

# Mutational analysis of the variant surface glycoprotein GPI-anchor signal sequence in *Trypanosoma brucei*

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## Summary

The variant surface glycoproteins (VSG) of *Trypanosoma brucei* are anchored to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. All GPI-anchored proteins are synthesized with a C-terminal signal sequence, which is replaced by a GPI-anchor in a rapid post-translational transamidation reaction. VSG GPI signal sequences are extraordinarily conserved. They contain either 23 or 17 amino acids, a difference that distinguishes the two major VSG classes, and consist of a spacer sequence followed by a more hydrophobic region. The  $\omega$  amino acid, to which GPI is transferred, is either Ser, Asp or Asn, the  $\omega+2$  amino acid is always Ser, and the  $\omega+7$  amino acid is almost always Lys. In order to determine whether this high conservation is necessary for GPI anchoring, we introduced several mutations into the signal peptide. Surprisingly, changing the most conserved amino acids, at positions  $\omega+1$ ,  $\omega+2$  and  $\omega+7$ , had no detectable effect on the efficiency of GPI-anchoring or on protein abundance. Several more extensive changes also had no discernable impact on GPI-anchoring. Deleting the entire

23 amino-acid signal sequence or the 15 amino-acid hydrophobic region generated proteins that were not anchored. Instead of being secreted, these truncated proteins accumulated in the endoplasmic reticulum prior to lysosomal degradation. Replacing the GPI signal sequence with a proven cell-surface membrane-spanning domain reduced expression by about 99% and resulted not in cell surface expression but in accumulation close to the flagellar pocket and in non-lysosomal compartments. These results indicate that the high conservation of the VSG GPI signal sequence is not necessary for efficient expression and GPI attachment. Instead, the GPI anchor is essential for surface expression of VSG. However, because the VSG is a major virulence factor, it is possible that small changes in the efficiency of GPI anchoring, undetectable in our experiments, might have influenced the evolution of VSG GPI signal sequences.

Key words: Glycosylphosphatidylinositol anchor, GPI, Mutation, Signal sequence, *Trypanosoma brucei*

## Introduction

Many eukaryotic cell surface proteins, including receptors, adhesion molecules, differentiation antigens and enzymes, are attached to the lipid bilayer via a glycosylphosphatidylinositol (GPI) anchor (Cross, 1990; Englund, 1993; Udenfriend and Kodukula, 1995a; Udenfriend and Kodukula, 1995b; Ferguson, 1999), whose structure was first determined for the variant surface glycoprotein (VSG) of *Trypanosoma brucei* (Ferguson et al., 1988). GPI-anchored surface proteins are particularly abundant in pathogenic protozoa (Ferguson, 1999).

Proteins destined to be GPI-anchored are translated with cleavable N- and C-terminal signal peptides. The N-terminal signal peptide directs the nascent polypeptide to the endoplasmic reticulum (ER) (Walter et al., 1984). The C-terminal signal peptide is replaced by a preformed GPI anchor in an immediately post-translational transamidation reaction catalyzed by an ER protein complex containing at least four distinct proteins: GPI8, GAA1, GPI16/PIG-T and GPI17/PIG-S (Benghezal et al., 1996; Meyer et al., 2000; Ohishi et al., 2000; Sharma et al., 2000; Fraering et al., 2001; Ohishi et al., 2001; Vidugiriene et al., 2001). In all of the GPI-anchored proteins, the C-terminal signal peptide has certain conserved features. It consists of a hydrophilic spacer sequence of 8-12 amino acids, followed by a more hydrophobic region of 8-20

amino acids. Mutational analyses of the C-terminal GPI signal sequences of placental alkaline phosphatase (Berger et al., 1988; Kodukula et al., 1992; Lowe, 1992), 5'-nucleotidase (Furukawa et al., 1994; Furukawa et al., 1997), decay accelerating factor (Caras, 1991; Moran and Caras, 1991a; Moran and Caras, 1991b), CD46 (Coyne et al., 1993), acetylcholinesterase (Bucht and Hjalmarsen, 1996; Bucht et al., 1999) and other proteins have demonstrated that the length of the hydrophobic region and the spacer sequence are important for GPI anchoring. The site of GPI attachment is called the  $\omega$  site (Micanovic et al., 1990). Comparison of known and predicted GPI addition sites suggest that the  $\omega$  site is restricted to six amino acids with small side chains, namely (in order of predominance) Ser > Asn > Asp > Gly, Ala and Cys, whereas  $\omega+2$  can be Ala > Gly > Ser, Thr and Val. The  $\omega+1$  position, where Ala > Ser > Asp > Thr, Arg, Cys, Met, Trp, is less restricted. Mutational studies on several mammalian GPI signal sequences, especially on placental alkaline phosphatase (Gerber et al., 1992; Kodukula et al., 1992; Kodukula et al., 1993; Udenfriend and Kodukula, 1995a), have examined the consequences of substituting about 10 of the possible 20 amino acids at the  $\omega+1$  and  $\omega+2$  sites. Only Ala or Gly functioned efficiently at the  $\omega+2$  position. Of the 10 amino acids tested at the  $\omega+1$  site, only Pro did not

function. Whether a protein will be GPI anchored can be predicted, with  $\geq 80\%$  accuracy, either by manual inspection or by computer algorithms on the basis of whether an N-terminal signal sequence can be predicted (von Heijne, 1986; Nielsen et al., 1997; Nielsen et al., 1999), then whether the C-terminus contains the expected sequence motifs for GPI anchoring, as defined by 'linear' sequence considerations derived from experimental studies (Julien Kronegg internet site <http://dgpi.pathbot.com>) or using a 'knowledge-based algorithm' based on sequence properties extracted from a set of known GPI-anchored proteins and mutants thereof (Eisenhaber et al., 1998; Eisenhaber et al., 1999). Theoretical considerations also suggested (Eisenhaber et al., 1998) that the region immediately upstream of the  $\omega$  site, which is not so conserved but is generally unstructured and hydrophilic, could influence GPI addition, a possibility that has not been experimentally tested. However, this region is highly structured in many mature VSGs, and GPI anchoring probably precedes folding and disulphide bonding of the region immediately upstream of the  $\omega$  site.

VSG comprises about 10% of total cellular protein, making *T. brucei* an excellent model for studying GPI anchoring and a potential expression system for high-value medically important mammalian GPI-anchored proteins, which have sometimes proved difficult to express in more conventional cell systems (Azzouz et al., 2000). In comparison with other GPI-anchored proteins, the VSG GPI signal sequence is remarkably conserved. The  $\omega$  position is always Ser, Asp or Asn, and the length of the signal sequence is either 17 ( $\omega$  Ser) or 23 ( $\omega$  Asp) amino acids. Two positions in the spacer sequence are also remarkably conserved.  $\omega+2$  is always Ser, and  $\omega+7$  is almost always Lys. This high conservation suggested that even a modest change in one of these amino acids would have an impact on GPI anchoring. The development of tools for reverse genetics in *T. brucei* (Wirtz et al., 1998; Wirtz et al., 1999) allowed us to initiate mutational studies of the VSG GPI signal sequence. Attempts to use various non-trypanosomal reporter proteins, including potentially GPI-anchored forms of placental alkaline phosphatase, green fluorescent protein and *Saccharomyces cerevisiae* prepro- $\alpha$ -factor, were frustrated by extremely low levels of expression, which are at least partly attributable to rapid degradation of these alien proteins (K. P. Davies, M. Engstler, U.B. and G.A.M.C., unpublished; U. Böhme, Ph.D. Thesis, Universität Tübingen). We therefore turned to expressing mutants of VSG itself, in cells that concomitantly expressed a different wild-type VSG, as VSG expression is essential for *T. brucei*

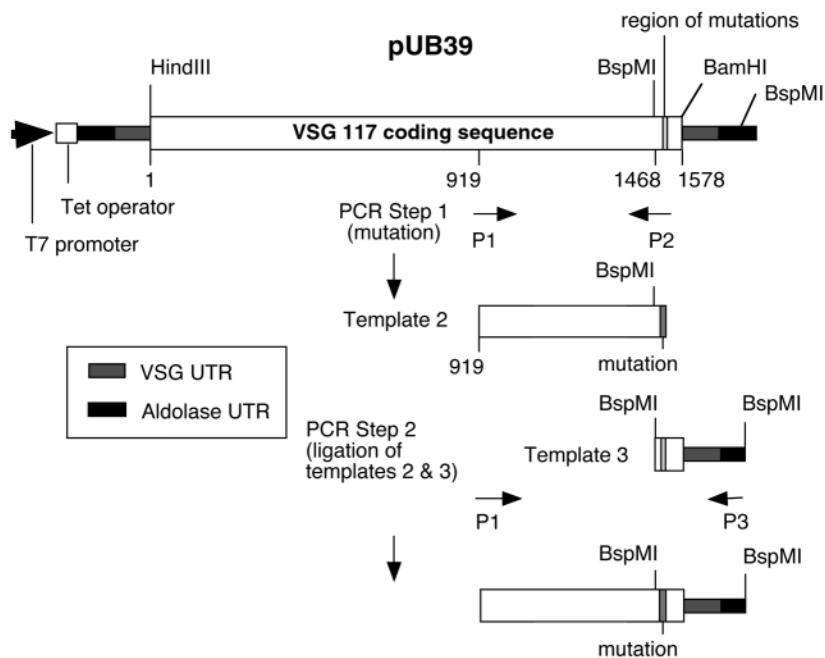
(V. B. Carruthers and G.A.M.C., unpublished) (Nagamune et al., 2000). We introduced various mutations into the 23 amino-acid C-terminal GPI signal peptide of VSG 117. In case of toxicity, the mutations were expressed under tetracycline regulation (Wirtz et al., 1998; Wirtz et al., 1999) in cells expressing wild-type VSG 221.

## Materials and Methods

### Generation of C-terminal mutations

All mutations (Table 1) were introduced by PCR and verified by standard sequencing methods (Protein/DNA Technology Center, The Rockefeller University). As an example, the procedure for introducing point mutations (Fig. 1) is described in detail for the S to G mutation at  $\omega+2$ . In the first PCR reaction, the mutation was introduced into VSG 117 with the forward primer (P1) 5'-TCCGGAGATGGCTGAAATACTGAAACTA-3' and reverse primer (P2) 5'-CTAGA-ATCCGGAATCTTTGCAA-3'. The second PCR reaction used the first PCR product and a *Bsp*MI fragment of pUB39 or pT7-VSG117 as templates. pUB39 is a derivative of the TetR- (*Tn10*-encoded tetracycline-responsive repressor) regulated T7-promoter-driven expression plasmid, pLew82 (Wirtz et al., 1998), containing the VSG 117 coding region separated from its 5' and 3' untranslated regions by *Hind*III and *Bam*HI sites, respectively. pT7-VSG117 is identical to pUB39 except for the absence of these *Hind*III and *Bam*HI sites. These untranslated regions are themselves flanked by *T. brucei* *ACTIN* untranslated regions derived from the parent expression plasmid pLew82. pUB39 allows two to threefold higher protein expression than pLew82. The forward primer used in this second PCR reaction was P1 and the reverse primer (P3) was 5'-CCAACCTGCAGGC-GCACCTCCCT-3'. The PCR-product was cut with *Bsp*MI and inserted into *Bsp*MI-digested plasmid pUB39 or pT7-VSG117. Because the product of the second PCR is a mixture of mutated and unmutated sequences, several clones were sequenced to identify the desired mutant derivative.

A series of deletions was also created by PCR. The forward primer for all deletions (P4) was 5'-CGAAGCTTATGGACTGCCATACAA-3', corresponding to the N-terminus of VSG 117. Specific reverse primers were used for each truncation: 5'-CGGGATCCTTTATCT-TTGAAGCATTATTT-3' for  $\Delta 504-526$ ; 5'-CGGGATCCTTATTC-



**Fig. 1.** Procedure for introducing mutations into the VSG 117 C-terminal GPI signal sequence. In the first PCR reaction, the mutation was introduced into VSG 117 with the wild-type and mutant primers P1 and P2. The second PCR reaction used the first PCR product (template 2) and a *Bsp*MI fragment (template 3) as templates. The forward primer in this reaction was P1 and the reverse primer P3. Because the final PCR product is a mixture of unmutated and mutated sequence, several plasmids were subjected to sequencing to identify the desired mutant derivative.

TTGGTTACTAGAATA-3' for  $\Delta$ 512-526; 5'-CGGGATCCTTAA-GCAGAAACCACGGTGAGG-3' for  $\Delta$ 519-526; 5'-CGGGATCCTTAAAAAAGCAAGGCCACAAATGCAGCAGAAACCACGGT-GAGGCGCAATAGAATAGAGGAATC-3' for  $\Delta$ 515-520; 5'-CGG-GATCCTTAAAAAAGCAAGGCCACAAATGCAGCAGAAACCA-CGGTGAGGCGCAAATCTTTGCAAGCATT-3' for  $\Delta$ 504-511. The PCR-products were cut with *Hind*III and *Bam*HI and inserted into *Hind*III- and *Bam*HI-digested pUB39.

To create VSG117TM, the transmembrane and cytoplasmic domain of a *T. brucei* 65 kDa invariant surface glycoprotein (ISG65) (Ziegelbauer et al., 1992) was amplified with the forward primer 5'-CGGGATCCGCAATGATTATATTAGCAG-3' and the reverse primer 5'-CGGGATCCTTACATTACCGCCTTTCCA-3'. We used plasmid p3'CRAM-XTM.CD as template (Yang et al., 2000) (a gift of Mary G.-S. Lee, New York University Medical School). The insert was cut with *Bam*HI and inserted into pUB73pre, which had been previously cut with *Bam*HI and treated with alkaline phosphatase. pUB73pre, which contains VSG 117 lacking the C-terminal signal peptide, is a derivative of pLew82 (Wirtz et al., 1998). For VSG117Ty, we used as a template pUB39 and as forward primer 5'-CGAAGCT-TATGGACTGCCATACAAA-3' and as reverse primer 5'-CGGG-ATCCTTAGTCAAGTGGGTCCTGGTTAGTATGGACTTCAAAA-AGCAAGGCCACAAATGCA-3'. The PCR product was cut with *Bam*HI and *Hind*III and inserted into pUB39, which was also cut with *Bam*HI and *Hind*III.

#### Culture and transfection of trypanosomes

Bloodstream-form *T. brucei* were cultured in HMI-9 at 37°C (Hirumi and Hirumi, 1989). The T7-promoter-driven Tet-operator-regulated VSG 117 cassettes were integrated into an rDNA spacer in trypanosome cell line 13-90, which expresses wild-type VSG 221, T7 RNA polymerase and the Tet repressor (Wirtz et al., 1999). All transfections were performed as described previously (Navarro and Cross, 1998). Expression of the selectable marker was induced by adding 2.5 ng/ml doxycycline to the medium. For maximum induction of mutant VSGs, doxycycline was added at 100 ng/ml. Genomic DNA was isolated from a representative selection of mutant cell lines, and the mutant sequences were amplified by PCR to verify that there was no reversion of the mutation.

#### GPIPLC release as an assay for GPI anchoring

$1 \times 10^7$  cells were resuspended in 200  $\mu$ l ice-cold water containing 0.1 mM TLCK and held on ice for 5 minutes. After centrifugation at 3,000 g for 5 minutes, the supernatant was discarded. The cell ghosts were resuspended in 200  $\mu$ l 10 mM sodium phosphate buffer, pH 8.0, containing 0.1 mM TLCK. After incubation at 37°C for 15 minutes, the sample was centrifuged at 16,000 g for 15 minutes and the different steps of the protocol were analyzed by western blotting. This protocol quantitatively releases cell surface GPI-anchored VSG, in the 37°C incubation, by activating an endogenous phospholipase C that is GPI specific under these conditions (Butikofer et al., 1996; Leal et al., 2001). Most cytoplasmic proteins are released in the initial 0°C lysis step (Cross, 1984).

#### Western blot analysis

Crude lysates of  $2 \times 10^4$  cells were loaded per lane in a 10% SDS-PAGE and transferred onto nitrocellulose membranes (Amersham-Pharmacia). Tris-buffered saline, pH 7.6, plus 0.1% Tween-20 (TBST), containing 5% non-fat dry milk, was used as a blocking solution, for 1 hour at room temperature. The membranes were subsequently incubated for 1 hour either with CRD-depleted rabbit anti-native VSG 221 or rabbit anti-rVSG-117 antibodies (Hoek et al., 1999) (dilution 1:10,000), washed in TBST, then incubated with the corresponding horseradish-peroxidase-conjugated goat-anti-rabbit

antibody for 1 hour (dilution 1:10 000). Proteins were visualized with the supersignal Pico chemiluminescence substrate (Pierce).

#### Northern blot analysis

Total RNA was isolated with RNA Stat-60 (Tel-Test Inc), electrophoresed on 2.2 M formaldehyde 1.5% agarose gels and transferred to nylon membranes. Filters were hybridized with [ $\alpha$ - $^{32}$ P]dATP random-primed full-length VSG 117. Final washings were performed at room temperature for 15 minutes and at 65°C for another 15 minutes with 2 $\times$ SSC, 0.1% SDS. The last wash was done at 65°C for 30 minutes with 0.1 $\times$ SSC, 0.1% SDS.

#### Immunofluorescence

For immunofluorescence analysis,  $5 \times 10^6$  cells were washed twice in phosphate-buffered saline (PBS), fixed with 2% formaldehyde in PBS for 10 minutes on ice. The cells were attached to glass cover slips by centrifugation at 400 g for 5 minutes at 4°C. For visualization of intracellular proteins, the attached cells were permeabilized with 0.2% NP-40 in PBS for 5 minutes. The cells were blocked twice for 10 minutes in PBG (PBS with 0.1% cold-water-fish-skin gelatin from Sigma and 0.5% BSA) and incubated overnight at 4°C with a 1:200 dilution of the primary rabbit antibodies to recombinant VSG 117 (Hoek et al., 1999), to native VSG 221 or mouse antibodies to *T. cruzi* BiP (a generous gift of D. M. Engman, Northwestern University, Chicago, IL, USA) or a 1:1000 dilution of mouse monoclonal antibody 280 to *T. brucei* p67 (a generous gift of J. D. Bangs, University of Wisconsin, Madison, WI, USA). After washing the cells six times with PBG for 5 minutes, the cells were treated for 2 hours at room temperature with fluorescein-conjugated goat or rhodamine-conjugated goat anti-rabbit or anti-mouse antibodies (1:200 in PBG). After treatment of the cells with DAPI to stain nuclear and kinetoplast DNA, cells were mounted with alkaline glycerol (45% glycerol, 50 mM Tris/HCl, pH8.0) containing p-phenylenediamine as anti-fade. Cells were observed with a Nikon epifluorescence microscope using a 100 $\times$  Fluor objective and the appropriate fluorescein or rhodamine filters. Images were captured with a Sony DKC5000 CCD or Spot 2.13 camera at ISO 100, using an 0.5 second integration time, and imported directly to Adobe Photoshop 6.0.

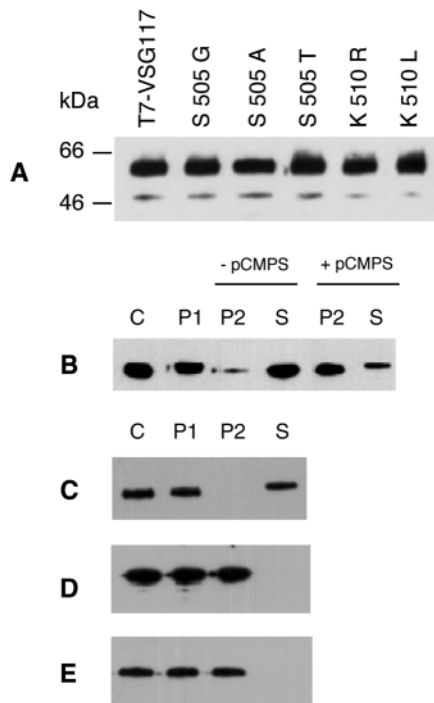
#### Dimerization analysis

$2 \times 10^8$  cells expressing mutations  $\Delta$ 504-526 and  $\Delta$ 512-526 were permeabilized with 500  $\mu$ l 20 mM HEPES, 0.15 M NaCl, pH 7.6, containing 1% (v/v) Nonidet P40. Insoluble material was removed by centrifugation (16 000 g for 15 minutes), and the supernatant was loaded onto a Sephacryl S-200 column (equilibrated with 20 mM HEPES, 0.15 M NaCl). Standards (Sigma) were as follows (250  $\mu$ g of each): alcohol dehydrogenase, 150 kDa; BSA, 66 kDa; carbonic anhydrase, 31 kDa; cytochrome C, 12.4 kDa. 500  $\mu$ l fractions were collected and VSG was detected in each fraction by immunoblotting after TCA precipitation and SDS-PAGE.

## Results

### Amino-acid substitutions in the GPI signal peptide

Mutations were first introduced at  $\omega+2$ , changing Ser to Gly, Ala, Thr or Pro, and at  $\omega+7$ , changing Lys to Leu or Arg (Table 1). The plasmids, containing the wild-type or mutated VSG in tandem with the phleomycin resistance gene, both under the control of the tetracycline-regulated T7 promoter, were transfected into *T. brucei* cell line 13-90, which constitutively expresses VSG 221 and the T7 RNA polymerase and Tet repressor (Wirtz et al., 1999). Upon low induction with doxycycline (2.5 ng/ml), to induce resistance to phleomycin



**Fig. 2.** (A) Western blot of T7-VSG 117 (wild-type VSG 117 expressed in the same context as the mutants) compared with mutants S505G, S505A, S505T, K510R and K510L. The cells were induced with 100 ng/ml doxycycline for 24 hours, and the equivalent of  $2 \times 10^4$  cells were loaded per lane. (B,C) Western blots of cell line T7-VSG 117 after endogenous GPIPLC activation. Aliquots representing  $2 \times 10^4$  cells were loaded per lane. Cells coexpressing VSGs 117 and 221 were osmotically lysed in ice-cold water and centrifuged. The cell ghosts (P1) were resuspended and incubated in 10 mM sodium phosphate buffer, pH 8.0, at 37°C for 15 minutes and centrifuged to generate pellet (P2) and supernatant (S) fractions. Lanes C, P1, P2 and S contain, respectively, whole cells, pellet after lysis and pellet and supernatant after GPIPLC activation. (B) VSG 117 release in the absence or presence of 5 mM pCMPS during PIPLC cleavage, probed with antibodies to VSG 117. (C) Analysis of the same samples, in the absence of pCMPS, with anti-VSG 221 antibodies. (D) Analysis with anti-BiP antibodies. (E) The same protocol was applied to a GPIPLC null-mutant cell line expressing VSG 221 (Leal et al., 2001) and the blot was probed with VSG 221 antibodies.

(2.5  $\mu\text{g/ml}$ ), several clones with stably integrated expression cassettes were obtained for each mutant except for S505P. Although numerous transfections were attempted, no S505P clones were obtained, suggesting that expression of this construct is toxic. The expression level of the mutated VSGs was determined. As observed by northern and western blotting, none of the mutations had a measurable effect on the expression level of VSG mRNA (data not shown) or protein (Fig. 2A) in comparison with wild-type VSG 117 expressed in the same context (T7-VSG117). The 46 kDa band that is seen to varying degrees in this and some subsequent figures is the typical (and normally very minor, under these conditions) product of VSG proteolysis: the VSG N-terminal domain. The 'hinge' region between the VSG N- and C-terminal domains is exquisitely sensitive to proteolysis (Johnson and Cross, 1979; Metcalf et al., 1988). There is some variation in the

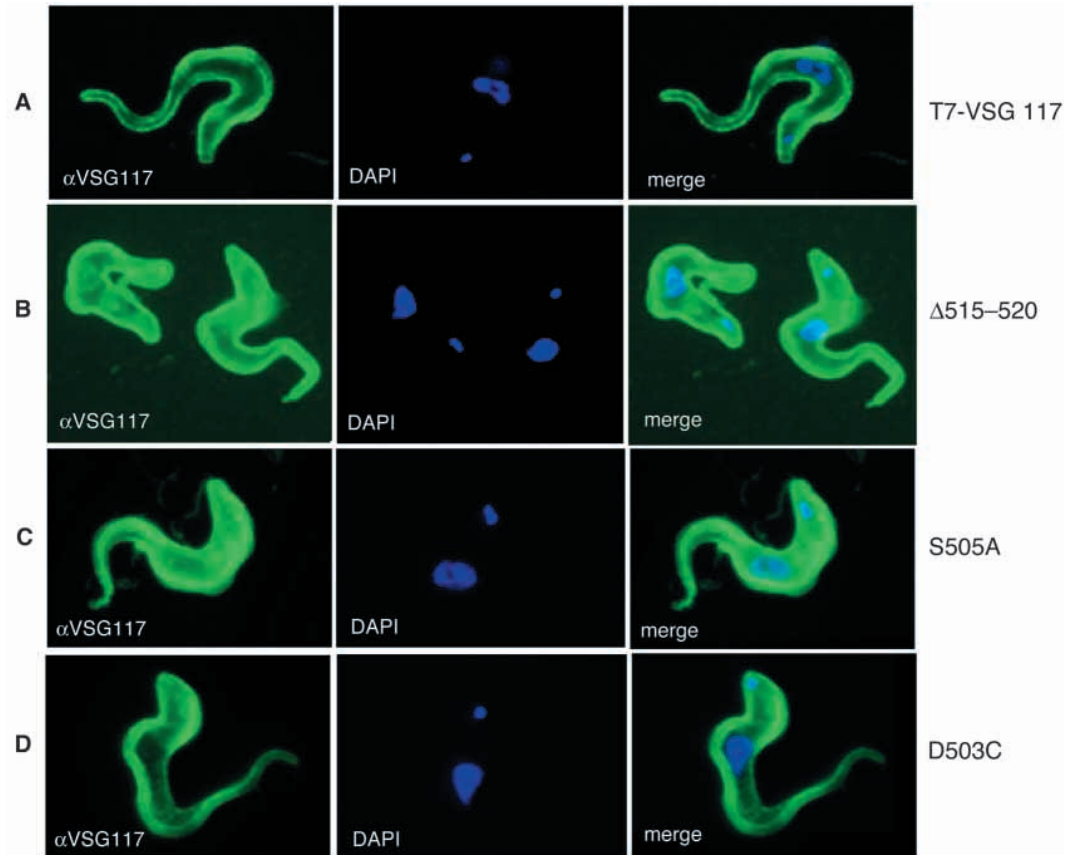
apparent amount of this product, in different experiments, some of which is attributable to different exposures of the western blot. We also had the impression that VSG overexpression increased the amount of this product. In these transgenic cells, we are producing twice the normal amount of VSG mRNA.

GPI modification of the mutated proteins was evaluated by measuring the GPI-phospholipase C (GPIPLC) releasable VSG 117 on western blots. Over 90% of the surface GPI-anchored VSG is released into the supernatant in this assay by activation of an endogenous PIPLC that is specific for GPI cleavage under these conditions (Butikofer et al., 1996). All of the point-mutated proteins were detected in the supernatant, suggesting that all VSGs with mutations at position  $\omega+2$  and  $\omega+7$  were GPI-anchored on the cell surface. To demonstrate that the GPIPLC release assay works reliably, several controls were performed (Fig. 2). Inhibition of GPIPLC with *p*-chloromercuriphenylsulfonate (pCMPS) (Butikofer et al., 1996) reduced VSG release by 90% (Fig. 2B). Since the 13-90 cell line is naturally expressing VSG 221, we used antibodies to reveal this VSG as a second control (Fig. 2C). Both the endogenous VSG 221 and ectopically expressed VSG 117 were GPI anchored and releasable to a similar degree. To determine whether this protocol releases luminal ER-associated proteins, we tested whether the ER chaperone BiP could be detected in the supernatant after release with GPIPLC, which it was not (Fig. 2D). This confirms that the protocol specifically releases GPI-anchored surface proteins, as previously shown (Ferguson et al., 1985; Bangs et al., 1986; Ferguson et al., 1986) and does not disrupt the ER membrane. Another control was performed with a previously acquired *GPIPLC* null-mutant cell line (Leal et al., 2001). As shown in Fig. 2E, no VSG was released into the supernatant of this cell line, which expresses VSG 221. The surface location of all the mutated VSGs was confirmed by indirect immunofluorescence with anti-VSG 117 antibodies (Hoek et al., 1999) (selected examples are shown in Fig. 3).

To see whether the  $\omega$  and  $\omega+1$  positions play a role in GPI signal sequence recognition, we introduced several mutations at these two positions (Table 1). The  $\omega$  position was mutated to Glu or Cys and the  $\omega+1$  position was mutated to Leu, His, Lys, Tyr or Phe. Surprisingly, with one exception, these mutated proteins were expressed at similar levels to wild-type VSG 117, and all were completely released by GPIPLC, indicating that they were anchored on the cell surface (summarized in Table 1; data for D503C and D503E are shown in Fig. 4A,B). Expression of the  $\omega$  position Cys mutant was reduced by about 90% (Fig. 4A). Indirect immunofluorescence confirmed cell surface expression of D503C (Fig. 3D).

#### Combination of mutations in the GPI signal peptide

Since the point mutations showed no effect on GPI anchoring, we introduced more extensive mutations (Table 1).  $\omega+3$ ,  $\omega+4$  and  $\omega+5$  were simultaneously substituted with Thr (506–508T) and  $\omega+7$  and  $\omega+8$  with Leu (510–511L). A stretch of eight Leu residues was introduced at the end of the hydrophobic region (519–526L). Expression levels of all three mutations were comparable to wild-type VSG 117, and all of them were GPI anchored on the cell surface, as shown by GPIPLC release and

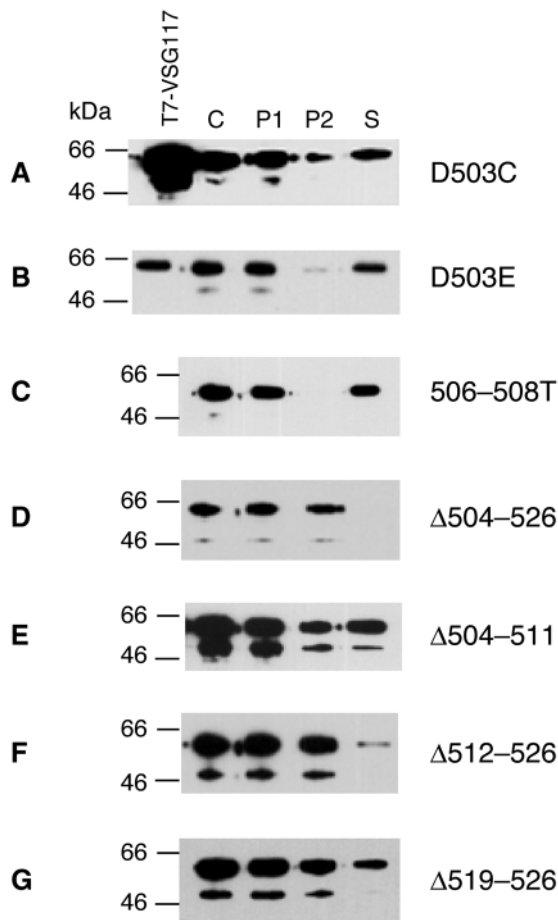


**Fig. 3.** Immunofluorescence of (A) T7-VSG117, (B)  $\Delta$ 515–520, (C) S505A and (D) D503C. Fixed cells were treated with rabbit anti-VSG 117 plus fluorescein-conjugated secondary antibody and DAPI (4',6-diamidino-2-phenylindole), and the images were digitally merged.

**Table 1. Summary of C-terminal sequences of VSG 117 mutants and their effects on GPI anchoring**

Plasmid	Mutant	$\omega$	$\omega+1$	$\omega+2$		Hydrophobic region	GPI
pUB39	wild-type	D	S	S	ILVTKK	FALTVVSAAFVALLF	+
pUB30	S505G	D	S	<b>G</b>	ILVTKK	FALTVVSAAFVALLF	+
pUB38	S505A	D	S	<b>A</b>	ILVTKK	FALTVVSAAFVALLF	+
pUB42	S505T	D	S	<b>T</b>	ILVTKK	FALTVVSAAFVALLF	+
pUB67	S505P	D	S	<b>P</b>	ILVTKK	FALTVVSAAFVALLF	NC
pUB61	S504L	D	<b>L</b>	S	ILVTKK	FALTVVSAAFVALLF	+
pUB62	S504H	D	<b>H</b>	S	ILVTKK	FALTVVSAAFVALLF	+
pUB63	S504K	D	<b>K</b>	L	ILVTKK	FALTVVSAAFVALLF	+
pUB64	S504Y	D	<b>Y</b>	S	ILVTKK	FALTVVSAAFVALLF	+
pUB65	S504F	D	<b>F</b>	S	ILVTKK	FALTVVSAAFVALLF	+
pUB69	D503C	<b>C</b>	S	S	ILVTKK	FALTVVSAAFVALLF	+
pUB70	D503E	<b>E</b>	S	S	ILVTKK	FALTVVSAAFVALLF	+
pUB40	K510R	D	S	S	ILVTRK	FALTVVSAAFVALLF	+
pUB41	K510L	D	S	S	ILVTLK	FALTVVSAAFVALLF	+
pUB51	506-508T	D	S	S	<b>TTT</b> TKK	FALTVVSAAFVALLF	+
pUB53	519-526L	D	S	S	ILVTKK	FALTVVSALLLLLLL	+
pUB48	510-511L	D	S	S	ILVT <b>LL</b>	FALTVVSAAFVALLF	+
pUB54	$\Delta$ 504-526	D	-	-	-----	-----	-
pUB58	$\Delta$ 512-526	D	S	S	ILVTKK	-----	-
pUB60	$\Delta$ 519-526	D	S	S	ILVTKK	FALTVVS----	+/-
pUB59	$\Delta$ 504-511	D	-	-	-----	FALTVVSAAFVALLF	+/-
pUB57	$\Delta$ 515-520	D	S	S	IL----	FALTVVSAAFVALLF	+
pUB55	511FK	D	S	S	ILVTKK	<b>FK</b> FALTVVSAAFVALLF	+
pUB39Ty	VSG117Ty	D	S	S	ILVTKK	FALTVVSAAFVALLFVALLFEV- HTNQDPLD	+
pUB73	VSG117TM	D	G	S	AMIILAVL	VPAILLVVTVAVAFFIMVKKRRRNN- SQVDVTGKAEQGVSSGKAVM	-

Mutated residues are highlighted in bold type and deletions are indicated by a dash. GPI anchoring was normal (+), absent (-) or partial (+/-). In one case (NC) the mutation appeared to be lethal, as no trypanosome clones containing this mutation could be obtained. The steady-state abundance of GPI-anchored mutants was not noticeably different from wild-type VSG 117, with the exception of D503C, which was  $\leq$ 10% of the wild-type level. The steady-state level of VSG117TM was about 1% of the wild-type but the truncated mutants that were not anchored were present at high levels, presumably owing to their retardation in the ER, prior to degradation.

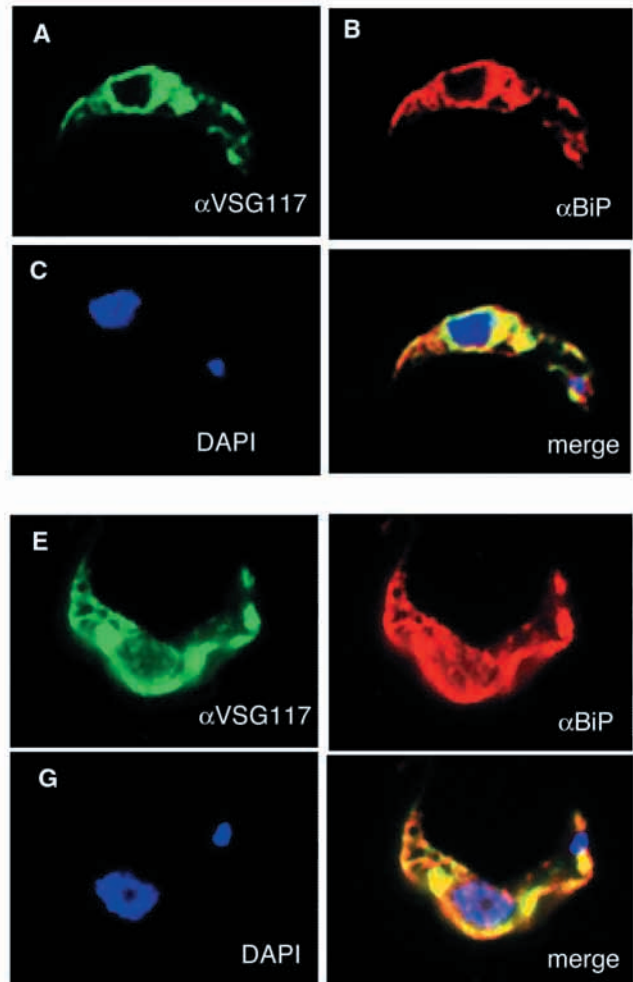


**Fig. 4.** Western blots showing VSG release for mutants D503C (A), D503E (B), 506-508T (C),  $\Delta$ 504-526 (D),  $\Delta$ 504-511 (E),  $\Delta$ 512-526 (F) and  $\Delta$ 519-526 (G). Cells were induced with 100 ng/ml doxycycline for 24 hours in HMI9 medium. Aliquots representing  $2 \times 10^4$  cells were loaded per lane. Lanes C, P1, P2 and S contain, respectively, whole cells, pellet after lysis, and pellet and supernatant after GPIPLC activation. In (A) and (B) the amount of mutated VSG is compared with unmutated VSG 117 (T7-VSG117). The exposure was 10 seconds for all panels except (A), which was exposed for 1 minute.

indirect immunofluorescence with anti-VSG 117 antibodies. A representative western blot for the GPIPLC release of mutant 506-508T is shown in Fig. 4C.

#### Deletions in the GPI signal peptide

To investigate the length requirements of the C-terminal signal peptide, several deletion mutants were constructed (Table 1). The effect of the mutations on the expression and location of VSG 117 was analyzed by western blotting. As expected, when the entire C-terminal signal peptide was deleted ( $\Delta$ 504-526), VSG was not anchored on the cell surface, as shown by the GPIPLC release assay (Fig. 4D). Deleting four amino acids from the spacer sequence ( $\Delta$ 515-520) did not affect release by GPIPLC, indicating that this mutant is GPI anchored. In contrast, deleting the entire spacer sequence ( $\Delta$ 504-511) showed a partial effect on anchoring: about 50% of the protein was found in the supernatant after GPIPLC activation (Fig.



**Fig. 5.** Immunofluorescence of  $\Delta$ 504-526 (A-D) and  $\Delta$ 512-526 (E-H). Fixed and permeabilized cells were treated with rabbit anti-VSG 117 and fluorescein-conjugated secondary antibody (A,E), with mouse anti-BiP and rhodamine-conjugated secondary antibody (B,F), with DAPI (C,G), and the three images were digitally merged (D, H), which results in a yellow pseudo-color in regions of VSG-BiP colocalization.

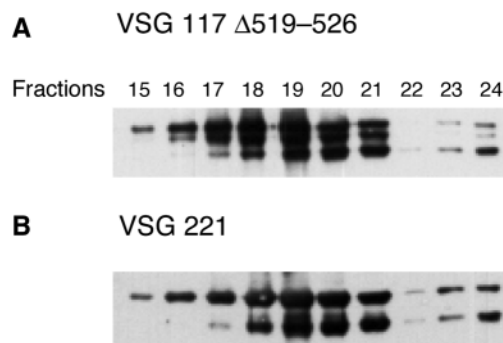
4E). Deleting the entire hydrophobic region ( $\Delta$ 512-526) prevented GPI anchoring (Fig. 4F), whereas deleting part of the hydrophobic region ( $\Delta$ 519-526) resulted in a partly anchored protein (Fig. 4G). This result indicates that the hydrophobic region has to be at least eight amino acids long for anchoring to occur.

Other investigators have shown that proteins lacking the GPI signal peptide are secreted into the medium (Furukawa et al., 1994; Bucht and Hjalmarsson, 1996; McDowell et al., 1998). We therefore examined the fate of unanchored proteins. After induction of mutants  $\Delta$ 504-526 and  $\Delta$ 512-526 for 48 hours, we resuspended the cells in culture medium containing 100 ng/ml doxycycline and only 1% fetal calf serum for 8 hours. Proteins in the medium were concentrated, electrophoresed, and VSG was revealed by western blotting. An equivalent of  $2 \times 10^6$  cells was loaded per lane. Although this loading would permit the detection of 1% of the normal amount of cell-surface VSG, neither mutant VSG could be detected in the medium

(data not shown), implying that they were entirely retained in the cell or extensively degraded.

To more precisely determine the intracellular location of  $\Delta 504$ -526 and  $\Delta 512$ -526, fixed and permeabilized cells were stained simultaneously with antibodies to VSG 117 and the ER-resident protein BiP. The pattern of staining seen with anti-BiP (Fig. 5B,F) is characteristic of ER morphology in trypanosomes (McDowell et al., 1998). The staining seen with anti-VSG 117 in the mutants (Fig. 5A,E) exactly matches the distribution of BiP. This colocalization can be seen best as a yellow pseudo-color in the merged images (Fig. 5D,H). Thus, mutated VSGs that are not GPI-anchored accumulate in the ER, the initial compartment of the secretory pathway. By contrast, mutations that were GPI anchored resulted in a typical surface staining (Fig. 3). As a control, we used the wild-type cell line, MITat 1.2, expressing VSG 221. No staining was observed with anti-VSG 117 antibodies (data not shown). In partly anchored mutants, the surface staining obscured any intracellular staining.

VSG exists as a dimer on the membrane. To determine whether the failure of  $\Delta 504$ -526 and  $\Delta 512$ -526 to exit the ER was due to misfolding and a lack of dimerization, we determined the oligomeric state by gel filtration on a Sephacryl S-200 column. Data for mutation  $\Delta 512$ -526 are shown in Fig. 6. The different fractions were subjected to SDS-PAGE and western blotting. By comparison with the molecular mass standards, VSG dimers should elute at fraction 19. As shown in Fig. 6, this is true for the co-expressed control (VSG 221) and mutant (VSG 117) proteins. This result shows that the accumulated mutant VSG is not misfolded and ER retention is only attributable to lack of a GPI anchor. The increased VSG degradation, revealed in the gel analysis of the column fractions, is attributed to the longer duration of the analysis, in which protease inhibitors were not included, and overexposure of the peak lanes in the western blot.

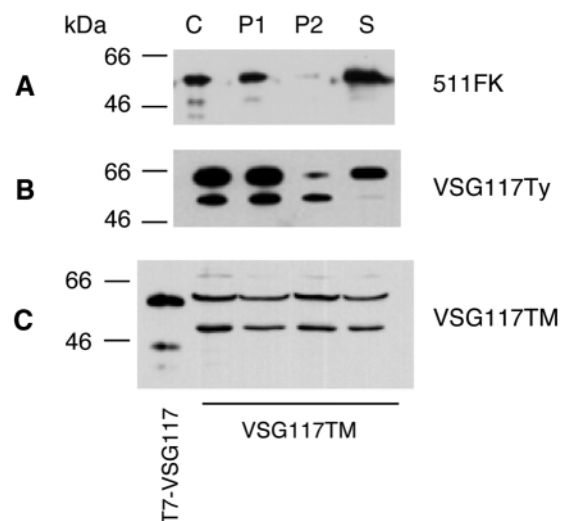


**Fig. 6.** Dimerization analysis of mutation  $\Delta 519$ -526.  $2 \times 10^8$  cells of cell line  $\Delta 519$ -526 were permeabilized with 500  $\mu$ l 20 mM HEPES, 0.15 M NaCl, pH 7.6 containing 1% (v/v) Nonidet P40. Insoluble material was removed by centrifugation (16 000  $g$  for 15 minutes), and the supernatant was loaded onto a Sephacryl S-200 column. Fractions were TCA-precipitated and analysed by SDS-PAGE. Western blot with anti-VSG 117 (A) or anti-VSG 221 (B) antibodies. As determined by running standards (see Materials and Methods), dimerized VSG should peak in fraction 19. The lower bands on the gel correspond to VSG degradation products, which are exaggerated in this long exposure.

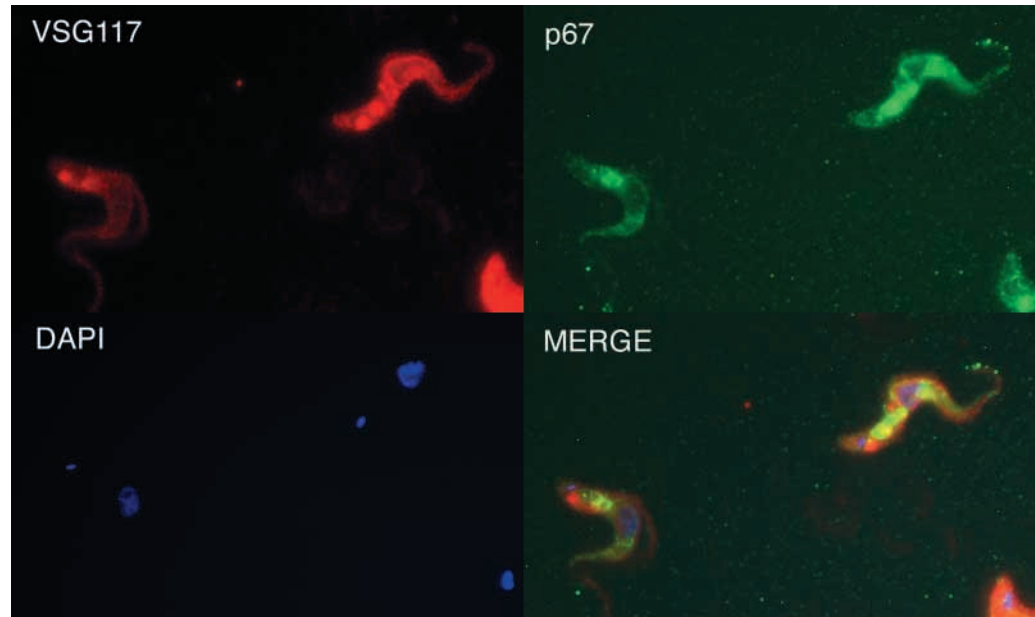
### Elongation of the GPI signal peptide

Two elongation mutants, 511FK and VSG117Ty, were constructed. Their expression levels were comparable with wild-type VSG 117. As determined by the GPIPLC release assay and indirect immunofluorescence, these two mutations were anchored and expressed on the cell surface (Fig. 7A,B). Although VSG117Ty has a Ty epitope tag at the C-terminus, we were not able to detect this mutant with the monoclonal antibody BB2 (Bastin et al., 1996). This result confirms the cleavage of the GPI-anchor signal peptide. This cell line could be useful for identifying the transient precursor prior to exchange of the signal peptide for the GPI anchor.

To investigate the effect of a transmembrane anchor on VSG localization, we replaced the GPI signal sequence with the C-terminal transmembrane plus cytoplasmic domains of the invariant surface glycoprotein 65 (ISG65) (Ziegelbauer et al., 1992) (Table 1). The expression level of this fusion protein (VSG117TM) was about 1% of wild-type GPI-anchored VSG (Fig. 7C). By northern blot, however, no difference was detected in the mRNA abundance between mutant and wild type (data not shown). To examine the cellular localization, we performed indirect immunofluorescence in trypanosomes expressing VSG117TM (Fig. 8). Although the chimeric protein contains a transmembrane domain, it was not found on the cell surface, instead it showed a faint staining characteristic of the ER and larger accumulations in compartments close to the flagellar pocket. Some of this staining did not overlap with the regions reacting with a mouse monoclonal antibody to the major lysosomal membrane protein, p67 (Kelley et al., 1999), suggesting that they might represent pre-lysosomal compartments. The staining pattern is similar to that reported for endocytosed transferrin.



**Fig. 7.** Effects of elongation mutations on GPI anchoring and expression level. A western blot with rabbit anti-VSG 117, showing the release of VSG117 511FK (A) and VSG117Ty (B). Aliquots representing  $2 \times 10^4$  cells were loaded per lane. Lanes C, P1, P2 and S contain, respectively, whole cells, pellet after lysis, and pellet and supernatant after GPIPLC activation. (C) Comparison of VSG expression in four independent clones of VSG117TM, which is not GPI anchored. Aliquots representing  $2 \times 10^4$  cells and  $2 \times 10^6$  cells were loaded for T7-VSG117 and VSG 117TM, respectively.



**Fig. 8.** Immunolocalization of VSG117<sup>TM</sup>. Fixed and permeabilized VSG117<sup>TM</sup> cells were treated with rabbit anti-VSG 117, mouse anti-p67 (a lysosomal marker) and DAPI, and the images were merged.

#### Effects of mutations on RNA and protein stability

To see whether mutations of the C-terminal signal sequence that showed significant effects on VSG expression or anchoring had affected RNA stability, we extracted RNA from these mutant cell lines at different times after transcriptional shutoff by actinomycin D. Compared to wild-type VSG 117, none of the mutations showed any effect on RNA stability (representative data for mutant  $\Delta 519$ -526 are shown in Fig. 9A). We then checked whether the mutated VSGs were degraded faster than wild-type VSG. A selection of cell lines expressing different mutated VSGs were cultured for 8 hours in medium containing cycloheximide to terminate protein synthesis. Samples were taken at intervals and analyzed on a western blot. Mutations  $\Delta 504$ -526 and  $\Delta 512$ -526 were degraded rapidly (Fig. 9B), compared with wild-type VSG 117, whose half-life is ~33 hours (Seyfang et al., 1990). Adding lactacystin, a proteasome inhibitor that is known to work in *T. brucei* (Mutomba et al., 1997; Fenteany and Schreiber, 1998) did not prevent degradation (Fig. 9C), suggesting that the unanchored truncated mutants are targeted for lysosomal rather than proteasomal degradation.

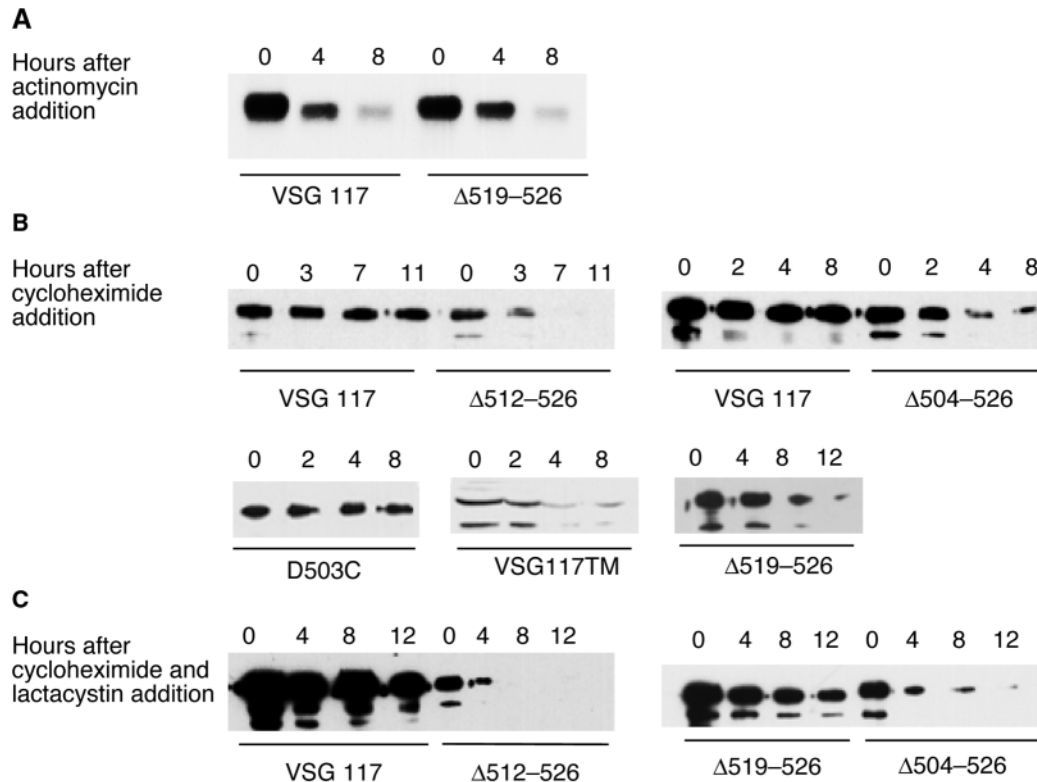
#### Discussion

We first introduced point mutations into the highly conserved GPI anchor signal peptide of VSG 117. Surprisingly, almost all of these mutations were GPI anchored and routed to the cell surface at a level comparable to the wild-type VSG (Table 1). Only two of these mutations detectably affected GPI anchoring. Changing  $\omega+2$  Ser to Pro resulted in no clones, suggesting that the expression of this mutant is very toxic, and mutating  $\omega$  Asp to Cys reduced expression by  $\geq 90\%$ . Mutating  $\omega$  Asp to Glu, surprisingly, did not affect VSG GPI addition or abundance. Glu has never been identified at any natural  $\omega$  site, although the number of experimentally proven sites remains small, and it is allowable by prediction (Eisenhaber et al., 1999). It seemed possible, therefore, that this substitution changed the position at which the GPI anchor was added,

although this explanation was not favored in experiments with the signal sequence from decay-accelerating factor, where Glu was inefficiently permissive and Cys was not allowed (Moran et al., 1991). Migration of the  $\omega$  site has been invoked, but never confirmed, in studies of mutated mammalian GPI anchoring sequences (e.g. Caras et al., 1989; Moller et al., 1992; Bucht and Hjalmarsson, 1996). The sequence preceding the wild-type VSG 117  $\omega$  Asp residue (CKWENNACK) contains several potential  $\omega$  sites, the most probable being Asn498. With recent advances in mass spectrometry, we had expected to easily confirm the site of GPI addition in the  $\omega$  Glu and Cys mutants. Unfortunately, despite extensive efforts by two collaborating laboratories, we were unable to do this. Reversion to more classic protein biochemistry methods for identifying GPI addition sites (Holder and Cross, 1981; Holder, 1983) was beyond the scope of the present investigations.

To address the minimum length of the spacer sequence and the hydrophobic region, we constructed a series of deletion mutants. Just two severe changes, which deleted the entire C-terminal signal peptide or the hydrophobic region, prevented GPI addition. Deleting the entire spacer region, or eight amino acids from the hydrophobic region, reduced the efficiency of anchoring. Previous work showed that the length of both the spacer and hydrophobic regions were important for efficient GPI anchoring (Caras, 1991; Moran and Caras, 1991b; Moran and Caras, 1991a; Lowe, 1992; Coyne et al., 1993; Furukawa et al., 1994; Bucht and Hjalmarsson, 1996; Furukawa et al., 1997; Bucht et al., 1999), whereas we found that a spacer sequence consisting of just four amino acids sufficed. However, inspection of the GPI signal sequence of VSG 117, coupled with our observation that KK in the spacer can be replaced by LL, suggests that there may be less distinction, in the VSG signal sequence, between the hydrophilicity/hydrophobicity of what have been designated as spacer and hydrophobic domains. In addition, it should be kept in mind that the VSG 117 wild-type signal sequence contains 23 amino acids, whereas the other major VSG subclass (represented by VSG 221, which is coexpressed in the cell line used for





**Fig. 9.** Effects of selected mutations on RNA and protein stability. (A) Degradation kinetics of unmutated VSG 117 and  $\Delta 519-526$  RNA. Cell lines were cultured for 8 hours in medium containing 10  $\mu\text{g/ml}$  actinomycin D to prevent transcription. Samples were taken at 4 hour intervals and a northern blot was probed with VSG 117. (B) Degradation kinetics of unmutated VSG 117,  $\Delta 504-526$ ,  $\Delta 512-526$ ,  $\Delta 519-526$ , D503C and VSG117TM. Cell lines were cultured for 8 hours in medium containing 50  $\mu\text{g/ml}$  cycloheximide to prevent translation. Samples were taken at different time points and analyzed on a western blot with anti-VSG 117 antibodies. (C) Degradation kinetics of wild-type VSG 117,  $\Delta 504-526$ ,  $\Delta 512-526$  and  $\Delta 519-526$  in the presence of lactacystin. Cell lines were cultured for 8 hours in medium containing 50  $\mu\text{g/ml}$  cycloheximide and 1  $\mu\text{M}$  lactacystin. Samples were taken at different time points and analyzed on a western blot with anti-VSG 117 antibodies. Aliquots representing  $2 \times 10^4$  cells were loaded per lane with the exception of VSG117TM where  $2 \times 10^6$  cells were loaded per lane.

the current experiments) has only a 17 amino-acid signal, suggesting that up to six amino acids might be dispensed with, from VSG 117, without affecting anchoring. In conclusion, our analysis suggests that the requirements for VSG, despite initial appearances, might be less stringent than for mammalian GPI-anchored proteins.

Our results, together with others (Engstler et al., 2000), suggest that there is a stringent quality control for VSG secretion in bloodstream-form *T. brucei* and that the structure of the VSG itself, and the presence of a GPI anchor, are both required for efficient secretion. Failure to add a GPI anchor to VSG results in ER retention followed by degradation. This result indicates that the GPI anchor is a strong facilitator of VSG secretion. This was suggested by earlier studies of a VSG 117  $\Delta 504-526$  mutation expressed in procyclic forms of *T. brucei* (McDowell et al., 1998), where the GPI-anchored wild-type VSG 117, although expressed at far lower levels than in bloodstream forms, was transported to the surface, but the  $\Delta 504-526$  mutation was retarded in the ER and secreted five-fold more slowly than the wild type. A similar result was subsequently reported for bloodstream forms, where degradation of  $\Delta 504-526$  following its retention in the ER was sensitive to inhibitors of lysosomal proteases (Triggs and Bangs, 1999). Something about VSG structure, other than the presence of a GPI anchor, is also important for efficient

secretion in *T. brucei*. Several investigators have failed to efficiently express alternative GPI-anchored reporters with VSG signal sequences or chimeras consisting of VSG or its subdomains fused to different reporter proteins, for example, placental alkaline phosphatase, *Saccharomyces cerevisiae* prepro- $\alpha$  factor and green fluorescent protein. None of these fusions accumulated on the cell surface (M. Engstler et al., 2000) (M. Engstler, unpublished; K. Davis, U.B., J. Wang and G.A.M.C, unpublished).

Degradation of proteins that fail ER quality-control tests has been described in a variety of situations and has been ascribed to retrotranslocation from the ER and proteasomal degradation (Lippincott-Schwartz et al., 1988; Bonifacino et al., 1990; Pilon et al., 1997; Plemper et al., 1997; Zhang et al., 1997; Ellgaard et al., 1999). There are several examples where misfolded proteins fail to dimerize and are eliminated by this route. In our experiments, truncated VSGs were dimerized, and degradation did not appear to occur via the proteasomal pathway. Rather, by considering all of our observations of non-GPI-anchored VSG mutants, we speculate that degradation may take place either via inefficient secretion followed by rapid endocytosis and lysosomal degradation or by ejection from a later stage of the secretory pathway (Golgi or TGN). Recent experiments suggest that the ER retention or retardation of mutant proteins that are unable to receive a GPI anchor may

be due to a sustained interaction with a component of the transamidase complex (Spurway et al., 2001; Vidugiriene et al., 2001).

The different results obtained with the transmembrane (VSG117<sup>TM</sup>) versus the VSG117<sup>Ty</sup> mutants are interesting. The  $\omega$ ,  $\omega+1$ ,  $\omega+2$  positions, and the length and composition of the spacer and hydrophobic domains of these two constructs, are similar. The main difference is in the degree to which the two proteins are extended, to form potential cytoplasmic domains. The Ty extension of 15 amino acids did not prevent its anchoring, whereas the transmembrane extension of 30 amino acids, onto what looks as if it would otherwise be an acceptable GPI signal sequence, prevents GPI anchoring. This transmembrane domain was also included in a mutational study of the C-terminal targeting sequence of the *T. brucei* flagellar-pocket protein CRAM (Yang et al., 2000). Interestingly, when the native CRAM sequence was truncated by 40 amino acids, CRAM was partly distributed over the trypanosome surface. Inspection of the truncated C-terminal sequence suggests the presence of several very probable GPI-attachment sites, between 20 and 38 amino acids upstream of the truncated C-terminus, suggesting that truncation revealed a latent signal sequence to the GPI machinery. These observations highlight the unanswered question of how the machinery for GPI addition distinguishes between internal and terminal hydrophobic sequences, as it must, if GPI addition at fortuitous internal signal sequences is to be avoided.

Variations in the efficiency with which different GPI signal sequences are recognized could contribute to the regulation of surface protein abundance. The ER quality-control mechanisms can destroy evidence of GPI anchoring failures, rather than allow aberrant versions of these proteins to proceed through the secretory pathway. Signal sequence requirements are similar but not identical in trypanosomes and mammalian cells, where the VSG GPI signal sequence functioned poorly in some studies (Moran and Caras, 1994) but better in others (White et al., 2000), suggesting that the reporter protein, or just the region immediately upstream of the  $\omega$  site (Eisenhaber et al., 1998), might influence the results.

GPI-anchored proteins may follow a novel secretory pathway, and the presence of a GPI anchor may influence endocytic and recycling pathways. Not all GPI-anchored proteins traverse the same recycling pathway (Nichols et al., 2001). Sphingolipid-rich microdomains ('lipid rafts') are probably important for the secretion and sorting of GPI-anchored proteins (Bagnat et al., 2000; Muniz and Riezman, 2000), and a recent study shows that, in yeast, GPI-anchored proteins exit the ER in different vesicles from other secretory proteins (Muniz et al., 2001). The existence of alternative secretory pathways remains to be investigated in *T. brucei*.

In conclusion, our results indicate that the high conservation of naturally occurring VSG GPI signal sequences is unnecessary for efficient VSG synthesis and anchoring, so the question of why these sequences are so conserved remains unanswered. It is possible that smaller changes than we could detect in GPI anchoring efficiency have played a subtle role in trypanosome virulence and have led to the evolutionary optimization of VSG GPI signals.

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