Bacteriophage lambda surface display of a bacterial biotin acceptor domain reveals the minimal peptide size required for biotinylation

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Abstract Phage display is a powerful technique for identifying specific ligands to a given target. In this work random peptides derived from the biotin accepting domain of the *Klebsiella pneumoniae* oxaloacetate decarboxylase were displayed on bacteriophage lambda heads to determine the minimal sequence length that is necessary to effect biotinylation in vivo. Phages with a functional biotinylation domain were identified after affinity purification with immobilised avidin. All biotinylated phages isolated this way were found to have a sequence of 66 amino acids from the parental protein in common. This minimal biotinylation domain is fully functional as a biotin acceptor and more resistant to proteolytic attack compared to domains of larger size derived from the same protein. The data present the first example of a posttranslational protein modification analysed in a phage display system. Moreover, a biotin domain of reduced size and improved stability was identified, that should be superior to the larger parental protein as a tag to generate biotinylated fusion proteins.

Key words: Biotin; Enzymatic biotinylation; Phage display; Bacteriophage lambda

1. Introduction

Due to the high affinity of biotin to avidin and streptavidin, biotin-based reagents and applications are widely used in molecular biology [1]. Examples include detection, localisation, purification and immobilisation of nucleic acids, proteins and other macromolecules. A large set of chemicals for the covalent biotinylation are available, but target molecules may be inactivated or not uniformly labelled by chemical reactions. An alternative approach to covalent biotinylation of proteins is to generate genetic fusions, in which the protein of interest is linked to a protein domain that is a substrate for protein biotin ligase [2]. This approach is facilitated by the high degree of sequence conservation among the known biotin enzymes, and indeed, protein biotin ligases have been shown to biotinylate acceptor proteins from very different species [2,3]. For the generation of fusion proteins, domains with 67 [4], 75 [2], 76 [5], 78 [6], 82 [7], 87 [8], 95 [9], 99 [10], and 105 [11] amino acids of biotinylated proteins were employed to direct addition of biotin to a fusion. To our knowledge, an unbiased approach to identify the minimal peptide size that is necessary to specify biotinylation has not been performed for any of these biotin accepting domains.

Phage display is a very powerful technique for identifying interacting molecules. It derives its versatility from the genetic diversity that can be generated in phage vectors and from the physical linkage of the peptide to its DNA. Phage display of antibody domains, for example, is frequently employed to identify antibodies with desired specificities or increased affinities to a given target. Vice versa, display of random or antigen-derived peptide sequences is highly efficient to map the epitopes of monoclonal antibodies.

Here we show that phage display is also useful to study posttranslational modifications of proteins. We used bacteriophage lambda surface display of the biotin acceptor domain of the *Klebsiella pneumoniae* oxaloacetate decarboxylase α-subunit (Kp_OAD) to analyse the sequence requirements for biotinylation in vivo. This domain is known to be readily biotinylated in *Escherichia coli* [2,12], *Saccharomyces cerevisiae* [13] and *Schizosaccharomyces pombe* [14], and can therefore be regarded as a model substrate for biotin ligases. DNA encoding this protein was randomly fragmented and cloned into λfooDc to express the DNA fragments as genetic fusions to the capsid protein D. The resulting phage libraries were screened for the presence of biotinylated particles, and positive phages were purified and sequenced. Alignment of the obtained sequences identifies the minimal domain size that permits biotinylation in vivo.

2. Materials and methods

2.1. Generation of the phage display libraries

Bacteriophage λfooDc is a phage vector that expresses insert DNA as fusion to the 3′ end of the D-gene that encodes the capsid protein D (gpD) [15]. DNA from bacteriophage λfooDc was prepared according to standard procedures [16], and cut with *Eco*RI and *Bam*HI. The polylinker-derived *Bam*HI-*Eco*RI fragment was removed by annealing the digested DNA at the cos ends and precipitation with 0.6 volumes of isopropanol in the presence of 0.3 M Na-acetate pH 6.0. DNA encoding amino acids 501–594 of the Kp_OAD sequence [17] was part of an *Eco*RI fragment encoding the fusion protein PmSUC2bio-his6 in the vector pSH213 [13]. Insert DNA was excised from pSH213 with *Eco*RI, and 6 μg of the purified fragment was digested with 10 U of DNase I (Merck, 116326) for 15 min at 14°C in 180 μl buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 50 μg/ml bovine serum albumin). DNase I-generated fragments were blunted with T_4-DNA polymerase and ligated to a 20-fold molar excess of the adaptors ERI (5′-AATTCGCGGCCGCT-3′ annealed to 5′-AGCGGCCGCG-3′) and BHI (5′-GATCCGCGGCCGCT-3′ annealed to 5′-AGCGGCCGCG-3′). Unligated adaptors were removed by electrophoresis through a 2% (w/v) agarose gel and fragments ranging in size from

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Abbreviations: Ec_BCCP, biotin carboxyl carrier protein of the *Escherichia coli* acetyl-CoA carboxylase; gpD, phage λ protein encoded by gene D; Hs_PCCA, human propionyl-CoA carboxylase α-subunit; Kp_OAD, *Klebsiella pneumoniae* oxaloacetate decarboxylase α-subunit; PCR, polymerase chain reaction; pfu, plaque forming unit; Ps_TC, *Propionibacterium shermanii* transcarboxylase 1.3S subunit; PMSF, phenylmethylsulfonyl fluoride; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; X-phos, 5-bromo-4-chloro-3-indolyl phosphate

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50 to 150 bp and from 150 to 250 bp were eluted, ligated to λfooDc arms and packaged. The resulting libraries were analysed in *E. coli* JM105 [18] by plating in the presence 0.2 mg/ml X-gal and amplified in *E. coli* Q358 [19] to induce generation of fusion proteins.

2.2. Affinity selection and sequencing of biotinylated phages

An affinity matrix for selection of phages with biotinylated capsid fusion proteins was created by coating the wells of an ELISA plate with avidin (Sigma A-9275, dissolved in water at 1 mg/ml) for 1 h at room temperature. Affinity selection was performed as described [19] using 3×10^8 pfu per well. Enriched phages were used to infect *E. coli* Q358 and plated on LB plates supplemented with 2 mg/l biotin. Plaques were lifted to nitrocellulose, filters were blocked (10 g/l non-fat dry milk in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20), decorated with avidin-alkaline phosphatase conjugate (Sigma, E-2636, diluted 1:5000 in blocking buffer), and developed (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.25 mg/ml nitroblue tetrazolium, 0.25 mg/ml X-phos). DNA sequences of positive phages were determined after PCR amplification of the insert with the primers lfDc-5 (5′-GACTTCGACCGTTGGGCC-3′) and rev (5′-AACAGCTATGACCAT-3′).

2.3. Western blotting of phage proteins

E. coli Q358 was infected with selected phages and plated on LB plates supplemented with 2 mg/l biotin. Phages were eluted from a confluent plate with 4 ml of lambda-dil, supplemented with 2 mM PMSF at 4°C. Phage proteins were separated on a 15% SDS gel [20], and transferred to a nitrocellulose membrane. Biotinylated proteins were detected with avidin-peroxidase (Amersham RPN1231, diluted 1:2000) using Amersham's ECL kit.

3. Results

3.1. Generation and screening of two libraries and sequencing of positive clones

Two lambda surface display libraries have been established that express fragments of Kp_OAD as C-terminal fusions to the capsid protein D. The library obtained with the larger fragments (150–250 bp) contained 1×10^6 pfu, of which 5% formed white plaques on X-gal plates. The library with the smaller inserts (50–150 bp) contained 5×10^5 pfu and 10% of them were white. Both libraries were amplified in *E. coli* Q358 to express the inserted DNA fused to gpD. Recombinant phages with functional biotin acceptor domains were enriched from the library by binding to avidin-coated microtitre wells. Of 700 plaques that were obtained from the large-insert library, 79 scored positive after plaque lifts and labelling with avidin-phosphatase conjugate. Positive signals were significantly stronger than the background staining that presumably resulted from binding of avidin-phosphatase to Ec_BCCP, the only biotinylated protein present in *E. coli* [21]. The inserts of 48 positive clones were sequenced. Positive clones could not be obtained from the small-insert library.

3.2. Sequence requirements for biotinylation

Sixteen independent sequences were obtained from the 48 clones analysed. Their derived amino acid sequences are given in Fig. 1. Identical clones most likely arose from the amplification of the library. The *K. pneumoniae* oxaloacetate decarboxylase α-subunit sequence is also given in Fig. 1.

The alignment reveals a stretch of 66 amino acids (boxed in Fig. 1) that is shared by all biotinylated fusion proteins. This sequence corresponds to amino acids 529–594 of Kp_OAD and has the biotinylated lysine residue (marked with an arrowhead in Fig. 1) in the centre. No out-of-frame fusions or sequences unrelated to the sequences aligned in Fig. 1 were identified in these experiments. This underlines both the specificity and the strength of the binding of avidin to biotin and the feasibility of this experimental approach.

Due to the cloning strategy, the biotin domains carried C-terminal extensions that were translated from the α-peptide encoding DNA downstream of the polylinker. Depending on the reading frame, these extensions ranged in length from 8 to 82 amino acids. As expected, clones that had the 82-amino acid α-peptide fused in-frame to the biotin acceptor domain (39.6% of the clones in Fig. 1) displayed a blue colour on X-gal indicator plates, whereas all others formed white plaques (data not shown). Due to this fact, the number of recombinant clones present in the library exceeds the number of white clones stated above.

3.3. Analysis of avidin-reactive phages by Western blotting

To address the degree of biotinylation, phages with various

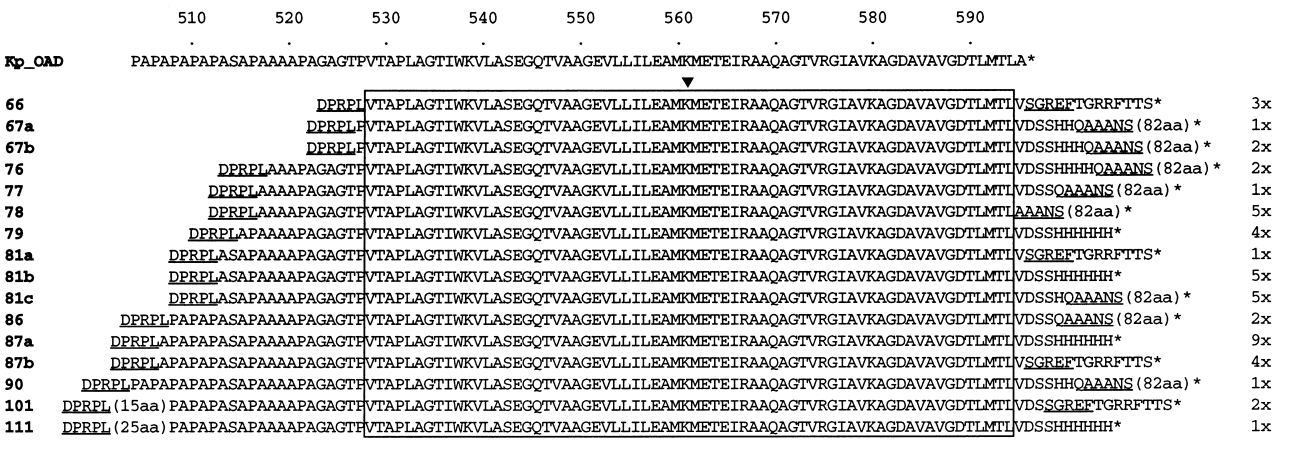

Fig. 1. Peptide sequences encoded by phage clones affinity-selected with avidin. Biotinylated phages were selected from the large-insert library and their inserts were sequenced. The derived protein sequences were aligned with the C-terminal sequence of Kp_OAD. The region found to be identical in all avidin-reactive clones is boxed, amino acids derived from linker sequences are underlined. The number of amino acids extending beyond this region is given in parentheses, the number of clones encoding identical sequences is indicated on the right. The position of the biotinylated lysine residue is marked by a triangle. The numbers on the left of the sequence indicate the number of amino acids that each protein has in common with Kp_OAD.

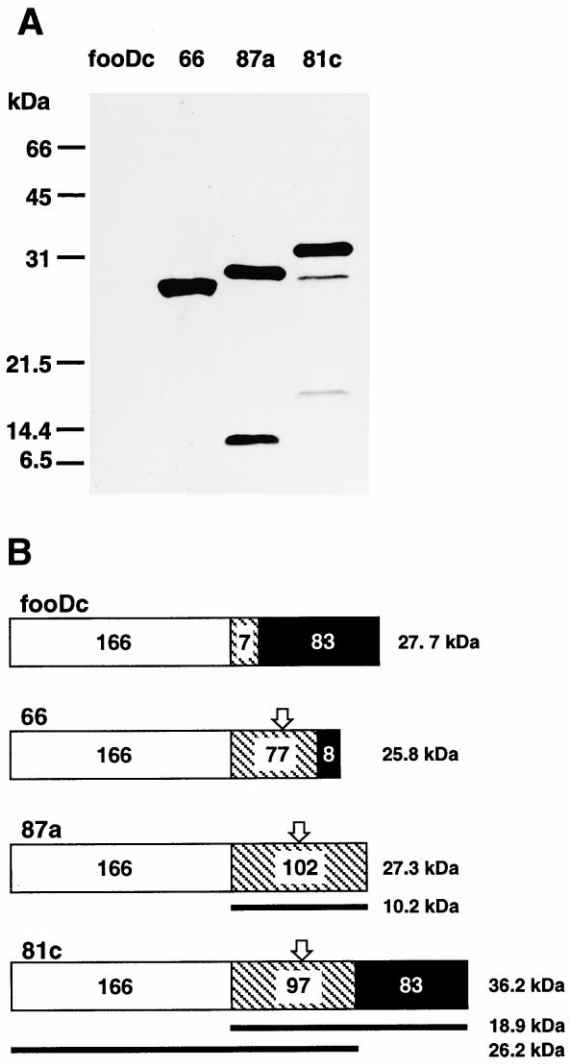

Fig. 2. Western blot analysis of biotinylated phage proteins and structural domain map of gpD fusion proteins. A: SDS-solubilised phage proteins corresponding to 5×10^5 pfu were loaded per lane. Biotinylated proteins were visualised with streptavidin-peroxidase and ECL chemistry. B: Schematic illustration showing the gpD fusions encoded by the phages analysed in A. White segments indicate the amino acids encoded by the D-gene up to the *Bam*HI insertion site, hatched segments indicate amino acids encoded between *Bam*HI and *Eco*RI insertion sites (i.e. DNase I-generated inserts or polylinker sequences). The position of the modified lysine residue within this domain is indicated by an arrow. Black segments indicate amino acids encoded by vector sequences downstream of the *Eco*RI insertion site. The numbers within the boxes indicate the number of amino acids present within the individual segments. Bars below the domain represent biotinylated fragments originating from protease degradation.

lengths of inserts and C-terminal extensions were analysed by Western blotting. The result of this analysis (Fig. 2A) shows no apparent differences in the extent of biotinylation of the fusion proteins. The smallest domain obtained in the screening (phage 66), which comprises only 66 amino acids of Kp_OAD, is as efficiently biotinylated as a larger domain (phage 87), which caries 87 amino acids of Kp_OAD. Moreover, the large C-terminal extension present on phage 81c, encompassing the complete α-peptide, does not restrict biotinylation. However, additional biotinylated bands of increased mobility are apparent in the lanes of phages 81c and 87a. The block diagram of the fusion proteins (Fig. 2B) shows that the observed bands at lower apparent molecular weight might well be the result of proteolysis of the fusion proteins in vivo or during isolation. The observed molecular weights of the fragments in of phages 87a and 81c are fully compatible with the idea that the junction points, at which the individual domains are connected in these fusion proteins, are the most sensitive to proteolysis. Our results indicate that the large fusions encoded by phages 87a and 81c are more prone to proteolytic degradation and that the small fusion protein encoded by phage 66, while apparently fully biotinylated, is also the most resistant to proteolysis.

4. Discussion

Bacteriophage lambda surface display was used to determine the minimal sequence requirements for enzymatic biotinylation. DNA encoding Kp_OAD was partially digested with DNase I and expressed as a fusion to gpD. The inserts of biotinylated phages were sequenced and a stretch of 66 amino acids was found to be common to all avidin-reactive phages isolated in the screening. Biotinylated phages were absent from the small-insert (50–150 bp) library, which has a coding capacity for up to 50 amino acids of Kp_OAD, although this library has successfully been employed in unre-

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                                                    ▼
Kp_OAD     528 PVTAPLAGTI W-------KV LASEGQTVAA GEVLLILEAM KMETEIRAAQ AGTVRGIAVK AGDAVAVGDT LMTLA*          595
St_OAD     523 PVTAPLAGNI W-------KV IATEGQTVAE GDVLLILEAM KMETEIRAAQ AGTVRGIAVK SGDAVSVGDT LMTLA*          590
Ps_TC       56 EIPAPLAGTV S-------KI LVKEGDTVKA GQTVLVLEAM KMETEINAPT DGKVEKVLVK ERDAVQGGQG LIKIG*          123
Hs_PCCA    635 VLRSPMPGVV V-------AV SVKPGDAVAE GQEICVIEAM KMQNSMTAGK TGTVKSVHCQ AGDTVGEGDL LVELE*          702
Ec_BCCP     82 IVRSPMVGTF YRTPSPDAKA FIEVGQKVNV GDTLCIVEAM KMMNQIEADK SGTVKAILVE SGQPVEFDEP LVVIE*          156
Hs_PYC    1111 QIGAPMPGKV I-------DI KVVAGAKVAK GQPLCVLSAM KMETVVTSPM EGTVRKVHVT KDMTLEGDDL ILEIE*         1178
Sc_PYC1   1111 HIGAPMAGVI V-------EV KVHKGSLIKK GQPVAVLSAM KMEMIISSPS DGQVKEVFVS DGENVDSSDL LVLLEDQVPV ETKA* 1178
Sc_ACC1    699 QLRTPSPGKL V-------KF LVENGEHIIK GQPYAEIEVM KMQMPLVSQE NGIVQLLK-Q PGSTIVAGDI MAIMTLDDPS KVKH   774
Rn_ACC     760 VMRSPSAGKL I-------QY IVEDGGHVFA GQCYAEIEVM KMVMTLTAVE SGCIHYVK-R PGAALDPGCV IAKMQLDNPS KVQQ   835
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Fig. 3. Alignment of sequences of biotin proteins. Protein sequences were retrieved from the SwissProt database and aligned according to Toh et al. [22]. The sequence of the minimal biotin accepting domain of Kp_OAD, which was identified in this work, is marked with a bar. Identical residues are boxed, the position of the biotinylated lysine residue is marked with an arrowhead. The abbreviations and SwissProt accession numbers are as follows: Kp_OAD, *Klebsiella pneumoniae* oxaloacetate decarboxylase α-subunit, P13187; St_OAD, *Salmonella typhimurium* oxaloacetate decarboxylase, Q03030; Ps_TC, *Propionibacterium shermanii* transcarboxylase 1.3S subunit, P02904; Hs_PCCA, human propionyl-CoA carboxylase α-subunit, P05265; Ec_BCCP, biotin carboxyl carrier protein of the *Escherichia coli* acetyl-CoA carboxylase, P02905; Hs_PYC, human pyruvate carboxylase, P11489; Sc_PYC1, *Saccharomyces cerevisiae* pyruvate carboxylase, P11154; Sc_ACC1, *Saccharomyces cerevisiae* acetyl-CoA carboxylase, Q00955; Rn_ACC1, rat acetyl-CoA carboxylase, P11497.

lated affinity selection experiments (J. Stolz, A. Ludwig, and N. Sauer, unpublished results). Based on this finding and on the large number of avidin-reactive phages that have been sequenced, it is concluded that we have identified the minimal unit of Kp_OAD that is capable of specifying addition of biotin in *E. coli*.

In Fig. 3 the sequence of Kp_OAD is aligned with amino acid sequences of biotin domains from different proteins and species. In addition to the MKM motif, where K is the biotin accepting lysine residue, several other residues are conserved throughout these domains. Our results on the sequence requirements for biotinylation of Kp_OAD compare well with studies on the biotinylation of other biotin enzymes. Using site-directed mutagenesis, Leon-Del-Rio and Gravel [4] showed that 67 amino acids of Hs_PCCA are necessary for biotinylation in *E. coli*. Stepwise truncations were also carried out on Ps_TC [23]. Removal of the last amino acid had no effect, whereas removal of two amino acids from the C-terminus completely abolished biotinylation. Their finding is in absolute agreement with the C-terminus of the minimal biotinylation sequence determined in this work. In contrast to this, a variant of Hs_PCCA with three C-terminal residues removed is still a substrate for biotin ligase [4].

Recently, the structure of amino acids 77–156 of Ec_BCCP was reported [24]. Within this domain, biotin is located at an exposed hairpin β-turn. The four conserved glycyl residues (boxed in Fig. 3) occupy structurally important sites of Ec_BCCP, and do not cluster on the protein surface [24]. When the corresponding glycyl residues of Hs_PCCA are replaced, biotinylation is reduced rather than abolished [4]. By sequence comparisons it is obvious that the 66-amino acid minimal domain identified here includes all of the residues that are necessary to build this structure. In conclusion, one may speculate that it is this structure, rather than individual amino acids, that is recognised by biotin protein ligases, a view also shared by Reche et al. [7]. However, this interpretation has recently been questioned by the identification of a peptide tag of only 13 amino acids, which is biotinylated in *E. coli* when linked to proteins at N- or C-terminal positions [25–27]. This peptide was identified in oligonucleotide-generated libraries that encoded some of the conserved residues of naturally biotinylated proteins (e.g. the MKM motif), and random amino acids at flanking positions [25]. At present it is unknown if this short peptide has structural similarity to the exposed surface turn of Ec_BCCP, and if it is also a substrate for protein biotin ligases in other organisms. On the basis of our results, however, it is highly unlikely that a 13-amino acid peptide derived from Kp_OAD is able to effect biotinylation in *E. coli*.

The presented data clearly demonstrate that bacteriophage lambda surface display is a powerful technique for the analysis of posttranslational protein modifications. In contrast to the more frequently used M13 phages, assembly of lambda phages takes place in the cytosol of *E. coli*, and thereby phage proteins are efficiently exposed to cytosolic enzymes and factors. A possible application of phage lambda display lies in the study of protein biotinylation vs. protein lipoylation. Both primary sequences and structures of biotin proteins and lipoyl proteins are related [24,28] and both contain a lysine residue as target for modification. This raises the question how the modifying enzymes determine whether a particular lysine residue will be biotinylated or lipoylated and thus provides a model system to study the interaction of protein modifying enzymes with their substrates [7,29].

Instability and degradation of genetically engineered fusion proteins are major drawbacks for protein purification. A biotinylated fusion protein of β-lactamase with 88 amino acids of the Ec_BCCP was found to be very unstable, and *E. coli* mutants lacking periplasmic proteases had to be used for expression [10]. Individual cases of proteolytic degradation of biotinylated fusion proteins have also been observed by others [2,23] and in the authors' lab (J. Stolz and N. Sauer, unpublished results). Proteolysis of Ec_BCCP has been intensely studied in vitro. Fall and Vangelos [30] showed that full-length BCCP (14 kDa) is hydrolysed to smaller fragments (8–9 kDa) by proteases. These fragments contained the bound biotin and were highly resistant to further proteolysis. Their findings support the idea that the small Ec_BCCP-derived proteins represent a stable folding unit and that proteolysis takes place at either side of this stable core. Some of the fusion proteins of gpD with the biotin domain of Kp_OAD obtained in our screening were also found to be degraded (Fig. 2A), but the shortest biotin domain identified (phage 66) was found to be resistant to proteolytic attack. Our findings suggest that in this small fusion protein protease recognition sites of Kp_OAD are lacking or that access of proteases is restricted due to the reduced distance between the individual domains. Because this domain is still fully functional as a biotin acceptor, use of this minimal sequence is expected to provide better stability to fusion proteins and thus greatly facilitate protein detection and purification.

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