Analysis of the Interaction of Platelet Collagen Receptor Glycoprotein VI (GPVI) with Collagen

A DIMERIC FORM OF GPVI, BUT NOT THE MONOMERIC FORM, SHOWS AFFINITY TO FIBROUS COLLAGEN*

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Glycoprotein VI (GPVI) is a platelet-specific glycoprotein that has been indicated to react with collagen and activate platelets. Its structure was recently identified by cDNA cloning (Clemetson, J. M., Polgar, J., Magnenat, E., Wells, T. N., and Clemetson, K. J. (1999) J. Biol. Chem. 274, 28019–28024). However, the mechanism of the interaction between collagen and GPVI has not been analyzed in detail because both collagen and GPVI are insoluble molecules. In this study, we expressed the extracellular domain of GPVI as soluble forms as follows: the monomeric form (GPVIX) and the dimeric form of GPVI fused with the human immunoglobulin Fc domain (GPVI-Fc2). Purified GPVIX strongly inhibited convulxin (Cvx)-induced platelet aggregation but only weakly inhibited that induced by collagen-related peptide. However, only GPVI-Fc2 and not GPVIX, inhibited collagen-induced platelet aggregation.

Collagen is one of the major components of the vessel wall. When the vessel wall becomes damaged, platelets adhere to

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EXPERIMENTAL PROCEDURES

Expression and Purification of Soluble GPVIs—The cDNA of GPVI containing the extracellular two Ig domains of human GPVI without the signal sequence (461 bp, residues 21–234) was obtained from the GPVI cDNA as the template and the oligonucleotide TTAAGCTTGGAGGGACCGCTCACCAAGCC (with the HindIII site underlined) and AACTTGGAGAAGGTGACTGTTGAGCT (with the XbaI site underlined) as the forward and reverse primer, respectively. Purified plasmid was digested with HindIII and XbaI and ligated with the pET-28b vector (Novagen). The resulting plasmid was transformed into the E. coli strains BL21 and XL1-Blue with an auto-inductor plate (Biolistic, Darmstadt, Germany). The plate lysates were filtered through 0.22-μm filters. The inclusion bodies were solubilized in buffer containing 6 M urea, 50 mM Tris-Cl pH 8.0, 500 mM NaCl, and 10% glycerol. The inclusion bodies were purified using a HiTrap Ni2+ affinity column (Amersham Biosciences) followed by a Superdex 200 column (Amersham Biosciences) equilibrated with 20 mM NaH2PO4, pH 7.4. The soluble protein was obtained by dilution of the inclusion bodies to 20 mM NaH2PO4, pH 7.4. The soluble protein was dialyzed against HEPES-Tyrode buffer (136 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 10 mM HEPES, pH 7.4). Then the soluble protein was purified using a QIAquick gel extraction kit (Qiagen, Tokyo, Japan). The purified protein was digested with trypsin, and the resulting peptides were purified by reverse-phase high-performance liquid chromatography using a C18 column (Phenomenex, Torrance, CA). The purified peptide was analyzed by matrix-assisted laser desorption mass spectrometry (Applied Biosystems, Foster City, CA) and N-terminal sequencing (Applied Biosystems). The protein concentration was determined using the Coomassie protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Interaction of GPVI with Collagen—The interaction of GPVI with collagen was determined by the bio-layer interferometry method (15). Stable cell lines expressing recombinant protein were obtained in the following manner: the cDNA of GPVI was digested with HindIII and XbaI and ligated with the pET-28b vector (Novagen). The resulting plasmid was transformed into the E. coli strains BL21 and XL1-Blue. The plate lysates were filtered through 0.22-μm filters. The inclusion bodies were solubilized in buffer containing 6 M urea, 50 mM Tris-Cl pH 8.0, 500 mM NaCl, and 10% glycerol. The inclusion bodies were purified using a HiTrap Ni2+ affinity column (Amersham Biosciences) followed by a Superdex 200 column (Amersham Biosciences) equilibrated with 20 mM NaH2PO4, pH 7.4. The soluble protein was obtained by dilution of the inclusion bodies to 20 mM NaH2PO4, pH 7.4. The soluble protein was dialyzed against HEPES-Tyrode buffer (136 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 10 mM HEPES, pH 7.4). The soluble protein was purified using a QIAquick gel extraction kit (Qiagen, Tokyo, Japan). The purified protein was digested with trypsin, and the resulting peptides were purified by reverse-phase high-performance liquid chromatography using a C18 column (Phenomenex, Torrance, CA). The purified peptide was analyzed by matrix-assisted laser desorption mass spectrometry (Applied Biosystems, Foster City, CA) and N-terminal sequencing (Applied Biosystems). The protein concentration was determined using the Coomassie protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

RESULTS

Production and Isolation of Soluble Monomeric and Dimeric GPVIs—the extracellular domain of GPVI (residues 21–234) was expressed as the monomeric (Myc-His6) tag fusion protein and as the dimeric human immunoglobulin Fc domain fusion protein. These GPVIs were expressed as secreted soluble proteins using a eukaryotic cell line, Hek 293 cells, which prevented misfolding and any non-glycosylation of the expressed proteins.
proteins, as occasionally happened when using E. coli. About 2 mg/liter of the recombinant GPVIs were obtained from the culture mediums for both forms of GPVI. The molecular mass of GPVlex is 41 and 42 kDa under non-reduced and reduced conditions in SDS-PAGE, respectively (Fig. 1, lanes 3 and 4). GPVI-Fc2 has a molecular mass of 150 and 68 kDa under the non-reduced and reduced conditions in SDS-PAGE, respectively (Fig. 1, lanes 1 and 2). The molecular mass of the purified GPVI-Fc2 was estimated to be ~180 kDa from gel filtration (data not shown), confirming that the purified GPVI-Fc2 is present as a dimer. Both biotinylated Cvx (12) and anti-human GPVI monoclonal antibody 204-112 recognize native GPVI and recombinant GPVlex and GPVI-Fc2 only under the non-reduced condition, suggesting that these recombinant GPVIs have conformations similar to that of native GPVI (data not shown).

Effect of Recombinant GPVIs on Platelet Aggregation—Next, we tested the activities of the monomeric and the dimeric forms of GPVI against platelet aggregation. As shown in Fig. 2, GPVlex did not inhibit collagen-induced platelet aggregation even at a high concentration (100 μg/ml), but it did inhibit cross-linked CRP-induced platelet aggregation, although very weakly (Fig. 3). In contrast, GPVI-Fc2 at 10 μg/ml strongly inhibited cross-linked CRP-induced platelet aggregation. Cvx-induced platelet aggregation was markedly inhibited by both GPVlex and GPVI-Fc2 at concentrations of 0.5 and 0.56 μM, respectively (data not shown).

GPVlex did not inhibit collagen-induced platelet aggregation even at a concentration of 100 μg/ml, but GPVI-Fc2 abrogated collagen-induced platelet aggregation at 20 μg/ml without pre-incubation (Fig. 2B). These results indicate that the two forms of GPVI show different binding affinities to collagen itself and the collagen-mimetic CRP.

Binding of Monomeric and Dimeric GPVIs to CRP and Collagen—The binding of monomeric and dimeric GPVIs to immobilized ligands was analyzed by the ELISA method (Fig. 4). Both proteins showed dose-dependent and saturable binding to the immobilized-CRP surface (Fig. 4A). EDTA did not affect the binding of either of the recombinant GPVIs to CRP and collagen (data not shown). Although it is impossible to compare quantitatively the extent of bindings of GPVlex and GPVI-Fc2 because we used different antibodies to detect these proteins, we could not detect any strong binding of GPVlex to type I and type III collagens (Fig. 4B). Even when the sensitivity was increased by using a longer color-developing time, only a small increase in color development was observed at a high concentration of GPVlex, 400 μg/ml (data not shown). In contrast to GPVlex, the bindings of GPVI-Fc2 to bovine type I and type III collagens were each dose-dependent, becoming saturated at 400 μg/ml (Fig. 4C). These results also indicated that collagen preferably binds to dimeric GPVI-Fc2.

The Effect of Dimerization of Monomeric GPVlex on Binding to Bovine Type I Collagen—The above results suggested that the dimerization of GPVI may be necessary to induce a specific conformation with a high affinity for collagen. To test this hypothesis, we cross-linked the monomeric GPVlex with the anti-Myc monoclonal antibody 9E10, which enabled us to make dimeric GPVlex because each chain of the antibody could bind to one GPVlex molecule through the Myc tag at its COOH-terminal. Fig. 5 illustrates the effect of dimerization on the affinity of GPVlex for collagen. GPVlex preincubated with 9E10 exhibited enhanced binding to collagen, with the binding enhancement depending on the molar ratio of 9E10 to GPVlex.

At the molar ratio of 1:2 (9E10:GPVlex), the binding enhancement was maximum, with a 3.4-fold higher amount of binding than that of GPVlex alone. When the binding of cross-linked GPVlex was compared with that of GPVI-Fc2 form, the developed color using the same measuring method, the binding of cross-linked GPVlex corresponded to the amount of binding obtained at about 160 nM GPVI-Fc2, indicating that the binding of the cross-linked GPVlex was about 10–20% of that of GPVI-Fc2 (data not shown). These curves also indicate that an excess amount of 9E10 rather decreases the binding of GPVlex to collagen, which would be explained by a decrease in the amount of dimerized GPVlex at the higher ratio of the antibody to GPVlex, where there is a higher chance for antibody reacting with only one GPVlex molecule, and thus not forming a dimer.

These results support the hypothesis that the dimerization of GPVI would be responsible for the high affinity binding of GPVI-Fc2 to collagen and negate the possibility that the high affinity is due to GPVI-Fc2 having a conformation different from that of GPVlex.

The Effect of CRP and Cvx on Dimeric GPVlex Binding to Bovine Type I Collagen—Because Cvx and CRP were reported to be specific ligands for GPVI, we determined their ability to compete with collagen for the binding to GPVI-Fc2 by the ELISA method. CRP inhibited the binding of GPVlex-Fc2 to immobilized collagen in a dose-dependent manner (Fig. 6A). The concentration required for 50% inhibition of binding (IC50) was calculated to be 4.3 ± 0.4 μg/ml. However, Cvx did not inhibit the binding of GPVlex-Fc2 to collagen but instead enhanced the apparent binding to collagen at low concentrations (Fig. 6B). A possible explanation for the enhancing effect of Cvx can be proposed on the basis of its multiple subunit structure (17). Cvx has multiple binding sites for GPVI, and GPVI binds with Cvx at sites different from the collagen-binding ones. As a result, Cvx would be able to bind multiple molecules of GPVI and thereby help to accumulate more GPVI at the collagen surface. Furthermore, Cvx did not inhibit GPVI-Fc2 binding to collagen, providing further evidence that the Cvx-binding site of GPVI is different from its collagen-binding site. Because the binding to type I collagen was almost completely inhibited by CRP, the collagen-binding site of GPVI may be the same or shared with that for CRP.

Binding Analysis of GPVlex and Collagen Using Surface Plasmon Resonance—Kinetic analysis of the binding of collagen and the GPVIs was performed by the SPR method. Collagen and CRP were immobilized on sensor chips under acidic conditions, and the interactions between flowing GPVlex-Fc2 or GPVlex and immobilized collagen or CRP were measured under physiological conditions. The sensorsgrams at different ligand concentrations were obtained and normalized by subtracting the background signals from the collagen (Fig. 7A) or CRP (Fig. 7B) responses. The kinetic data obtained from 3 independent experiments are summarized in Table I. In this

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dissociation constants were obtained by two calculation methods, from $k_{on}$ and $k_{off}$ and from the equilibrium binding equation using resonance units calculated at the equilibrium. Curve fitting indicated that our data fit better to a two-state model than a one-state one, suggesting that there may be a conformational change of the complex after the initial association of GPVI-Fc2 with collagen (Table II). However, even in the two-state model, the apparent $K_D$ values are not substantially different from those of the one-state model, and the transition rate is very small, suggesting a rather small contribution of the transition (conformational change) of the complex to GPVI binding. The $K_D$ value of GPVI-Fc2 indicated that dimeric GPVI has at least 10-fold higher affinity to collagen than CRP. The binding of GPVlex to the collagen surface was so weak that
the immobilized GPVI-Fc2 with soluble collagen, although the sensorgram indicates that there is no significant interaction by the SPR method. As shown in Fig. 9, the mean molar ratio of the antibody to GPVIex. Each data point is the mean ± S.E. of three determinations.

reliable kinetic parameters could not be calculated from the obtained experimental data (Fig. 8A). In contrast to collagen, the $K_D$ value of GPVIex for CRP was measurable, being $8.5 \pm 0.1 \times 10^{-7}$ M (Fig. 8B).

In the above experiments, the interaction of GPVI and immobilized collagen was analyzed. These immobilized collagens would contain some fibrous collagen because the SPR experiment was performed under physiological conditions, after the collagen was first immobilized to the chips under acidic conditions. To analyze the interaction of GPVI with only the soluble form of collagen, we prepared soluble type III collagen as described under “Experimental Procedures.” The $abscissa$ indicates the molar ratio of the antibody to GPVIex. Each data point is the mean ± S.E. of three determinations.

DISCUSSION

GPVI is a platelet-specific membrane protein whose structure was recently identified from cDNA cloning (11–13). Serving as a physiological collagen receptor on platelets, its function is to bind to collagen and activate platelets, as concluded from studies on GPVI-deficient platelets from patients (3, 4, 18). It has been hypothesized that the high affinity interaction of platelets with collagen through integrin $\alpha_\beta_1$, another collagen receptor, functions in platelet adhesion, and the lower affinity interaction between collagen and GPVI mainly serves to induce activation pathways in platelets (10, 19). Although quantitative studies on the interaction between integrin $\alpha_\beta_1$ and collagen have been performed (16, 20, 21), no quantitative analyses of the interaction between GPVI and collagen have been reported. In this study, we prepared soluble forms of recombinant GPVI, and we analyzed their interaction with collagen.

To obtain a sufficient amount of the recombinant proteins, we inserted the Ig $\kappa$ signal sequence instead of the original sequence in the pSecTag vector expression system. The Ig $\kappa$ signal sequence significantly increased the secretion of the recombinant GPVI, which could be ascribed to the short hydrophobic core in the signal sequence of GPVI (data not shown). We expressed the extracellular domain of GPVI conjugated with Myc and His tags at the COOH-terminal end (GPVIex) and the fused form of this extracellular domain with the IgG Fc domain (GPVI-Fc$_2$). GPVIex is a monomeric form, and GPVI-Fc$_2$ is a dimeric form, in which two GPVI-Fc molecules are cross-linked by disulfide bonds formed from the Cys in the Fc domain of each molecule (Fig. 1).

GPVI has been indicated to form a complex with Fc receptor $\gamma$-chain through the ionic bonds between Arg of GPVI and Asp of the FcR $\gamma$-chain in the transmembrane domains (12, 22). Because FcR $\gamma$-chain is present as a dimer cross-linked by a disulfide bond, GPVI should also be present as a dimer on the platelet surface. Jandrot-Perrus et al. (13) reported that the GPVI extracellular domain-Fc domain fusion protein inhibited collagen-induced platelet aggregation when collagen was pre-incubated with it. Our data (Fig. 2) indicating that only GPVI-Fc$_2$, and not GPVIex, inhibited collagen-induced platelet aggregation is consistent with the observations of Jandrot-Perrus et al. (13); however, preincubation with collagen was unnecessary for the inhibitory effect of our dimeric protein. In contrast, both the monomer and the dimer inhibited Cvx-induced platelet aggregation at a similar molar concentration. The inhibitory effect of the GPVI proteins on cross-linked CRP-induced aggregation was intermediate between its effects on collagen- and Cvx-induced platelet aggregations. The GPVIex weakly inhibits the cross-linked CRP-induced platelet aggregation. These results suggested that GPVI-Fc$_2$ has a binding affinity to collagen, whereas GPVIex, the monomeric form, does not.

It is possible that a difference in conformation may explain the different reactivity of GPVIex and GPVI-Fc$_2$, but several of our observations indicate that this is unlikely. In Fig. 5, we cross-linked GPVI ex by reacting the anti-Myc antibody 9E10 with the COOH-terminal Myc tag of GPVIex, and we showed that the cross-linked GPVIex also exhibited the ability to bind to immobilized collagen, although its binding activity was not the same as that of GPVI-Fc$_2$. The lower binding efficiency of the cross-linked GPVIex compared with that of GPVI-Fc$_2$ can be explained from the loosely cross-linked GPVIex. More importantly, we observed that the efficiency of binding becomes lower at higher antibody to GPVIex ratios, suggesting the importance of dimerization by the antibody. These results support the hypothesis that the amount of the dimerized form of GPVI is mainly correlated to the ability to bind collagen, thus dismissing the possibility that the collagen binding portion of GPVI-Fc$_2$ has a conformation intrinsically different from that of GPVIex.

ELISA and SPR were used to analyze the GPVI-collagen-binding reaction. Both methods indicated that GPVI-Fc$_2$ binds to collagen, but GPVIex has essentially no affinity for collagen. SPR experiments indicated that GPVI-Fc$_2$ has a $K_D$ of $5.76 \pm 0.64 \times 10^{-7}$ M (Table I). This value is markedly higher (lower affinity) than the $K_D$ value that we obtained for the soluble collagen-integrin $\alpha_\beta_1$ interaction, $6–86 \times 10^{-7}$ M (16, 20). This indicates that the activated integrin $\alpha_\beta_1$ is mainly related to the tight binding of platelets to collagen. GPVIex did not show any significant interaction with collagen by ELISA and SPR methods. Even very high concentrations of GPVIex could only bring about slight inhibitions of platelet aggregation or collagen binding (Fig. 3B). On the other hand, GPVIex could bind CRP in a dose-dependent manner (Fig. 4A) and inhibit Cvx-induced platelet aggregation. Thus, the CRP and Cvx binding abilities of GPVIex are similar to those of the dimeric GPVI-Fc$_2$, and it is only the affinity toward collagen that differs. In addition, dimerization of monomeric GPVIex with monoclonal antibody increases its binding to immobilized collagen. Taken
together, our results indicate that the high affinity to collagen is attributable to the dimeric structure of GPVI. Such enhancement of affinity induced by receptor dimerization has been reported for several Ig superfamily receptors (23).

In one of our previous papers (16), we showed that platelet binding to soluble collagen is characteristically very different from platelet binding to fibrous collagen. Platelet binding to soluble collagen is strongly dependent on divalent cations and

### Table I

**Kinetic parameters in the one-state model**

|        | $k_a$ | $k_d$ | $K_D$ | $K_D$ equilibrium |
|--------|-------|-------|-------|-------------------|
|        | M$^{-1}$ s$^{-1}$ | s$^{-1}$ | M | M |
| CRP    | 7.87 ± 3.21 × 10$^7$ | 3.22 ± 1.57 × 10$^{-2}$ | 5.27 ± 3.40 × 10$^{-6}$ | 5.26 ± 3.40 × 10$^{-6}$ |
| Collagen | 8.62 ± 0.66 × 10$^7$ | 5.04 ± 0.67 × 10$^{-3}$ | 5.78 ± 0.38 × 10$^{-7}$ | 5.76 ± 0.37 × 10$^{-7}$ |

*a* $L \rightleftharpoons L \cdot R$; $L$, ligand; $R$, receptor.

### Table II

**Kinetic parameters in the two-state model**

|        | $k_{a1}$ | $k_{d1}$ | $k_{a2}$ | $k_{d2}$ | $K_D^b$ |
|--------|---------|---------|---------|---------|---------|
|        | M$^{-1}$ s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | s$^{-1}$ | M |
| CRP    | 1.65 ± 0.54 × 10$^4$ | 6.38 ± 1.09 × 10$^{-2}$ | 1.04 ± 0.25 × 10$^{-3}$ | 1.91 ± 0.31 × 10$^{-3}$ | 2.71 ± 1.90 × 10$^{-5}$ |
| Collagen | 1.69 ± 0.40 × 10$^4$ | 3.12 ± 1.59 × 10$^{-2}$ | 2.19 ± 0.56 × 10$^{-3}$ | 1.36 ± 0.20 × 10$^{-3}$ | 8.91 ± 0.33 × 10$^{-7}$ |

*a* $L \rightleftharpoons L \cdot R \rightleftharpoons (LR)$; $L$, ligand; $R$, receptor.

$b$ $K_D = \frac{k_{d2} k_{a1}}{k_{a2} k_{d1}}$

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**Fig. 6.** Effects of CRP and Cvx on the binding of GPVI-Fc$_2$ to immobilized collagen. GPVI-Fc$_2$ (20 µg/ml) was preincubated with various concentrations of CRP (A) or Cvx (B) for 30 min and then reacted with immobilized collagen. The bound GPVI-Fc$_2$ was measured by the ELISA method as described under "Experimental Procedures." Each data point is the mean ± S.E. of three determinations.

**Fig. 7.** Sensorgrams of the interaction of GPVI-Fc$_2$ with immobilized collagen and CRP. A, the solution of GPVI-Fc$_2$ (31.25 to 500 nM) was flowed over the immobilized collagen and control surfaces, and the response from the immobilized collagen surface was subtracted by the response from the control surface. B, the GPVI-Fc$_2$ solution (31.25 to 1000 nM) was flowed over the immobilized CRP surface, and the sensorgram was obtained by a similar calculation as in A. RU, resonance units.
strongly inhibited by anti-integrin α,β1 antibodies, whereas platelet binding to fibrous collagen is independent of the former and only slight affected by the latter. The binding of GPVI-deficient platelets to fibrous collagen is decreased in the presence of EDTA (4), which suggests that GPVI does not bind to soluble collagen and specifically binds to fibrous collagen. To test this hypothesis, we measured the binding of soluble collagen to the immobilized GPVI-Fc2 by the SPR method, and we found that there is no interaction between GPVI-Fc2 and soluble collagen (Fig. 9). This also suggested that the GPVI binding to immobilized collagen measured by the ELISA or SPR method is not due to monomeric GPVI but reacts with Cvx at a concentration similar to that of monomeric GPVIex to these agonists. GPVIex does not bind to collagen, although the cells react with Cvx and showed that the RBL cells expressing GPVI on the cell surface at a similar density to that of native platelets can react with collagen and induce intracellular Ca2+ release (25). RBL cells expressing about 50% the amount of GPVI in platelets did not react with collagen, although the cells react with Cvx and with a similar concentration dependence to that of platelets. These GPVI-expressing cells show weak reactivity to CRP. The reactivities of these GPVI-expressing RBL cells to collagen receptor agonists are very similar to the reactivities of our monomeric GPVIex to these agonists. GPVIex does not bind to collagen but reacts with Cvx at a concentration similar to that reactive with GPVI-Fc2. GPVIex also reacts weakly with CRP. These results suggest that the GPVI expressed on the RBL cells would be present as a monomeric form complexed with the FcR γ-chain. Recently, the same group advanced their studies and showed that the RBL cells expressing GPVI on the cell surface at a similar density to that of native platelets can react with collagen and induce intracellular Ca2+ release (25). RBL cells expressing about 50% the amount of GPVI in platelets showed much weaker reactivity to collagen. If we assume that 100% density would indicate 100% formation of GPVI dimer, a 50% density would suggest that the most of the GPVI-FcR γ-chain would be in a 1-to-1 with monomeric GPVI. These results also support our hypothesis that the dimeric form of GPVI would be the active form of GPVI, and thus GPVI must be expressed at a high density to obtain an active GPVI in the cultured cells. However, platelets from the parents of a previously reported GPVI-deficient patient (4) and platelets from heterozygous FcR γ-chain-deficient mouse (25) expressed only the GPVI-FcR γ-chain complex that specifically produces a dimeric complex in platelets and megakaryocytes.

In this study, we prepared two forms of GPVI, the monomeric form GPVIex and the dimeric form GPVI-Fc2, and our data presented herein indicate that the dimeric GPVI-Fc2 has high
affinity to fibrous collagen. These results, along with other data from our laboratory and by other groups, suggested that GPVI would be present as a dimeric form in platelets. Our studies also show that the dimeric form of GPVI, GPVI-Fc2, could be useful for analyzing the interaction of GPVI with insoluble collagen.

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