Communication

Identification and Characterization of Src SH3 Ligands from Phage-displayed Random Peptide Libraries*

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We have used the Src homology 3 (SH3) domain to screen two phage-displayed random peptide libraries, each containing 2×10^8 unique members, and have identified a series of high affinity peptide ligands. The peptides possess similar proline-rich regions, which yield a consensus Src SH3-binding motif of RPLPPLP. We have confirmed this motif by screening a phage-displayed peptide library biased for SH3 ligands and identifying the same consensus sequence. Binding studies using synthetic peptides suggest that the RPLPPLP motif is important for SH3 binding and confers specificity for the Src SH3 domain, and that residues which flank the motif may also contribute to binding. Peptides that contain the RPLPPLP motif compete Src, but not Abl or phospholipase Cγ, SH3 interactions with SH3-binding proteins from cell lysates ($IC_{50} = 1$–5 μM). Furthermore, RPLPPLP-related peptides are able to accelerate progesterone-induced maturation of *Xenopus laevis* oocytes. A similar acceleration has been observed in oocytes treated with activated, but not normal, *Xenopus* Src, suggesting the possibility that the peptides are able to antagonize the negative regulation of Src activity by Src SH3 *in vivo*.

Src represents a family of at least nine non-receptor protein-tyrosine kinases; members of this family share an overall structural organization comprising a series of catalytic and non-catalytic domains (1). The carboxyl-terminal half of Src contains the protein-tyrosine kinase catalytic domain and a negative regulatory tyrosine (Tyr-527), whose phosphorylation results in the inhibition of kinase activity (2). The amino-terminal half of Src contains two highly conserved non-catalytic regions termed Src homology (SH)[1] domains 2 and 3. SH2 and

SH3 domains are composed of approximately 100 and 60 amino acids, respectively, are found in a variety of proteins with important roles in signal transduction, and have been shown to mediate critical protein-protein interactions in a number of signaling pathways (3). SH2 domains bind phosphotyrosine (Tyr(P)); residues that surround the Tyr(P) moiety determine SH2 specificity (4). SH3 domains recognize short proline-rich stretches of amino acids, although the basis for SH3 specificity remains unclear (5).

Many mutations that result in increased Src protein-tyrosine kinase and transforming activity map to the Src SH2 and SH3 domains (6–9), suggesting a negative regulatory role for these domains. There is evidence that both domains are involved in maintaining Tyr(P)-527-mediated inhibition of Src kinase activity (9–11). Whereas the discovery that SH2 domains bind Tyr(P)-containing sequences has established an explanation for the role of the SH2 domain in Tyr(P)-527-mediated inhibition of Src activity (12), the role of the SH3 domain in Src regulation remains unclear. Identification of Src SH3 ligands may provide insight into the role of the SH3 domain in regulating Src and may lead to the discovery of SH3-binding proteins critical for Src function and transforming activity.

Efforts to identify natural ligands for different SH3 domains have led to the characterization of a number of SH3-binding proteins, including the Abl SH3-binding proteins 3BP1 and 3BP2 (13); the Ras GTP-exchange factor SOS, which interacts with Grb2 (14, 15); p85 phosphatidylinositol 3'-kinase, which binds Src, Fyn, and Lck SH3 (16, 30); dynamin, which binds SH3 domains from Grb2, Src, Fyn, Fgr, and phospholipase Cγ (17); and the Src SH3-binding protein AFAP-110 (18). These proteins possess short proline-rich segments, some of which have been directly implicated in SH3 binding. Although a number of SH3 ligand consensus motifs have been proposed (13, 17), factors that govern the affinity and specificity of SH3 interactions remain poorly defined. Attempts to define SH3-binding consensus motifs and to elucidate rules for ligand specificity have been complicated by the small number of SH3 ligands characterized to date, the often low degree of similarity between ligands for the same SH3 domain, and the presence of multiple overlapping putative SH3-binding regions within the same protein.

Screening complex random peptide libraries has proved to be a powerful strategy for identifying peptide ligands for a variety of target molecules. Such libraries have been used to identify peptide epitopes for monoclonal (19) and polyclonal (20) antibodies, as well as ligands for a variety of proteins, including streptavidin (21, 22), calmodulin[2] (24), and the endoplasmic reticulum protein BiP (23).

To expand upon the limited repertoire of known SH3 ligands and to gain a more sophisticated understanding of the role of the SH3 domain in Src function, we have used the Src SH3 domain to isolate a series of peptide ligands from phage-displayed random peptide libraries. The peptides possess similar proline-rich regions, which yield a consensus sequence of RPLPPLP. Binding studies using synthetic peptides suggest that

that the RPLPPLP motif is important for SH3 binding and confers specificity for the Src SH3 domain, and that residues which flank the motif may also contribute to binding. Furthermore, RPLPPLP-related peptides act as potent and specific antagonists of Src SH3 interactions with SH3-binding proteins from cell lysates and accelerate progesterone-induced maturation of *Xenopus laevis* oocytes.

EXPERIMENTAL PROCEDURES

Preparation of GST-SH3 Fusion Proteins—PCR fragments encoding full-length Grb2 or c-Src SH3 (amino acids 87–143) were cloned into the *Bam*HI site of pGEX-2T. pGEX-derived constructs expressing GST fusion proteins containing the SH3 domains of Yes, Crk, Abl, and phospholipase Cγ were obtained from M. Sudol (Rockefeller University), M. Matsuda (NIH, Tokyo, Japan), A. M. Pendergast (Duke University), and S. Earp (University of North Carolina), respectively. Fusion proteins were prepared as described previously (26). Microtiter wells were coated with 5–20 µg of GST-SH3 fusion protein in PBS; blocked with 100 mM Na_2HCO_3, 1% bovine serum albumin; and washed with five applications of PBS, 0.1% Tween 20, 0.1% bovine serum albumin (Buffer A). The amount of protein bound to each well was quantified by anti-GST antibody-based ELISA (Pharmacia Biotech Inc.) or with a GST-binding peptide.[3]

Isolation and Characterization of Src SH3-binding Phage—Library screens were performed as described previously (20). Briefly, 5×10^{11} plaque-forming units of T9 (20), T12,[2] or T13[4] library in Buffer A were incubated in GST-Src SH3-coated wells for 2 h. The wells were washed five times with Buffer A, and bound phage were eluted with 50 mM glycine-HCl (pH 2.2). Recovered phage were amplified in DH5αF' *Escherichia coli* and affinity-purified twice more, as above. Binding phage were plated to yield isolated plaques, from which phage stocks and DNA were produced for binding experiments and dideoxy sequencing (27), respectively. Binding was confirmed by applying equal amounts of phage to wells coated with GST-Src SH3 or GST, washing the wells with Buffer A, and detecting bound phage by anti-phage antibody-based ELISA (Pharmacia Biotech Inc.).

In Vitro Peptide Binding Assays—Biotinylated peptides (Research Genetics, Birmingham, AL; Chiron Mimotopes, Victoria, Australia; Cytogen Corp., Princeton, NJ) were synthesized with a GSGS peptide linker between the biotin moiety and the NH_2 terminus of the indicated sequence. Peptide purity was assessed by high pressure liquid chromatography and/or mass spectrometry. Binding experiments were performed as above, except for the use of 1 µM peptide instead of phage. Bound peptide was detected by streptavidin-alkaline phosphatase-based ELISA (Sigma).

Peptide Competition of GST-SH3 Affinity Precipitations of Cell Lysates—NIH 3T3 cells were labeled overnight in Dulbecco's modified minimal medium, 10% dialyzed fetal calf serum, 80 µCi/ml Tran[35]S-label (ICN, Irvine, CA). Labeled cells were washed with PBS, lysed in RIPA buffer, and clarified as described (28). Lysate from 1.5×10^6 cells was incubated with 10 µg of glutathione-agarose-immobilized GST-SH3 fusion protein ± peptide in a final volume of 250 µl. Pelleted beads were washed with 1 ml each of RIPA; RIPA, 1% deoxycholate, 0.1% SDS; and PBS, then resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis (7.5%). Labeled proteins were detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Progesterone-induced Oocyte Maturation—Stage VI oocytes were prepared and injected as described previously (29). Oocytes were injected with 40 nl of 100 µM peptide, 1 mg/ml monoclonal antibody 327, or water. After injection, oocytes were incubated in 2 µg/ml progesterone (Sigma) and were scored at hourly time points for germinal vesicle breakdown.

RESULTS AND DISCUSSION

In an effort to identify peptide ligands for the Src SH3 domain, we undertook the screening of three different phage-displayed peptide libraries. Two of the libraries, T9 and T12, each contain 2×10^8 unique clones expressing 22- or 36-amino acid-long random peptides, respectively, fused to the amino terminus of M13 protein III. As such, they represent a vast source of molecular diversity and have the potential to encode

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[3] A. B. Sparks and B. K. Kay, unpublished observations.
[4] N. B. Adey, A. B. Sparks, and B. K. Kay, unpublished observations.

A

T9.2	SSFDQQDWDYSIAEKMHPIRPGF	RELPPLP	PSRASFGGGASRPSR
T9.4	SSSGYVVPKRLGDMREYNAHPGLHVPPN	SPLPPLP	THLQSSRPSR
T9.6	SSRGEGNNIISSRPFLSNSDPGVSNKLTGRW	GPLPPLP	NDSRPSR
T12.6	STAPWGLRVAHEGG<u>VLK</u>	<u>RPLPIPP</u>	<u>VTRPSR</u>
T12.4	STNVWVTGSVIARGAQS	RPLPIPP	ETRPSR
T12.3	STNDVDWMHMWNSG<u>GPH</u>	<u>RRLPPTP</u>	<u>ATRPSR</u>
T9.5	SSDNWARRVHASELIYTDLSPGILLAQ	RQLPPTP	GRDPSHSRPSR
T9.3	SSYNDLGTRPVSEVIKYDYF<u>PGYSQ</u>HVITPDGSYST	RPLPSRP	SR
T12.2	STRWSHSWPGYVG<u>GANPSPAT</u>	<u>RPLPTRP</u>	SR
T12.5	STAHSLWDWGTFSGVSHK	SRLPPLP	TRPSR
T9.7	PGYARIVSYRF	RALPSPP	SASRPSR
T9.1	SSESPLMYNRVGALQSLTSVPGSMMHFALQ	RRLPRTP	PPASRPSR
T12.7	STAVSFRFMPGGGGAFYST	RPVPPIT	RPSR
T12.1	STMYGVSWLSSGS<u>GG</u>	<u>ILAPPVP</u>	<u>PRNTRPSR</u>

Consensus **RPLPPLP**

B

T13.5	<u>STP</u>	<u>RPLPMLP</u>	<u>TTRP</u>
T13.6	ST	RPLPILP	RTTRP
T13.1	ST	RPLPSLP	ITTRP
T13.7	STS	RPLPSLP	TTRP
T13.3	STN	RPLPMIP	TTRP
T13.10	STP	RPLPLIP	TTRP
T13.8	ST	RPLPPTP	LTTRP
T13.2	ST	RSLPPLP	PTTRP
T13.9	ST	RPQPPPP	ITTRP
T13.4	ST	RQLPIPP	TTTRP

Consensus **RPLPPLP**

FIG. 1. **Deduced amino acid sequence of peptides displayed by Src SH3-selected phage.** *A*, sequence of peptides isolated from T9 and T12 random peptide libraries. *B*, sequence of peptides isolated from T13 biased peptide library. Residues within the peptides that match the consensus have been aligned. *Bold* residues are fixed in all library clones; sequences included in synthetic peptides are *underlined*.

virtually every possible 7-amino acid-long peptide. The third library, T13, contains approximately 1×10^6 unique members expressing 8-amino acid-long peptides encoded by the DNA sequence $((C/A)NN)_8$. This encoding scheme results in a biased peptide library wherein the constituent amino acids are represented in the following proportions: 6R:4P:4L:4T:3I:2H:2Q:-2K:2N:2S:1M. Many of these residues have been identified in naturally occurring SH3-binding sequences (13–17, 30).

Phage clones expressing SH3-binding peptides were affinity-purified with immobilized GST-Src SH3 fusion protein. After three rounds of purification, isolated clones were confirmed as binding to Src SH3 (data not shown). Fig. 1 lists the amino acid sequences of peptides expressed on Src SH3-binding phage; similar sequences within the peptides have been aligned and yield a consensus motif of RPLPPLP. Peptides derived from screening either the random peptide libraries (expressing all 20 residues at each position, Fig. 1*A*) or the biased peptide library (expressing 11 residues at each position, Fig. 1*B*) yield the same consensus motif. These data suggest that the sequence RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif. Support for this conclusion comes from a recent study in which a synthetic peptide library biased for SH3 ligands (of the form *XXXPPXPXX*) was used to identify Src SH3-binding peptides with the consensus motif RXLPPLPR (31). Our identification of the RPLPPLP motif from two random peptide libraries with no *a priori* bias for any particular sequence characteristic, together with the identification of the same motif from two different biased peptide libraries, provides strong evidence that the sequence RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif.

Biotinylated peptides corresponding to sequences displayed by Src SH3-binding phage were synthesized and assayed for direct binding to immobilized GST-Src SH3 fusion protein (Fig. 2*A*). Each of the library-derived peptides bound GST-Src SH3 over GST background. This binding was not simply a consequence of the proline-rich nature of the library-derived peptides, since a proline-rich peptide from the cytoskeletal protein

FIG. 2. **Binding strength and specificity of synthetic Src SH3-binding peptides.** *A*, relative binding of peptides to GST-Src SH3. ELISA signal after 30 min. *B*, relative binding of peptides to various GST-SH3 fusion proteins. ELISA signal after 30 min (peptides pT13.5 and pLC) and after 240 min (all other peptides). Peptides (see Fig. 1 for library-derived sequences; other sequences are as follows: pVIN, LAPPKPPLPEGEV; pSOS, GTVEPVPPPVPPRRRPESA; pSC, RPL-PPLP; pLC, STPRPLPPLPTTR) were bound to equal amounts of immobilized GST-SH3 fusion protein. Bound biotinylated peptide was detected with streptavidin-alkaline phosphatase ELISA. Each point was performed in triplicate; values are average absorbance at 405 nm above bovine serum albumin background ± S.D.
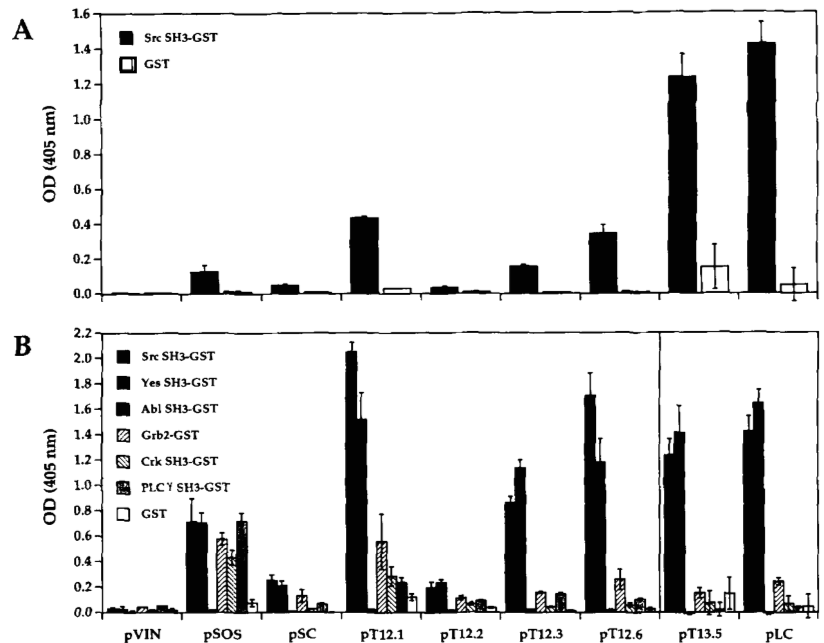
vinculin (pVIN) exhibited no binding. Furthermore, all but one of the library-derived peptides bound Src SH3-GST better than did a previously characterized SH3-binding peptide from SOS (pSOS) (14). We observed a strong correlation between the peptides' similarity to the RPLPPLP motif and their affinity for Src SH3; experiments comparing the relative binding of various phage clones produced equivalent results (data not shown). Moreover, while none of the clones expressed the sequence RPLPPLP, a peptide (pLC) consisting of the RPLPPLP motif flanked by residues encoded by clone T13.5 bound Src SH3 better than all other peptides tested. Taken together, these data provide further evidence that RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif.

The single exception to the correlation between RPLPPLP similarity and Src SH3 binding, pT12.1, bound well despite its lack of similarity to the RPLPPLP motif (Fig. 2A). As pT12.1 exhibits other unique characteristics (see below), it may represent a distinct solution to Src SH3 binding. Interestingly, pT12.1 resembles SH3-binding sequences in SOS and a set of Src SH3 ligands identified by Yu *et al.* (31) that did not match their consensus motif.

Whereas pLC produced the strongest Src SH3-binding signal, a peptide consisting of the RPLPPLP motif alone (pSC) bound poorly (Fig. 2A). Moreover, SH3-binding motifs tend to cluster near one end of the phage-displayed random peptides, adjacent to sequences that are fixed in every clone (Fig. 1A). This tendency has not been observed in binding populations selected with other proteins (20) and suggests that fixed flanking residues may facilitate SH3 binding by the phage. Thus, although RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif, sequences that flank RPLPPLP may contribute to SH3 binding. This contribution may be an indirect consequence of increased stabilization of the RPLPPLP binding conformation or a direct consequence of additional SH3-contact residues. The use of second generation random peptide libraries that fix all or part of the RPLPPLP motif and randomize flanking residues may help define additional residues important for Src SH3 binding.

To evaluate the specificities of the library-derived peptides, we determined their relative binding to SH3 domains from Src, Yes, Grb2, Crk, Abl, and phospholipase Cγ (Fig. 2B). Of the peptides that demonstrated significant Src and Yes SH3 bind-

ing, only pT12.1 and pSOS bound appreciably to SH3 domains from Grb2, Crk, and phospholipase Cγ. Thus, peptides related to the RPLPPLP consensus motif exhibit specificity for the Src SH3 domain (and the highly similar Yes SH3 domain), whereas the more divergent peptides pT12.1 and pSOS recognize a broad range of SH3 domains. That none of the peptides recognize Abl SH3 is consistent with the fact that they do not conform to a previously defined Abl SH3 consensus motif (13) and provides further evidence that SH3 domains possess distinct ligand specificities.

GST-SH3 fusion proteins may be used to affinity precipitate SH3-binding proteins from cell lysates. We therefore tested the ability of library-derived peptides to compete with cellular proteins for binding to the Src SH3 domain. As shown in Fig. 3, recovery of SH3-binding proteins with GST-Src SH3 could be blocked in a dose-dependent fashion (IC_{50} = 1 to 5 μM by PhosphorImager densitometry) by pT12.1, pT12.6, and pLC, although 100 μM pVIN had no effect. Similar results were obtained in experiments using GST-Yes SH3 (data not shown). This competition was specific for Src SH3, since none of the peptides blocked association with Abl SH3, and only pT12.1 significantly blocked binding to phospholipase Cγ SH3. These results are consistent with binding data presented in Fig. 2. Thus, the RPLPPLP-related peptides are able to specifically compete Src SH3 interactions with natural SH3-binding proteins *in vitro*, suggesting their potential for disrupting SH3-mediated events *in vivo*.

X. laevis oocytes injected with mRNA encoding constitutively active Src undergo progesterone-induced maturation at an accelerated rate relative to oocytes injected with water or c-Src mRNA (32). Since the Src SH3 domain is thought to be involved in the negative regulation of Src kinase and transforming activity (6–11), we have examined the effect of Src SH3-binding peptides upon oocyte maturation. Fig. 4 shows that maturation was accelerated by approximately 1 h in oocytes injected with pT12.6 or pLC, but not in oocytes injected with water, pVIN, the Src SH3-specific monoclonal antibody 327, or pT12.1. The magnitude of this effect is similar to that seen with injection of mRNA encoding constitutively active Src (32). Interestingly, only RPLPPLP-related peptides with specificity for Src SH3 were competent to accelerate maturation. Although the peptides may be exerting their effect via a target other than the Src
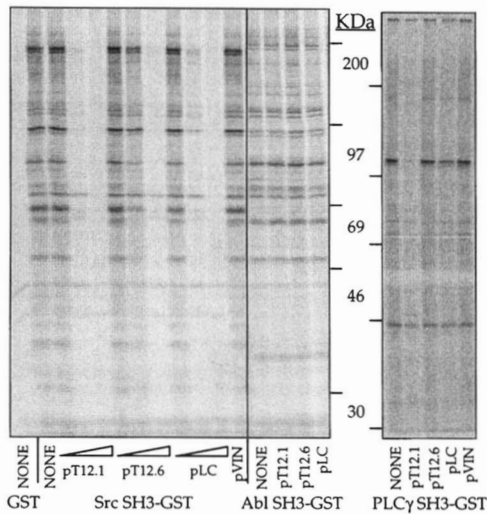
FIG. 3. **Peptide competition of Src SH3-GST precipitation of proteins from cell lysates.** ^{35}S-Labeled NIH 3T3 cell lysates were incubated with 1 µM glutathione-agarose-coupled GST-SH3 fusion protein ± peptide. Final peptide concentrations were 1, 10, and 100 µM for the series indicated by ◁ (otherwise 100 µM).
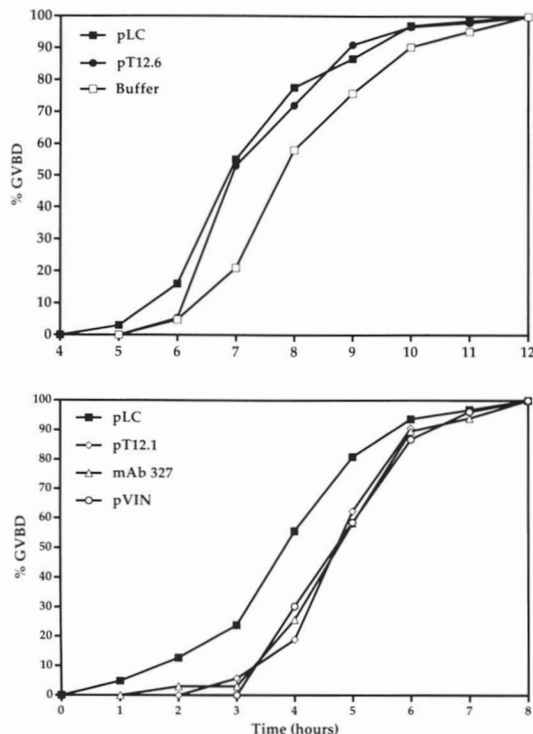

FIG. 4. **Peptide acceleration of progesterone-induced oocyte maturation.** Stage VI oocytes were injected with peptide, monoclonal antibody 327, or injection buffer. For each treatment, 100 oocytes were injected, treated with progesterone, and scored for germinal vesicle breakdown (*GVBD*) at hourly time points. Results are expressed as a percentage of oocytes that underwent germinal vesicle breakdown by the indicated time.

SH3 domain, these results suggest the possibility that the RPLPPLP-related peptides are able to antagonize the negative regulation of Src activity by Src SH3 *in vivo*. Future studies examining the effect of these peptides upon Src kinase activity, substrate phosphorylation, and protein-protein interactions may clarify the specific mechanism whereby the peptides exert their effect and may contribute to our understanding of the role of the Src SH3 domain *in vivo*.

We have used phage-displayed peptide libraries to identify the Src SH3-binding motif RPLPPLP. Because this motif was identified from libraries with no *a priori* bias for any specific sequence characteristics, we conclude that RPLPPLP represents at least a significant part of the preferred Src SH3-binding sequence. Second generation biased libraries may provide a means of extending the Src SH3-binding motif, as well as addressing the binding specificities of other SH3 domains. The isolation of biologically active Src SH3 ligands from libraries incorporating no information regarding natural SH3-binding sequences suggests that non-biased peptide libraries may represent a general and efficient means of discovering peptide ligands for, and characterizing binding specificities of, other domains thought to be involved in protein-protein interactions, such as pleckstrin homology domains (33) and armadillo repeats (25), even when no information is available regarding their natural ligands.

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