Rapid Communication

Activated Protein C Resistance: Molecular Mechanisms Based on Studies Using Purified Gln506-Factor V

By Mary J. Heeb, Yumi Kojima, Judith S. Greengard, and John H. Griffin

Gln506-factor V (FV) was purified from plasma of an individual homozygous for an Arg506Gln mutation in FV that is associated with activated protein C (APC) resistance. Purified Gln506-FV, as well as Gln506-FVAs generated by either thrombin or FXa, conveyed APC resistance to FV-deficient plasma in coagulation assays. Clotting assay studies also suggested that APC resistance does not involve any abnormality in FV-APC cofactor activity. In purified reaction mixtures, Gln506-FVAs in comparison to normal FVAs showed reduced susceptibility to APC, because it was inactivated ~10-fold slower than normal Arg506-FVAs. It was previously reported that inactivation of normal FVAs by APC involves an initial cleavage at Arg506 followed by phospholipid-dependent cleavage at Arg506. Immunoblot and amino acid sequence analyses showed that the 102-kD heavy chain of Glnm-FVa was cleaved at Arg506 during inactivation by APC in a phospholipid-dependent reaction. This reduced but measurable susceptibility of Gln506-FVAs to APC inactivation may help explain why APC resistance is a mild risk factor for thrombosis because APC can inactivate both normal FVAs and variant Gln506-FVAs. In summary, this study shows that purified Gln506-FV can account for APC resistance of plasma because Gln506-FVAs, whether generated by thrombin or FXa, is relatively resistant to APC.

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THE PROTEIN C anticoagulant pathway provides a major physiologic mechanism that regulates thrombosis, and defects in this pathway account for most currently known genetic risk factors for venous thrombosis. Hereditary heterozygous deficiency of protein C or of protein S is associated with increased risk for venous thrombosis in young adults, whereas severe deficiency (≤1%) of protein C or protein S is associated with purpura fulminans or massive venous thrombosis in the neonatal period. Another hereditary abnormality of the protein C pathway involves a laboratory finding of a poor anticoagulant response to activated protein C (APC), termed APC resistance. APC resistance is detected in 20% to 50% of venous thrombosis patients, depending on criteria for patient population selection. The genetic and molecular basis for APC resistance involves a mutation in the coagulation factor V (FV) gene at nucleotide 1691 that causes the mutation, Arg506Gln. Two reports of functional activity studies suggested that the variant Gln506-FVA is resistant to cleavage by APC. One report, using FV purified from two individuals heterozygous for the Arg506Gln-FV mutation, concluded that thrombin-activated Gln506-FV was APC resistant; however, another report, using unpurified adsorbed plasma from an individual homozygous for the Arg506Gln mutation, concluded that Gln506-FV was generated by Factor Xa (FXa) activation is APC resistant, whereas Gln506-FVAs generated by thrombin activation is not APC resistant. To date, no studies comparing purified Gln506-FV activated by FXa or by thrombin have been reported. To address this controversy and to assess the hypothesis that purified Gln506-FV indeed accounts for APC resistance in plasma, the present study based on purification of Gln506-FV from homozygous plasma was undertaken.

MATERIALS AND METHODS

Patient material. During testing of normal laboratory worker volunteers for APC resistance, a 32-year-old man was identified as homozygous for the DNA mutation predicted to give Gln506-FV using DNA analysis performed as previously described. Clotting assays of the proband’s plasma showed it to be markedly APC resistant with a ratio of (activated partial thromboplastin time [APTT] + APC)/(APTT) of 1.1 to 1.3 on different dates, compared with 2.6 for pooled normal plasma using APTT assays as previously described. The father (age 63 years), mother (age 63 years), and sister (age 26 years) of the individual were determined to have only normal APC resistance. The only notable thrombosis episode occurring before the age of 60 years in this family was a documented incident of thrombophlebitis and pulmonary embolism presented by the father of the probit at 49 years of age. The maternal grandparents and the maternal uncle of the probit was reported to have experienced deep vein thrombosis, stroke, and myocardial infarction after 60 years of age, respectively. Free protein S antigen (Asserachrom free protein S; Diagnostica Stago, Asnieres, France) and protein C amidolytic activity were within the normal ranges for the probit, his parents, and two siblings.

FV purification. Blood (450 mL) from the individual homozygous for the mutation Arg506Gln in FV was collected after informed consent into 50 mL of 0.11 mol/L trisodium citrate. Plasma was prepared by centrifugation at room temperature and made 50 μg/mL in soybean trypsin inhibitor, 10 mmol/L in benzamidine, and 100 U/mL in aprotinin and frozen at −70°C. A second unit (450 mL) of blood was similarly obtained 8 weeks later, and the plasma was treated with inhibitors and combined with thawed plasma from

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the first collection. Disopropylfluorophosphate and phenylmethylsulfonyl fluoride were each added to a concentration of 1 mM/L and the plasma was barium adsorbed as described. The supernatant solution was sequentially treated with 7% and then 13% polyethylene glycol-6000 (PEG); the precipitate from 13% PEG containing FV was further purified as described. FV was similarly prepared from 500 mL of plasma from an individual determined not to be APC resistant by APTT and from 1.5 L of pooled plasma from 6 individuals of undetermined APC resistance status. In experiments described below, normal FV from the single donor was used unless otherwise stated.

**FV characterization and activation.** FV was characterized by absorbance at 280 nm using A280 = 8.9, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), by enzyme-linked immunosorbent assay (ELISA) for FV antigen, and by functional assay. The sandwich ELISA was performed as described for protein S antigen using immobilized rabbit anti-FV IgG (Dako, Carpinteria, CA) at 7.5 μg/mL as the capturing antibody and monoclonal anti-FVa light chain antibody at 5 μg/mL (Hematologic Technologies, Essex Jct, VT) as the detecting antibody. Dilutions of normal plasma were simultaneously assayed and the data were used to construct a standard curve, assuming a plasma concentration of 30 nmol/L FV antigen. FV activity was determined in a FXa-1-stage assay in which 60 μL of FV-deficient plasma (George King Biomedical, Overland Park, KS) was mixed with 20 μL of HEPES-buffered saline, pH 7.4 (HBS), and 50 μL of cephalin (prepared as recommended by Sigma, St Louis, MO). After 2 minutes of preincubation of this reaction mixture at 37°C, 10 μL containing 20 mM/L FXa (Enzyme Research Laboratories, South Bend, IN) and 10 μL of a dilution of normal plasma or of a test sample as a source of FV or FVa were added. Finally, 50 μL of 33 mM/L CaCl2 in HBS containing 1% bovine serum albumin (BSA) was added and clot time was measured at 37°C using an ST4 coagulometer (American Bioproducts, Parsippany, NJ). FV activity in the test samples was calculated by reference to a standard curve constructed with dilutions of normal plasma. Activity was expressed in units, in which 1 U equals the FV coagulant activity in 1 mL of normal plasma. FV (100 mM/L) was activated at room temperature in HBS containing 0.5% BSA and 5 mM/L CaCl2 using either 1 mM/L thrombin (Enzyme Research Laboratories) or 10 mM/L F Xa and 25 µM/L phospholipid vesicles (20% phosphatidylserine and 80% phosphatidylycholine). Maximal activation time for FV was about 1 hour in each case, after which thrombin was neutralized and the sample was diluted in HBS-0.5% BSA for immediate further use.

**APC resistance assays.** APTT assays were performed as follows. FV-deficient plasma (45 μL) was mixed with 5 μL of test sample (consisting of the manufacturer, 1 mM/L final FVa, or 22 μM/L final FVa) and 50 μL of Platelin LS (Organon Teknika, Durham, NC). After 200 seconds of preincubation at 37°C, the mixture was re-calculated with 30 mM/L CaCl2 in HBS, 0.5% BSA at 37°C in the presence or absence of 16 mM/L APC in this solution. The ratio of (APTT + APC)/APTT was calculated. An FXa-1-stage assay was performed as indicated above, except that CaCl2 was added in the presence or absence of 48 mM/L final concentration of APC. The ratio of (clotting time + APC)/clotting time was calculated.

**APC inactivation of FVa.** FVa or Gln506-FVa was reacted with APC at various concentrations at room temperature in the presence of 25 μM/L phospholipid vesicles, HBS, 0.5% BSA, and 5 mM/L CaCl2. Aliquots were removed at suitable times and immediately assayed for residual FVa activity in prothrombinase assays or boiled for 1 minute in SDS-PAGE sample buffer for immunoblotting analysis.

### RESULTS

Volatile Gln506-FV was purified from plasma obtained from an APC-resistant subject who was homozygous for the DNA mutation of G to A at nucleotide 1691 that predicts the mutation Arg506Gln. Normal FV and Gln506-FV were ≥50% pure as judged by final antigen/protein ratio and ≥70% pure as judged by SDS-PAGE (data not shown). A purification of greater than 2,880-fold was achieved based on initial versus final antigedprotein ratio, with a recovery of 10% antigen.

To define the APC resistance of the variant Gln506-FV in comparison to normal FV, APTT assays were performed using FV-deficient plasma reconstituted with samples containing normal or variant forms of FV or FVa. Test samples including normal plasma, homozygous Gln506-FV plasma, purified normal FV or FVa, or purified Gln506-FV or Gln506-FVa were analyzed (Table 1). The APTT values for each test sample were determined in duplicate in the presence and in the absence of APC. The ratio of APTT in the presence of APC to the APTT value in the absence of APC was calculated in each case and the mean value for this APC resistance ratio is given in Table 1. When purified normal FV was used to reconstitute FV-deficient plasma, the APC resistance ratio was 3.1, similar to that seen for normal plasma, whereas the APC resistance ratio for the addition of

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Normal Arg506-FV</th>
<th>Variant Gln506-FV</th>
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<tbody>
<tr>
<td>Plasma</td>
<td>2.7</td>
<td>1.8</td>
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<tr>
<td>Purified FV</td>
<td>3.1</td>
<td>1.9</td>
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<td>Thrombin-activated FVa</td>
<td>4.6</td>
<td>3.0</td>
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<tr>
<td>FXa-activated FVas</td>
<td>3.2</td>
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purified Gln^506^-FV was 1.2, similar to that for whole plasma from the same subject homozygous for the Arg^506^-Gln mutation. This finding shows that purified Gln^506^-FV indeed conveys APC resistance to plasma, as predicted. Subsequently, experiments were performed to determine whether normal and variant FVAs generated by either thrombin or FXa activation conveyed APC resistance in APTT assays. FV-deficient plasma was reconstituted with the various FVAs species, as described under experimental procedures, and the APC resistance ratios were determined (Table 1). Normal FVAs, whether generated by thrombin or by FXa activation, was quite susceptible to APC as indicated by APC resistance ratios of 4.6 and 3.2, respectively (Table 1). The data in Table 1 indicate that the variant Gln^506^-FVAs, whether activated by thrombin or by FXa, was relatively resistant to APC, giving ratios of 1.6 and 1.5, respectively. These results indicate that, in comparison to normal FVAs, the purified variant Gln^506^-FVAs is relatively resistant to APC independent of whether it is activated by thrombin or FXa.

If the variant Gln^506^-FVAs were susceptible, ie, completely resistant, to APC, one would predict that the APC resistance ratio should be 1.00. However, values for APC resistance ratios of Gln^506^-FVAs in APTT assays were 1.05 to 1.6 (Table 1 and data not shown). Ratios greater than 1.0 could conceivably reflect inactivation of FVIIIa by APC during coagulation as monitored in APTT assays. To exclude any contribution of inactivation of FVIIIa by APC, FXa-1-stage assays were also used for APC resistance tests because these are independent of FVIIIa. The APC resistance ratios from the FXa-1-stage assays were also greater than 1.0, ie, 1.2 to 1.4 for Gln^506^-FV and Gln^506^-FV (Table 1), suggesting that Gln^506^-FV and/or Gln^506^-FVAs are not completely susceptible to APC.

Interestingly, in the FXa-1-stage assays, the APC resistance ratio of normal FV was 1.9, whereas for normal FVAs activated by FXa or thrombin the ratios were 2.8 and 3.0, respectively (Table 1). Similarly, when the APC resistance ratios were determined using APTT assays, the ratios for normal and variant FVAs were greater than those for normal or variant FV (Table 1).

Studies were made to define the influence on APC resistance ratios of the concentration of purified normal and variant FV that was added to FV-deficient plasma. When [FV] was varied from 0.9 to 4.5 mmol/L, the APC resistance ratios in FXa-1-stage assays for either normal FV or Gln^506^-FV were essentially constant (1.90 ± 0.05 or 1.15 ± 0.05, respectively). Similarly, the APC resistance ratios in APTT assays were constant, ie, independent of [FV] between 0.9 and 4.5 mmol/L (3.35 ± 0.35 or 1.03 ± 0.05; data not shown).

In the absence of APC, the FXa-1-stage clotting times for the mixture of FV-deficient plasma with normal FV varied from 55 to 35 seconds, and the clotting time for the FV-deficient plasma containing Gln^506^-FV varied from 51 to 34 seconds depending on the [FV] (0.9 to 4.5 mmol/L). The preparations of normal and variant FV used for these studies were mostly single-chain FV. Thus, the APC resistance ratio in FXa-1-stage and in APTT assays was independent of FV final concentration between 0.9 and 4.5 mmol/L, equivalent to an initial plasma FV level of 9% to 45%.

Studies were performed to compare the sensitivity of purified Gln^506^-FVAs with that of normal FVAs to inactivation by APC and to determine if these sensitivities were markedly different for thrombin-activated versus FXa-activated FVAs species. For these experiments, normal and variant purified FVAs were separately activated either by FXa or by thrombin. The time courses for maximal activation were similar in each case (data not shown). Each of the various species of FVAs was mixed with varying amounts of APC (0 to 1.33 nmol/L). Inactivation of normal and Gln^506^-FVAs that occurred during 3 minutes of incubation was quantitated by measuring the residual FV activity using a purified prothrombinase assay (Fig 1). Under the conditions used, thrombin-activated normal FVAs was inactivated by APC with the same dose response as FXa-activated normal FVAs (Fig 1, lower curves).

In comparison to normal Arg^506^-FVAs, both thrombin-activated and FXa-activated Gln^506^-FVAs were relatively resistant to APC, with the apparent sensitivity to APC of the variant FVAs species approximately 10-fold less than the normal FVAs species (Fig 1). Moreover, Gln^506^-FVAs activated by thrombin was indistinguishable from Gln^506^-FVAs activated by FXas in its relative resistance to APC. To compare further the relative sensitivities of each FVAs to APC, the time course of APC inactivation was examined at two doses of APC (Fig 2). At 100 pmol/L APC, 20% to 25% inactivation of Gln^506^-FVAs was observed within 30 minutes of incubation, whereas a similar extent of inactivation of normal FVAs was observed within approximately 2 to 3 minutes (Fig 2, left panel). The time for 50% inactivation of normal FVAs by 100 pmol/L APC was 4 to 7 minutes, whereas for 50% inactivation of Gln^506^-FVAs it was 8 minutes for 800 pmol/L APC (Fig 2, right panel). These comparisons suggest that, under the conditions of these experiments, Gln^506^-FVAs is approximately 8- to 10-fold less susceptible than normal FVAs to APC inactivation.

At 800 pmol/L APC, 80% inactivation of Gln^506^-FVAs was observed within 30 minutes, with a similar extent of inactivation of normal FVAs within about 5 minutes (Fig 2, right panel). These results were independent of whether the Gln^506^-FVAs or normal FVAs had been generated by thrombin or by FXa. In other experiments with longer times and higher APC concentrations, greater than 90% inactivation of Gln^506^-FVAs was observed.

To compare the proteolytic fragments of normal and variant FVAs produced by APC during inactivation, cleavage of the heavy chains of Gln^506^-FVAs and normal FVAs was monitored by immunoblotting using a polyclonal antibody against the human FVAs heavy chain (Fig 3). The first heavy chain fragments observed during incubation of normal FVAs with APC were of apparent molecular mass 76 and 30 kD (Fig 3 and data not shown), suggesting initial cleavage at Arg^506^- as reported by Kalafatis et al. Subsequent appearance of a fragment of approximately 44 kD, the size of a polypeptide containing residues 1-306, was consistent with a secondary cleavage at Arg^506^- in normal FVAs, in agreement with previous work. Generation of the fragment containing residues 1-306 would also be associated with release of a 30-kD fragment corresponding to residues 307-506, and indeed a
30-kD doublet was observed on this and other blots (data not shown). Because the 30-kD band did not increase in intensity after 3 minutes, it is possible that the original 30-kD fragment probably corresponding to residues 507-709 was further cleaved by APC at residue 679, as reported. However, no fragments smaller than 30 kD were observed with the particular anti-heavy chain antibodies used in this study.

In contrast to the results for normal FVa, the fragments that appeared upon incubation of Gln508-FVa with APC were at approximately 44 and 58 kD, consistent with a single cleavage at Arg679 (Fig 3). To identify the site of phospholipid-dependent cleavage of Gln508-FVa by APC, the heavy chain fragments of 44 kD and 58 kD were sequenced. The 44-kD fragment had the expected NH2-terminal sequence of residues 1-9 of FV, Lys-Gln-Leu-Thr-Gln-Phe-Tyr-Val-Ala. The 58-kD fragment had the sequence X-Leu-Lys-Lys-Ile-Thr-Arg-, corresponding to the expected sequence of residues 307-313, Asn-Leu-Lys-Lys-Ile-Thr-Arg-. Because cleavage at Arg679 of normal FVa is known to be phospholipid dependent, Gln508-FVa was treated with 700 pmol/L APC in the absence of phospholipid for 90 minutes. No inactivation (=10%) was observed and no heavy chain fragmentation was observed on immunoblots. In contrast, in the absence of phospholipids, ~25% inactivation of normal FVa was observed in 3 minutes, with no further loss of activity observed in 90 minutes (data not shown). These observations are consistent with inactivation of Gln508-FVa via phospholipid-dependent cleavage at Arg679.

**DISCUSSION**

APC resistance has been associated with a DNA mutation causing replacement of Arg679 by Gln in FV, and this mutation was shown to cosegregate with risk of thrombosis in familial APC resistance. To show that this DNA defect is indeed the direct biochemical cause of APC resistance, we purified FV from plasma of a subject homozygous for Gln508-FV. Coagulation studies (Table I) show that purified Gln508-FV imparts to plasma resistance to APC.

We previously reported experiments suggesting that thrombin-activated Gln508-FVa is relatively resistant to APC; in contrast, others suggested that FXa-activated Gln508-FVa, but not thrombin-activated Gln508-FVa, is resistant to APC. Studies described here show that Gln508-FVa conveys APC resistance to plasma and that Gln508-FVa is relatively resistant to APC independent of whether the Gln508-FVa is generated by thrombin or by FXa, thus clarifying this important conceptual conflict.

The molecular defect responsible for APC resistance was initially hypothesized to involve a new APC cofactor deficiency; subsequently, it was suggested to involve defective APC cofactor activity of FV in APC-resistant patients. The influence of the concentration of normal or variant FVa added to FV-deficient plasma on the response to APC was determined using APTT and FXa-1-stage assays, and the results showed that the APC resistance ratios are insensitive to FVa concentration. Because increasing the amounts of FVa does not improve the APC resistance ratio, ie, apparently does not enhance APC activity, these findings do not support the hypothesis that single-chain normal FV or Gln508-FV can act as a significant cofactor for APC anticoagulant activity in plasma under typical conditions of the clotting assays used.

Studies in which purified Gln508-FVa was added to FV-deficient plasma consistently gave APC resistance ratios greater than 1.00, suggesting that Gln508-FVa was not susceptible to APC.
response studies and time course studies at different concentrations of APC (Figs 1 and 2) confirmed that purified Gln$^{506}$-FVa is not completely resistant to APC and suggested that the variant Gln$^{506}$-FVa is approximately 10-fold less sensitive to APC than is normal FVa. These findings imply that the inactivation of Gln$^{506}$-FVa by APC can occur in vivo, albeit at a reduced rate compared with normal FVa. This implication may help explain why APC resistance appears to be a rather mild risk factor for venous thrombosis and why a combination of genetic risk factors for venous thrombosis is found in a significant fraction of symptomatic patients.

The APC-dependent cleavage patterns for the FVa heavy chains of normal FVa and Gln$^{506}$-FVa were compared using immunoblotting. The observed time-dependent cleavage patterns for normal FVa were consistent with a report showing that inactivation of normal FVa involves an initial cleavage at Arg$^{509}$ followed by a phospholipid-dependent cleavage at

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**Fig 2.** Time course of inactivation of thrombin-activated and FXa-activated normal FVa and Gln$^{506}$-FVa. Normal or variant FVa (200 pmol/L) was incubated with APC and phospholipids. At various times as indicated, aliquots were removed and assayed for residual FVa prothrombinase activity as described in Materials and Methods. For the left panel, APC concentration was 100 pmol/L. For the right panel, APC concentration was 800 pmol/L. (○) Thrombin-activated normal FVa, (▲) FXa-activated normal FVa, (●) thrombin-activated Gln$^{506}$-FVa, (●) FXa-activated Gln$^{506}$-FVa.

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**Fig 3.** Immunoblot of FVa and Gln$^{506}$-FVa heavy chains after APC inactivation. Normal FV or Gln$^{506}$-FV were each activated with thrombin and then incubated at 5 nmol/L with APC (350 pmol/L) and phospholipids/Ca$^{2+}$. At the times indicated, aliquots containing 60 ng of FVa were removed and subjected to boiling, SDS-PAGE, and immunoblotting for FVa heavy chain epitopes as indicated in Materials and Methods. FVa activity assays performed in parallel indicated that Gln$^{506}$-FVa was greater than 80% inactivated at 120 minutes and that normal FVa was greater than 90% inactivated at 60 minutes.
Arg506. In contrast to the pattern for normal FVa, inactivation of Gln506-FVa by APC was associated with appearance of polypeptide fragments of approximately 44 and 58 kD (Fig 3) because of cleavage at Arg506, as confirmed by NH2-terminal amino acid sequencing. These results show that cleavage by APC at Arg506 in FVa is sufficient to inactivate FVa, but that this cleavage is inefficient, approximately 10 times slower, unless prior cleavage at Arg506 occurs.

Baboon studies suggest that FVa rather than FV is the physiologic substrate for APC. APC at antithrombotic levels of 6 and 30 nmol/L circulating in vivo for up to 1 hour in baboons did not significantly decrease FV or FVIII levels even though the APTT was very markedly prolonged, eg, 36 to 169 seconds at 30 nmol/L APC. In contrast, when thrombin was infused at 2 U/kg/min into baboons for 1 hour, levels of FV and FVIII decreased significantly (40% to 60%). Infused thrombin activates FV and FVIII to FVa and FVIIIa, increases the level of circulating APC to ~7 nmol/L, and prolongs the APTT (eg, 32 to 158 seconds at 2 U thrombin/kg/h). Compared with normal FVa, Gln506-FVa has a relatively reduced rate of inactivation by APC in the presence of phospholipids. The fact that Arg506Gln mutation performed by Xiao Xu was cleaved at Arg306, and Arg506Gln mutation is clearly associated with increased risk of thrombosis12,24 and that cleavage of Gln506-FV by APC is similar to that of normal FV35 (Heeb, Kojima, and Griffin, unpublished data) further supports the concept that FVa rather than FV is the physiologic target of APC.

After this report was submitted, Kalafatis et al25 reported biochemical studies that are related to this study and that complement the coagulation experiments presented here. Using purified component prothrombinase assays with the fluorescent thrombin inhibitor DAPA, it was shown32 that purified thrombin-activated Gln506-FVa was inactivated by APC by cleavage at Arg506 at a rate slower than that of normal FVa that was cleaved at Arg506, Arg507, and Arg506.

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Activated protein C resistance: molecular mechanisms based on studies using purified Gln506-factor V

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