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Trypanosoma evansi: Identification and characterization of a variant surface glycoprotein lacking cysteine residues in its C-terminal domain

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ABSTRACT

African trypanosomes are flagellated unicellular parasites which proliferate extracellularly in the mammalian host blood-stream and tissue spaces. They evade the hosts' antibody-mediated lyses by sequentially changing their variant surface glycoprotein (VSG). VSG tightly coats the entire parasite body, serving as a physical barrier. In *Trypanosoma brucei* and the closely related species *Trypanosoma evansi, Trypanosoma equiperdum*, each VSG polypeptide can be divided into N- and C-terminal domains, based on cysteine distribution and sequence homology. N-terminal domain, the basis of antigenic variation, is hypervariable and contains all the exposed epitopes; C-terminal domain is relatively conserved and a full set of four or eight cysteines were generally observed. We cloned two genes from two distinct variants of *T. evansi*, utilizing RT-PCR with VSG-specific primers. One contained a VSG type A N-terminal domain followed a C-terminal domain lacking cysteine residues. To confirm that this gene is expressed as a functional VSG, the expression and localization of the corresponding gene product were characterized using Western blotting and immunofluorescent staining of living trypanosomes. Expression analysis showed that this protein was highly expressed, variant-specific, and had a ubiquitous cellular surface localization. All these results indicated that it was expressed as a functional VSG. Our finding showed that cysteine residues in VSG C-terminal domain were not essential; the conserved C-terminal domain generally in *T. brucei* like VSGs would possibly evolve for regulating the VSG expression.

1. Introduction

On the cell surface of *Trypanosoma brucei* and the closely related species *Trypanosoma evansi* and *Trypanosoma equiperdum*, variant surface glycoprotein (VSG) is by far the most abundant membrane protein, which accounts for more than 95% of the externally disposed cell surface protein, and is closely packed as a dense coat protecting the underlying invariant surface proteins from host's immune system (Turner, 1985). VSG itself is highly immunogenic and elicits specific, trypanocidal immune responses. During the course of infection, the parasites spontaneously express a series of immunologically distinct VSGs, thus allows them to keep ahead of the host immune system and to maintain a long-term infection (reviewed in Barry and McCulloch (2001), Donelson (2003) and Taylor and Rudenko (2006)).

VSG is translated with an N-terminal signal peptide and a C-terminal GPI-anchor-adding sequence. The former signal peptide guides the nascent polypeptide into the endoplasmic reticulum (ER), where the GPI anchor-adding sequence is replaced with a preformed GPI anchor through which VSG molecules are attached to the cell membrane. Mature VSG polypeptide has 350–400 amino acids with a molecular mass of 45–55 kDa (Carrington et al., 1991; Johnson and Cross, 1979). Most part of the sequence forms a single N-terminal domain of 350–400 residues, followed by one or two smaller C-terminal domains of 50–100 residues each. Based on conserved cysteine distribution, N-linked glycosylation sites, C-terminal residue and GPI-anchor-adding sequence, VSGs are grouped as three types of N-terminal domain (types A, B, and C) and six types of C-terminal domains (types 1–6) (Berriman et al., 2005; Carrington et al., 1991; Marcello and Barry, 2007). There is no apparent restriction on N- and C-terminal domain combination and the same N-terminal domain type can be functionally combined with different C-terminal domain type (Hutchinson et al., 2003).

The hypervariable N-terminal domain, which is the basis of antigenic variation, contains all the exposed epitopes; the C-terminal domain, hidden beneath the N-terminal domain in a living trypanosome, is relative conserved. Except for providing a GPI-anchor linking site, the role of N-terminal domain remains unclear. A full set of four or eight cysteines were generally observed in the expressed VSGs and were thought to be required for C-terminal domain correct folding (Chattopadhyay et al., 2005; Jones et al., 2008).

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In this study, we cloned and sequenced two cDNA sequences from two variants derived from *T. evansi* YNB stock, utilizing VSG-specific primers. One deduced amino acid sequence possessed the conserved cysteine residues near the mature VSG N-terminal domain, but, surprisingly, did not contain any cysteine residues in its C-terminal domain. The other shared all the conserved features of VSGs. We further characterized the corresponding gene of the first cDNA sequence encoding a functional VSG. Our finding indicated that cysteine residues in VSG C-terminal domain were not essential to maintain the function of VSGs. The predominant existence of VSG C-terminal domain, with a full set of four or eight cysteines, would possible evolve to regulate the VSG expression in trypanosomes.

2. Materials and methods

2.1. Animals, trypanosome cloning and parasites purification

Female ICR mice, male Wistar rat and lop-eared rabbit were obtained from the Center of Experimental Animals, Peking University Health Science Centre. They were fed and watered *ad libitum*. All experiments complied with current laws of China in which they were performed. Mice were immunosuppressed with cyclophosphamide treated (0.3 g/kg body weight; sigma) 24 h before trypanosome infection.

All trypanosome used in this study was derived from *T. evansi* YNB stock, originally isolated from a naturally infected buffalo in Yunnan province, China in 1987. This stock was kindly provided by Dr. Shen (Shanghai Institute of Animal Parasitology, Chinese Academy of Agricultural Sciences, Shanghai, China) and was passaged several times in mice in our lab before cloning (Yang et al., 2007).

ICR mice were initially injected with cryopreserved *T. evansi* YNB stock. When parasitemia reached peak ($\sim 10^8$ per ml), mice were sacrificed and single cell was picked optically and was expanded in cyclophosphamide treated (CY) mice by the triple-cloning procedure (Rosen et al., 1981). Following two expansions in CY mice, a clone population was obtained and was designated as YNBc1.

To obtain a second clone expressing a different VSG, a Wistar rat was injected with approximately 10^3 trypanosomes of YNBc1. The parasitemia in the rat was monitored daily and was allowed to relapse only once. Once a second peak (first relapse population) was detected, parasites were directly cloned as above (the clone population was designated as YNBc2). Both clone populations were cryopreserved as stabilates. Trypanosomes were expanded in CY mice and were purified from blood by DEAE cellulose chromatography, as described previously (Lanham and Godfrey, 1970). The purified parasites were used immediately.

2.2. VSG amplification and cloning

Total RNA was extracted from the trypanosomes ($\sim 10^8$) with Trizol reagent (Invitrogen), according to the manufacturer's instructions. cDNA was synthesized using oligodT and a RevertAid First Strand cDNA synthesis kit (MBI Fermentas). The subsequent PCR reaction were performed using Phusion High-Fidelity DNA polymerase kit (Finnzymes Oy.) with the following VSG-specific primers: 5′-GACTAGTTTCTGTACTAT-3′ (binds to splice leader) and 5′-CCGGGTACCGTGTTAAAATATATC-3′ (binds to a conserved sequence found in the 3′UTR of all VSGs) (Boothroyd et al., 2009). The reaction conditions were optimized and carried out as follow: 30 s of initial denaturation at 98 °C, followed by 28 cycles of 7 s of denaturation at 98 °C, 15 s of annealing at 50 °C, 25 s of extension at 72 °C, and a final 7 min final extension at 72 °C. RT-PCR product of the expected size ($\sim$1.5 kb) was gel recovered, cloned into a cloning vector (pEASY-Blunt, TransGen). Two independent clones of the RT-PCR product were sequenced both strands with the M13 universal primers.

2.3. Cloning and expression of a recombinant rYNBc1 in Escherichia coli

To test the prediction that the putative YNBc1 gene encoded a functional VSG protein, the recombinant YNBc1 (rYNBc1) was expressed in *E. coli*. Primers EP006 (5′-CGCGGATCCATGCTAACAGTG CTCTTAG-3′ BamHI site underlined) and EP007 (5′-CCGCTCGA GTACTGTGGTGTTTGTGGC-3′ XhoI site underlined) were designed to amplify the region encoding the N-terminal region (first 445 amino acids). The PCR product was digested with BamH I and XhoI, gel purified and ligated into the BamHI and XhoI sites of the expression vector pGEX 4T-1 (Pharmacia Biotech) to create a fusion protein with glutathione *S*-transferase (GST). The ligation mixture was first transformed into the DH5α competent cells to amplify the plasmid. Before transformation into the BL21 (DE3) expression cells for the protein expression, the correct construction of the plasmid was confirmed by sequencing. GST-YNBc1 fusion protein was induced and purified using Glutathione Sepharose 4B according to the protocol provided with the GST Gene Fusion System (Pharmacia Biotech).

2.4. Antibody production

Antisera against GST-fusion protein were produced in a 2.50 kg male lop-eared rabbit. The primary inoculation was intradermal with GST fused protein in Freund's complete Adjuvant (Sigma), followed by four subcutaneous inoculations at 2-week intervals with the fused protein in Freund's incomplete Adjuvant (Sigma). The rabbit was exsanguinated 10 days post final boost and the increase of antibody titer was controlled by ELISA test.

2.5. SDS–PAGE and western blot analysis

Crude lysates of 1×10^5 parasites were boiled in loading buffer (6.25 mM Tris–HCl pH 6.8, 2% SDS, 10% sucrose) for 5 min and then loaded per lane of a 12% SDS–PAGE gels. Following electrophoresis, separated proteins were visualized by Coomassie Blue staining or transferred onto Polyvinylidene fluoride (PVDF) membranes (immobilon –P, Millipore) for Western blot analysis.

For Coomassie Blue staining, gels were soaked in a solution of 0.2% Coomassie Brilliant Blue R250 (Amresco), 50% methanol and 10% glacial acetic acid for 1 h with shaking. The gels were then destained in 12% ethanol/7% glacial acetic acid. For Western blotting, proteins transferred onto PVDF membranes were blocked with 5% (w/v) skimmed milk powder in PBST (PBS/0.1% (v/v) Tween-20) for 1 h at room temperature before incubation with rabbit anti-rYNBc1 antisera (dilution 1:2000). After washing in TBST, the membrane was incubated with 1:2000 diluted goat anti-rabbit IgG HRP-labled secondary antibody (Signalway Antibody, Pearland, TX, USA) for 1 h. Proteins were visualized with ECL chemiluminescence reagents (Applygen Technologies Inc., Beijing, China).

2.6. Immunofluorescent staining

Immunofluorescent staining was performed as previously described (Miller and Turner, 1981), all the procedure was carried out on ice to reduce parasites' rapid endocytosis of VSG-immunoglobulin complexes (O'Beirne et al., 1998; Pal et al., 2003). Fresh purified parasites were diluted in ice-cold trypanosome dilution buffer (TDB) (Cross, 1975) to 10^7/ml and 100 μl aliquots of parasite suspension with equal volumes of rabbit anti-rYNBc1 antisera (diluted 1:100 in ice-cold TDBGF (TDB containing 10% glycerol and 10% fetal bovine serum)). Following incubating for 30 min on ice, parasites were immediately fixed with 4% formaldehyde in TDBGF