Tailoring echistatin to possess higher affinity for integrin $\alpha_{IIb}\beta_3$

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Abstract A mutant of echistatin, a disintegrin with a high affinity for the integrins, was constructed by substituting CRGDC for ARGDD in the Arg-Gly-Asp (RGD) region. The mutant was chemically synthesized, subjected to a folding process with air oxidation, and purified by reverse-phase HPLC. The peptide mapping and mass spectrometric analyses revealed that the two Cys residues introduced in the mutant are linked to each other, without any effect on the mode of the four disulfide bonds present in native echistatin, as expected. The mutant strongly inhibited the binding of human fibrinogen to its receptor, integrin $\alpha_{IIb}\beta_3$, with an IC_{50} value of 0.12 nM. This value shows that the mutant is twice as potent as the native form (IC_{50} = 0.23 nM). These results indicate that the native disintegrin molecule, which has been considered to possess the optimum affinity for the integrins, can be tailored to exhibit even higher affinity by introducing the conformational constraint into the RGD region. Monte Carlo simulations of KRCRGDCMD, the RGD region in the mutant, suggested that the disulfide bond constrains the RGD region to assume a type II′ β-turn, with Gly and Asp in positions 2 and 3 of the turn.

Key words: Echistatin; Arg-Gly-Asp; Integrin $\alpha_{IIb}\beta_3$; Conformational constraint; Monte Carlo simulation; Type II′ β-turn

1. Introduction

Recently, a number of potent integrin antagonists, the disintegrins, have been isolated from the venom of various vipers. These proteins include echistatin [1] from *Echis carinatus*, kistrin [2] from *Agkistrodon rhodostroma*, trigramin [3] from *Trimeresurus gramineus*, and others. They are cysteine-rich, 50–80 residue proteins, and all contain a functional Arg-Gly-Asp (RGD) sequence. It is known that they inhibit platelet aggregation, through the binding to integrin $\alpha_{IIb}\beta_3$, 500–1000 times more strongly than the GRGDS peptide [2,4].

Pierschbacher and Ruoslahti reported that G-Pen-GRGDSPCA, in which Pen, penicillamine, forms a disulfide bond with Cys, can inhibit the adhesion of normal rat kidney cells to vitronectin more effectively than the linear peptide, GRGDSPC [5]. It was also described that cyclo(GRGDSPA) is a much better inhibitor of the adhesion of B16 melanoma cells to vitronectin or fibronectin than the linear counterpart, GRGDSPA [6]. These results indicate that it is possible to increase the affinity to the integrins by introducing a conformational constraint into an RGD-containing peptide.

In this study, we examined whether this strategy is also applicable to a natural protein with a high integrin affinity. We have constructed a mutant molecule by placing two Cys residues at the positions flanking RGD in echistatin, a disintegrin composed of 49 amino acid residues [7]. Here we report the successful formation of a new disulfide bond between the introduced Cys residues in the mutant, and its functional evaluation. Modeling of the RGD conformation in the mutant by a Monte Carlo simulation is also shown.

2. Materials and methods

2.1. Chemical synthesis

Starting with 0.08 mmol (0.8 g) of Fmoc-Thr(tBu)-NovaSyn KA resin (0.1 mmol/g), the synthesis of mutant echistatin was carried out in a stepwise manner using the LKB automated peptide synthesizer, Biolynx 4170. The amino acids were introduced using the manufacturer's cartridges (0.25 mmol each). Side chain protection was arginine (Mtr), aspartic acid (OtBu), cysteine (Trt), glutamic acid (OtBu), histidine (Trt), lysine (Boc), serine (tBu), threonine (tBu), tyrosine (tBu) (Mtr, 4-methoxy-2,3,6-trimethylphenyl-sulfonyl; OtBu, *tert*-butyl ester; Trt, trityl; Boc, *tert*-butyloxycarbonyl; tBu, *tert*-butyl).

2.2. TFA cleavage and air oxidation

The assembled resin (1.3 g) was suspended in a mixture of TFA (42 ml), anisole (3 ml), and ethane-dithiol (2 ml), in a flat-bottomed flask. After stirring at room temperature for 12 h, the reaction mixture was concentrated on a rotary evaporator. The residue was triturated with cold ether, filtered, and washed three times with cold ether. The filtered residue was dissolved in 2 N acetic acid and freeze-dried. The crude product was purified on an A-303 ODS column (2×30 cm, YMC).

The air oxidation of the protein was carried out as described by Garsky et al. [8]. The solution was acidified to pH 3.0 by the addition of acetic acid and pumped directly onto a Shodex ODSpak F-411/S column (0.46×10 cm, Showa-dencoh, Japan). The oxidized protein product was eluted at a flow rate of 0.8 ml/min with a 60-min linear gradient of acetonitrile increasing from 5% to 25% in 0.1% TFA. The oxidized form of the mutant echistatin was obtained with a yield of 0.5 mg.

2.3. Binding assay

Native echistatin was purchased from Sigma. The concentrations of the mutant and the native echistatin were determined according to the results of the amino acid analyses. Two RGD-containing peptides, GRGDSP and cyclo(Arg-Gly-Asp-DPhe-Val), were purchased from Peptide Institute (Japan).

The inhibitory effects of these molecules on the binding of fibrinogen to immobilized integrin $\alpha_{IIb}\beta_3$ were examined as described previously [9].

2.4. Peptide mapping analysis

The mutant or the native echistatin (0.2 mg each) was dissolved in 1.0 ml of 25 mM Tris-1 mM EDTA, pH 8.3, and digested with 10 μg of endoproteinase Lys-C (Wako Pure Chemicals, Japan) at 37°C for 5 h. The BrCN treatment was carried out in 1.0 ml of 70% formic acid with 0.3 mg of BrCN at room temperature for 20 h. The resultant peptides were separated by reverse-phase HPLC and identified by amino acid analyses and N-terminal sequence analyses.

2.5. Analysis of N-terminal amino acid sequence

The N-terminal amino acid sequence was determined, using an Applied Biosystems Model 477A sequencer equipped with a 120A online PTH amino acid analyzer.

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Abbreviations: RGD, Arg-Gly-Asp; ESI, electrospray ionization; FAB, fast-atom bombardment; MC, Monte Carlo

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2.6. Analysis of amino acid composition

The amino acid composition was determined on a 24-h hydrolysate with 6 N HCl at 110°C in the presence of 4% thioglycolic acid. Amino acid analysis using ninhydrin was performed on a Hitachi Model 835 amino acid analyzer.

2.7. Mass spectrometry

The electrospray ionization (ESI) mass spectrum was measured with a JEOL JMS-HX110/110A double-focusing mass spectrometer equipped with an ESI ion source (Analytica, Branford, CT) [9]. Each of the echistatins (200 pmol) was dissolved in 20 μl of H_2O/acetic acid/acetonitrile (5:1:4) and infused into the ion source at a flow rate of 1 μl/min.

The fast-atom bombardment (FAB) mass spectrum was obtained using the above spectrometer equipped with an FAB ion source. The sample (0.5 nmol) was dissolved in water-acetonitrile (1:1), mixed with a liquid matrix (a mixture of dithiothreitol and dithioerythritol, 5:1 w/w), and bombarded with xenon atoms accelerated at a 6-kV potential in the ion source.

2.8. Monte Carlo simulation

The Monte Carlo (MC) simulations of the two RGD-containing peptide fragments, KRARGDDMD (residues 21–29) from the native echistatin and KRCRGDCMD (residues 21–29) from the mutant, were carried out by the program FEDER [10] with the ECEPP/2 force field [11] at 1000 K. In order to improve the acceptance ratio of the ring structure formation of the mutant, we adopted the scaled collective variable MC method [12]. This method effectively samples conformations that satisfy the ring closure of the disulfide bond between the two Cys residues. The high-temperature simulation allows sampling of all possible conformations. In addition to the ECEPP/2 potential, a harmonic distance restraint of 3 kcal/mol is imposed between the two Cα atoms of Lys-21 and Asp-29 to maintain the average distance of the NMR structures [13]. This had little influence on the RGD conformations. A total of 10^7 MC steps were accumulated for each peptide.

2.9. Classification of the sampled conformations

The sampled conformations were grouped into three classes in terms of the (φ, ψ) region of the Gly residue [14]. This classification is consistent with a cluster analysis of RGD conformations in terms of the root-mean square deviation, because the conformation is mostly determined by the (φ, ψ) angles of Gly. The three classes are characterized by the distance d between the Cβ atoms of Arg-24 and Asp-26, and by the conformation of Gly-25. Class 1: d=9.0–9.5 Å; Gly conformation=E, F, E*, or F*. Class 2: d=7.5–8.5 Å; Gly conformation=C* or D*. Class 3: d= 5.5–6.0 Å; Gly conformation=C, A*, or B* [9]. The conformational regions are designated according to Zimmerman et al. [14].

3. Results

The mutant echistatin with the RGD sequence flanked by two Cys residues was chemically synthesized, in vitro folded with air oxidation, and purified by reverse-phase HPLC, as described in section 2. The mutant thus obtained eluted as a single peak, with a similar retention time to that of native echistatin (from Sigma) on analytical reverse-phase HPLC. The N-terminal amino acid sequence of the mutant, as determined up to 20 residues by the automated Edman degradation method, was identical with that of the native form. The amino acid composition of the mutant was in good agreement with the theoretical values (data not shown).

On the electrospray mass spectrometric analysis, the mutant (Fig. 1A) and the native form (Fig. 1B) produced two multiply-charged ion signals, $[M+5H]^{5+}$ and $[M+6H]^{6+}$. These ion signals for the mutant and the native form gave calculated average molecular weights of 5434.7 ± 0.2 and 5416.7 ± 0.2, respectively. These values are in good accordance with their theoretical molecular weights, 5435.2 and 5417.1, indicating that no modification occurs on these molecules. In addition, no free thiol group was detected in the mutant by the dithiobis(2-nitrobenzoic acid) method [15]. These results show that all of the 10 Cys residues in the mutant are linked via five disulfide bonds.

The locations of the disulfide bonds were examined by peptide mapping analyses. When digested by endoproteinase Lys-C, peptide fragment L (see Fig. 2B), lacking FLK (residues 13–15) and GPAT (residues 46–49), was isolated by reverse-phase HPLC. Fragment L from the mutant was further treated with BrCN, resulting in the formation of fragment B-I, RCRGDCM (Fig. 2), while the same treatment of the native

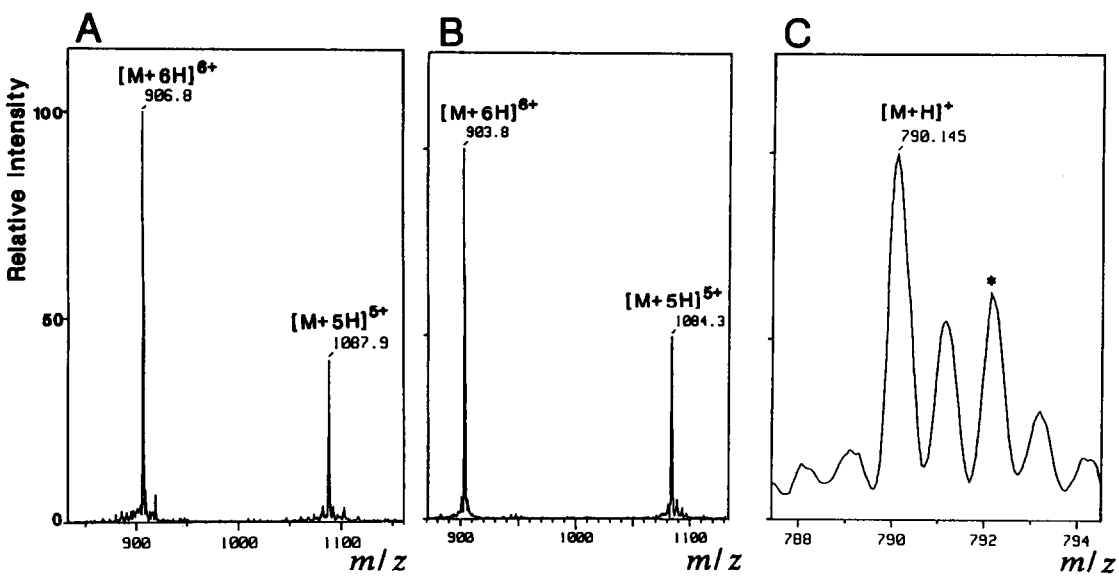

Fig. 1. Electrospray mass spectra of mutant (A) and native (B) echistatins. The mass of each echistatin was calculated from the m/z values of the two multiply-charged ion signals, $[M+5H]^{5+}$ and $[M+6H]^{6+}$. (C) Fast-atom bombardment mass spectrum of fragment B-I. The B-I fragment (see Fig. 2A) produced a main molecular ion ($[M+H]^+$) with an m/z value of 790.145, which is in good agreement with the theoretical value (790.3) of the cyclic form of RCRGDCM with C-terminal homoserine lactone in place of the Met residue. The signal marked by an asterisk is derived from the linear counterpart, which was produced by reduction of the disulfide bond during mass measurement.

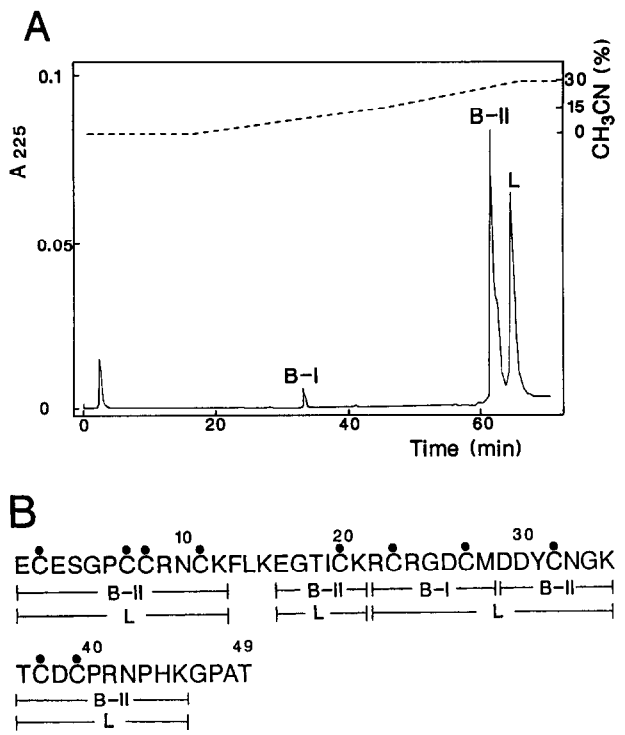

Fig. 2. BrCN treatment of the Lys-C digest (fragment L) from the mutant echistatin. (A) Elution pattern after BrCN cleavage of fragment L on reverse-phase HPLC. The BrCN-treated products from fragment L (50 μg) were applied to a Shodex ODSpak F-411/S column (0.46×10 cm). Elution was performed with a linear gradient of acetonitrile concentration in the presence of 0.1% TFA, at a flow rate of 0.8 ml/min. (B) Identification of each peptide fragment. Fragments B-I, B-II, and L were identified by amino acid analyses and N-terminal sequence analyses. Dots show the positions of Cys residues.

echistatin produced the fragment RARGDDM. The C-terminal Met residues from RCRGDCM and RARGDDM, of course, were converted into homoserine lactone after the BrCN reaction. In both cases, the recovery of the heptapeptides was about 70%, according to the amino acid analyses. This value is consistent with the fact that a considerable amount of the starting peptide fragment L was detected after the BrCN reaction. Fragment B-II (Fig. 2) eluted with the same retention time as the corresponding fragment from the native echistatin on the HPLC. These results reveal that the two Cys residues introduced in the mutant are linked to each other without any effects on the four disulfide bonds in the native form [7]. The presence of the new disulfide bond in the mutant was further confirmed by FAB mass spectrometry of the B-I fragment (Fig. 1C).

We examined the affinity level of the mutant echistatin to the integrins. For this purpose, integrin $\alpha_{IIb}\beta_3$ was purified from human erythroleukemia cell line HEL (ATCC TIB 180) by chromatography on columns of ConA-Sepharose, GRGDSPK-Sepharose, and Sephacryl S-300. After these steps, highly purified integrin $\alpha_{IIb}\beta_3$ was obtained with a yield of 1 mg per 10 liters of culture medium. We finally established a binding assay system using the integrin and biotinylated fibrinogen, as described in section 2. The binding assay showed that the mutant is twice as potent (IC_{50}=0.12 nM) as the native echistatin (IC_{50}=0.23 nM) (Fig. 3). The results indicate that the introduction of a conformational constraint into the RGD region of the echistatin molecule significantly increases the affinity to the integrin, as in an RGD-containing peptide. In addition, it should be noted that these echistatin molecules are much more effective than a short peptide, GRGDSP (IC_{50}=120 nM) or cyclo(Arg-Gly-Asp-DPhe-Val) (IC_{50}=6.8 nM) (data not shown).

The influence of the introduced disulfide bond on the RGD conformation was investigated by Monte Carlo simulations of the two RGD-containing peptide fragments, KRARGDDMD (residues 21–29) from the native echistatin and KRCRGDCMD (residues 21–29) from the mutant. The sampled conformations were classified into three structural classes in terms of the (ϕ, ψ) region of the Gly residue (see section 2): class 1 has a fully extended Gly residue; class 2 assumes a type II′ β-turn having a hydrogen bond between the C=O of Arg-24 and the H–N of Cys-27 (Asp-27 for the native form); and class 3 is characterized by a salt bridge between the two side chains of Arg-24 and Asp-26. The MC simulations show that classes 1, 2, and 3 were sampled with probabilities of 0.15, 0.29, and 0.22 for the native fragment and 0.13, 0.52, and 0.13 for the mutant fragment. The disulfide bond increases the occurrence of class 2 by about 100%, while the other two classes are rather suppressed. The results suggest that the most probable conformation of the mutant fragment belongs to class 2 assuming a type II′ β-turn, with Gly and Asp in positions 2 and 3 of the turn. A snapshot of the mutant fragment with the type II′ β-turn conformation is shown in Fig. 4.

4. Discussion

It has been shown that the cyclic form of an RGD-containing peptide has much higher affinity for integrins than the linear counterpart [5,6]. Recently, we constructed a mutant lysozyme, Cys-RGD4, by inserting RGDS flanked by two Cys residues, and found that the Cys-RGD4 possesses higher cell adhesion activity than the RGDS-inserted lysozyme, RGD4 [9,16]. In these cases, the conformational constraint in the RGD region effectively increased the affinity for the integrins. However, these RGD-containing cyclic molecules

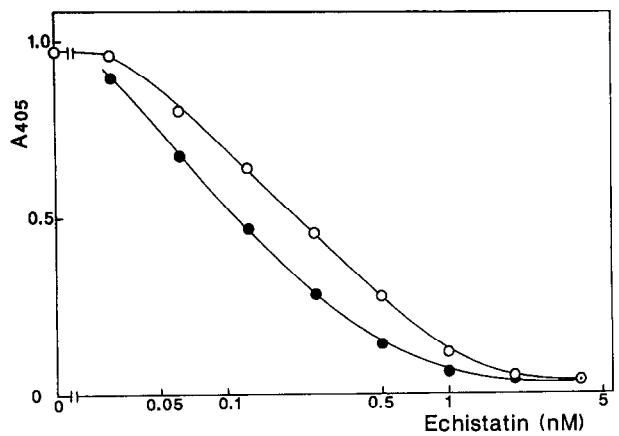

Fig. 3. Inhibitory effect of each echistatin on the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$. The plastic substrates were coated with 1 μg/ml integrin $\alpha_{IIb}\beta_3$, and 1 nM biotinylated fibrinogen was incubated on the substrates for 3 h at 30°C in the presence of different concentrations of the mutant (●) and the native echistatin (○). The IC_{50} value of each echistatin is defined as the concentration necessary to inhibit receptor binding by 50%.

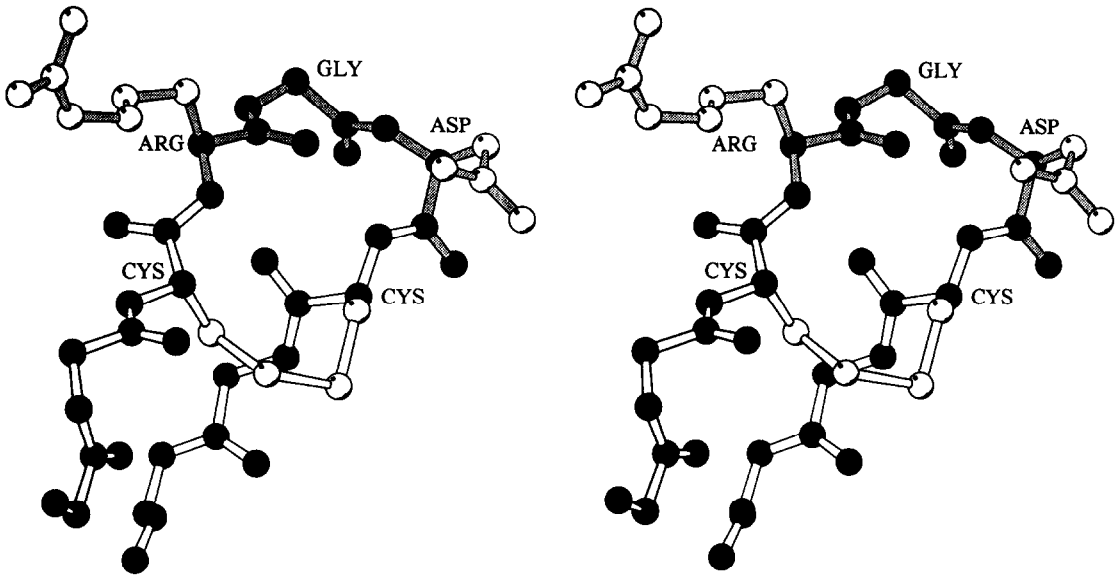

Fig. 4. Molscript drawing of a snapshot of KRCRGDCMD assuming the type II′ β-turn conformation [21]. Main chain atoms are represented by black balls. Only the side chains of CRGDC are shown as white balls. The RGD residues are indicated by shaded sticks. The type II′ β-turn is characterized by a hydrogen bond between the C=O of Arg-24 and the H–N of Cys-27; the distance between O of Arg-24 and N of Cys-27 is 2.9 Å in this structure.

have a 10-fold lower affinity than the natural cell adhesive proteins including the disintegrins [2,5,9]. The purpose of this study was to address the question of whether the conformational constraint works also in a natural disintegrin, echistatin. The results obtained here prove that this strategy is significantly effective in the disintegrin, which possesses an affinity comparable to that of the ligand protein, fibrinogen [1]. It is noteworthy that the affinity can be improved beyond the level that has been considered to be optimal.

Monte Carlo simulations suggest that the disulfide bond constrains the RGD region to assume a type II′ β-turn, with Gly and Asp in positions 2 and 3 of the turn (Fig. 4). It is easy to explain why the disulfide bond enhances the occurrence of the turn conformation. The disulfide bond forms a small 17-atom-membered ring in the RGD region. This covalent structure does not favor the distance d (Cβ of Arg–Cβ of Asp) to be either more than 9 Å or less than 6 Å. A value of d that is too large causes difficulty in maintaining the ring structure, and a d value that is too small may produce unfavorably dense atom packing. It is natural to conclude that the formation of the type II′ β-turn structure in RGD is the reason for the increase of the binding affinity of the mutant.

This conformation has been found in the RGD regions of several proteins. We have recently solved the X-ray crystal structure of the CRGDSC-inserted lysozyme, Cys-RGD4. The RGD sequence in this protein resides within a stable type II′ β-turn, with a hydrogen bond between the C=O of Arg and the H–N of Ser [17]. The fibronectin type III domain from human tenascin [18] and the leech protein decorsin [19], each of which does not contain a Cys residue neighboring the RGD, also have similar structures in their RGD regions.

It has been suggested that the C-terminal portion of echistatin, in addition to the RGD region, would be important in the interaction with integrin $\alpha_{IIb}\beta_3$ [13]. This speculation agrees with the idea that the integrin $\alpha_{IIb}\beta_3$ contains an unrecognized exosite other than the RGD binding site [20]. Thus, in the case of the present mutant echistatin, the RGD region with the type II′ β-turn conformation, in cooperation with the C-terminal portion, could bind to the integrin with an extremely high affinity.

In the present investigation, we have succeeded in tailoring echistatin to possess higher affinity for integrin $\alpha_{IIb}\beta_3$. The strategy described here can be also applied to other members of the disintegrins.

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