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Miniantibodies

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Introduction

The term "miniantibodies" describes artificial multivalent or multispecific recombinant antibody fragments. They resemble natural antibodies in carrying two or four binding sites with a flexible arrangement and a long "wing span" but they are much smaller, as they consist only of fusions of a scFv to an oligomerization module. Applications of multivalent and bispecific antibody fragments in a variety of formats have been reviewed (Plückthun and Pack 1997; Carter and Merchant 1997). Approaches to quantify the avidity gain achieved by the multivalency effect present in all the described miniantibody models have been reported (Crothers and Metzger 1972, Plückthun and Pack 1997, Müller et al. 1998b).

Briefly, there are three motivations to consider making miniantibodies:

- First, they will bind with significantly higher avidity than monomers to any surface carrying the antigen close enough that two (or more) binding sites can be engaged.
- Second, when these molecules are immobilized on plastic support, at least one binding site usually remains functional, while the other may denature upon binding to the plastic surface. In contrast, scFv fragments usually lose all binding under these conditions.
- Third, two different specificities can be combined, with numerous applications in biotechnology, diagnostics and, potentially, therapy.

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The basic element of all constructs is a fusion of a scFv fragment to an oligomerizing element, in the simplest case an amphipathic α-helix-forming stretch of amino acids (usually between 16 and 40 residues, but see Table 2 for details) via a flexible hinge region, giving the partners enough steric freedom to fold individually. As outlined schematically in Fig. 1, this leads to dimeric or tetrameric miniantibodies, depending on the oligomerization motif chosen. Most conveniently, the miniantibodies are expressed in the periplasm to allow the disulfide formation in the scFv part. This requires that the chosen oligomerization modules are compatible with periplasmic folding.

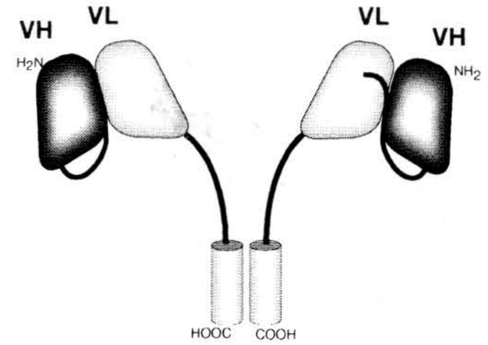
Dimeric miniantibody constructs

While all other principles for the formation of bivalent or bispecific antibody fragments (discussed in chapters 42 to 45) require a significant reconstruction of the format compared to the scFv, the generation of dimeric miniantibodies is simply achieved by adding a sequence to the C-terminus. Examples for such oligomerization modules are a naturally occurring dimerization helix from the yeast transcription factor GCN4 (O'Shea et al. 1991, Dürr et al. 1999), or C_H3/F_c-domains of antibodies (Chapter 44), or a synthetic 4-helix bundle element (Eisenberg et al. 1986).

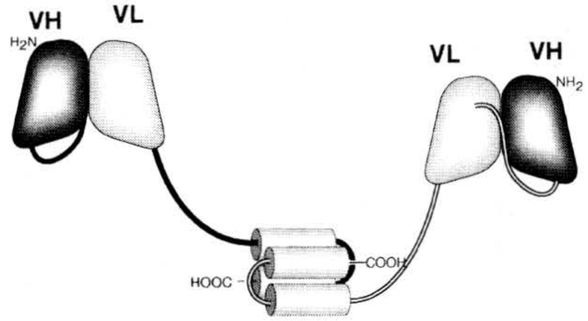
The 4-helix bundle motif is obtained by the alignment of two double-helix pairs, each pair being fused to one scFv and "clasping" each other. As these helices are in antiparallel orientation, this results in the two scFvs emerging from opposite sides of the bundle (see Fig. 1, scdHLX). Parallel alignment of the helices is obtained if GCN4 "leucine zipper" or other coiled coils like e.g. JUN/FOS are used as fusion partners (Fig. 1).

Bi*specific* miniantibodies can be obtained if two different scFvs are chosen as fusion partners and fused to modules which form a specific heterodimer. Not all heterodimerizing modules work well in vivo, as problems of homodimerization and proteolytic susceptibility are often not considered. Recently, the question of specific heterodimerization was tackled using a helix-library vs. helix-library-selection approach *in vivo* (Arndt et al. 2000). The work resulted in a pair of coiled coil helices which showed excellent behavior with regard to stability, heterospecificity, and resistance to proteases.

Dimeric miniantibodies

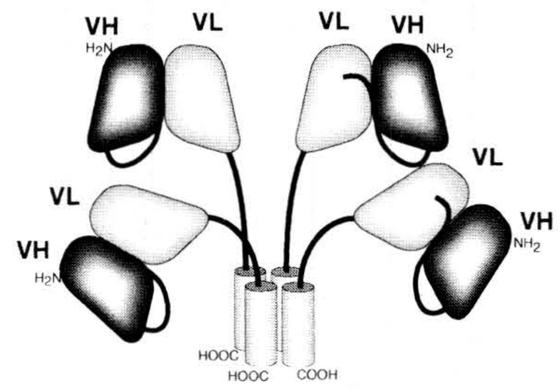


a scZIP

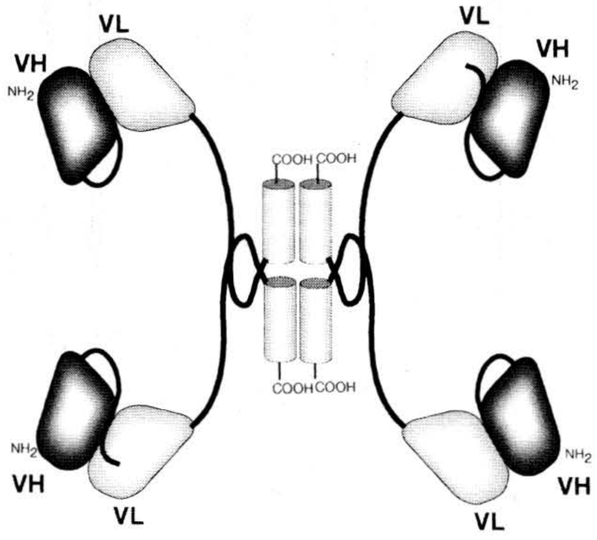


b scdHLX

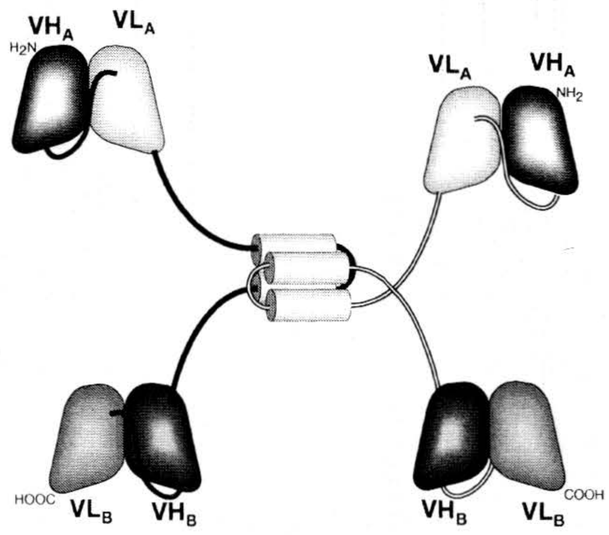
Tetrameric miniantibodies



c scTETRAZIP



d scp53



e di-bi-miniantibody

Fig. 1. Schematic representation of oligomeric miniantibody formats. VH and VL domains of the scFvs are represented in darker and lighter color, respectively. Linker and hinge regions are shown as black lines, and are either filled or not to indicate different polypeptide chains within one heterodimeric construct. Oligomerizing, helical elements are depicted as cylinders. The orientation is derived from the published crystal structures of the coiled coil (O'Shea et al. 1991), tetrazipper (Harbury et al. 1993), the NMR structure of the designed dHLX motif (Hill and DeGrado 1998) and the p53 tetramerization domain (Jeffrey et al. 1995). Dimeric miniantibodies: *(a)* GCN4 leucine zipper, scZIP; *(b)* four helix-bundle, scdHLX; Tetrameric miniantibodies: *(c)* modified GCN4 leucine zipper, scTETRAZIP; *(d)* oligomerizing domain of human p53, scp53; *(e)* $(scFv)_A$-hinge-dHLX-hinge-$(scFv)_B$ arrangement, di-bi-miniantibody.

Tetrameric miniantibody constructs

In principle, all fusion modules forming oligomers themselves appear to be suitable for designing oligomeric miniantibodies, provided their termini are accessible for attaching linker-scFv-cassettes and they are compatible with *in vivo* folding of the antibody.

Specific amino acid exchanges in the hydrophobic contact positions **a** and **d** of the GCN4 zipper results in the self-assembly of a stable tetrameric bundle (Harbury et al. 1993). A fusion of this zipper version to a scFv leads to tetrameric miniantibodies with improved affinity, compared to the corresponding dimeric construct (Pack et al. 1995, Fig. 1). The effect of amino acid exchanges in coiled coil helices on the oligomerization state have recently been reviewed (Müller et al. 2000).

Many fusions to enzymes and other proteins have been reported and have been summarized elsewhere (Plückthun and Pack, 1997). The lowest immunogenicity is to be expected with the fusion of a scFv to the oligomerization domain of human p53 (Jeffrey et al. 1995) via the human IgG3 hinge (Table 1; Burton 1985).

A combination of directed bivalency with bispecificity is obtained by using so-called "di-bi-miniantibodies" (Müller et al. 1998c). Here, a second scFv is fused after the dimerization motif, resulting in a $(scFv)_A$-hinge-dHLX-hinge-$(scFv)_B$ arrangement (Fig. 1).

In order to stabilize the oligomer formation by covalent linkage between either the zipper or the bundle-helix constructs, single cysteine residues may be added at their C-termini which lead to disulfide bridge formation (Pack and Plückthun 1992). It has to be noted, however, that also wrong disulfides may be formed with Fv-internal cysteine residues possibly leading to somewhat lower yields.

Materials

- French Pressure Cell (Aminco, Rochester, NY, USA)
- Molecular biology laboratory instrumentation

Procedure

Below, the bacterial expression of miniantibodies in shake flasks is described in detail. The procedure essentially follows the protocol as described in Pack and Plückthun (1992).

Table 1. Cross-references between oligomerizing elements and corresponding plasmid/literature.

construct	upper hinge	self-associating peptide	linker/tag	plasmid/s	reference
bivalent					
scHLX	murine IgG3	Helix		pACK01sc	C, D
scHLXc	murine IgG3	Helix	Cys tail	pACKHLXc	C, D, E
scdHLX	murine IgG3	Helix1-turn-Helix2		pACKdHLX, pHKK	B, I
scdHLX-His	murine IgG3	Helix1-turn-Helix2	spacer-His tail	pACKdHLXH, pAK500	F, J
scZIP	murine IgG3	GCN4 leucine zipper		pACKZIP	B, C, D, A, E
scZIPc	murine IgG3	GCN4 leucine zipper	Cys tail	pACKZIPc	C, D, E
bispecific					
scJUN	murine IgG3	JUN leucine zipper		pACKIHJUN	K, L
scFOS	murine IgG3	FOS leucine zipper		pACKFOS	K, L
CH1-CL	murine IgG3	CH1 and CL from IgG	His tail	pKM30245M1ChCl	G
tetravalent					
scTETRAZIP	murine IgG3	GCN4 leucine zipper, modified		pACKtZIP	A, E
scp53	human IgG3	oligomerization domain of human p53		pMS9-9 p53	K, L
scp53-His	human IgG3	oligomerization domain of human p53	spacer-His tail	pMStetp53His	F
tetravalent/bispecific					
di-bi	murine IgG3	Helix1-turn-Helix2	His tail after 2nd scFv	pKM310M1dhlx425h	H

Important elements of various miniantibody formats are listed as overview. For exact amino acid sequences of the elements, see Table 2. Vectors carrying miniantibody genes in these formats and references are given. Letters in the reference column denote: (A) Pack et al. 1995; (B): Pack et al. 1993; (C) Pack and Plückthun 1992; (D) Pack et al. 1992; (E) Ge et al. 1995; (F) Rheinnecker et al. 1996; (G) Müller et al. 1998a; (H) Müller et al. 1998b; (I) Horn et al. 1996; (J) Krebber et al. 1997; (K) Plückthun and Pack 1997; (L) Pack 1994.

Table 2. Amino acid sequences of hinges and oligomerizing elements.

element	amino acid sequence	construct
murine IgG3 upper hinge	PKPSTPPGSS	
human IgG3 upper hinge	TPLGDTTHTSG	
helix	GELEELLKHLKELLKG-EF	scHLX
helix-Cys tail	GELEELLKHLKELLKG-PRKANSRNC	scHLXc
helix1-turn-helix2	GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKG-EF	scdHLX or di-bi
helix1-turn-helix2-spacer-His tail	GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKG-GSGGAP-HHHHH	scdHLX-His
GCN4 leucine zipper	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER	scZIP
GCN4 leucine zipper-Cys tail	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER-GGCGG	scZIPc
JUN leucine zipper	RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNY	scJUN
FOS leucine zipper	LTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAAH	scFOS
GCN4 leucine zipper, modified	R**L**KQ**I**EDK**L**EE**I**LSK**L**YH**I**ENE**L**AR**I**KKL**L**GER	scTETRAZIP
oligomerization domain human p53	KPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEP	scp53
oligomerization domain human p53-spacer-His tail	KPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEP-GGSGGAP-HHHHH	scp53-His

Amino acid sequences (one-letter-code) of various oligomerizing modules and hinges are given. In the modified GCN4 leucine zipper, which leads to tetramerization, the exchanged amino acids are in bold-face. The amino acids EF and the end of some constructs were introduced for an *Eco*RI-restriction site. Cross-references to the corresponding vectors and literature are listed in Table 1.

Detailed information on high-cell-density fermentation is given in Horn et al. (1996), Schroeckh et al. (1996), Plückthun et al. (1996), and Pack et al. (1993).

1. Select one of the presented formats (Table 1) for the chosen scFv and pick the appropriate vector (Table 2 and Fig. 2).

2. If no compatible restriction sites are present, design suitable PCR-primers.

   Note: A discussion on how to PCR amplify an antibody with an unknown sequence and convert it into a scFv format compatible with this vector series is given in Chapter 2

3. PCR amplify and clone the scFv in the selected vector. Confirm the correct DNA sequence.

   Note: Add 1% glucose in all growth media (steps 3 through 6) in order to reduce expression from potentially leaky CAP regulated promoters prior to induction.

4. Transform a suitable *E. coli* host (e.g. JM83 (Yanisch-Perron et al. 1985), RV308 (Maurer et al. 1980) or SB536 (Bass et al. 1996)).

5. From a single colony inoculate ca. 25 ml preculture in LB medium. For this volume use at least a 250 ml shaking flask. Shake at 25°C overnight.

6. From this overnight culture inoculate the main culture in rich medium with 1% glucose to a starting OD_{550} of 0.1. Use a baffled shake flask for higher final cell densities. Shake at 25°C and add 1 mM IPTG (final conc.) at an OD_{550} of 0.5. Continue growth for another 3 h or until the cells stop growing.

   Note: If the vector carries the *skp* or *fkpA* gene, much higher cell densities can be obtained, as the cells usually do not lyse or stop growth after induction. For details, see Chapter 23.

7. Harvest the cells by centrifugation (4500 x g, 10 min, 4°C).

   Note: From now on, all steps should be carried out at 4°C in order to minimize protease activity and to stabilize the protein of interest.

8. Resuspend the cell pellet carefully in 1/100 column volume of loading buffer. This, of course, depends on which purification method is chosen for the miniantibody. If the construct carries a his tag for IMAC purification (as for some of the vectors in Table 1 or pAK500, Fig. 2), it is recommended to use cold 50 mM Tris-Cl, 1 M NaCl, pH 8.0 (Lindner et al. 1992).

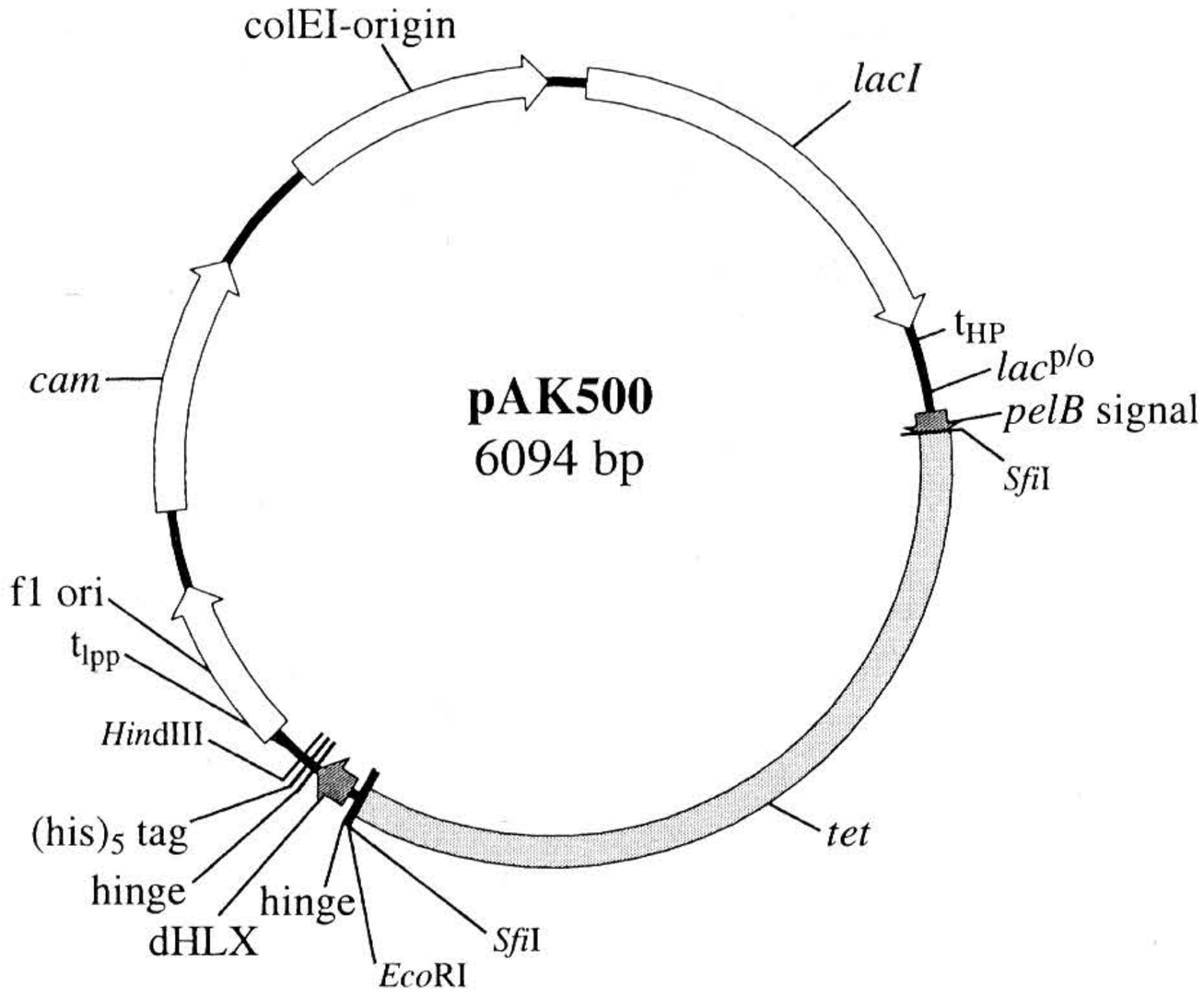


Fig. 2. pAK500 vector (Krebber et al. 1997, see also Chapter 2) containing the dHLX cassette and the tetracyclin-resistance cassette as stuffer. This stuffer, shown herre only schematically, contains the genes for *tetA* and *tetR*, and it does not make a fusion protein with upstream or downstream elements in the vector. For details, see Chapter 2. As an example for a potential miniantibody producing plasmid, the tet-cassette in pAK 500 was replaced by a scFv to build pAK5scFv. Size of the genetic elements is drawn to scale. *lacI*: *lac* repressor; t_{HP}: strong upstream terminator to prevent read-through from *lacI* expression; *lac* p/o: *lac* promoter/operator; *pelB* signal: leader sequence (pectate lyase gene of *Erwinia carotovora*), modified to contain a *Sfi*I site; *tet*: tetracyclin resistance "stuffer" cassette (contains *tetA* and *tetR*-genes; 2101 bp), which is to be replaced by the antibody gene; FLAG tag: short (DYKD) version of FLAG tag for Western blot detection (Knappik and Plückthun, 1994); VH-linker-VL scFv gene; hinge: murine IgG3 hinge region; dHLX: double helix element, see Table 2; $(his)_5$ tag: stretch of 5 histidine residues for IMAC purification (Lindner et al. 1992) and detection with an anti-his tag antibody (e.g. 3D5-phosphatase fusion (Lindner et al. 1997)); t_{lpp}: downstream terminator; f1 ori: intergenic region of phage f1 (for production of single-stranded DNA; *cam*: chloramphenicol-acetyl-transferase gene; colEI-origin: plasmid replication origin (derived from pUC-plasmid series).

9. Break the cells by French Press (20000 psi, pass cells through twice) or sonification. In both methods, take care that the cell suspension is not exposed to heating.

10. Centrifuge the crude extract in order to separate insoluble cell debris from soluble protein (48000 x g, 30 min, 4°C). Carefully separate supernatant from pellet.

Note: The soluble/insoluble distribution of the miniantibody expressed may be checked by performing a Western blot (see also Chapter 23). If the gene of interest was inserted in a pAK-vector (see Table 1 and Fig. 2) via *Sfi*I as described in Chapter 2, this will lead to periplasmically expressed protein carrying a short FLAG tag at its mature N-terminus ($^{+}H_3N$-DYKD...) which can be detected by the M1 anti-FLAG antibody (Kodak; Knappik and Plückthun 1994).

11. If the protein of interest is (mainly) in the soluble fraction, continue by applying the supernatant of step 10 to the appropriate chromatography column.

    Note: Purification of scFvs via IMAC on the BioCAD-instrument with a rapid two-column procedure (PerkinElmer, Wellesley, MA, USA) is described in detail in Chapter 23. Conventional purification of antibody fragments via IMAC is described in detail in Lindner et al. (1992).

12. If the protein of interest is (mainly) insoluble, you may follow several lines:
    i. Co-express a molecular chaperone which may increase the soluble fraction (Chapter 23; Bothmann and Plückthun 1998, 2000).
    ii. Refold the protein from inclusion bodies. First, reclone it without signal sequence in a plasmid with the strong T7-expression system (Ge et al. 1995). Refolding, however, has to be optimized for each protein individually (for initial guidance, see Huston et al. 1991; Rudolph and Lilie 1996). However, there is a refolding kit available commercially, which may facilitate the screening for optimal conditions (Hampton Research, Laguna Niguel, CA, USA).
    iii. Probably most efficiently, introduce mutations in the scFv gene which may support proper folding or transplant the CDRs to a well folding framework, thus leading to reduced aggregation (see e.g. Knappik and Plückthun 1995; Nieba et al. 1997; Jung and Plückthun 1997; Willuda et al. 1999; Lindner, Blank and Honegger, unpublished experiments).

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