Coagulation Factor V_a Binds to Human Umbilical Vein Endothelial Cells and Accelerates Protein C Activation

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Abstract. In vitro the rate of protein C activation by thrombin is significantly accelerated by two distinct cofactors (a) the endothelial cell surface protein, thrombomodulin, and (b) human coagulation Factor V_a. We have recently reported that the activity of Factor V_a is contained in the 78,000-D light chain. In this study we have investigated the effects of Factor V_a and its light chain on the activation of protein C in the presence of cultured endothelial cells. Thrombin-catalyzed protein C activation on human umbilical vein endothelial cells was enhanced by Factor V_a. The ability of Factor V_a to stimulate protein C activation on these cells was saturated at 50 nM Factor V_a and was observed at several protein C concentrations. Isolated Factor V_a light chain in concentrations up to 50 nM also accelerated protein C activation on endothelial cells, but higher concentrations inhibited the reaction. The effects of Factor V_a or its light chain on protein C activation were also shown on a mouse hemangioma cell line but not on human fibroblasts nor on a human amelanotic melanoma cell line. Protein C activation on endothelial cells was partially inhibited by a goat anti-thrombomodulin antibody and further addition of a polyclonal rabbit anti-Factor V(V_a) antibody resulted in additional inhibition. Endothelial cells grown in medium supplemented with human serum, devoid of Factor V coagulant activity, contained cell surface Factor V(V_a) (~15,000 molecules/cell) as measured by the binding of a monoclonal IgG directed against Factor V(V_a). These cells also bound an additional 6,000–10,000 molecules Factor V_a per cell as determined by direct binding studies using ¹²⁵I-Factor V_a. We suggest that thrombo-

modulin and Factor V_a act in concert to regulate protein C activation on the surface of endothelial cells.

Introduction

Protein C, a vitamin K-dependent protease, which circulates in plasma as a zymogen, is activated by thrombin (1, 2). Activated protein C exerts an anticoagulant effect by inactivating Factor V_a and VIII_a (3–5). Protein C plays an important role in the regulation of coagulation since patients congenitally deficient in protein C suffer thromboses (6, 7). The activation of protein C by thrombin, the only known physiologic activator, is relatively slow, so the physiological mechanism for the formation of activated protein C has been uncertain. Esmon and Owen (8) discovered that endothelial cells contain a cofactor for the activation of protein C by thrombin. This cofactor was subsequently purified from rabbit lungs and termed thrombomodulin (9). Recently, we reported that human Factor V_a can also accelerate the rate of protein C activation by thrombin in solution (10), and that this activity is contained in the light chain of Factor V_a (11).

In this study, we have examined the cofactor activity of Factor V_a and its light chain in the presence of human umbilical vein endothelial cells (HUVE)¹ and mouse hemangioma cells.

Methods

Materials. Except where indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Factor V (12), prothrombin (13), Factor X (13), protein C (4), and antithrombin III (14) were isolated from human plasma and activated as indicated. The light chain of Factor V_a was isolated as described (11). Factor V coagulant activity was measured in a one-stage assay by using purified coagulation factors (15). Thrombin was used to activate Factor V and ¹²⁵I-Factor V. Full (20- to ~50-fold) enhancement in Factor V coagulant activity was consistently obtained within 10 min at 37°C. Human anti-Factor V serum was from a patient who developed a monoclonal anti-Factor V antibody (16). The IgG fractions containing anti-Factor V activity and control IgG were purified using protein A Sepharose (17). Polyclonal anti-Factor V IgG was purified from immunized rabbit serum using protein A Se-

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1. Abbreviation used in this paper: HUVE, human umbilical vein endothelial.

pharose. This antibody was prepared using homogeneous human Factor V as antigen and was monospecific as determined by Western blots of plasma. Thrombomodulin from rabbit lung and anti-rabbit thrombomodulin goat IgG were from Drs. N. and C. T. Esmon. Factor V, monoclonal anti-Factor V IgG, and control IgG were radiolabeled using Na 125 I (Amersham Corp., Arlington Heights, IL) and IODO-GEN (Pierce Chemical Co., Rockford, IL). Radiolabeled proteins had the same biological activity as the starting materials and were labeled at 1,300–5,700 cpm/ng.

Unless otherwise stated the buffer used throughout this study was a 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl, 5 mM CaCl₂, 5.5 mM glucose, and 5 mg/ml bovine serum albumin.

Cell culture. Primary cultures of HUVE cells were prepared by the method of Jaffe et al. (18). The amelanotic melanoma cell line (CRL 1585) and human fibroblasts (IMR 90) were from Dr. N. L. Baenziger. Mouse hemangioma cells (19) were provided by Dr. J. C. Hoak. Cells were grown in plastic (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) 30–100 mm Petri dishes or 6–16 mm multi-well plates (Flow Laboratories, Inc., MacLean, VA) using medium 199 (KC Biological) containing 20% human serum (Flow Laboratories, Inc.), 2 mM glutamine (Gibco Laboratories, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco Laboratories) in a 5% CO₂ atmosphere.

To determine cell number, cells were harvested with a rubber policeman, lysed with cetrimide, and the nuclei were counted using a Coulter Counter model F (Coulter Electronics, Inc., Hialeah, FL) (20). In some experiments, confluent HUVE were grown for 1–24 h in medium containing 25 µg/ml sterile polyclonal anti-Factor V IgG or control IgG. Cell morphology was unchanged by the addition of immunoglobulins as determined by phase-contrast microscopy.

Binding studies. In initial studies we attempted to measure the binding of 125 I-Factor V_a and 125 I-anti-Factor V IgG to confluent monolayers of HUVE. Although binding could be detected by this method, nonspecific binding was >50% due to the low number of cells per volume of medium. To increase the cell concentration and thereby increase the number of specific binding sites, we carried out the binding studies by using suspensions of HUVE. Culture medium was aspirated from monolayers and the cells washed three times with the Tris-albumin buffer. The cells were harvested in the same buffer using a rubber policeman (No. 7835, Arthur H. Thomas Co., Philadelphia, PA), and the clumps dispersed by gently flushing the cells through a 19G needle. The cells were then centrifuged at 800 rpm and resuspended in polypropylene tubes (Eppendorf) at a concentration of $1-2 \times 10^7$ /ml in the same buffer. Binding was performed at 37°C by incubating the cells for 20 min with 125 I-Factor V_a, 125 I-anti-Factor V IgG, or 125 I-control IgG. Nonspecific binding was measured in the presence of a 100-fold excess unlabeled ligand. The cells were then collected by centrifugation in a microfuge, resuspended, and washed three times with ice-cold buffer, and finally the tips of the tubes containing the cells were cut off and counted in a Biogamma II scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Cells suspended in this way were 70–90% viable as examined by Trypan Blue exclusion. However, the binding observed in various experiments did not vary with cell viability as measured in this way.

Protein C activation. Medium was removed from confluent monolayers of cells and the cells were washed three times with buffer. Protein C activation was measured directly on cells in 6-mm culture wells or in polypropylene tubes (Eppendorf) containing cell suspensions prepared as described above. Reaction mixtures contained protein C, thrombin, Factor V_a and other additions as indicated, in a final volume of 40 µl of Tris-albumin buffer. Incubations were carried out for 30–60 min at

37°C after which reactions were terminated by addition of 5 µl of a mixture of hirudin (360 U/ml) and antithrombin III (3.15 mg/ml). The supernatant was collected and frozen for subsequent assay of activated protein C using benzoyl arginine ethyl ester (10). Control incubations minus protein C were carried out to correct for small amounts of benzoyl arginine ethyl esterase activity released from cells.

Results

Effect of calcium ions on Factor V_a-stimulated protein C activation in solution. We previously reported that human Factor V_a stimulated protein C activation by thrombin (1 nM) in solution with an apparent Michaelis constant for Factor V_a of 14 nM (10). Saturation of the activation rate occurred at 0.1 µM Factor V_a and activated protein C was formed at a rate of 50 pmol/ml per h. These experiments were carried out in the presence of EDTA. We have recently performed similar experiments in 2.5 mM CaCl₂ and found that much higher Factor V_a concentrations are required to stimulate protein C activation. There was no saturation of the rate of protein C activation using up to 1 µM Factor V_a. For example, using 1 µM protein C and 1 nM thrombin, activated protein C was formed at a rate of 55 pmol/ml per h at 1 µM Factor V_a, but only 6 pmol/ml per h were formed at 0.1 µM Factor V_a. The different rate of protein C activation in reaction mixtures containing calcium ions vs. those containing EDTA was not observed when isolated Factor V_a light chain was used in place of Factor V_a (11). In the experiments using cultured cells, all reactions were carried out in 2.5 mM CaCl₂ under conditions where protein C activation in the absence of cells is minimal.

Effect of Factor V_a on protein C activation by thrombin on cultured cells. Protein C activation by thrombin in the presence of HUVE (either monolayers or cells in suspension) was enhanced by Factor V_a. Fig. 1 shows the effect of additions of various concentrations of Factor V_a on protein C activation by thrombin in the presence of suspensions of HUVE. Using 0.5

Figure 1. Factor V_a-dependent protein C activation by thrombin on human umbilical vein endothelial cells. Reaction mixtures contained Factor V_a at the indicated concentrations, 0.5 µM protein C, 1 nM thrombin, and a suspension of 2×10^5 HUVE in the Tris-albumin buffer. The arrow indicates cofactor activity of HUVE treated with 2.5 mM EDTA for 3 min.

μM protein C, 1 nM thrombin, and 2.5 mM CaCl_2 , activated protein C was formed at a rate of 2 pmol/ml per h in the absence of cells and 40 pmol/ml per h in the presence of 2×10^5 of HUVE. The addition of Factor V_a further accelerated protein C activation to 125 pmol/ml per h at 100 nM Factor V_a . Suspended HUVE were treated with 2.5 mM EDTA for 3 min and washed once without EDTA in an attempt to remove endogenous Factor V_a that might contribute to the basal HUVE co-factor activity. In the presence of such EDTA-treated cells protein C was activated at 16 pmol/ml per h. Addition of 100 nM Factor V_a to these cells increased protein C_a formation to 88 pmol/ml per h. Both HUVE and mouse hemangioma cells accelerated protein C activation that was further enhanced by Factor V_a as shown in Fig. 2. No acceleration of protein C activation by thrombin was detected in the presence of human fibroblasts (IMR 90) or the amelanotic melanoma line (CRL 1585) as also shown in Fig. 2. Factor V_a did not accelerate protein C activation on these two cell lines that lack thrombomodulin activity.

Factor V_a stimulated protein C activation at all concentrations of added protein C, as shown in Fig. 3. Similar results were obtained with either HUVE or the mouse hemangioma cell line.

Isolated Factor V_a light chain also enhanced thrombin-catalyzed protein C activation in the presence of HUVE or mouse hemangioma cells, as shown in Fig. 4. Protein C activation was stimulated by low concentrations of light chain (<50 nM) but higher concentrations inhibited protein C activation. Factor V_a light chain in high concentrations also inhibits the activity of rabbit thrombomodulin in solution (22).

Binding studies. The presence of endogenous Factor $\text{V}(\text{V}_a)$ on HUVE was estimated by using an ^{125}I -monoclonal anti-Factor V IgG as shown in Fig. 5. The cells used in these experiments were grown for several days in human serum, which is devoid of Factor V activity. Approximately 15,000 molecules of IgG were bound per cell (mean of three experiments). Assuming that one antibody binds one Factor V_a molecule, this would yield 0.012 U of Factor $\text{V}_a/10^6$ cells (assuming that Factor V_a is 1.5 U/ μg protein [12]). This compares to 0.014 U of Factor $\text{V}_a/10^6$ cells, as detected by coagulation assay of frozen-thawed

Figure 2. Factor V_a -dependent protein C activation on cultured cells. Protein C activation using various cell types is shown by using 0.5 μM protein C, 1 nM thrombin, and a suspension of 2×10^5 cells. The effect of Factor V_a (50 nM) is indicated by the hatched bars.

Figure 3. Effect of protein C concentration on protein C activation on endothelial cells. The experiments were carried out by using a 2×10^5 of HUVE (A) or mouse hemangioma cells (B) in suspension, 1 nM thrombin, the indicated concentrations of protein C in the presence (●, ▲) or absence (○, △) of 50 nM Factor V_a .

HUVE. Similar results were obtained in three separate experiments. Specific binding of ^{125}I -control IgG to HUVE was not observed under the same conditions and unlabeled control IgG did not displace any ^{125}I -monoclonal anti-Factor V IgG, while unlabeled monoclonal antibody did displace the radioactivity (data not shown). Under similar conditions there was no specific binding of anti-Factor V IgG to either human fibroblasts or amelanotic melanoma cells. These results suggest that a small amount of Factor V_a is associated with HUVE. It is possible that the antibody detects inactive Factor V adsorbed from human serum, although the finding that extracts of HUVE contain a comparable amount of Factor V_a measured by coagulation assay

Figure 4. Effect of Factor V_a light chain on protein C activation on cultured endothelial cells. Monolayers of mouse hemangioma cells (3×10^4) or suspensions of 2×10^5 HUVE were incubated at 37°C with 1 nM thrombin, 0.5 μM protein C, and the indicated concentrations of Factor V_a light chain. There are two experiments using different light chain preparations and mouse hemangioma cells, ($\bullet$ and $\circ$). The third experiment was performed by using HUVE ($\square$). The "x" marks the rate of protein C activation using intact Factor V_a with mouse hemangioma cells in the same experiment as shown by $\bullet$.

is consistent with the hypothesis that the bound Factor V_a is active.

HUVE were also able to bind additional exogenous Factor V_a as shown in Fig. 6. We found 6,000–10,000 molecules of

Figure 5. Binding of anti-Factor V monoclonal IgG to HUVE. Increasing concentrations of ^{125}I -anti-Factor V (V_a) monoclonal IgG were incubated with 6×10^5 HUVE in 40 μl of Tris-albumin buffer as described in Methods. $\bullet$, total binding; $\circ$, specific binding; $\blacksquare$, nonspecific binding.

Figure 6. ^{125}I -Factor V_a binding to cultured human endothelial cells. Increasing concentrations of ^{125}I -Factor V_a were incubated with suspensions of 5×10^5 HUVE in 40 μl of Tris-albumin buffer as described in Methods. $\bullet$, total binding; $\circ$, specific binding; $\blacksquare$, nonspecific binding.

^{125}I -Factor V_a specifically bound per cell in several experiments. Binding was rapid and reached equilibrium within 5 min. Bound Factor V_a was rapidly displaced by excess unlabeled V_a to the level of nonspecific binding within 5 min. Addition of 2.5 mM EDTA resulted in displacement of $\sim 40\%$ of specifically bound Factor V_a in 5 min. The concentration dependence of binding of Factor V_a cannot be directly compared with the concentration of Factor V_a required to stimulate protein C concentration since the presence of protein C_a in the latter case leads to varying and decreasing concentrations of Factor V_a . Although we were unable to measure endogenous Factor V_a on the mouse hemangioma cells because of the lack of an appropriate anti-Factor V antibody, we did show binding of additional ^{125}I -Factor V_a to these cells similar to that found using HUVE. No specific binding of ^{125}I -Factor V_a was detected when human fibroblasts or amelanotic melanoma cells were used. Both the 110,000- M_r heavy chain of Factor V_a and the 78,000- M_r light chain bind to endothelial cells as shown in Fig. 7. The 150,000- M_r activation peptide of Factor V and intact Factor V itself did not bind. We cut out the heavy and light chain from the gels in lanes 2 and 4 to determine the relative proportion of light chain and heavy chain bound to endothelial cells. The ratio of light chain/heavy chain radioactivity in the supernatant ^{125}I -Factor V_a (lane 4) was 1.6. The ratio of radioactivity in the chains bound to endothelial cells (lane 2) was 3. These results suggest that the light chain is preferentially bound under the conditions used. Similar results were obtained in another experiment; the ratio of light chain to heavy chain radioactivity in the Factor V_a in the supernatant solution was 2, while that bound to the cells was 5.6.

It is not clear whether the excess light chain reflects a higher affinity of HUVE for free light chain or whether some heavy chain is lost during washing of the cells before electrophoresis. We previously found that platelets bind light chain and heavy chain in a ratio of 1/1 (23), and reconfirmed this result by using the same ^{125}I -Factor V_a used in Fig. 7.

Inhibition of protein C activation on human umbilical vein endothelial cells treated with anti-Factor V IgG and anti-rabbit thrombomodulin (TM) IgG. Protein C activation by thrombin on HUVE cells previously cultured for 24 h in medium containing a polyclonal rabbit anti-Factor V IgG was inhibited slightly, compared with cells treated with control rabbit IgG. This inhibition was seen consistently in several experiments. Incubation of HUVE with goat rabbit thrombomodulin IgG inhibited the protein C activation by 65% and the inhibition was increased to 88% when cells were cultured in the presence of both antibodies (Table I). Similar inhibition was noted when additional Factor V_a was included during the protein C activation reaction. It is interesting to note that when endothelial cells were preincubated with anti-Factor V antibody, the addition of Factor V_a to the protein C activation reaction did not completely reverse the inhibition due to the anti-Factor V antibody (compare Exp. 8 with Exp. 7 in Table I), implying that on these cells the binding sites for Factor V_a are not fully available.

Discussion

We previously reported that human Factor V_a and its isolated light chain can accelerate protein C activation by thrombin in

Figure 7. Autoradiography of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of HUVE incubated with Factors V and V_a . HUVE grown to confluence in 60-mm petri dishes (5×10^5) were incubated with $1 \mu\text{g}$ ^{125}I -Factor V or V_a (5,700 cpm/ng) in 1 ml of Tris-albumin buffer containing 0.1 mM dansyl arginine piperazine amide. After 20 min at room temperature the reaction mixtures were removed; the cells were washed three times with buffer, scraped from the dish with a rubber

policeman, and sedimented through a mixture of Apiezon oil and *n*-butyl phthalate (15) in Eppendorf tubes. The cell pellets were suspended in 0.1 ml 2% sodium dodecyl sulfate containing 5% 2-mercaptoethanol, boiled, and subjected to electrophoresis and autoradiography (23). Lane 1, endothelial cells incubated with ^{125}I -Factor V (50 μl of pellet); lane 2, endothelial cells incubated with ^{125}I -Factor V_a (50 μl of pellet); lane 3, supernatant of reaction mixture from lane 1 (2.7 μl); lane 4, supernatant of reaction mixture from lane 2 (2.7 μl).

Table I. Inhibition of Protein C Activation on Human Umbilical Cord Vein Endothelial Cells by Anti-Factor V IgG and Anti-Rabbit Thrombomodulin IgG

Exp.	Additions to cultured cells			Protein C _a formation	
	Rabbit IgG	Goat IgG	Factor V_a	pmol/ml/h	% Inhibition
1	Control	Control	—	49	—
2	Anti-Factor V	Control	—	41	16
3	Control	Anti-TM	—	17	66
4	Anti-Factor V	Anti-TM	—	6	88
5	Control	Control	+	71	—
6	Anti-Factor V	Control	+	65	8
7	Control	Anti-TM	+	42	30
8	Anti-Factor V	Anti-TM	+	25	65

Confluent monolayers of HUVE grown in 6-mm microwells were incubated 24 h in medium containing either 25 $\mu\text{g}/\text{ml}$ control rabbit IgG or rabbit anti-Factor V IgG. Medium was removed and cells were washed three times in buffer (20 mM Tris-HCl, pH 7.4, containing 5 mg bovine serum albumin/ml, 0.15 M NaCl, 5.5 mM glucose, and 2.5 mM CaCl_2). Then medium containing either control goat IgG or goat anti-rabbit thrombomodulin IgG (1.5 mg/ml) was added. After 1 h at 37°C the cells were washed three times using the same buffer and then protein C activation was measured using 0.5 μM protein C, 1 nM thrombin, 2.5 mM CaCl_2 , and 50 nM Factor V_a where indicated.

solution (10, 11). Factor V_a does stimulate protein C activation when blood clots *in vitro*, which implies that this reaction may occur under conditions that exist *in vivo* (10). The different rate of protein C activation by Factor V_a in reaction mixtures containing CaCl_2 vs. those containing EDTA was not observed when isolated light chain was used in place of Factor V_a . Thus, optimal protein C activation by Factor V_a light chain occurred in 2.5 mM CaCl_2 and the reaction was saturated at 50–100 nM Factor V_a light chain. Since the chains of Factor V_a (the heavy and light chain) are held together by calcium ions, it is possible that the observed effects of EDTA on the Factor V_a catalyzed activation of protein C are mediated by separating the chains and allowing the light chain to interact with protein C and thrombin. Even though Factor V_a in solution causes a 50-fold increase in the rate of protein C activation by thrombin it is unlikely that this reaction accounts for protein C activation *in vivo*. The reaction is relatively slow and requires high concentrations of Factor V_a . In another study we compared Factor V_a light chain with the other known cofactor for protein C activation, the endothelial cell surface protein thrombomodulin (22). In solution, solubilized rabbit lung thrombomodulin stimulated the rate of protein C activation about 1,000-fold which is 20-fold greater than the stimulation by Factor V_a light chain. Surprisingly, we found that Factor V_a light chain blocked the action of thrombomodulin in solution. The fact that inhibition was observed at nanomolar concentrations of both proteins suggested that they may function in concert to regulate protein C activation. The current work supports the concept that the physiologic role of Factor V_a in protein C activation occurs on endothelial cells. Thus, we find that physiologic concentrations of

Factor V_a stimulate protein C activation on HUVE in the presence of calcium ions. It appears that the stimulation depends on thrombomodulin since cells that have this activity (HUVE and mouse hemangioma cells) display Factor V_a -stimulated protein C activation while two other cell lines that lack thrombomodulin activity did not show that effect. Endothelial cells also have bound Factor V_a and additional Factor V_a binding sites. We were not able to precisely correlate Factor V_a binding activity with rates of protein C activation because as protein C is activated it destroys Factor V_a , thereby changing its concentration. That the Factor V_a reaction occurs on the cell surface is supported by the inhibition of protein C activation by antibodies directed against Factor V_a . Even when the antibodies were removed by washing and additional Factor V_a was added, inhibition of protein C activation remained.

The physiological significance of the finding that low concentrations of Factor V_a light chain stimulated protein C activation while inhibition was observed at high concentrations is not clear. On the basis of our findings we propose that Factor V_a may serve to regulate protein C activation on the endothelial surface by the following mechanism: when thrombin is formed in regions of vascular injury, some Factor V is converted to Factor V_a , which in turn binds to endothelial cells at or near sites containing thrombomodulin. The bound Factor V_a enhances the activity of thrombomodulin and increases its affinity for protein C, thereby accelerating the formation of activated protein C. Once formed activated protein C proteolyzes the 110,000- M_r heavy chain of Factor V_a (3, 4, 10) thereby destroying its coagulation activity and preventing further clot formation. In the course of this reaction the heavy chain of bound Factor V_a is cleaved, thereby potentially generating free light chain that remains bound to the cells. The light chain could then inhibit thrombomodulin, preventing further protein C activation. In this way Factor V_a could both limit coagulation by stimulating protein C activation and also prevent the excessive generation of activated protein C by blocking its further formation once the need to limit coagulation has ended. We plan further experiments to evaluate the validity of this hypothesis.

A number of uncertainties remain: (a) What is the source of the Factor V_a on endothelial cells? Is it synthesized by the cells or is it derived from plasma? Small amounts of Factor V_a may be synthesized by endothelial cells (24) although it appears that the protein is mainly synthesized by hepatocytes (25) and megakaryocytes (26). (b) Does endothelial cell Factor V_a participate in prothrombin activation? Recent work by Rogers and Shuman (27) indicates that prothrombin activation on bovine aortic endothelial cells is dependent on cell surface Factor V_a . (c) What is the intrinsic activity of thrombomodulin in the absence of Factor V_a ? While EDTA treatment of HUVE reduced thrombomodulin activity, substantial activity remained. We are not certain that the treatment removed all Factor V_a from the cells. In the direct binding studies only ~40% of bound ^{125}I -Factor V_a was removed by EDTA. The fact that thrombomodulin in solution does not require Factor V_a and the finding that antibodies to Factor V_a only partially inhibited protein C ac-

tivation on HUVE suggest that thrombomodulin is active without Factor V_a .

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