV, tobacco etch virus; Nb, nanobody; Ni-NTA, nickel-nitrilotriacetic acid; SSB, single strand DNA-binding protein; TUTase, 3′ terminal uridylyltransferase; VHH domains, variable domain of heavy chain llama antibodies.

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RNAs transcribed from genes encoded on the mitochondrial DNA of these organisms (Panigrahi et al., 2003; Schnaufer et al., 2003; Simpson et al., 2004; Stuart et al., 2005). In this editing process, a so-called pre-messenger RNA (pre-mRNA) is edited in a large series of steps according to sequence information from relatively small RNA's, called guide RNA's (gRNA's). Guided by information from many different gRNA's, many more U's are inserted than deleted by the editosome into the mRNA.

This U-insertion/deletion RNA editing process in the mitochondria of trypanosomatids requires a large number of proteins that are encoded on nuclear DNA. Once inside the mitochondrion, many of these proteins assemble into several large multi-protein complexes (Aphasizhev et al., 2003; Lukes et al., 2005; Rusche et al., 1997; Weng et al., 2008). One of these complexes is the ~20S editosome complex, hereafter called the editosome (reviewed in Simpson et al. (2004) and Stuart et al. (2005)). Recent electron microscopy studies have revealed an elongated shape of the editosome with dimensions of ~80 by ~140 by ~200 Å (Golas et al., 2009; Li et al., 2009). Evidence has been provided for the presence of three different types of editosomes that share a common core of 12 proteins (Panigrahi et al., 2006; Carnes et al., 2005, 2008) (Supplementary Fig. 1(a)). Multiple nomenclatures for the proteins in the editosome are summarized in Supplementary Fig. 1(b). The editosome core contains a large number of proteins which can be grouped as follows (Supplementary Fig. 1):

(i) The four enzymes KREX2, KRET2, KREL1 and KREL2. The enzyme KREX2 is a 3′ → 5′-exonuclease which removes U's from the cleaved pre-mRNA (Ernst et al., 2009). KRET2 is a 3′ terminal uridylyltransferase (TUTase) adding U's to the cleaved pre-mRNA. The crystal structure of *T. brucei* KRET2 revealed the structural basis of its U-specificity (Deng et al., 2005). KREL1 and KREL2 are two related RNA editing ligases which seal the mRNA after removal or addition of U's (Deng et al., 2004).

(ii) Two proteins with an RNase III-like domain, KREPB4 and KREPB5, which are most likely critical for linking the editosome core with the two or three specific extra proteins per type of editosome (Carnes et al., 2008).

(iii) Six so-called "interaction proteins", KREPA1 to KREPA6. These six proteins vary greatly in length, yet each contains a predicted "OB-fold" near the C-terminus (Brecht et al., 2005; Drozdz et al., 2002; Kang et al., 2004; Law et al., 2007, 2008; Panigrahi et al., 2006, 2001; Salavati et al., 2006; Schnaufer et al., 2003; Worthey et al., 2003). A key interaction protein is KREPA6, the three-dimensional architecture of which is the focus of the current paper.

Trypanosomatid KREPA6 contains 164–229 amino acids depending on species (Fig. 1(a)) and is a remarkable multifunctional protein, central to the integrity of the entire editosome (Tarun et al., 2008). It interacts with four other proteins: KREPA1, KREPA2, KREPA3 and KREPA4 (Schnaufer et al., 2003, 2010) (Supplementary Fig. 1(a)). In addition, KREPA6 interacts with ssRNA (Tarun et al., 2008). KREPA6 belongs to the large single strand nucleic acid-binding superfamily of OB-folds (Worthey et al., 2003), hereafter also called SSB domains and SSB proteins (Arcus, 2002; Murzin, 1993; Theobald et al., 2003). In eukaryotes, SSB-domains tend to be incorporated into multi-domain proteins (Bochkarev and Bochkareva, 2004; Bochkareva et al., 2002; Horvath et al., 1998; Yang et al., 2002) and participate in highly variable manners in several key cellular processes. In mitochondria and prokaryotes, OB-folds occur in SSB proteins which are involved in DNA repair, recombination, and replication (Anderson and Kowalczykowski, 1998; Chase and Williams, 1986; Eggington et al., 2004; Lohman et al., 1988; Meyer and Laine, 1990). Mitochondrial and prokaryotic SSBs are generally encoded as one OB-domain containing polypeptide chains. Some of these exist as single domain proteins in solution (Kerr et al., 2003). However, most form dimers via an anti-parallel pair of β-strands at the interface, and usually two such dimers are arranged as tetramers with D2 symmetry. Examples are *Escherichia coli* SSB (EcSSB) (Raghunathan et al., 1997, 2000) and *Helicobacter pylori* SSB (HpSSB) (Chan et al., 2009). The so-called P-dyad of the canonical SSB tetramer is shared by the two dimers (Saikrishnan et al., 2005), but a considerable variation occurs regarding the orientation of the two dimers with respect to each other (Chan et al., 2009; DiDonato et al., 2006; Jedrzejczak et al., 2006; Raghunathan et al., 1997; Saikrishnan et al., 2003, 2005; Yang et al., 1997). An interesting case regarding multimerization is *E. coli* PriB (EcPriB) which can form typical SSB-dimers (Liu et al., 2004; Lopper et al., 2004; Shioi et al., 2005) and tetramers (Shioi et al., 2005). Hence, SSB proteins have been shown to be able to exist as monomers, dimers and tetramers.

Here we describe crystal structures of *T. brucei* KREPA6 in complex with three different single domain antibodies (VHH domains) derived from llamas (Desmyter et al., 2002; Goldman et al., 2006; Muyldermans, 2001). The use of VHH domains as crystallization chaperones was critical in obtaining well-diffracting crystals of KREPA6 in complex with VHH domains. Two of the complexes showed that CDR2 and framework 3 of the VHH domain form a β-strand which engages in parallel pairing with a β-strand of KREPA6, a mode of interaction not seen before in protein-VHH domain complexes to the best of our knowledge. Surprisingly, all three VHH domains exhibited the capacity in solution to break the KREPA6 tetramer and form $(KREPA6)_2$:$(VHH\text{-}domain)_2$ heterotetramers. The hypothesis that the C-terminal tail of KREPA6 might be involved in KREPA6 tetramer formation was confirmed by solution studies. The implications of these findings for the possible modes of interactions of KREPA6 with other proteins in the core of the editosome are discussed.

2. Materials and methods

2.1. KREPA6 cloning, expression and purification

The gene fragment corresponding to KREPA6 (residues 19–164; the first 18 amino acids are the predicted mitochondrial import signal (Tarun, 2008)) was PCR amplified from genomic DNA of *T. brucei* and cloned into pRSF vector (Invitrogen) for expression with an N-terminal hexahistidine tag followed by a TEV protease cleavage site. KREPA6 with an N-terminal cleavable His-tag was expressed in *E. coli* BL21(DE3). Further details are provided in Supplementary material.

2.2. Single domain antibody selection, cloning, expression and purification

2.2.1. Nanobody generation

A llama was immunized six times with 330 μg of purified recombinant KREPA6/KREL1/KREPA2 protein complex over a period of 6 weeks. From the anti-coagulated blood of the immunized llama, lymphocytes were used to prepare cDNA which served as template to amplify genes coding for the variable domains of the heavy-chain antibodies. The procedures were essentially as described elsewhere (Lam et al., 2009) and summarized in Supplementary material.

2.2.2. Solid-phase ELISA

Maxisorb 96-well plates (Nunc) were coated with 100 μl purified *T. brucei* KREPA6 overnight at 4 °C at 1 μg/ml in sodium bicarbonate buffer pH 8.2. Residual protein binding sites in the wells