

Identification of Amino Acids at the Junction of Exons 3 and 7 That Are Used for the Generation of Glycosylation-related Human CD45RO and CD45RO-like Antigen Specificities

By Rafael Pulido, Stuart F. Schlossman, Haruo Saito, and Michel Streuli

From the Division of Tumor Immunology, Dana-Farber Cancer Institute, and the Departments of Pathology, Medicine and Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Summary

The CD45 transmembrane protein tyrosine phosphatase plays an essential role in lymphocyte activation. In humans, CD45 is composed of five isoforms that are generated by alternative splicing of three exons of a common precursor mRNA. Expression of the smallest molecular mass 180-kD CD45 isoform (CD45-O) results from splicing out of exons 4(A), 5(B), and 6(C), which encode peptide regions near the NH₂ terminus, and is regulated during T cell maturation and activation. Two monoclonal antibodies (mAb), UCHL1 (anti-CD45RO) and A6 (anti-CD45RO-like), were studied that selectively bind to murine transfectant cells expressing the human CD45-O isoform. The anti-CD45RO-like A6 mAb, but not the anti-CD45RO UCHL1 mAb, also weakly reacted with transfectant cells expressing the human CD45 isoforms that contained exons 4 and 5(AB), or exon 5(B) encoded sequences. The structural basis of the antigen specificities of these two different human anti-CD45RO mAbs was investigated at the molecular level by using potential glycosylation-defective CD45-O isoform variants containing amino acid substitutions at the junction of exons 3 and 7. Replacement of the threonine residue at position 8 (last amino acid encoded in exon 3 and a putative O-linked carbohydrate anchorage site) by an alanine, completely abrogated the reactivity of the UCHL1 mAb, but did not affect that of the A6 mAb. Conversely, replacement of either the asparagine at position 174 or the serine at position 176 (the first two putative carbohydrate anchorage sites in exon 7) by alanine, abrogated the reactivity of the A6 mAb, but not that of the UCHL1 mAb. Both the UCHL1 and A6 epitopes were dependent on the presence of O-linked carbohydrates; and the UCHL1, but not the A6 epitope, was dependent on the presence of sialic acid. These results demonstrate a pivotal role for the amino acids encoded at the junction of exons 3 and 7 for the generation of glycosylation-related CD45RO epitopes that are expressed in a cell lineage- and activation-regulated fashion.

The transmembrane protein tyrosine phosphatase CD45 is critically important for the activation of T cells through the TCR-CD3 complex (1-3), and may also be necessary for regulation of signaling via the Ig antigen receptor in B cells (4). Potential physiological substrates for CD45 include the tyrosine-phosphorylated negative regulatory sites of the protein tyrosine kinases p56^{lck} and p56^{lyn} (5, 6). Several molecules have been shown to associate with CD45, including intracellular and plasma membrane proteins, as well as cytoskeletal components (7-10). Although recent reports suggest that the extracellular portion of CD45 is not essential for signaling through the TCR (11), both the structural and functional properties of CD45 support the possibility that its cytoplasmic protein tyrosine phosphatase (PTPase) activity can

be regulated by interaction of its extracellular region with specific ligand(s), as it has been found for transmembrane receptors with tyrosine kinase activity. Furthermore, studies with anti-CD45 mAbs also demonstrate a role for CD45 in both early and late events of B and T cell activation (1, 2), and regulation of CD45 PTPase activity (12), suggesting that the binding of specific mAbs on CD45 could mimic the interaction of this molecule with natural ligand(s).

Human CD45 is composed of five isoforms that vary in their NH₂-terminal region as a result of tissue-specific alternative splicing. Regulation of CD45 alternative splicing requires exon-specific *cis*-elements and negative regulatory *trans*-acting splicing factors (13, 14). The three alternatively used CD45 exons, exons 4, 5, and 6, encode peptide segments

designated A, B, and C, respectively, and CD45 cDNAs have been isolated that contain all three alternatively used exons (the ABC isoform), two of the three exons (the AB and BC isoforms), only one exon (the B isoform), or lack all three alternatively used exons (the O isoform) (1, 2). Whereas CD45 is abundantly expressed on most hematopoietic cells, specific isoforms are expressed in a cell lineage- and activation-dependent fashion (1, 2). Furthermore, studies with genetically engineered mice lacking expression of the higher molecular weight isoforms suggest that different CD45 isoforms have distinct functions when expressed in various leukocyte lineages (15). Because the cytoplasmic PTPase domains and the majority of the extracellular domains are the same for all of the isoforms, the possibility exists that the alternative splicing regulated A, B, and C regions could mediate differential interactions between CD45-ligands and particular CD45 isoforms selectively expressed on certain cell types. However, the isoform selectively expressed in the majority of leukocytes, including the memory T cell subset which responds to recall antigen and provides help for the synthesis of Ig by B cells, is the 180-kD CD45-O isoform. This isoform lacks the NH₂-terminal A, B, and C peptide segments, and thus contains the least amount of primary sequence information (1, 2). A unique feature of the CD45-O isoform is the juxtapositioning of the sequences encoded by exons 3 and 7 which generates the CD45RO antigen specificities (16). To determine the molecular basis for the unique structural properties of the CD45-O isoform, amino acid residues required for CD45RO and CD45RO-like mAb binding were identified using a reversed genetic approach.

Materials and Methods

mAbs and Immunofluorescence. The anti-CD45RO UCHL1 and the anti-CD45 GAP8.3 mAb have been previously described (16) and were used as ascites fluid at a dilution of 1/500. The anti-CD45RO-like A6 mAb (17; Zymed Laboratories, Inc., South San Francisco, CA) was used at 5 µg/ml. MsIgG1 mAb (Coulter Immunology, Hialeah, FL) was used as negative control. The expression of CD45RO epitopes was analyzed by flow cytometry using FITC-conjugated goat anti-mouse as secondary Ab, on a Profile EPICS cell sorter (both from Coulter Immunology).

Plasmid Constructions and Cell Lines. Construction of the five full-length human CD45 cDNAs, fl-LCA.6 (CD45-ABC), fl-LCA.338 (CD45-AB), fl-LCA.260 (CD45-BC), fl-LCA.623 (CD45-B), and fl-LCA.1 (CD45-O) has been described previously (16). These cDNAs were inserted into the cloning site of a slightly modified version of the pcDL-SRα296 expression plasmid (18), termed pSP65-SRα.2. Amino acid substitution mutants were generated by oligo-directed mutagenesis, and mutants were confirmed by nucleotide sequencing. Amino acid substitutions are given in Fig. 2 A. Cell lines expressing the various CD45 isoforms or CD45-O mutants were established as described previously (16) using the murine pre-B lymphocyte cell line, 300-19, and the pSP65-SRα2-CD45 expression plasmids.

Purification of CD45-O Glycoproteins, Radiolabeling, Glycosidase Treatments, and Immunoprecipitation. CD45-O glycoproteins were purified from 300-19/CD45-O cells by affinity chromatography with the anti-CD45 GAP8.3 mAb, and radiolabeled in solution with tetrachloro-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rock-

ford, IL) as previously described (19). For neuraminidase digestion, the ¹²⁵I-labeled purified CD45-O glycoproteins were incubated at 37°C for 30 min in PBS containing 20 mU/ml of neuraminidase from *Clostridium perfringens* (Boehringer Mannheim, Mannheim, Germany). For O-glycosidase treatment, CD45-O proteins were boiled in 10 mM phosphate buffer, pH 7, containing 1% NP-40 and 0.1% SDS, and then were incubated at 37°C for 16 h with 35 mU/ml of O-Glycanase (Genzyme Inc., Cambridge, MA) and 5 mU/ml of neuraminidase. For N-glycosidase treatment, CD45-O proteins were boiled in 10 mM phosphate buffer, pH 7, containing 1% NP-40, 0.5% SDS, 8 mM EDTA, and 0.8% 2-ME, and then were incubated at 37°C for 16 h with 5 U/ml of endoglycosidase F/N-glycosidase F (Boehringer Mannheim). For immunoprecipitation, equal amounts of input radioactivity of ¹²⁵I-labeled proteins were incubated with the mAb. Immune complexes were isolated using protein G Sepharose (Pharmacia LKB, Uppsala, Sweden).

Results and Discussion

Alternative splicing of the human transmembrane protein tyrosine phosphatase CD45 results in the developmental and tissue-specific expression of five different CD45 isoforms that differ from one another only in the NH₂-terminal region (1, 2). To understand better the molecular heterogeneity of the splicing-regulated NH₂-terminal portion of human CD45, epitopes were mapped that are selectively carried on the 180-kD CD45 glycoprotein (CD45-O). Two different mAbs were studied because of their selective reactivity by flow cytometry with transfected 300-19 pre-B mouse cells expressing the recombinant human CD45-O isoform (300-19/CD45-O): the anti-CD45RO UCHL1 mAb (20); and A6 mAb, herein designated as anti-CD45RO-like (17) (Fig. 1, panels 23 and 24, respectively). Whereas the anti-CD45RO UCHL1 mAb bound only to the 300-19/CD45RO cells, the anti-CD45RO-like A6 mAb weakly reacted with transfectant cells expressing human CD45 isoforms that contained sequences encoded by exons 4 and 5 (300-19/CD45-AB) (55% positive cells) or by exon 5 (300-19/CD45-B) (22% positive cells) (Fig. 1, panels 12 and 20, respectively), suggesting that other CD45 isoforms could weakly express the A6 epitope. Immunoprecipitation experiments using cell surface radioiodinated T cells revealed that both the UCHL1 and A6 mAb precipitated only the 180-kD CD45 polypeptide (data not shown). Previous studies demonstrated that the UCHL1 mAb is able to preclear all the 180-kD CD45 glycoproteins from PBLs, as recognized by a conventional anti-CD45 mAb, indicating the existence of only one 180-kD CD45 protein (21). Furthermore, the A6 epitope was found to be present on a larger number of T cells than the UCHL1 epitope, and also to be coexpressed with the CD45RA 2H4 epitope on nonactivated "naive" T cells with inducer-suppressor activity in vitro (17, 22, and our unpublished results). Possibly, the A6 epitope is only weakly acquired on the CD45-AB and CD45-B isoforms, due to the sequence requirements for its generation (see below), making difficult its detection by immunoprecipitation techniques.

Carbohydrates located on the NH₂-terminal portion of the CD45 isoforms, including CD45-O, are involved in the generation of restricted CD45 epitopes (23). To analyze at

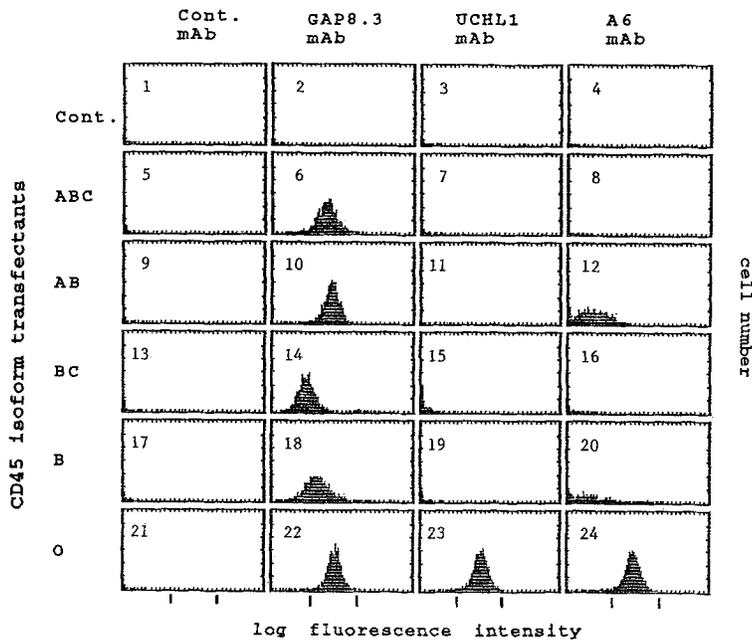


Figure 1. Reactivity of anti-CD45RO mAbs with murine pre-B cells expressing individual human CD45 isoforms. 300-19 pre-B murine cells expressing the distinct human CD45 isoforms, termed CD45-ABC, -AB, -BC, -B, and -O, or a control cell line (vector transfected only) were incubated with anti-CD45 GAP8.3, anti-CD45RO UCHL1, anti-CD45RO-like A6 mAbs, or Ig negative control mAb, and binding was measured by flow cytometry.

the molecular level the epitopes recognized by both the UCHL1 and the A6 mAb, cDNAs were generated that contain site-directed mutations at the junction of exons 3 and 7 that potentially encode glycosylation-defective human CD45-O variants. The mutations generated are shown in Fig. 2 A, and designated so as to indicate the wild-type residue mutated, the amino acid position, and the missense residue. CD45 cDNAs harboring the mutations were transfected into the mouse pre-B cell line 300-19, and CD45 biosynthesis and cell surface expression was monitored by using the conventional pan-anti-CD45 GAP8.3 mAb. Immunoprecipitation of lysates from [³⁵S]methionine-labeled cells demonstrated that the distinct CD45-O variants were properly synthesized and expressed after transfection into the 300-19 cell line (data not shown). The reactivity of both the UCHL1 and the A6 mAb with the panel of cells expressing the CD45-O variants was analyzed by flow cytometry (Fig. 2 B). Cells transfected with a CD45-O cDNA in which the threonine codon at position 8 (putative O-linked carbohydrate anchorage site) was replaced by an alanine codon (T8A) did not bind the UCHL1 mAb, but did bind the A6 mAb (Fig. 2 B, panels 15 and 16, respectively). Conversely, cells containing CD45-O mutants in which either the asparagine residue at position 174 (putative N-linked carbohydrate anchorage site) or the serine at position 176 (putative O-linked carbohydrate anchorage site) was replaced by an alanine residue (N174A and S176A, respectively), did not bind the A6 mAb (Fig. 2 B, panels 20 and 24, respectively), but were recognized by the UCHL1 mAb (Fig. 2 B, panels 19 and 23, respectively). As expected, neither the UCHL1 nor the A6 mAb stained cells expressing the double mutant T8A-N174A (Fig. 2 B, panels 27 and 28, respectively). The other CD45-O amino acid substitution mutations tested (T4A, S6A, and S186A) did not affect the recognition of the CD45-O isoform by either mAb (Fig. 2

B). Biochemical confirmation of the carbohydrate dependence of the UCHL1 and A6 epitopes was obtained by analyzing the reactivity of both mAbs with glycosidase-digested, purified CD45-O proteins (Fig. 3). The UCHL1 epitope was destroyed by neuraminidase (cleaves sialic acid residues from carbohydrate chains) and O-glycosidase (cleaves O-linked carbohydrates) digestions (Fig. 3, lanes 6 and 7, respectively), in agreement with our earlier observations (23). In contrast, the A6 epitope was resistant to neuraminidase treatment (Fig. 3, lane 10), but was destroyed by O-glycosidase digestion (Fig. 3, lane 11). Both the UCHL1 and A6 epitopes were resistant to N-glycosidase (cleaves N-linked type carbohydrates) digestion (Fig. 3, lanes 8 and 12, respectively). Because our results demonstrate that the N-174 residue is required for generation of the A6 epitope, the possibility exists that this residue is necessary for the anchorage of an O-linked carbohydrate molecule on the S-176 residue, or that N-174 itself is directly involved in the generation of the A6 epitope. In accordance with the proximity of residues T-8, N-174, and S-176 in the CD45-O isoform, mAb binding cross-inhibition experiments demonstrated that the UCHL1 and A6 epitopes overlap (17, and our unpublished results). These results demonstrate that specific amino acid residues in the sequence encoded by the junction of exons 3 and 7 are necessary for the selective anchorage of O-linked carbohydrates, thus, generating different CD45RO and CD45RO-like antigen specificities.

The important contribution of carbohydrates to the biochemical composition of mature CD45 glycoproteins has been documented (1). CD45 is recognized by mitogenic lectins, and CD45 carbohydrates are involved in the regulation of leukocyte functions (1). Herein, we have mapped the amino acid residues used for the isoform-specific anchorage of carbohydrate molecules on the human CD45-O isoform. Our results demonstrate that highly regulated, isoform-specific

A

	1	4	6	8	170	174	176		186																	
		↓	↓	↓		↓	↓		↓																	
CD45-O wt	Q	S	P	T	P	S	P	T	D	A	Y	L	N	A	S	E	T	T	T	L	S	P	S	G	S	
		Exon 3					Exon 7																			
T4A	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
S6A	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
T8A	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
N174A	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	
S176A	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	
T8A, N174A	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	
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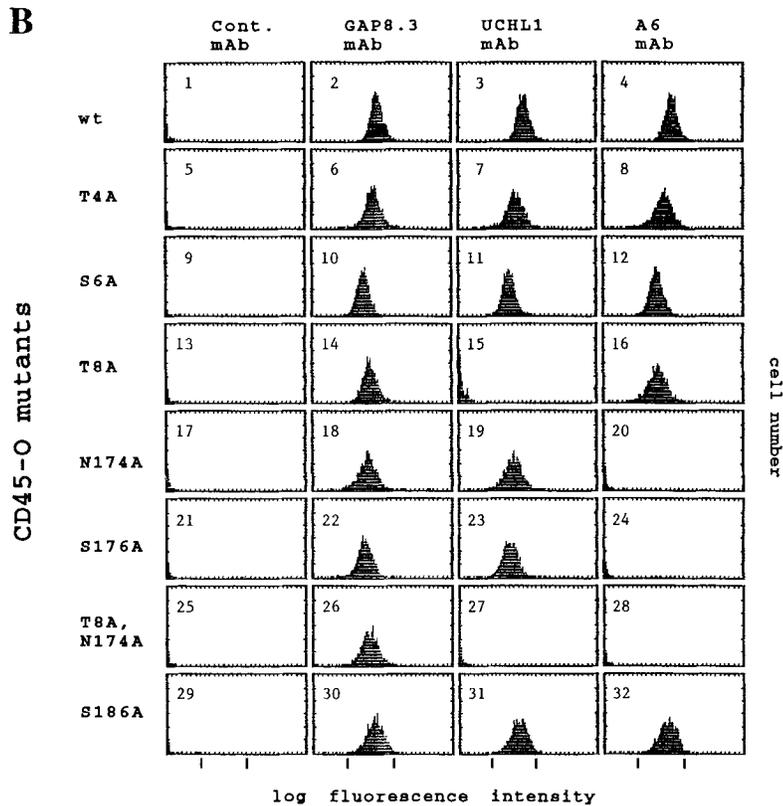
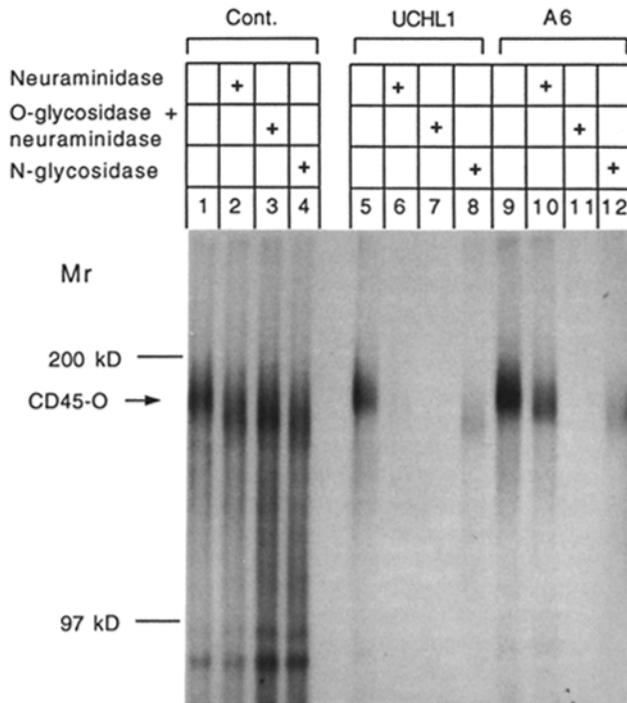


Figure 2. Molecular mapping of glycosylation-related CD45RO epitopes. (A) The amino-terminal sequence of the human CD45-O isoform is given at the top of the figure using the standard one letter amino acid code, and below are listed the amino acid substitutions (dashes indicate identical amino acids). The numbering of the amino acids is according to Streuli et al. (25), with amino acids 9–169 being derived from the alternatively used exons 4, 5, and 6. Mutations are designated, using the one-letter code, with the wild-type amino acid, its residue number and the amino acid substitution. For example, the threonine residue at position 4 was mutated to an alanine residue, and is termed the T4A mutant. (B) Reactivity of anti-CD45RO mAbs with murine pre-B cells expressing potential CD45-O glycosylation defective mutants. 300–19 pre-B murine cells expressing wild type (wt) or amino acid substituted-CD45-O mutants were incubated with the anti-CD45 GAP8.3, anti-CD45RO UCHL1, anti-CD45RO-like A6 mAbs, or Ig negative control mAb, and binding was measured by flow cytometry.

glycosylation contributes to the generation of structural variability within the distinct CD45 glycoproteins, even when no additional amino acid sequences are present (such as happens with CD45-O), in a manner that is ultimately directed by the alternative splicing of the CD45 mRNA. Comparison of the CD45 human, murine, and rat sequences contained at the junction of exons 3 and 7 (Fig. 4) reveals that the human residues T-8, N-174, and S-176 are not conserved in the murine and rat CD45 counterparts, suggesting that murine and rat CD45RO epitopes analogous to the human epitopes would be absent. In agreement with this hypothesis, neither anti-mouse nor anti-rat CD45RO mAbs have been described so far. It should be also noted that both human UCHL1 and

A6 carbohydrate-dependent epitopes were acquired in the B cell mouse system here used, as well as in NIH/3T3 cells (our unpublished results), indicating that both murine B cells and nonlymphoid cells contain the machinery required for such glycosylation events. In addition, differences have been found between the alternative splicing patterns of human and murine CD45 (14, 24). Thus, it appears that the regulation of the variability present on the NH₂-terminal portion of CD45 molecules is extremely complex and different between species. The understanding of this variability at the molecular level will be helpful to elucidate the mechanisms by which CD45 PTPase activity and CD45-mediated activation events are regulated in vivo.



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+1           ↓           ↓ ↓
Human Q S P T P S P T•D - A Y L N A S E T T L S P
Mouse  Q T P T P S - - D•G A S L - - - - T T L T P
Rat   Q G T T D - - - D•G A N L - - - - T T P A P

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Figure 4. Alignment of the NH₂-terminal amino acid sequences of the human, murine, and rat CD45-O isoform (1). The human residues T-8, N-174, and S-176, which are involved in the generation of CD45RO glycosylation-related epitopes, are indicated by arrows. Dots indicate the junctions between exons 3 and 7 in the distinct species; dashes indicate gaps in the sequence alignment.

Figure 3. Reactivity of anti-CD45RO mAbs with glycosidase-treated CD45-O isoform purified proteins. ¹²⁵I-labeled CD45-O isoform protein untreated (lanes 1, 5, and 9), neuraminidase-treated (lanes 2, 6, and 10), O-glycosidase plus neuraminidase-treated (lanes 3, 7, and 11), or N-glycosidase-treated (lanes 4, 8, and 12) was immunoprecipitated by the UCLH1 mAb (lanes 5-8) or the A6 mAb (lanes 9-12). Immune complexes were isolated and subjected to SDS-6% PAGE using reducing conditions. Lanes 1-4 correspond to purified CD45-O proteins after the various treatments. Molecular mass standards in kilodaltons (kD) are shown at the left.

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Address correspondence to M. Streuli, Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

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