Stable Expression of Mosaic Coats of Variant Surface Glycoproteins in *Trypanosoma brucei*

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The paradigm of antigenic variation in parasites is the variant surface glycoprotein (VSG) of African trypanosomes. Only one VSG is expressed at any time, except for short periods during switching. The reasons for this pattern of expression and the consequences of expressing more than one VSG are unknown. *Trypanosoma brucei* was genetically manipulated to generate cell lines that expressed two VSGs simultaneously. These VSGs were produced in equal amounts and were homogeneously distributed on the trypanosome surface. The double-expressor cells had similar population doubling times and were as infective as wild-type cells. Thus, the simultaneous expression of two VSGs is not intrinsically harmful.

In its bloodstream stage, *Trypanosoma brucei* evades the host immune response by a process of antigenic variation, in which the surface coat, consisting of about 10^7 copies of a glycosylphosphatidylinositol (GPI)–anchored VSG, equivalent to 10% of the total cellular protein, is periodically changed (*1*). Despite a repertoire of more than 1000 *vsg* genes (*2*), only one is normally expressed at any time. The expressed *vsg* is always located at a telomeric expression site (ES). However, there are probably at least 20 potential ESs (*3*), which consist of a polycistronic transcription unit containing several ES-associated genes (*esags*) (*4*) and one *vsg*. Antigenic variation is generally achieved either by gene conversion of a duplicated *vsg* into an active ES, or by in situ activation of one ES and concomitant inactivation of another (*5*), by an unknown mechanism. Therefore, only one ES, with one *vsg*, is productively transcribed at any time.

Trypanosoma brucei, like other species of African trypanosomes, is transmitted by *Glossina*, the tsetse. Naturally occurring *T. brucei* cells expressing two VSGs can be found as a minor population in rats infected with metacyclic forms from the tsetse (*6*), during the first infection cycle, when there is rapid switching from metacyclic to bloodstream-form *vsgs*. These double-expressing cells do not multiply for long, probably because metacyclic *vsgs* form a subclass of telomeric *vsgs* that are transcribed monocistronically and are not linked to *esags* (*7*). Some *esags* are essential for trypanosome survival in the mammalian host (*8*). There has been one report of *T. equiperdum* simultaneously expressing two VSGs from two ESs, in a situation that was stable for about 30 generations in vitro but could not be propagated beyond a few generations in mice (*9*).

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The questions of whether simultaneous expression of two VSGs is disadvantageous for replication or infection of trypanosomes or whether constraints intrinsic to VSG synthesis or coat assembly might enforce the singularity of *vsg* expression have never been experimentally tested in a systematic way, despite continuing speculation about the possible consequences (*10*, *11*). Using recently developed molecular tools, we have started to explore these questions experimentally by inserting a second *vsg* into an active ES.

A second *vsg* [MITat 1.5 clone 117, MITat 1.6 clone 121, or ILTat 1.24 (*12*, *13*)] was inserted into the active *vsg* 221 ES of *T. brucei* strain 427 clone 221a (*14*), in tandem with a neomycin phosphotransferase gene (*neo*), conferring resistance to G418 (Fig. 1). Correct insertion of the *vsg-neo* cassette was confirmed by Southern (DNA) blotting. The sequences of these VSGs are known (*13*, *15*), and the three-dimensional structures of the NH_2-terminal domains of VSGs 221 and ILTat 1.24 have been determined (*16*, *17*). Two major VSG classes have been distinguished on the basis of sequence conservation at their COOH-termini (*13*, *18*). The mature sequences of these two VSG classes terminate in either aspartate (for example, VSGs 117, 121, and ILTat 1.24) or serine (for example, VSG 221), forming the GPI attachment site, after cleavage of a 23– or 17–amino acid signal sequence, respectively.

Immunocytological analysis demonstrated that the two VSGs were homogeneously distributed on the cell surface of the three recombinant double-expressor populations (Fig. 2). Control cells (parental lines expressing VSG 117, 121, 221, or 118) showed no cross-reaction among the antibodies used. Protein immunoblotting showed that each of the recombinant cell lines expressed both VSGs in substantial amounts (Fig. 3A). Mobility differences between VSGs facilitated identification after SDS–polyacrylamide gel electrophoresis (SDS-PAGE). When purified VSGs, released from either parental or recombinant lines by the endogenous GPI-specific phospholipase C (*19*, *20*), were stained with Coomassie brilliant blue, they appeared to be present in similar amounts (Fig. 3B, upper panel). This result was confirmed by biosynthetic labeling followed by autoradiography (Fig. 3B, lower panel) and quantitative phosphorimaging. When these paired VSGs were treated with VSG-specific antibodies, there was no coprecipitation of the second VSG (Fig. 3B). Therefore, despite the similarity in their three-dimension-

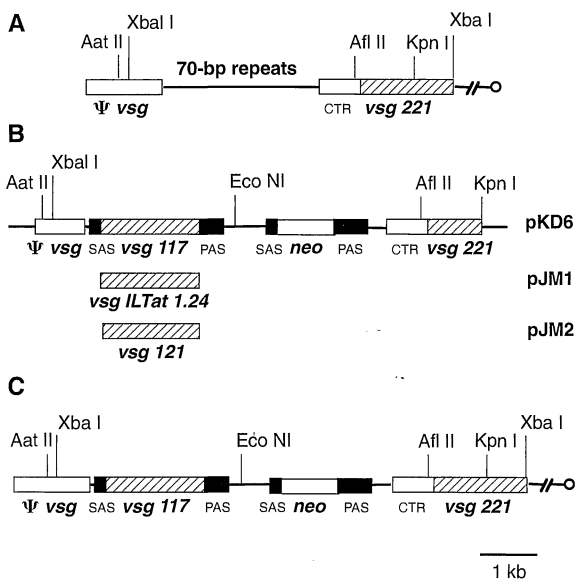

Fig. 1. Integration of *vsg-neo* cassettes into the active *vsg* 221 expression site. Maps of (**A**) the telomere-proximal region of the wild-type 221 ES, (**B**) the three plasmids constructed to insert each *vsg-neo* cassette, and (**C**) map of an ES after cassette insertion by homologous recombination. CTR is the *vsg* cotransposed region (*28*). *neo* was flanked by a splice acceptor site (SAS) and a polyadenylation site (PAS) from a *T. brucei* aldolase gene, and *vsgs* were flanked by a SAS and PAS from a *T. brucei* actin gene. Only relevant restriction sites are indicated. Linear plasmid DNA (5 μg) was used to transfect 2.4×10^7 bloodstream-form *T. brucei* by electroporation (*29*). Trypanosomes were recovered in HMI-9 (*30*) and incubated at 37°C for 18 hours. G418 (2 μg/ml) was added, and cells were incubated until growth of transfectants was observed (7 to 14 days). Clones were isolated on agarose plates containing G418 (*31*).

al structures (*13*, *17*), these VSGs do not form detectable heterodimers. This observation is consistent with the finding of naturally occurring double expression in *T. equiperdum*, in which each VSG was expressed from a different ES (*9*), and with observations of purified VSGs attached to nitrocellulose membranes, in which only homomeric interactions were detected (*21*). The NH_2-terminal domains of VSGs 221 and ILTat 1.24 (*17*) display a tight dimer interface, suggesting that VSGs would be unlikely to form heterodimers of similar stability.

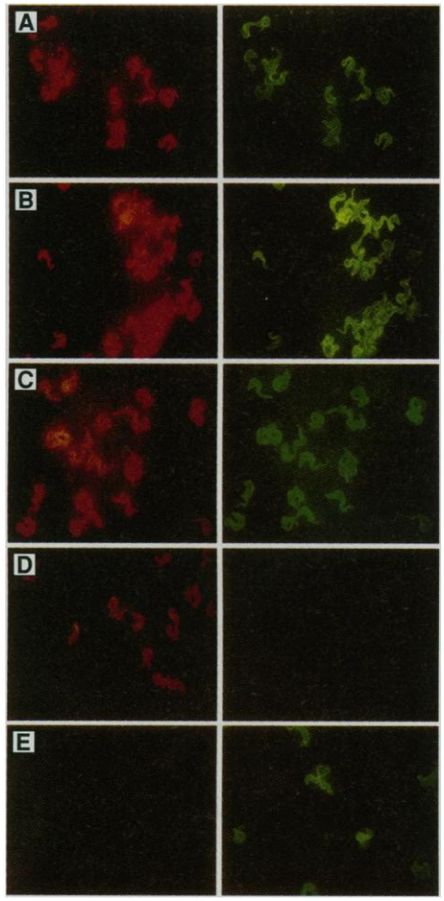

Fig. 2. Immunofluorescence of double-expressing and wild-type trypanosomes. Bloodstream forms were grown in vitro to a density of 10^6 parasites/ml, fixed, and dried onto microscope slides. Rabbit antibodies against VSG 117, 121, and ILTat 1.24 were individually combined with chicken anti–VSG 221. Goat anti-chicken IgG coupled to fluorescein isothiocyanate and goat anti-rabbit IgG coupled to rhodamine were used as second antibodies. The red fluorescence in the left panel of each pair represents the second VSG being expressed on the double expressors (**A**) 221-117, (**B**) 221-ILTat 1.24, and (**C**) 221-121. (**D**) Wild-type VSG 121. The green fluorescence in the right panels identifies the constitutively expressed VSG 221 in all the double expressors and in wild-type 221 cells (**E**). No reaction was detected on the wild-type 221 cells when incubated with antibodies for 121 (E, left) or on the wild-type 121 cells incubated with antibodies for 221 (D, right). All of the ~200 double-expressor cells observed exhibited the green and the red fluorescence simultaneously, confirming the homogeneity of cell populations expressing both VSGs.

Trypanosomes expressing two VSGs grew at the same rate (population doubling time ~6 hours) as wild-type parental cells in vitro, independent of the presence of G418 in the medium. In vivo, in the absence of G418, double expressors were as infective as the wild-type parental 221 line (Fig. 4). In each instance, two successive peaks of parasitemia were produced in rats, the second peak being fatal. At the first peak of parasitemia, the two VSGs were coexpressed on the surface of all members of the population, as determined by immunofluorescence analysis of fixed cells. No significant differences were observed between the growth kinetics of double expressors and wild-type 221 cells in different rats and in different experiments. We therefore concluded that parasites expressing two VSGs have no intrinsic growth disadvantage in vivo or in vitro.

Switching intermediates in the animal host probably occur too infrequently to be observed (*22*), except during the initial metacyclic to bloodstream-form transition (*6*, *23*). As previously reported, VSG has a half-life of 30 to 40 hours and VSG mRNA has a half-life of 4.5 hours (*24*). Therefore, during the process of antigenic variation, cells that simultaneously express two VSGs presumably remain in the bloodstream for a significant period of time. The engineering of trypanosomes to express two VSGs, thereby prolonging an intermediate in antigenic variation that could not hitherto be studied, provides a tool to investigate several aspects of antigenic variation, including the interplay between the trypanosome and the immune response. The availability of double expressors also offers the possibility of studying the extent to which the distinctive glycosylation of individual VSGs used in this study (*25*) is intrinsic to each VSG or is influenced by the genetic background in which a VSG is expressed.

Previously it was suggested that two ESs could be simultaneously active (*26*), but transcription of one ES terminated at an insertion before the *vsg*. It was also reported that simultaneous expression of different VSGs from two ESs was an unstable situation in *T. equiperdum* in vivo: the double expressors were outgrown by cells expressing only one of the two VSGs (*9*). These precedents suggested the existence of some intrinsic obstacle to the simultaneous expression of two VSGs. In the present studies, however, we observed no discrimination, affecting replication or infectivity, against cells expressing two VSGs. Our re-

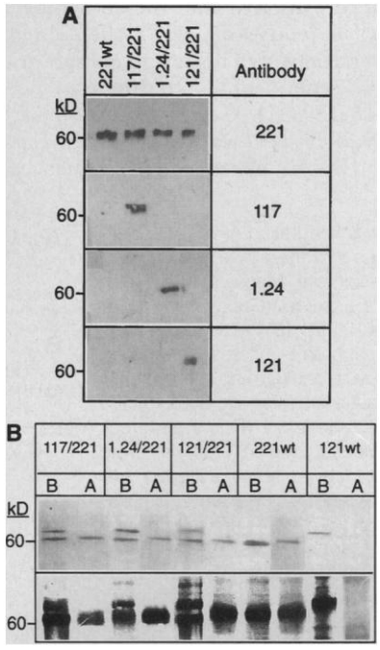

Fig. 3. Coexpression of VSGs by recombinant *T. brucei* lines. (**A**) Protein immunoblots of VSG preparations separated by SDS-PAGE (*20*). (**B**) Immunoprecipitation of partially purified ^{35}S-labeled VSGs with Sepharose-coupled anti–VSG 221 (*20*). The gels were stained with Coomassie brilliant blue (upper panel) and then dried and exposed to film for 5 days (lower panel).

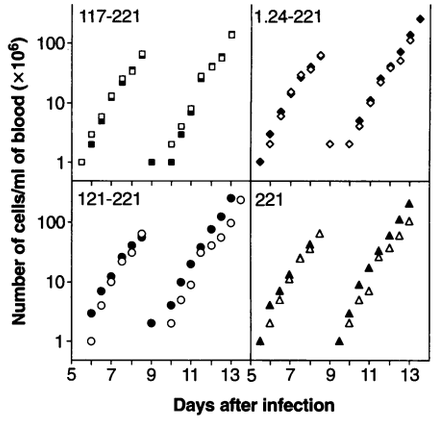

Fig. 4. Infection of rats with *T. brucei* 221 wild-type and double expressors. Pairs of rats (300 g) were each infected with about five wild-type or double-expressor trypanosomes. From the fifth day, the parasitemia in each rat was checked every 12 hours by tail puncture and hemocytometer counting. In each panel, closed and open symbols represent the course of parasitemia in individual rats. The lower limit of counting was set at 10^6 cells/ml. An initial peak of parasitemia was seen 8 to 9 days after infection, when all of the parasites were shown, by immunofluorescence, to be expressing both VSGs on their surfaces. A second peak of parasitemia was observed 13 to 14 days after infection, which the rats were unable to control.

sults indicate that the singularity of VSG expression and the semiordered appearance of VSGs in *T. brucei* infections (*10*, *27*) are probably determined at the level of ES transcription rather than imposed by intrinsic constraints on VSG compatibility. This interpretation is consistent with the previously demonstrated instability of cells expressing two VSGs from different ESs (*9*). Perhaps expressing one VSG at a time has evolved as simply the most economical strategy of immune evasion, but some other product of an ES is responsible for imposing singularity, for reasons that remain to be identified.

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20. Partially purified preparations of solubilized VSG were obtained by endogenous GPI-PLC digestion essentially as described [G. A. M. Cross, *J. Cell. Biochem.* **24**, 79 (1984)] but on a microscale. Briefly, 1×10^8 trypanosomes were pelleted and washed two times with trypanosome dilution buffer (*12*), resuspended in deionized water at 0°C, and incubated on ice for 5 min. Cells were centrifuged, resuspended in 100 μl of 10 mM phosphate buffer (pH 8.0), and incubated for 5 min at 37°C. Cells were pelleted again, and one-tenth of the supernatant was used for SDS-PAGE. The 10% polyacrylamide gels were blotted onto nitrocellulose filters, which were reacted with rabbit antibodies raised against individual VSGs, by standard procedures, followed by peroxidase-conjugated goat antibody to rabbit (anti-rabbit) immunoglobulin G (IgG), according to ECL kit specifications (Amersham, UK). The rabbit antibodies to VSGs 121 and 221 had been depleted of cross-reactive antibodies to the GPI anchors by passage over a column of VSG 117. For metabolic labeling, 1×10^8 bloodstream-form *T. brucei* grown in vitro were transferred into 10 ml of methionine-free RPMI (Gibco BRL, Gaithersburg, MD) supplemented with 10% (v/v) dialyzed fetal bovine serum, 10% dialyzed Serum Plus (JRH Biosciences, Lenexa, KS), and incubated with 50 μCi of ^{35}S-labeled methionine-cysteine mixture for 2 hours. VSGs were extracted as described above and samples were immunoprecipitated with cross-reacting determinant–depleted rabbit antibodies to VSG221 coupled to protein A–Sepharose (Pharmacia-LKB).
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Binding of Zinc Finger Protein ZPR1 to the Epidermal Growth Factor Receptor

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ZPR1 is a zinc finger protein that binds to the cytoplasmic tyrosine kinase domain of the epidermal growth factor receptor (EGFR). Deletion analysis demonstrated that this binding interaction is mediated by the zinc fingers of ZPR1 and subdomains X and XI of the EGFR tyrosine kinase. Treatment of mammalian cells with EGF caused decreased binding of ZPR1 to the EGFR and the accumulation of ZPR1 in the nucleus. The effect of EGF to regulate ZPR1 binding is dependent on tyrosine phosphorylation of the EGFR. ZPR1 therefore represents a prototype for a class of molecule that binds to the EGFR and is released from the receptor after activation.

The EGFR is a transmembrane glycoprotein with an extracellular ligand-binding domain and a cytoplasmic tyrosine kinase domain (*1*). EGF treatment causes increased EGFR tyrosine kinase activity. Substrates for the activated receptor tyrosine kinase include the COOH-terminal region of the receptor (*1*). The tyrosine-phosphorylated EGFR binds to modular signaling proteins that contain SRC homology 2 (SH2) or PTB (phosphotyrosine-binding) domains (*1*, *2*). However, before the formation of the receptor signaling complex with SH2-PTB proteins, the EGFR may interact with other proteins (*1*). The identities of the proteins within this complex have not been defined, but these components may include actin and other cytoskeletal proteins (*3*). Proteins bound to the EGFR may also include signaling molecules that are released from the receptor after activation.

The purpose of this study was to identify proteins that bind to the nonactivated

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