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Natural Actin and Tropomyosin from Molluscan Catch Muscle

Girich UV, Lazarev SS, Vyatchin IG, Matusovsky OS and Sheludko NS*

Laboratory of Cell Biophysics, A.V. Zhirmunsky Institute of Marine Biology, National Scientific Center of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, 17 Palchevsky Str., Vladivostok 690041, Russia

Abstract

In this work, the main proteins of thin filaments (actin and tropomyosin) were isolated from the catch muscle of the mussel *Crenomytilus grayanus* and the rabbit skeletal muscles. Rabbit actin and tropomyosin and mussel tropomyosin were isolated by traditional methods as opposed to "natural" mussel actin (isolation of the mussel actin from acetone powder is impossible). These proteins were used to reconstruct actin+tropomyosin complexes in non-hybrid and hybrid manner. A non-hybrid complex, reconstructed from rabbit actin and tropomyosin, has higher reduced viscosity than complex of mussel proteins where reduced viscosity was nearly indistinguishable from the intrinsic viscosity. Both rabbit and mussel actin were purified by polymerization-repolymerization cycles followed by gel filtration chromatography. During purification, the viscosity of both actins increased, and the difference in viscosity between them decreased. Based on the SDS-electrophoresis, we did not find any of the other proteins in the chromatographic fractions, except actin. However, obtained chromatographic fractions have significant differences in viscosity and rate of polymerization. We believe that these properties caused by the presence of an "ending factor" (such as β-actinin or Cap Z) in actin preparations. The data obtained indicate that isolation of "natural" actin is accompanied by a co-extraction of an unknown or known "ending factor" in amounts that may be greater than those obtained from the acetone powder of rabbit skeletal muscles.

Keywords: Molluscan catch muscle; Natural actin; Tropomyosin; Viscosity

Introduction

The main postulate of muscle motility is the theory of sliding filaments, according to which the muscles contract as a result of the interaction of thin and thick filaments and relax when this interaction ceases. In this respect, the catch muscles of bivalve mollusks are not an exception, but they are unique in their ability to be not in two but in three states: namely relaxed, contracted and catch states. In the catch state, the mussel can support their shells in a closed state for a long time with little, if any, energy input. This phenomenon has been known for more than 100 years, but its mechanism remains unexplained. It is generally accepted that the catch is based on the linkages between thick and thin filaments. To date, there are two hypotheses that postulate either protein myorod [1] or protein twitchin [2] to be able to establish "catch linkages".

Myorod is found only in muscles that are capable of catch-contraction. It is localized on the surface of thick filaments and can be phosphorylated [3]. In this state, myorod in the absence of Ca^{2+} does not interact with thin filaments. However, in the presence of Ca^{2+}, dephosphorylated myorod is able to interact with thin filaments [4]. Thus, myorod can form adjustable linkages between thick and thin filaments. Unlike myorod, twitchin can be present also in non-catch muscles [5]. It is also located on the surface of thick filaments and can interact with all proteins of thick filaments-myosin, paramyosin and myorod, as well as with thin filaments. Phosphorylation of twitchin does not affect its interaction with proteins of thick filaments, but radically weakens its interaction with F-actin and thin filaments; this leads to dissociation of twitchin-actin complexes [2,6,7]. Thus, twitchin can also be considered as a candidate for the role of an adjustable catch-link between thick and thin filaments. Both catch hypotheses involve participation of thin filaments in this phenomenon. To date, the composition of proteins in thin filaments of the catch muscle is fairly well studied [8], and methods have been developed for isolation of these proteins: natural actin [9], tropomyosin [10], troponin [11], calponin [12], filamin [13], α-actinin [14]. In this work, we isolated and purified the main proteins of thin filaments, actin and tropomyosin, from the catch muscle of bivalve and from the skeletal muscles of vertebrates. Using these proteins, we formed hybrid und non-hybrid actin+tropomyosin complexes and found differences in their viscosities. Probably, the possible functional differences between skeletal and catch muscles are connected with these differences of thin filaments.

Materials and Methods

Isolation of rabbit proteins

Skeletal muscle actin and tropomyosin were isolated from rabbit back and leg muscles [6]. The rabbit skeletal muscle tissues were donated from the Pacific Institute of Bioorganic Chemistry (Vladivostok, Russia). All procedures were approved by the Animal Care Committee of A.V. Zhirmunsky Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences (Protocol N 25 from 12.03.2017).

Isolation of natural mussel actin

Natural mussel actin was prepared from the posterior adductor of the mussel *Crenomussel grayanus* by the non-Straub's method, modified for the simultaneous isolation of actin and tropomyosin [9]. 100 g of fresh minced adductors of the mussel were glycerinated for 24 hours with 5 volumes of glycerol solution (50% glycerol, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 3 mM NaN_3, 0.1 mg/ml trypsin inhibitor, 0.5 mM PMSF, 1 mM DTT, and 20 mM Imidazol-HCl, pH 6.2) by constant

***Corresponding author:** Sheludko NS, Laboratory of Cell Biophysics, A.V. Zhirmunsky Institute of Marine Biology, National Scientific Center of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences, 17 Palchevsky Str., Vladivostok 690041, Russia, Tel: +79025210922; E-mail: sheludko@stl.ru

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agitation with an overhead stirrer. After, glycerinated muscles were ground in a meat grinder, diluted up to 4 L with the standard solution (75 mM KCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 2 mM NaN_3, 0.5 mM DTT, 20 mM imidazole-HCl, pH 6.5) and centrifuged at 4000 rpm for 20 minutes The pellets were resuspended in 1 L of standard solution and homogenized with Polytron PT 2500E at 5000 rpm thrice for 1 minutes each time. The homogenate was centrifuged as above. The residue was supplemented with 300 ml of the extraction solution (75 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 15 mM ATP, 5 mM pyrophosphate, 1.0 mM DTT, 0.1 mM PMSF, 2.5 minutes g/mL leupeptin, 10 mM imidazole-HCl, pH 7.0) and extracted for 30 minutes. The residue was precipitated at 4000 rpm for 20 min; the extract was clarified at 25,000 rpm (rotor 50.1, Beckman) for 30 minutes The clarified extract was subjected to ultracentrifugation for 120 minutes at 40,000 rpm (rotor 50.1, Beckman). The process resulted in precipitation of the complex of actin and tropomyosin (Figure 1, lane 3). This complex was suspended in 80-100 mL of the extraction solution, and the solution was brought to homogeneity with the use of a Gilson Microman pipette. 3 M KCl was supplemented to the solution up to 0.6 M KCl. Then solution was ultracentrifuged for 120 minutes at 50,000 rpm (rotor 70.1, Beckman). As a result, tropomyosin fully remained in the supernatant (Figure 1, lane 5), while actin completely precipitated (Figure 1, lane 4). Lane 1, the fraction enriched with actin and tropomyosin; Lane 2, the fraction depleted of actin and tropomyosin; Lane 3, the fraction containing predominantly actin and tropomyosin; Lane 4, the fraction containing actin; Lane 5, the fraction containing crude tropomyosin; Lanes 6 and 7, contaminants removed from the tropomyosin preparation during purification procedure; Lane 8, purified mussel tropomyosin; Lane 9, purified rabbit tropomyosin. Natural mussel G-actin (GA_{mus}) was obtained by natural mussel F-actin (FA_{mus}) depolymerization. Pellets of FA_{mus} were rinsed with G-buffer (2 mM tris-HCL, 0.5 mM DTT, pH 7.5), homogenized in this solution with a Microman pipette and dialyzed for two days in ice water against 20 volumes of this solution with one change. The dialysate was clarified at 50,000 rpm (rotor 70.1, Beckman) for two hours and used for purification of actin by gel filtration and for testing the properties of GA_{mus}. GA_{mus} was polymerized by adding 0.11 volume of FA-buffer (500 mM KCl, 20 mM $MgCl_2$, and 10 mM ATP) and was used up to 7 days when stored in ice. For storage purpose, GA_{mus} can be freeze-dried without lost in function.

Isolation of mussel tropomyosin

The ultra-supernatant (Figure 1, lane 5) is a source of mussel tropomyosin (TM_{mus}). Solid ammonium sulfate was added to this ultra-supernatant up to 50% saturation at 0°C and pH 7.0. After staying for 30 minutes, the precipitate was removed by centrifugation, and the supernatant was brought to 70% saturation with solid ammonium sulfate. KCI was added to 50-70% precipitate up to 1M, pH was brought to 4.7, and the formed flakes of TM_{mus} were collected by centrifugation. TM_{mus} was dissolved in 10-15 mL of the standard solution and dialyzed against this solution. The dialyzed solution was clarified at 40,000 rpm for 30 minutes (Figure 1, lane 8). The Figure shows also rabbit TM, composed of two isoforms (Figure 1, lane 9). For storage purpose, both tropomyosins can be freeze-dried or frozen.

Viscosity measurements

Viscosity measurements were carried out at 22°C using a homemade falling-ball device, as described by Pollard and Cooper [15]. While measuring the viscosity of actin-tropomyosin complexes, we controlled the degree of saturation of actin filaments with tropomyosin. To achieve it, mixtures of actin and tropomyosin in a weight ratio of

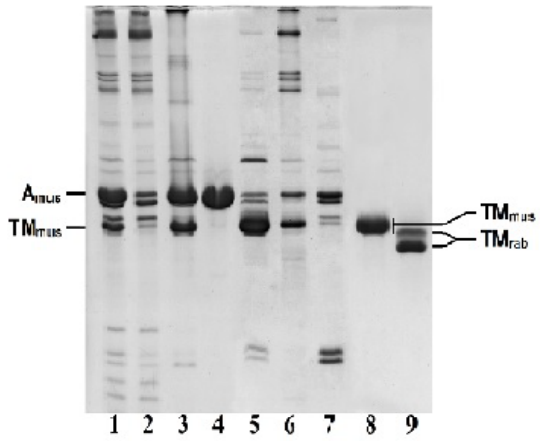

Figure 1: The stages of preparation of natural actin and tropomyosin from the catch muscle of the mussel *Crenomytilus grayanus*.

FA: TM=4.2: 1 were precipitated by ultracentrifugation for 90 min at 40,000 rpm (rotor 50.1, Beckman) in the medium of 75 mM KCl, 2 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM DTT, and 20 mM Tris-HCl, pH 7.0 at 22°C. The results were judged by the change of protein concentration in the test and the control tubes before and after centrifugation.

Light-scattering measurements

Light-scattering measurements were performed on a Spex Fluorat-02 "Luomex" spectrofluorometer. The intensity of light scattered at 90°C was measured at 400 nm.

Results

Figure 2 shows the dependence of the reduced viscosity (η_{red}) of various actin preparations on their concentration. Extrapolation of these dependencies to zero concentration allows us to determine the intrinsic viscosity ([η]) of natural mussel actin (FA_{mus}) and rabbit actin (FA_{rab}). It turned out than [η] values of rabbit actin were 8 times more than values of natural mussel actin, as can be seen in Figure 2a. Figure 2b shows the effect of rabbit tropomyosin (TM_{rab}) and mussel tropomyosin (TM_{mus}) on the reduced viscosity of mussel actin (FA_{mus}). Both tropomyosins greatly increased the slope of the relationships of reduced viscosity with concentration, without influence on the intrinsic viscosity. The maximum effect on the slope of the reduced viscosity relationship was observed in the non-hybrid FA_{rab}+TM_{rab} complex (Figure 2c).

- A, Comparison of viscosities of GFA_{rab} (polymerized globular rabbit actin from acetone powder) and nFA_{mus} ("natural" *in vivo* polymerized rabbit actin).
- B, Influence of TM_{rab} and TM_{mus} on FA_{mus} viscosity.
- C, Influence of TM_{rab} and TM_{mus} on FA_{rab} viscosity.
- Conditions: 50 mM KCl, 2 mM $MgCl_2$, 1.2 mM ATP, 0.2 mM $CaCl_2$, 1 mM DTT, 3 mM NaN_3, 2 mM Tris-HCl, pH 7.5 at 23°C.

Representative curves are given for three (in A), five (in B), and three (in C) experiments with different protein preparations. Each data point represents the mean of three experiments with the same proteins.

Interestingly, combination of TM_{mus} and FA_{rab} (hybrid complex)

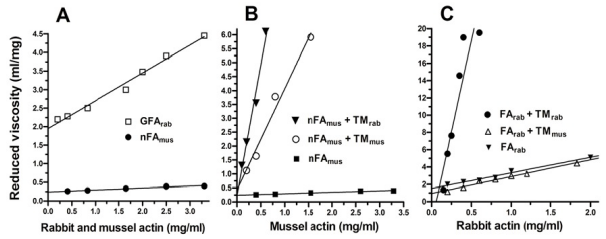

Figure 2: The reduced and intrinsic viscosities of mussel and rabbit actins in the presence and absence of mussel and rabbit tropomyosins.

does not increase the slope of the reduced viscosity relationship. It seems that the properties of the reconstructed complexes (FA+TM) depend on the both origin of proteins and their combination in complexes. The possible reason of dependence of actin-tropomyosin complexes viscosity from an origin of proteins, ways of their reconstruction and environmental conditions is dependence of these proteins interaction on the specified conditions. Such opportunity has been tested by ultracentrifugation of the Fibrillar Actin (FA) and tropomyosin mixed at equimolar ratio. Tropomyosin, unlike a fibrillar actin, does not precipitate at these conditions and, therefore, its presence in the pellet unambiguously confirms interaction of these proteins under specified conditions. As can be seen in Figure 3, ultracentrifugation (120 minutes at 50,000 rpm) of the mixtures of the mussel actin with either rabbit or mussel tropomyosin leads to almost completely sedimentation of actin-tropomyosin complexes.

- Lane 1, the mussel actin;
- Lane 2, the mixture of mussel actin with rabbit tropomyosin;
- Lane 3, the mixture of mussel actin with mussel tropomyosin.
- Lane 4, the supernatant of mussel actin;
- Lane 5, the supernatant of mixture of mussel actin with rabbit tropomyosin;
- Lane 6, the supernatant of mixture of mussel actin with mussel tropomyosin.
- Lane 7, the pellets of mussel actin;
- Lane 8, the pellets of mixture of mussel actin with rabbit tropomyosin;
- Lane 9, the pellets of mixture of mussel actin with mussel tropomyosin.

Conditions: mussel actin or rabbit actin, 1.5 mg/ml; concentration of tropomyosins was calculated from the weight ratio FA: TM=4.2:1; before centrifugation the mixtures were incubated at 22°C for 30 minutes; the composition of the solution was the same as in the legend to Figure 2.

Replacement of mussel actin with rabbit actin doesn't change the results of this experiment (not shown). Viscometric data for intrinsic and reduced viscosity of actin-tropomyosin complexes reconstructed variously are provided in Table 1. The maximum reduced viscosity observed for the rabbit actin-tropomyosin complexes, while the minimum reduced viscosity for the mussel ones. Purification of actin includes polymerization–depolymerization cycles and gel filtration chromatography [16]. The results of gel filtration of natural mussel actin and rabbit actin compared in Figure 4. The elution profiles for these proteins are fully symmetrical and coincide (Figure 4a). For receiving symmetrical and coincide elution curves during chromatography of rabbit actin we used the modified method of extraction of an actin from acetone powder [16,17]. Based on the data of SDS-electrophoresis, we found no differences between the chromatographic natural mussel actin and the chromatographic rabbit actin. Furthermore, according to SDS-electrophoresis, the chromatographic actin nearly did not differ from the pre-chromatographic actin, which was purified only by reprecipitations. However, the chromatographic fractions after actin polymerization showed significant differences in viscosity. The obtained fractions were divided into three parts (indicated in Figure 4a): the right edge of the peak (red), the middle part of the peak (blue), and the left edge of the peak (green). The relationships of reduced viscosity with concentration shown for the above fractions shown in Figures 4b and 4c (rabbit actin, mussel actin). In addition, these Figures show the viscosity of the original preparations. The viscosity of the actin preparations with various degrees of purification increased in the following sequence: the left edge of the peak (green), the original preparation (black), the middle part of the peak (blue), and the right edge of the peak (red). This sequence was similar for the mussel and the rabbit actin preparations; nevertheless, there was a significant difference in viscosity values between the corresponding preparations (Figures 4b and 4c).

- A, overlapping elution curves of the mussel and the rabbit actins. The peaks are divided to three parts: the right edge of the peak (red), the middle part of the peak (blue), and the left edge of the peak (green).
- B, Viscosity of preparations of rabbit actin from different parts of the chromatographic peak.
- C, Viscosity of preparations of the mussel actin from different parts of the chromatographic peak.
- Conditions the same as in Figure 2. Representative curves of two experiments are shown.

In all cases, the intrinsic viscosity of the rabbit actin preparations was 2-5 times higher than that of the mussel actin preparations. The low-viscosity and high-viscosity preparations of both rabbit actin and mussel actin differed in character of polymerization curves. Figure 5 showed the polymerization curves for three mussel actin preparations: with high, medium, and low viscosities. The curves are indicated above as the right edge of the peak-red, the middle part of the peak-blue, and the left edge of the peak-green. The high-viscosity preparation was polymerized slowly but achieved a high degree of polymerization. According to the thixotropy test [15] the solutions of polymeric actin obtained from chromatographic elution possess a clearly pronounced thixotropy. On the contrary, the low-viscosity preparation had the high rate of polymerization, low the polymerization degree, and thixotropy was almost lacking. The medium-viscosity preparations showed intermediate properties (Figure 5, middle part of the peak). Conditions the same as in the legend in Figure 2. Typical traces are shown (two independent experiments were performed).

Discussion

Similar effects were observed earlier in the case of hybrid and non-hybrid actomyosin models in the study of the effect of tropomyosin on the Mg-ATPase activity. It has been shown that replacement of rabbit tropomyosin with mussel tropomyosin leads to inhibition of Mg-ATPase activity instead of its activation [18]. It could be assumed that this inhibition occurs when mixing of actin and tropomyosin

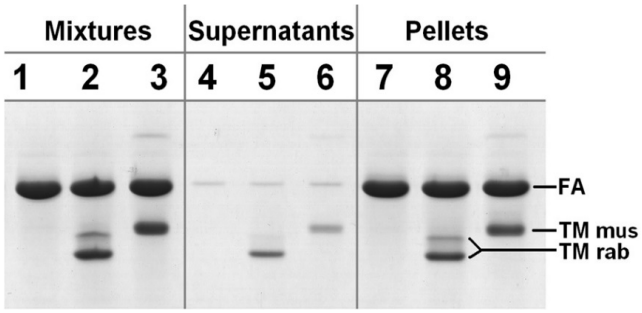

Figure 3: Co-precipitation of natural mussel actin with rabbit and mussel tropomyosins.

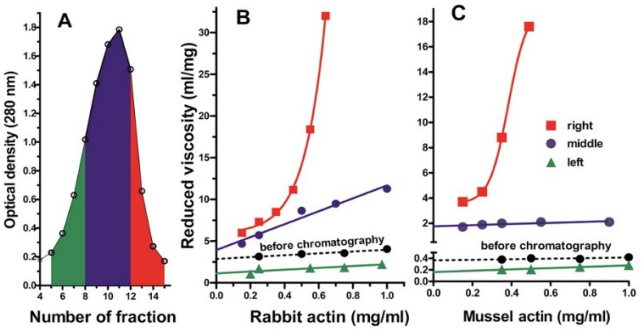

Figure 4: Purification of the mussel and the rabbit actin by gel-filtration chromatography.

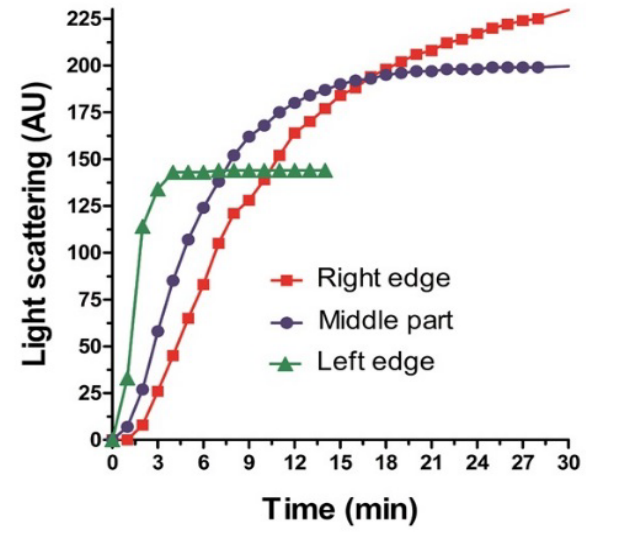

Figure 5: Kinetic polymerization of mussel actin from different chromatographic fractions: the right and the left edges and the middle part of the peak.

Components	Non-hybrid complex		Hybrid complex	
	$TM_{rab}+FA_{rab}$	$TM_{mus}+FA_{mus}$	$TM_{rab}+FA_{mus}$	$TM_{mus}+FA_{rab}$
η/c	37 ± 4.1	0.5 ± 0.4	4.8 ± 1.1	10.2 ± 1.7
[η]	1.5 ± 0.6	1.0 ± 0.5	0.25 ± 0.07	1.0 ± 0.1

Table 1: The dependence of viscosity of actin+tropomyosin complexes upon the conditions of their formation, Conditions the same as in Figure 2.

does not result in formation of a complex and, consequently, does not lead to a rise of viscosity. However, it is not so in the case. Analysis of the pellets after ultracentrifugation showed that both tropomyosin precipitate almost completely (i.e., they interact with actin) (Figure 3). The reason why complex of $FA_{rab}+TM_{rab}$ has a tens of times higher reduced viscosity than complex of $FA_{mus}+TM_{mus}$ is unclear. Most likely it is related to the isoforms of these proteins or to the minor impurities of actin-tropomyosin complexes.

According to the Staudinger equation, the intrinsic viscosity is in fact the reduced viscosity in very *diluted solutions*. Therefore, an increase in $(\eta_{red})/c$ with a simultaneous increase in the F-actin concentration should be interpreted as the result of intensified interaction between actin filaments. Indeed, in all cases, when the viscosity of our preparations is relatively low, this interaction increases linearly (Figure 2). However, as the degree of purification of actin preparations rises with an increase in viscosity and the appearance of the signs of thixotropy, the linear relationship is broken: the reduced viscosity increases faster than the concentration of actin does (Figure 4). This indicates the presence of additional factor, which intensifies the interaction of actin filaments. As mentioned above, the formation of the complex of F-actin with tropomyosin results in an abrupt increase in the reduced viscosity with a constant intrinsic viscosity, which is numerically equal to the segment on the axis $(\eta_{red})/c$, cut by the straight line of the reduced viscosity (Figure 2b). This, in turn, significantly affects the rheological properties of actin. For example, the stiffness of actin filaments with and without tropomyosin is 65.3 and 43.7 pN/nm, respectively [19].

At the first stage of isolation, the rabbit skeletal actin was purified by the reversible polymerization method: extraction of G-actin from acetone powder and its polymerization, F-actin pelleting, re-suspending and depolymerization, G-actin clearing, and polymerization. The intrinsic viscosity of this preparation was 6-8 times as high as that of the natural mussel actin. This ratio nearly did not change after the reversible polymerization without loss of proteins (Figure 2a). It means that the low viscosity of natural mussel actin, as compared to the rabbit actin, is not related to probable features of *in vivo* polymerization of the mussel actin. The subsequent purification of the natural mussel actin and the rabbit actin through gel filtration chromatography was accompanied by an increase in viscosity of the actin preparations and by reduction of the differences in viscosity between the actins (Figures 2 and 4). Nevertheless, the remarkable differences remained as previously: in any case, the highest possible viscosity of the mussel-actin preparation was two-times lower than that of the similar rabbit actin preparation. Also, the analysis of chromatographic fractions and polymerization kinetic indicated the presence of trace amounts of an active factor in "natural" mussel actin, whose molecular weight is somewhat higher than the actin weight. This factor most likely is similar to the capping protein (CP), which is a heterodimer with a molecular weight of about 60 kDa [20]. Earlier, the actin-capping activity was found in the residue of acetone powder, after extraction of actin [21]. The obtained data suggest that isolation of natural mussel actin is accompanied by co-extraction of CP in amounts, which can be larger than those obtained from acetone powder of rabbit skeletal muscles.

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