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Structure of membrane-bound human factor Va

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Abstract Coagulation factor Va is an essential cofactor which combines with the serine protease factor Xa on a phospholipid surface to form the prothrombinase complex. In the present study, the structure of factor Va interacting with lipid surfaces containing phosphatidylserine was studied by electron microscopy. Two-dimensional crystals of factor Va were obtained on planar lipid films under quasi-physiological conditions. The two-dimensional projected structure of factor Va was calculated at a resolution of 2 nm, revealing dimers of factor Va arranged on the surface lattice with the symmetry of the plane group p2. Average unit cell dimensions are a = 14.4 nm, b = 8.8 nm, γ = 107°. Each factor Va molecule presents two distinct domains of protein density consisting of one small domain, of 3 nm in diameter, connected to a larger domain of about 6 nm × 4.5 nm. The projected structure of factor Va covers an area equivalent to about fifty phospholipid molecules. In addition, edge-on views of factor Va molecules bound to liposomes reveal a globular structure connected through a thin stem to the liposome surface. A three-dimensional model of membrane-bound factor Va is proposed.

Key words: Factor Va; Two-dimensional crystal; Electron microscopy; Phosphatidylserine; Liposome

1. Introduction

Factor Va, an essential constituent of the prothrombinase complex, is derived by a proteolytic cleavage of factor V by thrombin [1,2]. The resulting factor Va product is a heterodimer composed of a heavy chain (M_r = 105,000) and a light chain (M_r = 74,000) derived from the NH_2-terminal and COOH-terminal ends of the factor V molecule, respectively. The two chains are non-covalently associated in the presence of divalent cations [2–5]. It is now well established that the interaction between factor Va and a lipid membrane requires the presence of negatively charged phospholipids [6,7] and is not dependent on the presence of exogenously added calcium ions [6]. A wealth of information concerning this interaction has been gathered by means of complementary biochemical and biophysical approaches (review in [8]). However, a detailed structure of membrane-bound factor Va has not been resolved.

The specific interaction between factor Va and negatively charged lipids renders this system suitable for study by the lipid-layer two-dimensional (2D) crystallization technique [9]. This technique has already proven to be very powerful for structural studies of proteins interacting with membranes, enabling the resolution of their structure in the membrane-bound state [10–13]. In addition, this approach has the potential for high-resolution structure determination by electron crystallography [14] provided that well-ordered 2D crystals are obtained [15].

Here, we report the 2D crystallization of factor Va on planar lipid films consisting of a mixture of dioleoylphosphatidylserine (PS) and dioleoylphosphatidylcholine (PC). The projected structure of factor Va was resolved at a resolution of 2 nm. In addition, binding of factor Va to liposomes provides complementary edge-on views. We propose a three-dimensional (3D) model of human factor Va bound to a lipid membrane.

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy; PC, dioleoylphosphatidylcholine; PS, dioleoylphosphatidylserine; UA, uranyl acetate.

2. Materials and methods

2.1. Chemicals

Phospholipids were purchased from Avanti-Polar Lipids (Alabaster, AL). *N*-octyl β-D-glucopyranoside was from Sigma (St. Louis, MO). Other chemicals were of the best available commercial grade.

2.2. Factor Va purification

Human factor Va was a gift from Haematologic Technologies, Inc (Essex Junction, VT, USA) and was prepared as previously described [16,17].

2.3. 2D crystallization

2D crystals of factor Va were prepared as described by Mosser et al. [10] in the case of annexin V. Crystallization experiments were carried out in teflon wells (4 mm in diameter, 1 mm deep) filled with 17 μl of a 100 μg/ml factor Va solution in either buffer A (20 mM Tris-HCl, 150 mM NaCl, 2 mM $CaCl_2$, 3 mM NaN_3, pH 7.3) or buffer B (20 mM Tris-HCl, 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM NaN_3, pH 7.3). A 0.6 μl drop of a 150 μM PS – 450 μM PC lipid solution in chloroform/hexane (1:1, v/v) was deposited on top of the liquid surface with a microsyringe. Incubation was allowed for 12 h at room temperature.

2.4. Liposome-bound factor Va

Large unilamellar phospholipid vesicles were prepared by detergent dialysis [18] as follows. 250 μl of a 40 mg/ml PC solution in chloroform were mixed with 200 μl of a 5 mg/ml PS solution in chloroform, together with 275 μl of chloroform-solubilized *N*-octyl β-D-glucopyranoside (20%, w/v). Chloroform was evaporated under reduced pressure. One ml of buffer made of 10 mM HEPES, 150 mM NaCl, 3 mM NaN_3, pH 7.3 (buffer C) was added and the mixture was allowed to stand at room temperature for 1 h. The solution was poured into Spectra/Por no.1 dialysis tubing (Medicell Int., London, UK) and dialyzed against buffer C at room temperature. Three bath changes were performed over two days. The liposomes were stored at 4°C until use. For binding experiments, 20 μl of factor Va at 360 μg/ml in buffer A were mixed with 4 μl of a liposome solution at 0.8 mg/ml total phospholipid content. This solution was incubated for 1 h at room temperature. In order to

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separate liposome-bound factor Va from unbound protein, liposome suspensions were passed through a CL-4B Sepharose gel filtration column (molecular weight cut-off: 40×10^6 Daltons; 3.5 cm height, 0.7 cm inner diameter) equilibrated and eluted with buffer A.

2.5. Electron microscopy

All electron microscopy (EM) observations were made by negative staining. In the case of factor Va 2D crystals, lipid films were picked up on carbon coated grids which had been deposited for 5 min on top of the incubating solution, carbon side facing the lipid film. The grids were washed with several drops of distilled water and negatively stained with 1% uranyl acetate (UA), pH 3.5, for 30 s. In the case of liposome-bound factor Va, a 5-μl drop of a liposome-factor Va solution was adsorbed onto carbon-coated grids rendered hydrophilic by glow discharge in air under reduced pressure [19] and stained with 2% sodium phosphotungstate, pH 7.2, for 2 min. EM was carried out with a Philips CM12 electron microscope operating at 100 kV. Low-electron dose irradiation was used for imaging 2D crystals, while no special care was made to decrease the electron irradiation when imaging liposomes. Electron micrographs were recorded at 45,000 × magnification on Kodak SO163 films and developed under standard conditions.

2.6. Image analysis

The best crystalline areas were selected by optical diffraction and digitized at the Sertit (Strasbourg, France) using a rotating drum microdensitometer Wirth Dios (Frankfurt am Main, Germany) with step and spot sizes of 25 μm. Image processing was performed by standard methods [20] using an Imagic software system [21] extended with programs of crystallographic analysis developed by V. Mallouh (L.G.M.E., Strasbourg).

3. Results

3.1. Factor Va 2D crystals

2D crystals of factor Va were grown on planar lipid films made of PS and PC (1/3 molar ratio) when Ca^{2+} ions were present in the incubation buffer (buffer A, see section 2) (Fig. 1a). Factor Va 2D crystals presented a wide range of size and morphology, as commonly observed with other soluble proteins crystallized on lipid layers [22]. The best crystalline areas presented diffraction peaks arranged on an oblique lattice and extending up to a resolution of 2 nm (Fig. 1b). The unit cell dimensions, defined by the geometry of the reciprocal lattice, are: $a = 14.4 \pm 0.7$ nm, $b = 8.8 \pm 0.4$ nm, $\gamma = 107 \pm 2°$. Further analysis of the Fourier transform showed that phases were close to 0° and 180°, indicating that the symmetry of the plane group is p2 [23].

When incubation was performed in the absence of Ca^{2+} (buffer B), 2D domains of protein–lipid complexes were observed which presented an overall aspect similar to those obtained in the presence of Ca^{2+} (data not shown). However, none of these domains was of crystalline nature, under our experimental conditions.

The 2D projected structure of factor Va was calculated at a resolution of 2 nm from the four best images (Fig. 2). Factor Va molecules are packed as dimers related by dyad axes normal to the crystal plane. Each factor Va molecule presents an asymmetrical distribution of protein density with overall dimensions 8.5 nm × 6 nm. Two well-separated protein domains are resolved within each factor Va molecule. One small domain (domain 1 in Fig. 2), of high protein density, presents a circular shape of about 3 nm in diameter. The other domain (domain 2) presents a uniform and lower density and has a rectangular shape of about 6 nm × 4.5 nm. It is noteworthy that for each processed image domain 1 consistently presented the highest density in the map, a feature which suggests the presence of an elongated structure viewed end-on. Domains 1 and 2 are connected through a short arm of low protein density, less than 1 nm long. The closest distances beween monomers within a dimer, and thus the most likely contact areas, relate domain 1 from one monomer to domain 2 from the other monomer. The surface covered by one factor Va molecule is about 34 nm^2. This surface is equivalent to about fifty lipid molecules, assuming an average lipid molecular surface of 0.7 nm^2.

3.2. Liposome-bound factor Va

We attempted to visualize factor Va molecules bound to

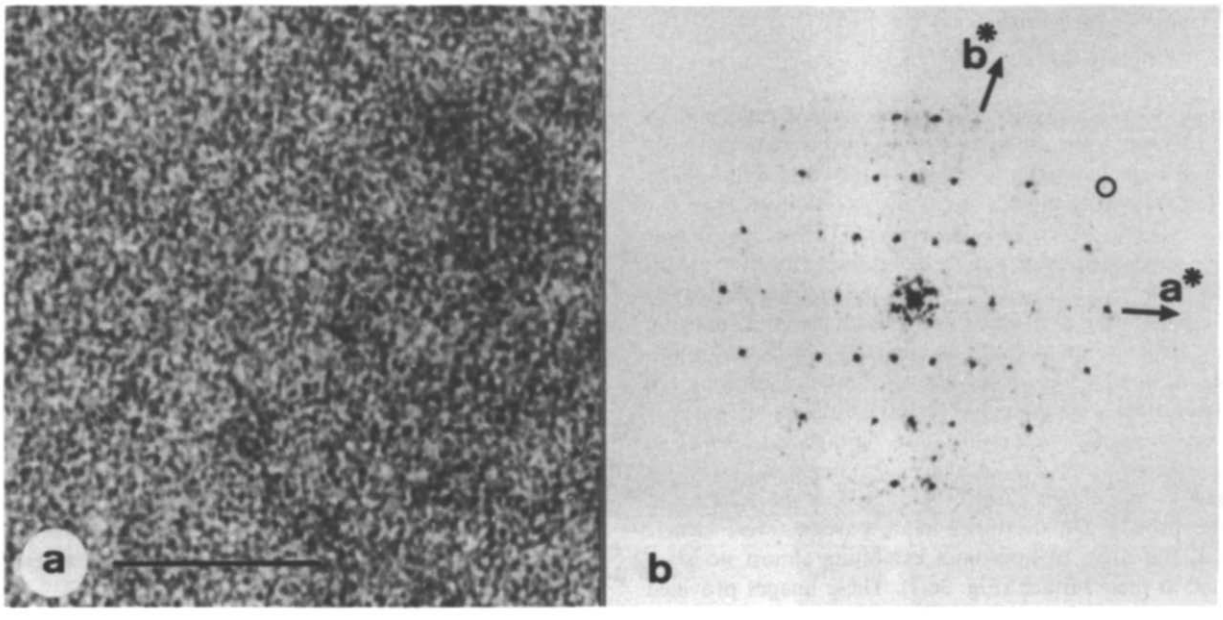

Fig. 1. (a) Electron image of an UA-negatively stained 2D crystal of factor Va. Faint stain striations extend over the entire area (this is better appreciated by viewing at glancing angle). Scale bar: 50 nm. (b) Distribution of Fourier transform amplitudes from a crystalline area (140×140 nm^2). Diffraction peaks are arranged onto an oblique lattice. The (4,2) reflection, at 1/2.3 nm^{-1}, has been encircled. Scale: 1 cm = 0.156 nm^{-1}.

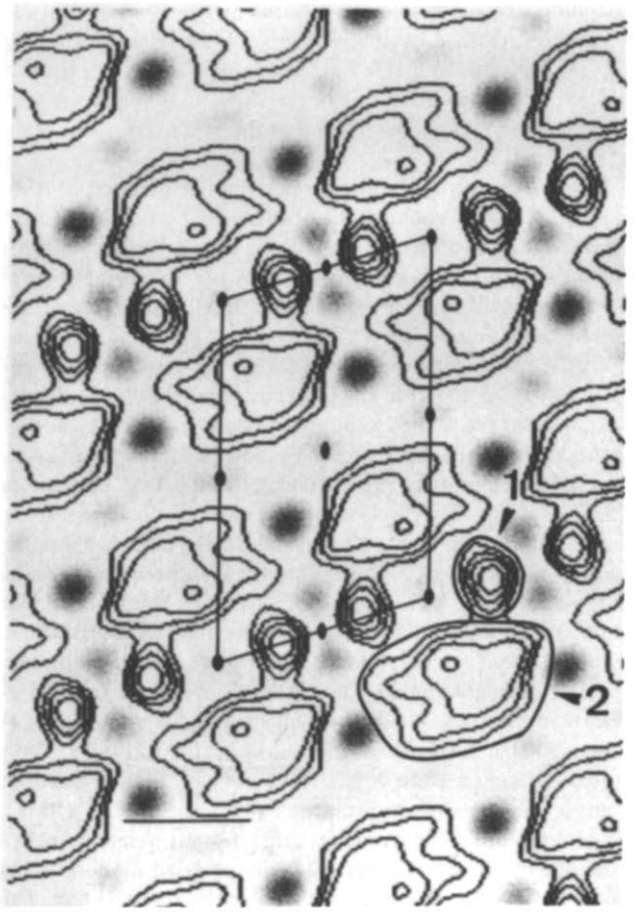

Fig. 2. 2D projection map of factor Va 2D crystals at 2 nm resolution. The map was calculated by Fourier synthesis after averaging the amplitudes from the four best images and imposing p2 symmetry to phases (mean phase residual: 19°). A unit cell has been outlined (a = 14.4 nm, b = 8.8 nm, γ = 107°) with p2 symmetry elements. Protein densities are represented with 4 contour levels (the increment between successive levels is equal to 1/4 th of the maximal value in the map). Stained areas are represented on a continuous grey scale. Domains 1 and 2 of one factor Va molecule are indicated. Scale bar: 5 nm.

liposomes, in order to verify whether the dimeric organization of factor Va was a genuine feature or resulted from a favorable packing arrangement within the 2D crystals. Liposomes made of PS and PC, with a PC/PS molar ratio of 10, were used for these experiments. Cryo-EM observations [19] indicated that these liposomes were mostly unilamellar (data not shown). Liposomes presenting a variety of staining patterns were observed (Fig. 3), illustrating a difficulty often encountered in imaging liposome structures by negative staining ([24]; M. Schmutz and A. Brisson, unpublished observations). Particles covering the liposome surface were mainly observed with liposome suspensions not submitted to gel filtration chromatography (Fig. 3a,b). However, detailed molecular images could not be distinguished, due to the high noise level induced by the presence of unbound protein. On the other hand, particles were clearly visible at the edges of liposomes exhibiting almost no stain deposited at their surface (Fig. 3c–f). These images provided views of membrane-bound factor Va molecules perpendicular to that of factor Va molecules in the 2D crystal. The main characteristic feature was the presence of protein densities aligned with a centre to centre distance of about 10 nm and separated from the liposome edge by a region filled with stain. The distance between the centre of mass of the protein density and the liposome edge was about 7 nm. Factor Va presented globular shapes with various aspects, due to the different rotational orientations of the molecules. The average size was 6 nm × 5 nm, the largest dimension being parallel to the liposome surface. A thin and elongated stem connecting the globular particle to the liposome surface was resolved in some images (arrows in Fig. 3f). These stems were most clearly resolved in liposome suspensions submitted to gel filtration chromatography, although this treatment resulted in a displacement of most of liposome-bound factor Va molecules.

3.3. *Model of membrane-bound factor Va*

The two orthogonal projection views reported here were combined to produce a 3D model of membrane-bound factor Va (Fig. 4). Each factor Va molecule consists of two domains. Domain 1 exhibits a cylindrical shape, 3 nm in diameter, which extends perpendicularly to the membrane plane over a distance of 10 nm. It is connected to a box-shaped domain 2 of dimensions 6 nm × 4.5 nm × 5 nm, positioned 4 nm from the membrane surface.

4. Discussion

The present study describes the structure of human factor Va interacting with lipid surfaces determined by electron microscopy. The 2D projected structure of factor Va observed along a direction normal to the membrane was determined by image analysis of 2D crystals. We found that factor Va molecules are arranged into dimers and present an asymmetrical distribution of protein density with two distinct domains (domains 1 and 2 in Fig. 2). In addition, complementary edge-on views of factor Va molecules bound to liposomes were obtained, allowing us to merge both projections into a 3D model.

Several EM studies have concordantly revealed a multi-domain structure for factor V [25–28], yet previous available structural data for factor Va were limited and the results were difficult to fit with a globular protein of M_r = 179,000. The main advantage of the present study is that 2D crystals were obtained, which enabled averaging over a large number of molecules observed along a single orientation.

We postulate that domains 1 and 2 correspond to the factor Va light chain and heavy chain, respectively, for the following reasons: (1) the molecular masses calculated from the volumes of domains 1 and 2 are 59,000 Da and 112,000 Da, respectively. These values are close to the known M_r of the subunits, 74,000 and 105,000, respectively; (2) the fact that domain 1 consistently appears as a very dense structure led us to assume that the stem connecting the globular core to the membrane was part of domain 1. This is in keeping with the fact that factor Va light chain is known to be responsible for the interaction with the lipid membrane [29–31]; (3) domains 1 and 2 are well separated (Fig. 2), a feature which is consistent with the fact that the non-covalent association between both chains is mediated by Ca^{2+} ions, and may not involve extensive contact areas as in classical oligomeric assemblies.

It could not be unambiguously concluded from the 2D crystal analysis alone whether the repeating dimeric motif corresponded to one or two factor Va molecules. However, the latter

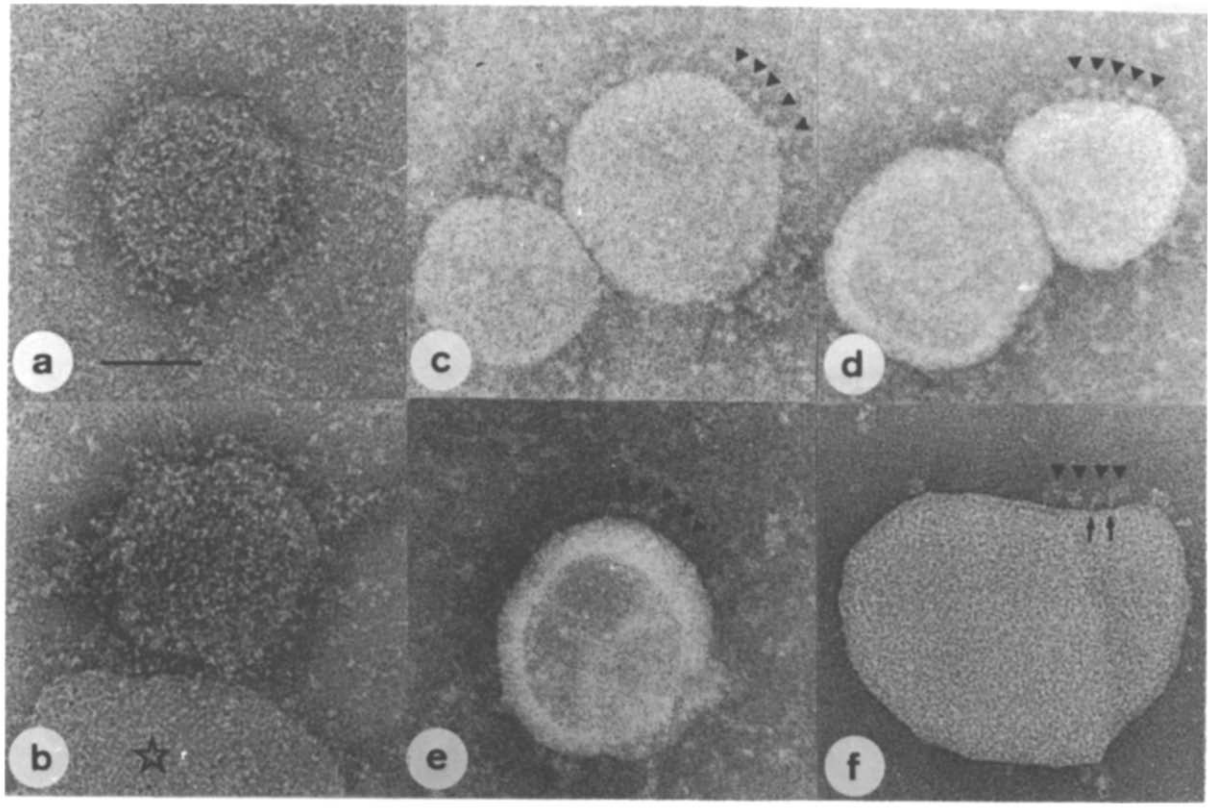

Fig. 3. Images of factor Va bound to (PS-PC) liposomes. (a,b) Liposomes densely covered with particles. No molecular details can be distinguished. In (b), a liposome devoid of particles presents a different staining aspect (☆). (c,d,e,f) Liposomes exhibiting characteristic edge-on views of factor Va molecules. Alignments of factor Va molecules are visible (triangles). In (f), arrowheads point to thin stems connecting the main core of factor Va particles to the liposome edge. The liposome in (f) is from a suspension purified by gel filtration chromatography, explaining the lower background level. Scale bar: 50 nm.

hypothesis is strongly supported by the morphological considerations developed above. In addition, the projected surface of a motif made of domains 1 and 2 covers an area equivalent to about 50 lipid molecules, which value is close to the value of 42 lipid molecules determined at saturation for factor Va by Krishnaswamy and Mann [32]. Furthermore, no internal two-fold symmetry in the amino-acid sequence of factor Va has ever been postulated. The dimeric organization of factor Va could, however, simply be a result of ordered packing of molecules on the lipid surface under saturating conditions. Although attempts to visualize dimers on liposomes were unsuccessful, binding experiments showing a positive cooperativity with a Hill coefficient of 2 support the concept of factor Va dimerization [33].

The absence of 2D crystals noticed in the presence of EDTA deserves some comments, as it is known that factor Va binds to PC/PS lipids independently of Ca^{2+}, and that factor Va light chain binds independently of heavy chain [6,7]. A simple interpretation of EM results is that 2D crystals are stabilized by heavy chain–heavy chain or heavy chain–light chain interactions which can only occur when the factor Va heterodimer is intact. It is important to note that 2D domains were observed in the presence of EDTA, which indicates that factor Va light chain binds to the lipid film and in addition that factor Va light chains self-organize within 2D domains. The formation of 2D crystals constitutes an all-or-none process and requires that subtile parameters are found which enable the establishment of favorable interactions. Several studies have shown that binding of factor Va light chain to membranes involves both hydrophobic and electrostatic interactions [34–36]. Two regions of the factor Va light chain, located in the A3 domain and in the C1–C2 domains respectively, have further been identified as responsible of these two types of interaction [31]. Although the resolution of our analysis thus far is too limited for identifying domains within the molecule, our results suggest that these two regions are at a close distance, as the area covered by factor Va light chain corresponds to only ten lipid molecules.

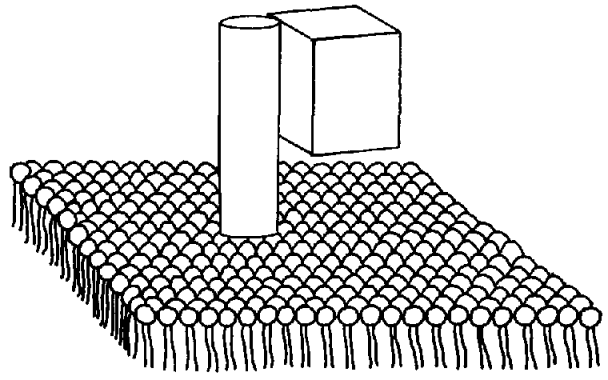

Fig. 4. Model of a molecule of factor Va bound to a PS-containing lipid monolayer.

A complete 3D description of factor Va is under progress, and the present approach will be extended to structural studies of complexes formed by factor Va and other protein members of the coagulation cascade.

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