Recombinant hirudin variants have been designed which inhibit \( \alpha \)-thrombin by the hirudin mechanism and which in addition exhibit disintegrin activity. These proteins, called "hirudisins," have been engineered by replacing the Ser-Asp-Gly-Ser sequence at the tip of hirudin's finger-like structure (residues 32–35) by Arg-Gly-Asp-Ser (RGDS) to yield hirudisin and Lys-Gly-Asp-Ser (KGDS) to obtain hirudisin-1. Comparison of thrombin inhibition activities showed that hirudisin is 2-fold more potent \( (K_i = 160 \pm 70 \text{ fm}) \) than hirudisin-1 \( (K_i = 370 \pm 44 \text{ fm}) \) and recombinant \( (r) \)-hirudin \( (K_i = 270 \pm 50 \text{ fm}) \). \( \alpha \)-Thrombin-stimulated platelet aggregation was effectively inhibited by \( r \)-hirudin, hirudisin, and hirudisin-1 with IC\text{sub}_50 of 5.7 to 6.8 nm. Unlike \( r \)-hirudin, hirudisin inhibits ADP-induced platelet aggregation \( (IC_{50} = 65 \text{ \mu M}) \) 3- to 5-fold stronger than the linear GRGDS- and RGDS-peptide. Direct interaction of hirudisin with purified glycoprotein Ib-IIIa demonstrated that antplatelet aggregation activity is due to the integrin-directed RGD motif. Disintegrin activity of hirudisin relative to that of reduced and carboxymethylated hirudisin suggests that the conformational strain favors binding to integrins. On the basis of these results, hirudisins appear to be interesting molecules for the design of potential antithrombotic agents with antithrombin as well as antiplatelet aggregation activities.

The thrombin-specific inhibitor hirudin is a polypeptide of 65 amino acid residues isolated from the leech Hirudo medicinalis (Markwardt, 1970; Bagdy et al., 1976; Dodt et al., 1986a; Harvey et al., 1986; Tripièr, 1988). On the basis of its structure and mechanism of thrombin inhibition this protein appears to be unique among serine proteinase inhibitors. From solution studies on thrombin-hirudin complex formation (Stone and Hofsteenge, 1986; Braun et al., 1988) it became evident that hirudin is basically a bivalent inhibitor comprising two binding domains. These are located in the disulfide-bonded NH\text{-terminal part (residues 1–49) and the COOH-terminal region (residues 54–65). Ionic interactions of negatively charged residues in the COOH-terminal part of hirudin with a positively charged surface groove on thrombin have been shown to play an important role in the first step of complex formation (Braun et al., 1988; Stone et al., 1989; Betz et al., 1991). In the second step, interactions of the NH\text{-terminal core and especially of the NH\text{-terminal 3 amino acid residues (Wallace et al., 1989; Lazar et al., 1991) with the active site cleft inhibit the enzyme's hydrolytic activities. Hirudin-derived COOH-terminal peptides with a minimal length of 12 amino acid residues exhibit anticoagulant activities (Mao et al., 1988; Maraganore et al., 1989) but only slightly modulate the enzyme's hydrolytic activity towards peptide-p-nitroanilides (Naski et al., 1990; Schmitz et al., 1991; Liu et al., 1991). Thus, based on the desired therapeutic application hirudin and derivatives are potent potential therapeutic anticoagulants.

Adhesion of platelets to vessel walls, their activation, and aggregation is a central primary event in blood coagulation. On activated platelets the glycoprotein IIb/IIIa (GP IIb-IIIa), a member of the integrin family, functions as a receptor for fibrinogen, fibronectin, von Willebrand factor, vitronectin, and thrombospondin (Hynes, 1987). Binding of GP IIb-IIIa to fibrinogen or von Willebrand factor mediates platelet aggregation, whereas binding to the other proteins may additionally allow platelet adhesion and spreading. The capability of GP IIb-IIIa to bind to adhesive proteins is due to its ability to recognize RGD motifs within their sequences (Russelathi and Pierschbacher, 1987; Phillips et al., 1988). Peptides containing the RGD sequence inhibit the binding of fibrinogen or von Willebrand factor to GP IIb-IIIa to platelets, thus inhibiting platelet aggregation (Kiefert and Phillips, 1990). However, not only the primary structure around the RGD motif but also its conformation, maintained by the appropriate cysteine pairing, is necessary for full expression of disintegrin activity (Dennis et al., 1990). Pierschbacher and Russelathi (1984) have already suggested that the RGD motif of adhesive proteins may be located at the tip of a \( \beta \)-turn. Recently, this proposal was established by \( 3 \)H NMR studies on the platelet aggregation inhibitors kistrin and echistatin from snake venoms (Adler et al., 1991; Cooke et al., 1991; Dalvit et al., 1991; Saudek et al., 1991). The RGD binding motif of these proteins is located at the end of a long arm consisting of two antiparallel strands which are connected by a \( \beta \)-turn with high flexibility.

Recently, a chimeric peptide has been described consisting of the cell adhesion sequence RGDG followed by the COOH-terminal region of hirudin (residues 53–64) (Church et al., 1991). This peptide was found to exhibit both disintegrin and antithrombin activities. It displays the corresponding activities to the same extend as its individual constituents. From the crystal structure of the thrombin-hirudin complex it is known that a finger-like structure points outwards from the

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complex into solution (Rydel et al., 1990; Grütter et al., 1990). The finger is formed by an antiparallel β-sheet comprising residues 27–31 and 36–40 which are connected by a highly flexible β-turn (residues 32–35). Since hirudin obviously shares this structural element with the snake venom proteins, we have decided to replace residues 32–35 of hirudin by the fibronectin-derived adhesion sequence RGDS. Introducing this sequence into an appropriate environment should enhance its disintegrin activity relative to that of the linear RGD peptides, yielding anticoagulants on the basis of hirudin which exhibit both antithrombin and platelet aggregation inhibitory activity. In this paper we provide data that the chimeric hirudin, called hirudisin, is a thrombin inhibitor comparable to r-hirudin as well as an inhibitor of platelet aggregation by direct interaction with the integrin GP IIb-IIIa. Thus, these proteins are of potential use as integrin-targeted antithrombotic agents.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were obtained from the following sources: Tos-Gly-Pro-Arg-AMC and the peptides Gly-Arg-Gly-Asp-Ser (GRGDS) and Arg-Gly-Asp-Ser (RGDS) from Bachem; trypsin (sequencing grade), T7 DNA polymerase, restriction enzymes, ADP, and streptavidin-peroxidase conjugates from Boehringer Mannheim; DEAE-Sephadex A-25 and PD10 columns from Pharmacia LKB Biotechnology Inc.; human fibrinogen from Kabi Vitrum; the murine biotinylated monoclonal antibody to human GP IIb from DiaNova; 3,3',5,5'-tetramethylbenzidine (TMB) from Fluka.

**Oligonucleotide-directed Mutagenesis**—Site-directed mutagenesis was performed according to Kunkel et al. (1987). A DNA fragment coding for the tac promoter, the alkaline phosphatase signal sequence, and the hirudin gene was inserted into the phasmid pUC 118 (Vieira and Messing, 1982). An M13-containing template single-stranded DNA was prepared using the Escherichia coli strain RZ1032 (American Type Culture Collection No. 39737). Oligonucleotides of 32 bases were used as mutagenic primers. *In vitro* DNA synthesis was performed with T7 DNA polymerase and T4 DNA ligase. E. coli BMH71-18 mutS (Kramer et al., 1984) was transformed with the mutagenized phasmid double-stranded DNA. Mutants were identified by sequencing single-stranded DNA according to Sanger et al. (1977).

**Protein Purification**—Human prothrombin was isolated according to Mann (1976) and converted to α-thrombin using the venom of Oxynurus scutellatus (Owen and Jackson, 1973). α-Thrombin concentrations were determined by active-site titrations (Jameson et al., 1979), the purity was >98%. Recombinant hirudisins were isolated from the periplasm of E. coli by anion-exchange chromatography on DEAE Sephadex A-25 and reverse-phase high performance liquid chromatography on a Shandon ODS Hypersil column (Dodt et al., 1996b). Protein sequence analyses (Hunkapiller et al., 1983) of 0.1–0.8 nmol of the recombinant proteins or of trypsic peptides of the reduced and carboxymethylated hirudisins were performed in a gas-phase sequenator (Applied Biosystems Model 470A connected to a Model 120A analyzer) and confirmed the correct sequence for hirudisins. GP IIb-IIIa was prepared by the method of Fitzgerald et al. (1985). After removing salts and reagents by chromatography on a PD10 column, reduced and carboxymethylated hirudisin (cm-hirudisin) was isolated by reverse-phase high performance liquid chromatography for inhibition studies.

**Fibrinogen/GP IIb-IIIa ELISA**—The ELISA was performed according to Dennis et al. (1990). Microtiter plates were coated with human fibrinogen (1 mg/ml) in 0.1 M sodium carbonate buffer, pH 9.0, overnight and then incubated with 3% BSA (5%, w/v, nonfat dry milk) in phosphate-buffered saline. After washing with TBST (50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 10 mM CaCl₂, 0.05% Tween 20) the samples to be evaluated and then GP IIb-IIIa (1 mg/ml in TBST) were added. After 1 h of incubation the plate was washed and then labeled with the biotinylated monoclonal antibody (1 μg/ml). Bound antibodies were detected with a streptavidin-peroxidase complex (50 milliunits/ml).

**Platelet Aggregation Assays**—Platelet aggregation assays were performed in human platelet-rich plasma (PRP). PRP was prepared as described by Dennis et al. (1990). For the assays, PRP was diluted to 250,000 platelets/μl with platelet-poor plasma. PRP (200 μl) plus 25 μl of sample in 0.9% NaCl or plus 25 μl of 0.9% NaCl alone was incubated in an aggerometer at 37 °C for 5 min. The aggregating agent (25 μl of α-thrombin (1 NIH unit) or 25 μl of 100 mM ADP) was added and the light transmission recorded. Transmission was set at 100% for platelet-poor plasma and 0% for PRP.

**Amidoctyl Assays of Thrombin Activity**—Assays were performed in polycrylate cuvettes at 25 °C in 0.1 M Tris-HCl, pH 8.5, containing 0.2 M sodium chloride and 0.05% Triton X-100 (Dodt et al., 1988). Tos-Gly-Pro-Arg-AMC was used as substrate at a concentration of 50 μM. Under the specified conditions, the Kₚ of the substrate was 5.0 ± 0.2 μM. In tight-binding inhibition experiments, 50 μM α-thrombin was preincubated with 10-200 μM inhibitor and the steady-state velocity was measured. In slow-binding inhibition experiments, 200-1000 PM inhibitor was incubated with 50 μM substrate and the reaction was started by the addition of 20 μM enzyme. Assays were performed with a Perkin Elmer LS50 spectrofluorometer (λₑ = 370 nm; λₛ = 450 nm), and fluorescence intensities were calibrated with 7-amino-4-methylcoumarin (100 nm).

**Data Analysis**—Data analysis was performed with the nonlinear regression program GraFit (Leatherbarrow, 1990). In slow-binding inhibition experiments for each of the inhibitors a set of progress curves at several inhibitor concentrations was obtained and the data were fitted to Equation 1 by nonlinear regression analysis (Morrison, 1982; Morrison and Stone, 1985).

\[
P = c(t + c_\infty - c_\infty)(1-e^{-t+k_{\text{app}}})/K_{\text{app}} + d
\]

The symbols cₜ, c₀, and Kₚ represent the initial velocity, steady-state velocity, and an apparent first-order rate constant; d is a displacement term to account for the case that at t = 0 the fluorescence is not known accurately. Values for Kₚ were plotted versus [I] and fitted to Equation 2 to obtain Kₚ (Morrison, 1982).

\[
K_{\text{app}} = c_{\text{app}}'/[1 + ([I]/K_{\text{app}})] + c_{\text{app}}
\]

Equilibrium dissociation constants Kₚ were obtained from tight-binding inhibition experiments by fitting the data to Equation 4 (Cha, 1975; Williams and Morrison, 1979).

\[
v = v_0 \frac{[E]}{[E]_0} = K_v' + [K_v - [E]]^2 + 4K_v'[E] - [K_v + [E]]
\]

with the following relationship.

\[
K_v' = K_v' \left(1 + [S]/K_v\right)
\]

ICₜₐₒ values of inhibitors in platelet aggregation assays as well as fibrinogen/GP IIb-IIIa ELISA were determined by numerical analysis of the data according to Equation 5 (Leatherbarrow, 1990).

\[
IC_{\text{tₐₒ}} = \frac{(E-d) \times [I]}{E - d - a}
\]

Where E is the response variable, a the maximum range of y values, b the background y value, c the slope factor, and I represents the inhibitor concentration.

**RESULTS**

**Interaction of Hirudisin with Human α-Thrombin**—Amino acid residues Ser-Asp-Gly-Glu at position 32–35 of hirudin were replaced by the cell adhesion motif Arg-Gly-Asp-Ser of fibronectin to generate hirudisin. In order to determine the effect of the mutation at a site which should not be involved in the thrombin-hirudin interaction (Rydel et al., 1990; Grütter et al., 1990), we studied the inhibitory properties of hirudisin in slow-binding as well as tight-binding inhibition experiments. From tight-binding experiments a 2-fold decrease in the dissociation constant Kₚ for the chimeric protein (Kₚ = 0.16 ± 0.06 pm) was obtained which corresponds well to the dissociation constant Kₚ = 0.27 ± 0.03 pm of r-hirudin (Table 1). In slow-binding inhibition experiments a set of progress curves was obtained at a fixed enzyme concentration (20 pm) and several inhibitor concentrations (200-800 pm). Curves were analyzed according to Equation 1 to yield the apparent first-order rate constants Kₚ. From the slope of a plot of Kₚ.
Table I

Kinetic parameters of the interaction of human α-thrombin with hirudisins and hirudins

| Compound           | $K_{i}$ ($10^{-11}$ M) | $k_{on}$ ($10^{9}$ M$^{-1}$ s$^{-1}$) | $k_{off}$ ($10^{-4}$ s$^{-1}$) |
|-------------------|-----------------------|-------------------------------------|-------------------------------|
| r-Hirudin         | 0.27 ± 0.03           | 5.7 ± 0.2                           | 1.5 ± 0.1                     |
| Hirudisin         | 0.16 ± 0.06           | 13.1 ± 3.2                          | 2.1 ± 1.4                     |
| Hirudisin-1       | 0.37 ± 0.06           | 6.8 ± 0.3                           | 2.4 ± 0.4                     |
| Hirudin(Δ2-35)$^a$| 0.36 ± 0.11           | ND$^b$                              | ND$^b$                        |

$^a$ From tight-binding inhibition experiments.

$^b$ From slow-binding inhibition experiments.

$^c$ Hirudin(Δ32-35), hirudin with amino residues 32 to 35 deleted.

$^d$ ND, not done.

![Graph](image1.png)

**Fig. 1. Inhibition of α-thrombin-stimulated platelet aggregation.** Dose-dependent inhibition of platelet aggregation was measured in the presence of hirudisin (Δ), cm-hirudisin (□), r-hirudin (○), and hirudin(45-65) (△).

**versus [I]** the association rate constant $k_{on} = 1.5 ± 0.08 \times 10^9$ M$^{-1}$ s$^{-1}$ has been determined for hirudisin. As the intercept with the y axis was too close to zero we estimated the dissociation rate constant $k_{off} = 1.54 ± 0.33$ s$^{-1}$ using the $K_i$ from tight-binding and the $k_{on}$ from slow-binding experiments ($k_{off} = k_{on} \times K_i$). Hirudisin-1, a variant of hirudisin with Arg$^2$ replaced by Lys, has also been analyzed for thrombin inhibitory properties and was found to display similar kinetics as determined for r-hirudin. Even deletion of the Ser-Asp-Gly-Glu sequence, which forms a flexible β-turn connecting strand II (residues 27-31) and strand II’ (residues 36-40) of the second antiparallel β-sheet of hirudin (nomenclature according to Folkers et al., 1989), does not interfere with thrombin-inhibitor interaction (Table I). Thus, modification of the amino acid sequence at the tip of the finger-like structure of hirudin can be performed without any loss of thrombin inhibitory activity.

Inhibition of α-Thrombin-stimulated Platelet Aggregation—

We examined hirudisin, hirudisin-1, reduced and carboxymethylated hirudisin (cm-hirudisin), r-hirudin, and the COOH-terminal fragment hirudin(45-65) for dose-dependent inhibition of platelet aggregation. α-Thrombin-stimulated platelet aggregation was most effectively inhibited by the native inhibitors hirudisin, hirudisin-1, and r-hirudin with IC$_{50}$ values of 5.7–6.8 nM (Table II). Hirudin(45-65), an exosite-directed inhibitor of α-thrombin (Ma0 et al., 1988; Dodt et al., 1990), blocked the enzyme-induced platelet aggregation with an IC$_{50}$ of 3.3 μM. Carboxymethylated hirudisin, containing this COOH-terminal hirudin sequence as well as the linear unfolded NH$_2$-terminal domain, inhibited platelet aggregation slightly better than hirudin(45-65). The 9-fold lower IC$_{50}$ appears to be due to additional contacts with thrombin. The GRGDS peptide did not inhibit thrombin-induced platelet aggregation up to 10 μM. However, at higher concentrations the peptide showed the same dose-dependent aggregation inhibition as in ADP-stimulated assays. This observation has to be assigned to the RGD-mediated route.

Inhibition of ADP-induced Platelet Aggregation—

GP Ib-IIIa antagonist activity was measured in vitro by dose-dependent inhibition of ADP-stimulated platelet aggregation in human platelet-rich plasma (Fig. 2). Disintegrin activity is most effectively exhibited by hirudisin (IC$_{50}$ = 65 μM). The cell adhesion sequence of this protein is about 3-fold more potent than that of cm-hirudisin and the linear GRGDS peptide (IC$_{50}$ = 180 μM) as well as about 6-fold more potent than the RGD-containing peptides (IC$_{50}$ = 330 μM). The GRGDS sequence of hirudisin-1 exhibited much weaker GP Ib-IIIa antagonist activity (Table II). Under assay conditions only slight inhibition of ADP-induced platelet aggregation was observed with 120 μM hirudisin-1.

Inhibition of GP Ib-IIIa-Fibrinogen Interaction—

Using a solid-phase integrin binding assay (Dennis et al., 1990) we determined the relative potency of hirudisin, r-hirudin, and...
RGDS peptide, to inhibit the binding of GP IIb-IIIa to immobilized fibrinogen (Fig. 3). Hirudisin binds 9-fold weaker to GP IIb-IIIa in solution (IC₅₀ = 13.6 μM) than has been observed for the linear RGDS peptide (IC₅₀ = 1.5 μM). Up to 35 μM r-hirudin did not inhibit the receptor-ligand interaction. It is noteworthy that the binding potency of the RGDS peptide is 200-fold enhanced in the ELISA as compared to the platelet aggregation assay, whereas the binding potency of hirudisin is only 4-fold increased (Table II). The different results are probably caused by differences in the affinity of purified GP IIb-IIIa for the disintegrins relative to the affinity in intact platelets.

**DISCUSSION**

We have generated hirudin-based thrombin-specific inhibitors with RGD-mediated disintegrin activity. The major findings of this study are summarized as follows. First, modifications of the β-turn (residues 32-35) in the finger-like structure of hirudin appear not to be crucial to the inhibitor's interaction with α-thrombin. Second, replacing the SDGE sequence (residues 32-35) by the cell adhesion sequence RGDS results in the acquisition of disintegrin function. Third, enhanced inhibition of ADP-induced platelet aggregation by native hirudisin relative to cm-hirudisin illustrates a considerable contribution of the tertiary structure to integrin binding. Fourth, thrombin-stimulated platelet aggregation shows that combining antithrombin and disintegrin activity does not result in a cooperative effect for hirudins available at present.

Recently, the solution structures of the GP IIb-IIIa antagonists kistrin (Adler et al., 1991) and echistatin (Cooke et al., 1991; Dalvit et al., 1991; Saueder et al., 1991) from snake venoms illustrated tertiary structure requirements of functional RGD adhesion sequences. The recognition sequence of echistatin lies in a loop of 11 residues joining the two strands of an antiparallel β-sheet and protrudes from the tightly packed core of the molecule. The RGD adhesion site of kistrin is located at the end of a long arm (residues 49-51) connecting two antiparallel strands (residues 41-48 and 52-59). Since hirudisin displays a related structural element in the loop (residues 32-35) linking two antiparallel β-strands (residues 27-31 and 36-40) (Folkers et al., 1989; Grutter et al., 1990; Haruyama and Wätrich, 1989; Rydel et al., 1990), we decided to introduce the RGD motif into this site of the inhibitor to generate hirudins. From the previously published work of Church et al. (1991) it was already known that chimeric molecules could be constructed combining the RGDS sequence and hirudin-derived COOH-terminal peptides which exhibit both antithrombotic and anti-adhesive activities. Our results demonstrate that hirudisin (IC₅₀ = 65 μM) is a 3- to 5-fold better inhibitor of ADP-induced platelet aggregation in PRP than RGDS or GRGDS peptides, whereas a careful interpretation of our present data, maximum inhibitory potency of cm-hirudisin is the same as that of the RGDS peptides. However, the 200- to 2000-fold poorer activity of the cell adhesion motif in hirudisin relative to that in disintegrins from snake venoms like echistatin (IC₅₀ = 32 nM; Garsky et al. (1989)), flavoridin (IC₅₀ = 40 μM; Musial et al. (1990)), kistrin (IC₅₀ = 130 nM; Dennis et al. (1990)), albolobrin (IC₅₀ = 220 nM; Calvete et al. (1991)), and barbourin (IC₅₀ = 300 nM; Scarborough et al. (1991)) may have two reasons: (i) the RGD sequence is not in the proper conformation for optimal binding to GP IIb-IIIa, or (ii) the linear sequence or the environment around the adhesion sequence does not satisfy all requirements. Comparison of amino acid sequences of the RGD containing loops of snake venom proteins (Fig. 4) reveals some conserved properties. There are positively charged amino acid residues on the NH₂-terminal side of the RGD motif and an accumulation of negatively charged amino acid residues on the COOH-terminal side of the binding sequence. In the hirudisin molecule a positively charged lysyl residue follows on the COOH-terminal side of the adhesion motif. Thus, the charge distribution around the RGD motif of hirudisin may be responsible for lower disintegrin activity with respect to the snake venom proteins. However, some conformational contribution to the integrin-directed cell recognition is indicated by the 3- to 5-fold better inhibition of ADP-induced platelet aggregation of hirudisin compared to cm-hirudisin and RGD peptides.

Since RGD-containing peptides have been shown to block binding of fibrinogen to GP IIb-IIIa and to prevent formation of platelet thrombin, these peptides appear to be promising candidates as antithrombotic agents (Kieffer and Phillips, 1990; Phillips et al., 1991). However, a possible therapeutic application of RGD-containing peptides requires distinct specificity because a large number of integrins are known to bind RGD peptides (Kieffer and Phillips, 1990) which may increase the risk of side effects. The snake venom disintegrin barbourin has been found to exhibit a GP IIb-IIIa-directed specificity due to a RGD motif instead of the RGD sequence (Scarborough et al., 1991). Substitution of Lys for Arg in the disintegrin eristociphin converts a nonselective into a GP IIb-IIIa-specific ligand and demonstrates that specificity is achieved merely by a single amino acid replacement (Scarborough et al., 1991). In order to gain such specificity we replaced Arg²⁷...
by Lys to generate hirudisin-1. However, careful interpretation of our results (maximum concentration of hirudisin-1 in ADP-induced platelet aggregation assay was 120 μM) shows that this inhibitor is an approximately 5-fold less active antagonist of GP Ib-IIIa than hirudisin. This finding is in agreement with earlier studies that have shown that peptides containing a KGD sequence are much weaker antagonists of integrins (Ginsberg et al., 1985; Scarborough et al., 1991).

One purpose of this study was the generation of a targeted hirudisin. Hirudins are designed to bind to platelet surfaces via their cell adhesion sequence, simultaneously acting as disintegrins and as inhibitors of thrombin functions just at the site where thrombin is generated. These proteins are considered to be potential anticoagulants with enhanced pharmacological properties. However, to avoid bleeding complications due to hirudisin’s strong thrombin inhibitory activity, its RGD motif should exhibit disintegrin function in the same therapeutic window of 10–100 nM in human plasma as has been proposed for hirudisin (Markwardt, 1989). In order to increase hirudisin’s disintegrin activity and specificity we will focus on engineering the charge distribution around the RGD adhesion sequence as well as on the complete replacement of hirudisin’s disintegrin-related loops.

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