Characterization of the ligand-binding site of the transferrin receptor in *Trypanosoma brucei* demonstrates a structural relationship with the N-terminal domain of the variant surface glycoprotein

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The *Trypanosoma brucei* transferrin (Tf) receptor is a heterodimer encoded by *ESAG7* and *ESAG6*, two genes contained in the different polycistronic transcription units of the variant surface glycoprotein (VSG) gene. The sequence of *ESAG7/6* differs slightly between different units, so that receptors with different affinities for Tf are expressed alternatively following transcriptional switching of VSG expression sites during antigenic variation of the parasite. Based on the sequence homology between pESAG7/6 and the N-terminal domain of VSGs, it can be predicted that the four blocks containing the major sequence differences between pESAG7 and pESAG6 form surface-exposed loops and generate the ligand-binding site. The exchange of a few amino acids in this region between pESAG6s encoded by different VSG units greatly increased the affinity for bovine Tf. Similar changes in other regions were ineffective, while mutations predicted to alter the VSG-like structure abolished the binding. Chimeric proteins containing the N-terminal dimerization domain of VSG and the C-terminal half of either pESAG7 or pESAG6, which contains the ligand-binding domain, can form heterodimers that bind Tf. Taken together, these data provided evidence that the *T.brucei* Tf receptor is structurally related to the N-terminal domain of the VSG and that the ligand-binding site corresponds to the exposed surface loops of the protein.

Keywords: antigenic variation/parasite adaptation/transferrin receptor/*Trypanosoma brucei*/variant surface glycoprotein

Introduction

The variant surface glycoprotein (VSG) is the major surface antigen of the bloodstream form of *Trypanosoma brucei*, and continuous variation of this antigen allows the parasite to escape the immune defences of its mammalian host (for recent reviews, see Borst *et al.*, 1993; Pays *et al.*, 1994; Cross, 1996). The VSG is contained in a telomeric expression site (ES), together with other genes termed expression site-associated genes (*ESAGs*) (Cully and Cross, 1985; Kooter *et al.*, 1987; Pays *et al.*, 1989). The trypanosome genome contains ~20 ESs which share similar

but not identical copies of each *ESAG* (Pays *et al.*, 1989; Hobbs and Boothroyd, 1990; Zomerdijk *et al.*, 1990; Do Thi *et al.*, 1991; Lodes *et al.*, 1993; Navarro and Cross, 1996). As a rule, only a single ES is transcribed at a time, but occasional switching between ESs leads to the alternative expression of different sets of *ESAGs* and VSGs.

ESAG7 and *ESAG6* encode the heterodimeric receptor for transferrin (Tf), which is attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor present at the C-terminus of pESAG6 only (Ligtenberg *et al.*, 1994; Salmon *et al.*, 1994; Sterverding *et al.*, 1994, 1995). Although both pESAG7 and pESAG6 can homodimerize *in vitro*, only heterodimers bind Tf (Salmon *et al.*, 1994). Since the pESAG7 and pESAG6 sequences are almost identical, this heterodimerization suggests that the combination of a few elements specific to each subunit is necessary to generate the ligand-binding site. These elements are not contained in the C-terminal region of the subunits (Salmon *et al.*, 1994). pESAG7/6 heterodimers encoded by different ESs show widely different affinities for a Tf from a given mammalian host, the dissociation constants for bovine Tf varying from 2 nM up to almost 1 µM (Ligtenberg *et al.*, 1994; Salmon *et al.*, 1994; Sterverding *et al.*, 1994, 1995; Borst *et al.*, 1996), all values being compatible with the physiological concentration of Tf in the serum (30–40 µM: Shih *et al.*, 1990). Clearly, the slight differences between *ESAG7/6* from different ESs strongly influence the binding of Tf.

The proteins encoded by *ESAG7* and *ESAG6* share significant sequence homology with the N-terminal domain of some VSGs (Hobbs and Boothroyd, 1990). This may indicate a requirement for a VSG-like tertiary structure linked to the accommodation of non-VSG proteins within the dense and highly ordered VSG coat, since various surface proteins of *T.brucei* share with the VSG some amino acids involved in the building of structural motifs (Carrington and Boothroyd, 1996). Along the same line, the three-dimensional structure of two VSG N-terminal domains, determined by X-ray crystallography, was found to be very similar despite extensive sequence divergence (Blum *et al.*, 1993). Both adopt an extended configuration due to the folding into two long antiparallel α -helices separated by a turn.

We took advantage of (i) the known structure of the VSG, (ii) the sequence homology between pESAG7/6 and some VSGs and (iii) the differences between the Tf receptors encoded by different ESs to test the hypothesis that pESAG7/6 are structurally related to the N-terminal domain of the VSG. In addition to mapping the ligand-binding site, the results reported here are consistent with an evolutionary and structural relationship between the Tf receptor of *T.brucei* and the major VSG.

Results

Sequence homology between pESAG7/6 and VSGs

A significant sequence homology was reported between pESAG7/6 and the MVAT4 VSG of *T. rhodesiense* (20% identity, and, taking conservative substitutions into account, 60% similarity in a stretch of 224 amino acids) (Hobbs and Boothroyd, 1990). The MiTat 1.5 VSG of *T. brucei* (Carrington *et al.*, 1991) shows even higher homology (Figure 1, top three lines). In the N-terminal domain of the VSG (357 amino acids), the levels of amino acid identity and similarity with pESAG6 are respectively 23 and 76%. The probability of obtaining such a score when comparing pESAG6 with 100 randomized versions of the VSG sequence (prdf test of FASTA; Pearson and Lipman, 1988) is 5×10^{-15} .

This observation prompted us to undertake a detailed sequence comparison between pESAG7/6 and VSGs whose structure is known (Blum *et al.*, 1993). As shown in Figure 1, most of the structurally important features of the N-terminal domain of VSGs of class A, including 80% of the 81 key amino acids (Blum *et al.*, 1993; Cohen *et al.*, 1984; Carrington and Boothroyd, 1996; Lupas, 1996) are conserved in pESAG7/6. These include hydrophobic residues (in light grey) comprising the quasi-periodic repeats forming coiled-coils [heptad repeats in the two long α -helices perpendicular to the cell surface (A and B) and in the α -helix D] (asterisks), several proline and glycine residues defining conserved peptidic turns (between the A and B helices, positions 60–63, and at the end of helix B, position 124) (in hatched bars), a β -sheet (positions 232–241) with a central turn of glycine between sheets 1 and 2 (position 237), most of the cysteine residues, in particular those forming the two intrachain disulfide bridges which define class A VSGs (in dark grey boxes), a loop between positions 129 and 135, and even the typical GRIDE motif with its conserved glycine involved in the symmetrical contact of the B2 helices of the VSG dimer (boxed around position 110). In the case of MiTat 1.5, the sequence conservation with pESAG7/6 is not limited to amino acids generally shared by the different VSGs. It also include two cysteines (positions 67 and 315) and 34 additional residues (small dots). Interestingly, the C-terminus of pESAG7 corresponded in the VSG to the hinge region which separates the N- and C-terminal domains, as defined by the location of the main cleavage site by trypsin in the AnTat 1.1 VSG (arrowhead below position 333) (Pays *et al.*, 1985). This region is also the preferential 3'-limit of segmental gene conversion between AnTat 1.1 and AnTat 1.10 (horizontal open bar below positions 330–340) (Pays *et al.*, 1985). Therefore, pESAG7 can be superimposed on the VSG N-terminal domain, and its gene can be viewed as a VSG gene conversion domain.

A structural model for the transferrin receptor

The alignments in Figure 1 predicted that the four blocks containing the major differences in the sequence of pESAG7 and pESAG6 (boxes I–IV, between positions 200 and 270), thus presumably representing the ligand-binding domain (Salmon *et al.*, 1994), should correspond to the most exposed surface loops of the VSG (termed l, m, o and p in Blum *et al.*, 1993) which exhibit the

trypanosomal surface variant epitopes (Baltz *et al.*, 1991). To evaluate this structural prediction, we took advantage of the observation that the affinity of the AnTat 1.3A receptor for bovine Tf is very low ($K_d \sim 900$ nM), and well below the affinities of Tf receptors from other ESs (K_d s 2–30 nM) (Salmon *et al.*, 1994; Steverding *et al.*, 1995; Borst *et al.*, 1996). According to the model, it was predicted that exchanging amino acids in the putative surface loops of pESAG6/7 from the AnTat 1.3A ES with those found at the same positions in Tf receptors from other ESs should improve the affinity for bovine Tf, while similar changes in other regions of the proteins would not be expected to affect the affinity significantly. Finally, on the basis of the VSG structure, it is also possible to predict mutations that should affect the folding of the surface loops or the dimerization of the subunits, possibly resulting in the loss of ligand binding.

Figure 2A presents the amino acid differences in the putative ligand-binding site of different copies of pESAG6 and pESAG7, and indicates the substitutions that were performed by site-directed mutagenesis in boxes I and II of pESAG6 and pESAG7 from the AnTat 1.3A ES. Mutations 1–4 convert sequences from the AnTat 1.3A receptor into those found in natural variants, whereas mutations 5pp and 6pp consist of the replacement of the original glycine pairs by pairs of prolines, which would be expected to alter the peptide structure. No substitutions were performed in boxes III and IV, because these sequences are essentially invariable between ESs. Changes were also performed in other regions (Figure 2B). Mutations 7 and 8 (positions 141–142 in Figure 1) were designed to probe a region which, according to the model, is not part of the ligand-binding site. These changes are of particular interest since they are in a region believed to be responsible for antigenic variation of the receptor (Borst, 1991). Mutations 9.1–9.4 occur at the end of the heptad repeat region (position 124–125 in Figure 1), and are located in one of the few spots, apart from the four central boxes and the C-terminus, where the sequence diverges between pESAG7 and pESAG6 (these spots are represented by large dots above the sequence alignments in Figure 1). Finally, mutation 10 consists of the replacement of the key glycine of the GRIDE motif (position 108 in Figure 1) by a charged residue (glutamic acid).

The wild-type and altered versions of ESAG7/6 were transcribed *in vitro*, and the mRNAs were injected into *Xenopus laevis* oocytes which were used to assay Tf binding. As shown in Table I, the affinity of the AnTat 1.3A receptor for bovine Tf was increased 22-fold in mutant 1, while mutations 2, 3 and 4 only slightly improved the binding. The combination of mutations 1 and 2 resulted in a 25-fold increase in affinity, and the combination of mutations 1, 2 and 3 improved the binding nearly 50-fold, to a K_d of 20 nM. Since the change of the dipeptide VR into PT in box II was the most effective, we evaluated the relative contribution of each one of these two amino acids (mutations 1.1 and 1.2). The single replacement of the valine at position 233 by proline increased the affinity 12-fold. Thus, this residue in box II of pESAG6 strongly influences the binding parameters, although amino acids in box I from both pESAG6 and pESAG7 also contribute. As expected, the disruption of the peptide structure in these regions, obtained by replacing

Fig. 1. Sequence alignment between pESAG7/6 and several VSGs. A first alignment was performed using the pileup program of GCG (GapWeight: 3; GapLengthWeight: 0.1), then this alignment was refined according to Blum *et al.* (1993), taking the known three-dimensional structure of the MiTat 1.2 and ILTat 1.24 VSGs into account. DSSP 1.2 and 1.24 refer to the secondary structure, determined by the DSSP program, of MiTat 1.2 and ILTat 1.24 respectively (a = α -helix; b = β -sheet; 3 = 3/10 helix) (the boxes represent regions of structural conservation between the two VSGs). The nomenclature given to the VSG structural elements by Blum *et al.* (1993) is mentioned under the sequence alignments, and is also used in the scheme of Figure 2B. The values in parentheses before the sequences indicate the number of amino acids from the initiation codon to the first amino acid in the alignments; these amino acids form the N-terminal signal sequence of each protein. In the alignments, the residues in light grey are conserved between at least 80% of the 10 VSGs examined by Blum *et al.* (1993) as well as in pESAG7/6; the residues marked with asterisks belong to heptad repeats forming coiled-coil structures; the boxes in light grey contain the conserved cysteines; the hatched residues represent prolines and glycines defining conserved peptidic turns; the boxed Asns are putative *N*-glycosylation sites; the box around position 110 contains the GRIDE motif (see text); and the four boxes in dark grey between positions 200 and 270 are blocks of sequence divergence between pESAG7 and pESAG6. Other differences between pESAG7 and pESAG6 are indicated by large dots above the sequences, whereas small dots point to amino acids specifically conserved between pESAG6/7 and the MiTat 1.5 VSG. Also indicated above the sequences are relevant restriction sites in ESAG7/6 (*Hind*III and *Sac*I, see text). Finally, the open bar under the 330–340 region indicates the preferential 3'-end region of intragenic conversion events in AnTat 1.1/1.10 VSGs, whereas the arrowhead below position 333 defines the main trypsin cleavage site in the AnTat 1.1 VSG (see text).

glycine pairs with proline pairs (mutants 5pp and 6pp, in box I and II respectively), completely abolished the binding of Tf. Substitutions in the region defined as 'hypervariable' (Borst, 1991) only slightly improved the efficiency of binding (mutations 7 and 8). Different substitutions performed at the end of the heptad repeats led to complete loss of binding. These substitutions included the replace-

ment, in pESAG6, of the arginine located immediately downstream from the conserved glycine at position 124 (see Figure 1) (mutation 9.1), as well as the replacement, in either pESAG6 or pESAG7 or both, of the glycine itself by a charged residue (mutations 9.2–9.4). Presumably the turn generated by the glycine at this position is essential for proper folding of the surface loops. Finally,

Table I. Relative K_d s of different mutants of the receptor for bovine Tf from *T.b.brucei*, variant AnTat 1.3A

		$K_d \pm \text{SD (nM)}$	Affinity relative to the wild-type	Nature of the mutation and location
Natural variant	ESAG 6+7	970 $\pm$ 360	1	
Mutant				
1	ESAG 6A +7	44.2 $\pm$ 4.9	21.9	²³³ VR→PT in box II from ESAG 6
1.1	ESAG 6A1 +7	78.9 $\pm$ 19.2	12.3	²³³ V→P in box II from ESAG 6
1.2	ESAG 6A2 +7	665 $\pm$ ND	1.4	²³⁴ R→T in box II from ESAG 6
2	ESAG 6B +7	461 $\pm$ 258	2.1	²¹⁴ RH→KD in box I from ESAG 6
1+2	ESAG 6C +7	38.6 $\pm$ 3.8	25.1	= 1 + 2
3	ESAG 6D +7	461.8 $\pm$ 17.4	2.1	²⁰⁵ AE→VK just before the box I from ESAG 6
1+2+3	ESAG 6E +7	19.9 $\pm$ 4.8	48.7	= 1 + 2 + 3
4	ESAG 6+ 7F	299 $\pm$ 121	3.2	²¹² GE→RK in box I from ESAG 7
5 _{pp}	ESAG 6I +7 ^a	5840 $\pm$ ND	0.2	²¹⁰ GG→PP in box I from ESAG 6
6 _{pp}	ESAG 6J +7 ^a	–	0	²³⁰ GG→PP in box II from ESAG 6
7	ESAG 6+ 7H ^a	612 $\pm$ 18	1.6	¹⁵⁷ HG→QN in the ‘hypervariable’ region from ESAG 7
8	ESAG 6K +7	258 $\pm$ 27	3.7	¹⁵⁷ HG→QN in the ‘hypervariable’ region from ESAG 6
9.1	ESAG 6N +7	5982 $\pm$ 323	0.2	¹⁴¹ R→G at the end of the heptad repeat region (α -helix) from ESAG 6
9.2	ESAG 6+ 7M	–	0	¹⁴⁰ G→D at the end of the heptad repeat region (α -helix) from ESAG 7
9.3	ESAG 6N + 7M	–	0	¹⁴⁰ G→D and ¹⁴¹ R→G at the end of the heptad repeats from ESAG 7 and 6 respectively
9.4	ESAG 6G +7 ^a	3782 $\pm$ 658	0.3	¹⁴⁰ GR→DG at the end of the heptad repeat region (α -helix) from ESAG 6
10	ESAG 6L +7 ^a	–	0	¹²⁶ G→E in ESAG 6, in the sequence ‘GRLEE’ homologous to ‘GRIDE’ from the VSG

^aAmino acid substitutions not found in the known TfR variants.

Fig. 3. Analysis of pESAG7 and pESAG6 expression in *Xenopus laevis* oocytes. Oocytes co-injected with the wild-type *ESAG7* mRNA and either wild-type or two different altered versions of *ESAG6* mRNA were incubated overnight with [^{35} S]methionine. The labelled proteins were extracted with either *n*-octylglucoside (1.5%, w/v) (total protein extract, **A**) or Triton X-114 (1%) according to Bordier (1981) (detergent phase, **B**). The samples were subjected to SDS-PAGE and autoradiography as described by Salmon *et al.* (1994). The arrows designate the proteins translated from the injected mRNAs.

as expected on the basis of its importance in the VSG structure, the glycine in the GRIDE motif was also essential in the Tf receptor, since its replacement by glutamic acid abolished the binding of Tf (mutation 10).

The expression of the modified proteins in oocytes was assessed in the case of mutations which abolished Tf binding in order to ensure that the lack of Tf binding was not due to a deleterious effect on the expression or processing of the proteins. Significantly, when Tf binding was abolished, both pESAG7 and the mutated pESAG6 proteins were expressed at the same level as for oocytes co-injected with the wild-type mRNAs (Figure 3A). Moreover, when pESAG7 was co-expressed with either the wild-type pESAG6 or a version containing a mutation in the binding domain, i.e. 6pp, it was also recovered along with pESAG6 in the detergent phase after treatment with Triton X-114 (Bordier, 1981), presumably due to formation of a heterodimer with either version of the GPI-anchored pESAG6 (Figure 3B). Thus, alterations in the region of the binding site, although abolishing Tf binding, did not affect the processing of pESAG6 nor the formation of heterodimers with pESAG7. However, in the case of alterations in the GRLEE motif of pESAG6 (mutation 10), only pESAG6 was recovered in the detergent phase, which was consistent with the expected inability of this mutant to form heterodimers with pESAG7 and consequently the lack of Tf binding.

Constructing a VSG-based transferrin receptor

Based on the prediction that the structure of pESAG7/6 is similar to that of a VSG, we investigated the novel possibility of converting a VSG into a Tf receptor. A PCR approach was employed to construct hybrid genes encoding proteins containing the C-terminal half of pESAG7 and pESAG6 which contain the ligand-binding site, i.e. downstream from Ser188 (*Hind*III site in Figure 1), linked to the N-terminal half (amino acids 1–185) of the MiTat 1.5 VSG (scheme in Figure 4). According to the proposed model, these constructs should result in the replacement of the exposed surface loops of the VSG by those of pESAG7 and pESAG6, which generate the Tf-binding site. The simultaneous injection into *Xenopus*

K_D for bovine Tf

- VSG/pESAG6 alone:	(no binding)
- VSG/pESAG7 alone:	(no binding)
- VSG/pESAG6 + VSG/pESAG7:	$1.20 \mu\text{M} \pm 0.27$
- pESAG6 + pESAG7:	$0.97 \mu\text{M} \pm 0.36$

Fig. 4. The heterodimer associating the VSG/pESAG7 and VSG/pESAG6 chimeras efficiently binds transferrin. The diagrams represent the chimeric genes, with indication of the *Hind*III site (H) used as a hinge between the MiTat 1.5 5'-terminal half (left) and the ESAG6 or ESAG7 3'-terminal half (right). The *Mi*Tat 1.5 sequence is represented by a hatched box, with the sequence encoding the N-terminal signal peptide in grey. The four blocks of sequence divergence between *ESAG6* and *ESAG7* are represented as black boxes. The sequences of *ESAG7/6* located downstream from the *Sac*I site (Ss) are not necessary for Tf binding (Salmon *et al.*, 1994), and that encoding the C-terminal hydrophobic tail of pESAG6 (stippled) is responsible for surface anchoring of the receptor.

oocytes of these *Mi*Tat/*ESAG7* and *Mi*Tat/*ESAG6* chimeric mRNAs led to efficient binding of Tf (similar K_d for bovine Tf as pESAG7/6), whereas the injection of either chimeric mRNA alone did not result in Tf binding (Figure 4).

Discussion

Through amino acid sequence comparison, it was hypothesized that the components of the Tf receptor of *T.brucei*, pESAG7/6, are likely to be folded in a similar fashion to the N-terminal domain of the VSG and that the ligand-binding domain corresponds to the exposed surface loops of the heterodimer. This proposal was supported by the results from several experiments involving the measurement of Tf binding to the receptor after site-directed mutagenesis and the generation of heterodimers of chimeric proteins consisting of the N-terminal domain of a VSG fused to the C-terminal half of either pESAG6 or pESAG7. First, the affinity of binding of the Tf receptor was increased nearly 50-fold after substitutions performed in two small stretches (boxes I and II, amino acids 209–213 and 223–237), which, according to the hypothesis, correspond to the most exposed loops of the VSGs as determined by X-ray crystallography (loops I and m, roughly between amino acids 200 and 230; Blum *et al.*, 1993) and by experimental mapping of the cell surface epitopes (Baltz *et al.*, 1991). Second, amino acid substitutions performed in other regions of the Tf receptor were either relatively neutral or abolished Tf binding. Third, abolition of Tf binding was not due to a deleterious effect of the mutation on the expression or processing of the

modified proteins but was probably due to changes in the folding of the binding domain or the inability to form a heterodimeric complex. Finally, chimeric protein constructs consisting of the C-terminal half of pESAG7 or pESAG6, which together form the ligand-binding domain, fused to the N-terminal dimerization domain of the VSG were processed correctly and associated successfully to form membrane-bound heterodimers that efficiently bind Tf.

While the sequence homology between pESAG7/6 and different VSGs of class A was usually restricted to residues which are key structural determinants, in some instances, particularly in the case of the MiTat 1.5 VSG, the homology was more extensive. This sequence homology between the Tf receptor and the VSGs is consistent with a common evolutionary origin for both proteins, which are probably subjected to similar structural and organizational constraints in the surface membrane (Carrington and Boothroyd, 1996). Interestingly, *ESAG7/6* also share homology with *PAG1*, a gene only expressed during the insect stage of the life cycle of these parasites (Koenig-Martin *et al.*, 1992). This observation may be consistent with the proposal of an original ancestral gene for a surface protein in primitive parasites of insects (Maslov and Simpson, 1995). If so, such an ancestral gene was probably limited to the N-terminal domain of the VSG, since this region appears to behave as a mini-gene. First, segmental gene conversion occurring between extensively homologous VSGs is preferentially restricted to this domain (Pays *et al.*, 1985). Second, the comparison of several VSG sequences strongly suggests shuffling of N-terminal domains between different classes of VSG (Carrington *et al.*, 1991). Therefore, the sequence encoding the N-terminal domain appears to recombine and evolve independently of that of the C-terminal domain, and it is conceivable that the VSG may have evolved from the ancestor by the acquisition of a C-terminal domain, rather than the Tf receptor from a VSG by the loss of the C-terminal domain. In this respect, it is interesting to note the case of the VSGs of *T. congolense*, a closely related species of African trypanosome. These VSGs are among the smallest described to date, typically containing on average 435 residues (close to the size of the pESAG6 average of 401 residues), and possess an N-terminal domain closely related to the B domain type of *T. brucei* VSGs but lack a defined VSG C-terminal domain (Rausch *et al.*, 1994; Urakawa *et al.*, 1997). It is tempting to speculate that, like the Tf receptor, the smaller VSG of *T. congolense* may also be folded in a manner similar to the N-terminal domain of the VSG of *T. brucei*.

The presence of the Tf receptor genes in the different VSG ESs, possibly due to telomere conversion (Pays *et al.*, 1983), ensures the expression of this receptor independently of the ES employed for antigenic variation. It also allowed an independent evolution of the different gene copies, perhaps through the accumulation of point mutations during gene conversion, as may occur for the VSG (Lu *et al.*, 1993). Thus, since each trypanosome contains a repertoire of ~20 different ESs, it also possesses up to 20 genes for Tf receptors. This work allowed the identification of a few amino acids which are crucial for ligand binding and demonstrated that the variability of these amino acids between proteins encoded by different

VSG ESs may account for the important differences in ligand-binding parameters observed between trypanosome variants. Since different receptors are expressed upon switching of VSG ESs, the alternative use of the different ESs leads to the expression of receptors with different affinities for Tf, which may contribute to explaining the growth differences observed between trypanosomes expressing different VSGs (Van Meirvenne *et al.*, 1975; Borst *et al.*, 1996).

Materials and methods

All materials and methods, including the Tf-binding assays in *X. laevis* oocytes, were as described in Salmon *et al.* (1994).

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