Thrombomodulin Changes the Molecular Surface of Interaction and the Rate of Complex Formation between Thrombin and Protein C*

Hong Xu‡, Leslie A. Bush‡, Agustin O. Pineda‡, Sonia Caccia§, and Enrico Di Cera‡¶

From the ‡Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110 and §Dipartimento di Scienze e Tecnologie Biomediche, Università’ di Milano, 20122 Milan, Italy

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The interaction of thrombin with protein C triggers a key down-regulatory process of the coagulation cascade. Using a panel of 77 Ala mutants, we have mapped the epitope of thrombin recognizing protein C in the absence or presence of the cofactor thrombomodulin. Residues around the Na⁺ site (Thr-172, Lys-224, Tyr-225, and Gly-226), the aryl binding site (Tyr-60a), the primary specificity pocket (Asp-189), and the oxyanion hole (Gly-193) hold most of the favorable contributions to protein C recognition by thrombin, whereas a patch of residues in the 30-loop (Arg-35 and Pro-37) and 60-loop (Phe-60h) regions produces unfavorable contributions to binding. The shape of the epitope changes drastically in the presence of thrombomodulin. The unfavorable contributions to binding disappear and the number of residues promoting the thrombin-protein C interaction is reduced to Tyr-60a and Asp-189. Kinetic studies of protein C activation as a function of temperature reveal that thrombomodulin increases >1,000-fold the rate of diffusion of protein C into the thrombin active site and lowers the activation barrier for this process by 4 kcal/mol. We propose that the mechanism of thrombomodulin action is to kinetically facilitate the productive encounter of thrombin and protein C and to allosterically change the conformation of the activation peptide of protein C for optimal presentation to the thrombin active site.

Thrombin is the only enzyme in the blood capable of activating protein C (1). Activated protein C is a natural anticoagulant that inactivates factors Va and VIIa with the assistance of protein S, thereby promoting the down-regulation of the coagulation cascade (2). In addition to its anticoagulant role, protein C has recently emerged as a regulator of inflammatory response and as an anti-apoptotic agent (3). This has in turn expanded the roles of thrombin in blood coagulation and established an intriguing link between thrombosis and inflammation via the protein C pathway (2, 3).

The interaction of thrombin with protein C has been studied in considerable detail. Under physiologic concentrations of Ca²⁺, thrombin has only marginal affinity for protein C in the absence of thrombomodulin (4–8). The presence of thrombomodulin increases the $k_{cat}/K_m$ of thrombin for protein C >1,000-fold (4–8). In the absence of Ca²⁺, thrombomodulin has only a modest effect on protein C activation by thrombin and the reaction proceeds with a $k_{cat}/K_m$ comparable to that observed under physiologic conditions of Ca²⁺ and thrombomodulin (4–8). Because Ca²⁺ has no effect on thrombin structure and function (7) but drastically changes the conformation of the activation peptide of protein C (6), it is reasonable to assume that an important component of thrombomodulin action is to allosterically affect protein C to relieve the inhibitory effect of Ca²⁺ binding on the conformation of the domain containing the scissile bond. However, it is unlikely that such an allosteric effect would explain a >1,000-fold change in $k_{cat}/K_m$.

Several hypotheses on the action of thrombomodulin have been presented in the literature. Some groups favor the idea that thrombomodulin changes the conformation of thrombin to enable efficient protein C activation. Support to this proposal comes from the observation that binding of thrombomodulin to thrombin alters the spectral properties of probes tethered to the active site of the enzyme (9). Furthermore, several mutations of thrombin have been found to enhance protein C activation in the absence but not in the presence of thrombomodulin (10, 11). These findings suggest that thrombomodulin changes the conformation of thrombin in a way that can be mimicked by ad hoc amino acid substitutions. However, changes in spectral probes are qualitative in nature and may bear little on the mechanism of protein C activation by thrombin. As to the thrombin mutations that increase protein C activation in the absence of thrombomodulin, the effects are small and nowhere close to the large (>1,000-fold) enhancement induced by thrombomodulin. Furthermore, these effects are reproduced entirely by mutations of protein C affecting domains interacting with thrombin (12, 13), thereby raising the possibility that thrombomodulin affects the structure of protein C rather than thrombin. In either case, no evidence has so far been presented that the large enhancement of protein C activation by thrombin induced by thrombomodulin can be reproduced entirely by single or multiple mutations of thrombin or protein C.

In contrast to previous claims of a significant effect of thrombomodulin on the active site of thrombin (9), analysis of the hydrolysis of several chromogenic substrates by thrombin shows little dependence on thrombomodulin binding (14). More importantly, the effect is reproduced both qualitatively and quantitatively by hirugen binding to exosite I, the major determinant of thrombomodulin recognition by thrombin (15), even though hirugen has no effect on the activation of protein C. The crystal structure of thrombin bound to a fragment of thrombomodulin required for protein C activation reveals no changes in the conformation of thrombin (16). Although the structure contains an inhibitor bound to the active site, which may obscure changes within this domain (17), it shows no evidence that thrombomodulin affects the conformation of thrombin away from the active site region.

The foregoing analysis of the literature reveals that no consensus has been reached so far on how thrombomodulin increases the specificity of thrombin toward protein C. Practi-
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Thrombomodulin was derived from the linkage expression (34) shown to extract the value of saturating conditions of thrombomodulin (TM), respectively. The value highly selective for activated protein C (32), and its hydrolysis monitors protein C interaction.

The first time the contribution of the cofactor to the kinetic step of the conditions tested. Experimental conditions were as follows: 5 mM Tris; 0.1% PEG1; 145 mM NaCl; 5 mM CaCl2; pH 7.4 at 37 °C. The pH was precisely adjusted at room temperature desired to obtain the value of 8.0 at the desired temperature. This buffer has a pH of 7.5 at 25 °C and a temperature coefficient of ∆pH/ΔT = -0.027 (38). Those properties ensured buffering over the entire temperature range examined. Under these conditions, the Kd for thrombomodulin binding is around 1 nM and changes little with temperature (14). Measurements of s in the presence of 100 nM thrombomodulin are therefore excellent estimates of s, (see Equation 2). The explicit expression for s as a function of temperature (35–37) is shown in Equation 3.

\[
s = k_1 \exp \left( - \frac{E_1}{R} \left( \frac{1}{T_1} - \frac{1}{T} \right) \right) \frac{\exp \left( \frac{E_1}{R} \left( \frac{1}{T} - \frac{1}{T_1} \right) \right)}{1 + \exp \left( \frac{E_1}{R} \left( \frac{1}{T} - \frac{1}{T_1} \right) \right)}
\]  

(Eq. 3)

where R is the gas constant, T the absolute temperature, and α = k_1/k_2 defines the “stickiness” of substrate as the ratio between k_o (the rate of acylation) and k_1 (the rate of dissociation of the enzyme-substrate complex into the parent species). The Arrhenius terms E_1 and E_o = E_1 - E_o define, respectively, the activation energy for substrate bind to the active site of the enzyme and the difference between the activation energies for substrate dissociation and acylation. The values of k_o and α refer to the reference temperature T_0 = 298.15 K. The measurements of s as a function of temperature resolve k_o, α, E_1, and E_o in Equation 3 provided that the plot shows curvature. The curvature is indicative of a change in the rate-limiting step for substrate hydrolysis due to the shift from k_o >> k_1 at low temperatures to k_1 >> k_o at high temperatures. The shift is caused by the drastic difference in activation energies for substrate acylation and dissociation when E_1, >> E_o or E_o >> 0.

RESULTS

Seventy-seven residues of thrombin located in exosite I, exosite II, the 60-loop region, the specificity sites S1–S4, and the Na⁺ binding site were targeted by mutagenesis. For each mutant, the value of Kd for thrombomodulin binding was determined as well as the values of s0 and s (see Equation 1) for protein C activation (Fig. 1). These are the three independent parameters defining the linkage expression in Equation 1. The measurements of the Kd for thrombomodulin binding are criti-
ical to the definition of the boundaries of the epitope-recognizing protein C in the presence of cofactor. The results presented in Fig. 1 extend our recent mapping of the thrombin epitope recognizing thrombomodulin (15) by adding information on the role of the 60-loop region and the 220 and 186 loops defining the recognition. These residues cluster around the Na$^+$ site of thrombin in Fig. 2. There are significant differences in the functional epitopes for protein C recognition in the absence or presence of thrombomodulin as depicted on the surface of thrombin in Fig. 2. There are significant differences in the structure of the epitopes that underscore molecular changes in the thrombin-protein C interface due to cofactor binding. In the absence of thrombomodulin, there are several residues of thrombin critically involved in protein C recognition. These residues cluster around the Na$^+$ site (Thr-172, Tyr-184a, Lys-224, Tyr-225, and Gly-226) and define the primary specificity pocket (Asp-189), the oxyanion hole (Gly-193), and portions of the aryl binding site in the 60-loop region (Tyr-60a). With the exception of Lys-224, none of these residues was targeted by previous Ala-scanning mutagenesis studies (24). Contrary to previous claims (24), mutations of Trp-60d, Glu-217, and Arg-221a were found to have a small effect on protein C recognition and certainly not comparable to that of the critical residues identified in this study and are labeled in yellow and red in Fig. 2. A striking property of the epitope is that it contains residues that once mutated to Ala produce a mutant that is activated 16-fold faster by thrombin in the absence but not in the presence of thrombomodulin. They suggested that the cluster experiences unfavorable contacts with this region upon binding to thrombin. Indeed, Grinnell et al. (13) have identified a cluster of positively charged residues in the pruned sites of protein C (Lys-174, Arg-177, and Arg-178) that once mutated to Glu produces a mutant that is activated 16-fold faster by thrombin in the absence but not in the presence of thrombomodulin. They suggested that the cluster experiences electrostatic repulsion with the positively charged region of exosite I of thrombin. Given that exosite I makes no contribution to protein C recognition (Fig. 2), it is likely that some of these positively charged residues of protein C clash with Arg-35 and the 30-loop region of thrombin.

The scenario changes significantly when thrombomodulin binds to thrombin (Figs. 1 and 2). Accurate measurements of the $K_d$ for thrombomodulin binding enabled estimation of $s_d$ in Equation 1 from which a detailed picture of the epitope could be obtained unambiguously for the first time. Recognition of protein C by thrombin involves a much smaller set of residues compared with the absence of cofactor. Practically only Asp-189 in the primary specificity pocket and Tyr-60a in the aryl bind-
Substrate encounters feature (right) of thrombomodulin. Bottom left oxyanion hole (Gly-193) and the Na⁺ type) contribution to binding. The critical role played by the values of $E_s$ linked to small activation energies ($<10^7$ M⁻¹ s⁻¹) is diminished when thrombomodulin binds. The unfavorable contribution from residues in the 30-loop region and the distal portion of the 60-loop region disappears in the presence of thrombomodulin. Because the binding of thrombomodulin depends little on residues located in the 30 loop, the 60 loop, the oxyanion hole, and the Na⁺ site (Fig. 1), the change in the epitope is not due to hijacking of these domains by the cofactor. The drastic difference in the epitopes is due to the mode of interaction of protein C with thrombin and proves that the thrombin-protein C interface changes when thrombomodulin is bound. The interface is reduced to the primary specificity pocket and the aryl binding site around Tyr-60a in the presence of cofactor.

A crucial component of any recognition process is the formation of the enzyme-substrate complex. The rate constant, $k_1$, defining this step (see Equation 3) is limited by diffusion (39) or conformational rearrangements that facilitate docking. Its energetic balance is determined by the Arrhenius energy of activation $E_A$ (see Equation 1). Typically, diffusion-limited enzyme-substrate encounters feature $k_1$ values in excess of $10^7$ M⁻¹ s⁻¹ linked to small activation energies ($E_A < 10$ kcal/mol) (39). Values of $E_A$ that exceed 20 kcal/mol are conducive to structural rearrangements of the enzyme-substrate complex that facilitate productive complex formation and are usually linked to slower ($<10^7$ M⁻¹ s⁻¹) values of $k_1$. Therefore, information on $k_1$ and $E_A$ is quite valuable in establishing mechanisms of enzyme-substrate interaction. In the case of thrombin interacting with protein C, these values can be used to establish the effect of thrombomodulin on how protein C docks on the thrombin active site to enable productive binding and activation. Fig. 3 presents the Arrhenius plots of log $s$ versus $1/T$ from which the values of $k_1$ at 25 °C and $E_A$ can be extracted using Equation 3. The specificity constant $s$ increases three orders of magnitude in the presence of cofactor. The shape of the Arrhenius plot changes little in the presence of thrombomodulin (see discontinuous line). The value of $E_A$ decreases by 4 kcal/mol, and $k_1$ approaches the diffusion-limited regime (Table I). The data in Fig. 3 proves that thrombomodulin improves diffusion of protein C into the thrombin active site and that this effect is linked to a small but significant conformational change that reduces the energetic barrier for formation of the productive enzyme-substrate complex by nearly 4 kcal/mol. The large value of $E_A$ in the absence of thrombomodulin indicates substantial structural strains linked to the formation of the thrombin-protein C complex. The strains are partially relieved by the presence of cofactor. The main function of thrombomodulin is to facilitate diffusion of protein C into the thrombin active site. An ancillary

**Fig. 2.** Space-filling model of thrombin in the Bode orientation (active site, center; exosite I, right; exosite II, top left; Na⁺ site, bottom left; 60-loop, top) depicting the structural organization of the epitopes recognizing protein C in the absence (left) or presence (right) of thrombomodulin. Residues affected by Ala replacement are color-coded based on the log change in the value of $s$ for protein C activation (see Fig. 1). Blue, $-1.5/-0.5$ units (1/30- to 1/3-fold change in $s$); cyan, $-0.5/0.5$ units (1/3- to 3-fold change in $s$); green, 0.5/1.5 units (3- to 30-fold change in $s$); yellow, 1.5/2.5 units (30- to 300-fold change in $s$); red, $>2.5$ units (>300-fold change in $s$). Residues not subject to Ala-scanning mutagenesis are in gray. Crucial residues are labeled. The epitope in the absence of thrombomodulin is split into one domain providing favorable interactions (residues in yellow and red; visible are Tyr-60a, Thr-172, Asp-189, Gly-193, and Lys-224) and a second domain providing steric/electrostatic hindrance (residues in blue: from top to bottom, Phe-60b, Arg-35, and Pro-37). In the presence of thrombomodulin, the shape of the epitope changes drastically and only Tyr-60a and Asp-189 make significant contribution to protein C recognition. The region of unfavorable contributions to recognition (residues in blue at left) disappears.

**Fig. 3.** Arrhenius plots of the specificity constant $s = k_{cat}/K_m$ for the cleavage of protein C by thrombin in the absence (○) or presence (●) of 100 nM thrombomodulin over the temperature range from 5 to 45 °C. Values in the absence of cofactor could only be measured accurately at temperatures $>20$ °C. Note the use of the decimal logarithm in the ordinate. Experimental conditions are as follows: 5 mM Tris; 0.1% PEG; 100 mM NaCl; 5 mM CaCl₂; pH 8.0. Continuous lines were drawn according to Equation 3 with best-fit parameter values listed in Table I. The discontinuous line depicts the difference in the temperature dependence of log $s$ between the two sets of data and gives a direct representation of the effect of thrombomodulin on the hydrolysis of protein C by thrombin. This difference is almost temperature independent and underscores the dominant role of thrombomodulin as facilitating the diffusion of protein C into the thrombin active site.
function is to induce changes in the molecular surface of recognition between thrombin and protein C.

The parameters $\alpha$ and $E_\alpha$ in Equation 3 reveal further aspects of the thrombin-protein C interaction. Protein C behaves as a "sticky" substrate with the rate of acylation at 25 °C exceeding that of substrate dissociation by one order of magnitude. The stickiness of protein C is not altered by the presence of thrombomodulin, suggesting that the cofactor does not influence the propensity of the enzyme-substrate complex to undergo acylation rather than dissociation. On the other hand, the value of $E_\alpha$ changes significantly in the presence of thrombomodulin. The large positive nature of this term signals a substantial difference in the energetic cost of dissociating the substrate compared with acylating it. Thrombomodulin reduces this difference, presumably by lowering the barrier for diffusion of protein C back into solution once in complex with thrombin. This effect echoes the energetical facilitation of diffusion of protein C into the thrombin active site and further thrombin. This effect echoes the energetic facilitation of diffusion of protein C back into solution once in complex with thrombomodulin. The large positive nature of this term signals a conformational change linked to complex formation. The value of activation energy decreases 4 kcal/mol and underscores a conformational change linked to binding. The conformation of thrombin and/or protein C must change drastically when thrombomodulin binds to thrombin, and only Asp-189 and Tyr-60a make a significant contribution to protein C recognition.

The Ala scan unravels the functional epitope for recognition, which often but not always overlap with the structural epitope (22). To first approximation, the Ala substitution of any residue is an "adiabatic" perturbation that shaves the side chain down to the $\beta$-atom while retaining the chirality (with the exception of Gly) (40). Under this assumption, the functional epitope revealed by Ala scans should overlap substantially with the structural epitope determined by crystallographic investigation. In the case of thrombin, the current information on the possible overlap between functional and structural epitopes favors the conclusion that Ala scans reproduce quite well the boundaries emerged from structural investigation. This is particularly evident in the case of the thrombin-thrombomodulin interaction (15) for which relevant crystallographic information has recently emerged (16). Furthermore, recent structures of Ala mutants of thrombin reveal perturbations that are confined to the site of mutation even when they cause substantial disruption of catalytic activity (17, 41). These findings support the conclusion that our Ala scan (Fig. 2) reveals to a first approximation the topology of the thrombin-protein C interface and that the surface of recognition between the two proteins is drastically reduced in the presence of thrombomodulin. This represents a new insight into the action of the cofactor.

Studies on the temperature dependence of $s$ for protein C activation indicate that the major effect of thrombomodulin is to facilitate diffusion of protein C in the thrombin active site. A small but significant change was observed in the value of $E_1$ linked to complex formation. The value of activation energy decreases 4 kcal/mol and underscores a conformational change that facilitates binding. This energetic signature complements the changes in the epitope documented by Ala-scanning mutagenesis. Because the epitope changes in the presence of cofactor, the conformation of thrombin and/or protein C must change as necessary. If thrombomodulin only acted to enhance diffusion of protein C into the thrombin active site, the residues recognizing protein C in the absence of thrombomodulin would be the same as those recognizing the substrate in the presence of cofactor. In this case, the Ala scan would have returned nearly identical consequences for Ala replacements of thrombin residues on the activation of protein C in the absence or presence of thrombomodulin.

We conclude that thrombomodulin promotes diffusion of protein C into the thrombin active site, which is indicated eloquently by the drastic change in the value of $k_1$ when thrombomodulin binds. Additional contributions must come from conformational rearrangements of either thrombin or protein C as evidenced by the small but significant decrease in the value of $E_1$. Because this parameter changes with different substrates (Table I), it is quite likely that thrombomodulin changes the conformation of protein C rather than thrombin. This possibility was first voiced by Hayashi et al. (42) and is consistent with a large body of kinetic, thermodynamic, and structural data (9, 14, 16, 43) as well as with the recent observation that $Ca^{2+}$ and $Na^+$ binding affect the conformation of the activation peptide of protein C (6). The flexibility of this domain documented by fluorescence spectroscopy suggests that thrombo-

### Table 1

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|                | PC   | PC + TM | Fibrinogen | PFR |
|----------------|------|---------|------------|-----|
| $k_1$ (μM⁻¹ s⁻¹) | 27 ± 1 x 10⁻⁶ | 0.10 ± 0.02 | 19 ± 3 | 120 ± 15 |
| $\alpha$ | 11 ± 3 | 10 ± 4 | 2.6 ± 0.4 | 2.6 ± 0.3 |
| $E_\alpha$ (kcal/mol) | 30 ± 1 | 26 ± 1 | 12 ± 2 | 19 ± 2 |
| $E_\alpha$ (kcal/mol) | 33 ± 1 | 24 ± 2 | 28 ± 3 | 36 ± 3 |

* Data from reference (36).

** Data from reference (37).

Abbreviations: PC, protein C; PFR, H-o-Phe-Pro-Arg-p-nitroanilide.

**DISCUSSION**

In this study, we have identified in greater detail the epitope of thrombin recognizing protein C, and for the first time, we have provided evidence that the epitope changes significantly upon thrombomodulin binding. Recognition of protein C involves two main regions of thrombin that contribute favorably and unfavorably to binding in the transition state. The region contributing favorably to protein C recognition encompasses residues of the Na⁺ site (Thr-172, Tyr-184a, Lys-224, Tyr-225, and Gly-226), the primary specificity pocket (Asp-189), the oxyanion hole (Gly-193), and portions of the aryl binding site (Tyr-60a). The region providing unfavorable contribution to protein C recognition is shaped as a small patch in the 30-loop region (Arg-35 and Pro-37) and the adjacent distal portion of the 60-loop region (Phe-60h). The role of this region has partially been uncovered in previous studies (11, 24). The spatial contiguity of Phe-60h, Arg-35, and Pro-37 (Fig. 1) suggests that an extended domain of protein C is sterically or electrostatically hindered in its docking onto the thrombin surface. This domain may coincide with the cluster of positively charged residues identified by Grinnell et al. (13). The epitope changes drastically when thrombomodulin binds to thrombin, and only Asp-189 and Tyr-60a make a significant contribution to protein C recognition.

The Ala scan unravels the functional epitope for recognition, which often but not always overlap with the structural epitope (22). To first approximation, the Ala substitution of any residue is an “adiabatic” perturbation that shaves the side chain down to the $\beta$-atom while retaining the chirality (with the exception of Gly) (40). Under this assumption, the functional epitope revealed by Ala scans should overlap substantially with the structural epitope determined by crystallographic investigation. In the case of thrombin, the current information on the possible overlap between functional and structural epitopes favors the conclusion that Ala scans reproduce quite well the boundaries emerged from structural investigation. This is particularly evident in the case of the thrombin-thrombomodulin interaction (15) for which relevant crystallographic information has recently emerged (16). Furthermore, recent structures of Ala mutants of thrombin reveal perturbations that are confined to the site of mutation even when they cause substantial disruption of catalytic activity (17, 41). These findings support the conclusion that our Ala scan (Fig. 2) reveals to a first approximation the topology of the thrombin-protein C interface and that the surface of recognition between the two proteins is drastically reduced in the presence of thrombomodulin. This represents a new insight into the action of the cofactor.

Studies on the temperature dependence of $s$ for protein C activation indicate that the major effect of thrombomodulin is to facilitate diffusion of protein C in the thrombin active site. A small but significant change was observed in the value of $E_1$ linked to complex formation. The value of activation energy decreases 4 kcal/mol and underscores a conformational change that facilitates binding. This energetic signature complements the changes in the epitope documented by Ala-scanning mutagenesis. Because the epitope changes in the presence of cofactor, the conformation of thrombin and/or protein C must change as necessary. If thrombomodulin only acted to enhance diffusion of protein C into the thrombin active site, the residues recognizing protein C in the absence of thrombomodulin would be the same as those recognizing the substrate in the presence of cofactor. In this case, the Ala scan would have returned nearly identical consequences for Ala replacements of thrombin residues on the activation of protein C in the absence or presence of thrombomodulin.

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modulin may change the orientation of the scissile bond, which should cause the change in $E_1$ observed experimentally. As the scissile bond is presented faster and optimally to thrombin by the action of the cofactor, the contacts made by protein C with thrombin are reduced to the primary specificity pocket (Asp-189) and portions of the ary binding site (Tyr-60a). Because these domains are involved in protein C recognition regardless of the presence of thrombomodulin, it is reasonable to expect no functionally relevant changes in the active site region of thrombin upon thrombomodulin binding as indeed found experimentally (14). The effect of thrombomodulin on the molecular surface of recognition between thrombin and protein C is felt mainly at the Na$^+$ site region and at the 30-loop region (Fig. 2) to which chromogenic substrates do not have access. Future analysis of the transport of thrombomodulin should be aimed at providing information on how the cofactor influences allosterically the conformation of the activation peptide of protein C. We predict that thrombomodulin reverses the negative influence of Ca$^{2+}$ on the conformation of this peptide (6) and that this change results in the reduction or abrogation of contacts with the Na$^+$ site and the 30-loop region of thrombin. This conformational change, together with the facilitated diffusion provided by the thrombomodulin scaffold, enables protein C to be efficiently activated by thrombin.

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