Identification of Human Plasma Kallikrein Gene Polymorphisms and Evaluation of Their Role in End-Stage Renal Disease

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Abstract—Kallikreins are serine proteases that release kinins from kininogens. Kinins, via their effects on cardiovascular and renal function, may be involved in the pathogenesis of hypertension and renal failure. Two groups of kallikreins exist, glandular or tissue kallikrein and plasma kallikrein. In this study, we examined the human plasma kallikrein gene KLK3 to determine whether it contributed to end-stage renal disease (ESRD) susceptibility. We identified two novel polymorphic sequences closely linked to the KLK3 gene, designated KLK3b and KLK3c (heterozygosities: 0.64 to 0.68 and 0.48 to 0.52, respectively). We mapped the KLK3 gene and the marker KLK3c to the long arm of human chromosome 4 between F11 and D4S426 using a radiation hybrid panel. The study population consisted of 142 sibling pairs concordant for ESRD from 121 African American families. The 142 sibling pairs were stratified into 78 pairs with hypertension- and chronic glomerulonephritis–associated ESRD and 64 with non–insulin-dependent diabetes mellitus–associated ESRD. Linkage analyses, using SIBPAL of SAGE, and exclusion analysis, using MAPMAKERS/SIBS, were performed. Linkage analysis of affected sibling pairs did not reveal any evidence of linkage of KLK3 to ESRD in all 142 sib-pairs or in the two stratified subsets. Exclusion analysis indicated that the KLK3 gene could be excluded from contributing to ESRD at a relative risk of 3 when the maximum log of the odds score of −2 was used as the criterion for exclusion. However, an association analysis using the relative predispositional effect technique showed that alleles 7 and 9 of KLK3b were consistently associated with ESRD. Alleles 7 and 9 were present in 11.2% and 10.8% of the 113 unrelated ESRD probands and in 6.6% and 6.6% of the 204 race-matched control subjects without renal disease (allele $P=.0041$ and .0016, respectively). Alleles 7 and 9 were also present in 13% and 10.4% of the proband's first siblings (allele $P=.00014$ and .0087, respectively). The association of KLK3b alleles with ESRD raises the possibility that polymorphisms in KLK3 may play a role in ESRD susceptibility. The lack of linkage might reflect our relatively small family set. **(*Hypertension*. 1998;31:906-911.)**

Key Words: kallikrein ■ renal disease ■ African Americans ■ genetics ■ diabetes mellitus

Kallikreins are serine proteases involved in the posttranslational processing of polypeptides. By generating hormone peptides, kallikreins are important regulators in processes such as organ perfusion, systemic blood pressure, sodium and water homeostasis, and inflammation.[1–3] There are two groups of kallikreins: glandular or tissue kallikrein and plasma kallikrein. Plasma kallikrein differs from tissue kallikrein in that it is synthesized in the liver as plasma prekallikrein, secreted into the blood, and converted into plasma kallikrein.[2] In addition, the substrates for these two enzymes are slightly different.[4] Plasma and tissue kallikreins are encoded by different genes. In humans, tissue kallikrein is encoded by one member of the kallikrein-like gene cluster (KLK1) located on chromosome 19q13.3-13.4[5,6] and the plasma kallikrein gene (KLK3) has been localized to chromosome 4q34-35.[1,7]

Kallikreins release kinins and other vasoactive peptides that contribute to regulation of blood pressure and renal perfusion.[1,8,9] The plasma and tissue kallikrein genes can be considered candidate genes for essential hypertension and chronic renal failure. Previous reports reveal a strong negative correlation between the level of kallikrein expression and blood pressure in hypertensive rats.[10–12] Urinary kallikrein excretion demonstrates familial clustering in hypertensive families, with two dominant genotypes.[13] However, no confirmed association exists between blood pressure and kallikrein gene polymorphisms.[9,14,15] We have previously conducted a genetic linkage analysis between the glandular kallikrein gene (KLK1) and ESRD in African Americans.[16] No evidence for linkage was detected.[16] In this report, we examine the plasma kallikrein gene (KLK3) in a similar study population.

Methods

Development and Mapping of KLK3 Markers

A pair of primers, Pr4-2153/2154 of SHGC4-1080, were synthesized for the KLK3 gene based on sequence information in the Genomic

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Selected Abbreviations and Acronyms

ESRD = end-stage renal disease
IBD = identity by descent
KLK3 = plasma kallikrein gene
LOD score = log of the odds score
PCR = polymerase chain reaction
STS = sequence tagged site
YAC = yeast artificial chromosome

Data Base (gdbwww.gdb.org). SHGC4-1080 from genomic DNA amplified a sequence segment (position 2045 to 2259) near the 3′ end of the cDNA insert of Lambda PK129 coding for human plasma prekallikrein.[2] These primers were used to screen the CEPH (Centre d'Etude du Polymorphism Humain) human YAC DNA pools to identify a YAC clone that contained the KLK3 gene. Both the YAC DNA pools and the identified YAC clone, yh620E7, were purchased from Research Genetics (Huntsville, Ala). Subcloning of YAC yh620E7 was carried out using the Lambda ZAPII/*Eco*RI/CIAP cloning kit (Stratagene). Filters containing plasmid subclones of the YAC were hybridized with radiolabeled oligonucleotide composed of the dinucleotide repeat (CA)n. The (CA)n hybridizing human clones were identified, shotgun-cloned, and sequenced. Sequencing was carried out using an ABI 377 automated sequencer. Primers were designed based on this sequence information and used to amplify the (CA)n regions and as markers for KLK3.

We performed somatic hybrid mapping and radiation hybrid mapping using the NIGMS human/rodent somatic hybrid mapping panel #1 (Coriell Institute for Medical Research) and the Stanford G3 radiation hybrid panel version 1 (Research Genetics) to confirm that the developed markers were in the chromosomal region of KLK3.

Study Population and Genotyping

KLK3 polymorphisms were evaluated in 142 sibling pairs concordant for ESRD from 121 African American families. The clinical features in these patients have been described previously.[16,17] Of the 142 affected sib-pairs, 64 were concordant for non–insulin-dependent diabetes mellitus–associated ESRD and 78 were concordant for nondiabetic causes of ESRD (primarily hypertension- or chronic glomerulonephritis–associated ESRD). Nonrenal disease control subjects included 88 healthy African Americans employed at the North Carolina Baptist Hospital (NCBH, Winston-Salem, NC) and 116 healthy African Americans from Minnesota (DNA samples from the Minnesota control subjects kindly provided by Dr Richard Anderson, Department of Comparative Medicine, Wake Forest University Baptist Medical Center).

We developed two polymorphic KLK3 markers, KLK3b and KLK3c, from YAC yh620E7. Genotyping of these two markers in ESRD patients was carried out using PCR. The method of PCR used was similar to that of Yu et al[16] and Freedman et al.[17] Thermocycling was carried out for 30 cycles of denaturing at 94°C, annealing at 54°C for KLK3b and at 55°C for KLK3c, and extension at 72°C. Each step was maintained for 1 minute. There were 5 minutes of denaturing at the beginning of the cycles and 5 minutes of extension at the end. Forward primer for KLK3b and reverse primer for KLK3c were end-labeled with [^{32}P]dATP before PCR amplification.

The method of PCR used for the NIGMS and G43 mapping panels was essentially the same as for the ESRD patient DNA, with the exception that radioactivity was not involved and the PCR products were separated on 2% agarose gels (1:1 between regular and low melting agarose) instead of sequencing gels. The gels were photographed after staining with ethidium bromide. In the visual scoring of the gels, bands with reduced intensity compared with that of the human control clone (clone RM in the G3 panel) were considered ambiguous. Others were scored as either present or absent.

Data Analysis

The chromosomal location was determined using radiation hybrid mapping. Genotype data from KLK3 primers were amplified on a panel of radiation hybrid cell lines (G3 from the Stanford Human Genome Center). Linkage analysis was carried out with the Mapping Program RHMAP[18,19] to locate KLK3 within the existing framework of markers on chromosome 4q.

Two separate linkage analyses were performed on the KLK3 genotype data obtained from the ESRD population. Initially, we performed a sib-pair analysis, which is based on the mean proportion of marker alleles shared IBD in the affected sib-pairs. At a marker locus, an average IBD of 0.5 is expected for pairs of full siblings. Significant deviation from 0.5 at a marker locus is considered as evidence that the marker is linked to the disease that the affected sib-pairs share. The affected sib-pair analysis was carried out using the computer program SIBPAL of SAGE.[20]

The second linkage analysis was an exclusion analysis using the computer program MAPMAKER/SIBS.[21] The exclusion analysis takes relative risk into consideration. The relative risk is the genetic risk to the sibling of a patient compared with the risk in the general population. At a certain relative risk, a gene can be excluded from contributing to a disease when the maximum LOD score is at a selected value or less. This method is most useful when the relative risk of a disease is known. Even when the relative risk is unknown, this analysis is informative in terms of the certainty of concluding that a gene does not contribute to a disease.

The exclusion analysis is a multipoint analysis, which enabled us to fully use the multipoint linkage information provided by the separate KLK3b and KLK3c polymorphisms. Using the pulse field gel electrophoresis of the YAC DNA, we determined that the KLK3 containing YAC yh620E7 is 340 kb (kilobase) long. The distance encompassing KLK3, KLK3b, and KLK3c is therefore $<$0.34 Mb (megabase). One Mb is equal to approximately 1 cM (centimorgan). The data were analyzed in two ways: the two markers were set at 0 cM, ie, next to each other, and the two were set at the maximum distance, ie, 0.34 cM. We used the maximum LOD score of -2 as the exclusion criterion. In both the affected sib-pair and the exclusion analyses, we used allele frequencies calculated from sampling a single affected sibling (the proband from each family). This ensured the use of the most conservative approach in calculating the allele frequencies.

Because association analysis may be a more sensitive method for detecting genetic contribution to disease susceptibility,[22] the KLK3 markers were also evaluated for allelic association with ESRD. We carried out this analysis by assessing the relative predispositional effect of alleles[23] using the computer program GAS (A. Young, Oxford University, England, 1995). This technique calculates the χ^2 and P values for the frequency change of a specific allele and for the overall allele distribution. Those alleles with significantly higher frequencies in a disease population for both individual allele and overall allele distribution can be considered to have predispositional effect on and to be associated with the disease. The race-matched control subjects were 204 healthy African Americans from NCBH and Minnesota. The two control groups were combined because there was no significant difference in allele frequencies for KLK3b or KLK3c. We divided the ESRD-affected siblings into two groups, the proband group and the sibling group. The sibling group included the first siblings of the probands. Other siblings were not used in the association analysis. Thus, both the proband and the sibling groups are composed of unrelated individuals.

Results

Development and Mapping of KLK3 Markers

We identified the YAC clone yh620E7 as a candidate to contain the KLK3 gene, through PCR amplification of pools of YAC DNA clones using SHGC4-1080 primers (Table 1). Sequencing the SHGC4-1080 PCR product amplified from this YAC confirmed that it contained KLK3; the sequence of this PCR product was identical to that of the KLK3 gene.[2] In subcloning from this YAC, two separate potentially polymorphic (CA)n-positive subclones were identified and se-

TABLE 1. Primers Used in Study

Marker	Primer	Sequence	Product Length, bp
SHGC4-1080	Pr4-2153	TGAGTTCAAGTCAAATTCTGAGCC	
	Pr4-2154	CCATTTTATTTCACAACACACAGTC	214
KLK3a	Forward	GTCTTGGAGTGAGCGTGACC	
	Reverse	AAATAGTCTACCCACCCCCC	537
KLK3b	Forward	GTCTTGGAGTGAGCGTGAC	
	Reverse	ACACAGAGATACATGAAACACG	209
KLK3c	Forward	TGAAAGCTACACAGGCAAC	
	Reverse	AGCCATGGAACACAGATG	170

quenced. Based on the sequence information, two sets of primers, KLK3b and KLK3c, were designed to amplify the two (CA)n repeat sequences. Their product lengths from yh620E7 were 209 and 170 bp, respectively (Table 1). Genotyping of the ESRD patient population and the control subjects revealed that both markers were polymorphic.

There were no significant differences in the allele frequencies for KLK3b or KLK3c between the two groups of African American control subjects from NCBH and Minnesota. Thus, the NCBH controls and the Minnesota controls were combined. Heterozygosity in these 204 control subjects was 0.64 for KLK3b and 0.48 for KLK3c (Table 2). The heterozygosity calculated from the ESRD probands was higher: 0.68 for KLK3b and 0.52 for KLK3c.

TABLE 2. Allele Frequencies for Markers KLK3b and KLK3c

Marker and Allele	Length, bp	ESRD Probands	Controls*
KLK3b			
1	219	0.004	0.012
2	217	0.013	0.022
3	215	0.000	0.002
4	213	0.009	0.015
5	211	0.280	0.260
6	209	0.466	0.532
7	207	0.112	0.066
8	205	0.004	0.025
9	203	0.108	0.066
10	201	0.004	0.000
Individuals, n		116	204
Heterozygosity		0.68	0.64
KLK3c			
1	172	0.099	0.105
2	170	0.254	0.211
3	168	0.638	0.682
4	160	0.009	0.000
5	164	0.000	0.002
Individuals, n		116	204
Heterozygosity		0.52	0.48

*Healthy African Americans from both North Carolina Baptist Hospital (n=88) and Minnesota (n=116).

TABLE 3. Multipoint Radiation Hybrid Map of Region Containing KLK3 in 1000:1 Bin 191 on Chromosome 4

	Distance to Next Marker	
Marker Locus	cR_{8000}*	kb†
D4S3275	7.5	225
D4S546	7.5	225
D4S475	22.8	684
F11	22.6	678
KLK3c	6.6	198
KLK3 (SHGC4-1080)	68.4	2052
D4S426 (or D4S321)	27.1	813
D4S862	. . .	. . .

*Based on the maximum likelihood method of multipoint analysis in RHMAXLIK (branch and bound ordering, equal retention model).

†Estimates based on 30Kb/cR (Reference 24).

To exclude the possibility that yh620E7 was chimeric and consequently the polymorphisms in KLK3b and KLK3c might not be from the same chromosomal locus as the KLK3 gene, we performed somatic cell hybrid mapping to make sure that KLK3b and KLK3c were on chromosome 4, where they are expected to be located. KLK3b was mapped using another marker, KLK3a (amplifying the same but longer sequence as KLK3b) (Table 1). The pattern of presence and absence of the PCR products for KLK3a and KLK3c exactly matched that of chromosome 4 in the 18 somatic cell hybrid lines of the NIGMS somatic mapping panel. Thus, KLK3b and KLK3c were from the same chromosome as the KLK3 gene.

The specific locations of KLK3b, KLK3c, and the original KLK3 marker SHGC4-1080 on chromosome 4 were further determined by radiation hybrid mapping. Due to the presence of a comigrating hamster-specific PCR product, KLK3b (and KLK3a) could not be genotyped for the G3 panel. Other results, however, indicated that SHGC4-1080 was linked to D4S475 (LOD score, 3.99) and KLK3c to D4S321 (LOD score, 3.87). Both D4S475 and D4S321 were in 1000:1 bin 191 of chromosome 4 of the G3 map (version 1; Stanford Human Genome Center). There were 23 markers in this bin. Two-point linkage analysis of these markers with SHGC4-1080 and KLK3c delineated 14 markers tentatively linked with both SHGC4-1080 and KLK3c. The two-point distance between SHGC4-1080 and KLK3c was 6 cR (centiray) (LOD score, 10.47). Based on an estimate of 1 cR=30 kb,[24] KLK3c was at the same chromosomal locus as KLK3.

Multipoint analysis of SHGC4-1080, KLK3c, and these 14 markers was carried out. Several orders, all with a minimum number of breaks (n=37), were identified. We excluded 7 markers between and including D4S1554 and D4S171, which had unique placements in these orders. This reduced the number of markers for further analysis to 7. For these 7 markers, SHGC4-1080, and KLK3c, we carried out the maximum likelihood analysis using the orders identified in the minimum breaks analysis as candidate locus orders to determine one best order and marker distance. The derived best order is shown in Table 3. In this map, both markers were

TABLE 4. Affected Sib-Pair Analysis Using SIBPAL of SAGE

	All Families			Diabetic Families			Nondiabetic Families		
Marker	n	IBD	*P*	n	IBD	*P*	n	IBD	*P*
KLK3b	141	0.464	1.00	63	0.488	1.00	78	0.446	1.00
KLK3c	141	0.507	.35	64	0.532	.11	77	0.487	1.00

n indicates number of sib-pairs; IBD, estimated mean proportion of alleles shared identical by descent.

closer to F11 than to any other markers. The distance of KLK3c to F11 was 22.6 cR or ≈678 kb. F11 encodes factor XI in blood coagulation, where the plasma prekallikrein gene is also involved.[2] On the other side of KLK3 was D4S426, with a distance of 68.4 cR or 2052 kb to SHGC4-1080. D4S321 was at the same position as D4S426 in the G3 radiation map.

Linkage Analysis

Results of linkage analysis using SIBPAL are shown in Table 4. In the total population of ESRD sib-pairs, KLK3b and KLK3c did not show any evidence of linkage to ESRD. The estimated mean proportion of marker alleles shared IBD was 0.464 ($P=1.00$) for KLK3b and 0.507 ($P=.35$) for KLK3c. This was also the case when the sib-pairs were stratified into diabetic and nondiabetic subsets. In these subgroups, IBD varied from 0.446 to 0.532, with the associated *P* values ranging from 1.00 to 0.11 (Table 4).

Results of the exclusion analysis using MAPMAKER/SIBS are shown in Table 5. We could exclude KLK3b/c (when the two markers were set at 0 cM apart) from contributing a relative risk of 1.5 to ESRD in the total population. The maximum LOD score at a relative risk of 1.5 was −4.68, lower than the −2 exclusion criterion. In the diabetic subset of families, 3 was the highest relative risk of having the maximum LOD score lower than −2 (actual maximum LOD score, −3.51). Thus, KLK3b/c could be excluded at a relative risk of 3 in the diabetic sib-pairs. Similarly, KLK3b/c could be excluded at a relative risk of 1.5 in the nondiabetic sib-pairs (actual maximum LOD score, −2.47). When KLK3b and KLK3c were set at 0.34 cM apart, similar results were obtained.

Allele Association Analysis

In the allele association analysis using the GAS program, we found that alleles 7 and 9 of KLK3b had a significant predispositional effect on ESRD (Table 6). These alleles were more frequent in the patient population and were positively associated with ESRD. Alleles 7 and 9 were each present in 6.6% of the 204 control subjects (Table 2), whereas they accounted for 11.2% and 10.8% (Table 2) of the ESRD probands (allele $P=.0041$ and .0016, respectively) (Table 6), and 13% and 10.4% of the first siblings of the ESRD probands (allele $P=.00014$ and .0087, respectively) (Table 6). When the ESRD proband and sibling groups were stratified into diabetic and nondiabetic subgroups, neither allele was significant in the diabetic subgroups (Table 6). In the nondiabetic subgroups, allele 9 had a significant effect in the ESRD probands (allele $P=.0032$), whereas allele 7 had a significant effect in the first siblings (allele $P=.00066$).

For KLK3c, allele 4 consistently showed a significant positive effect on ESRD (data not shown). However, the frequency of this allele was too low to make a meaningful conclusion.

Discussion

We have developed two novel markers, KLK3b and KLK3c, for the plasma kallikrein gene (KLK3). In this study, we used these markers in the linkage analysis of KLK3 with ESRD in African Americans. The role of KLK1 in hypertension has been evaluated extensively. The two plasma kallikrein genetic markers developed in this study may be useful in the genetic analysis of essential hypertension, cardiovascular disease, and the Fletcher trait (prekallikrein deficiency).[4]

KLK3b has a heterozygosity of 0.64 to 0.68, and KLK3c has a heterozygosity of 0.48 to 0.52. If these two markers are used together in a multipoint linkage analysis, their informativeness can be increased. For example, in single-point analysis using GENEHUNTER,[25] the information content was 0.65 and 0.60 for KLK3b and KLK3c, respectively.

TABLE 5. Exclusion Analysis of KLK3 Linkage to ESRD Using MAPMAKER/SIBS

		Maximum LOD Score at Lambda					
Markers*	Type of Family	1.2	1.5	2	3	5	10
KLK3b/c	All families	−1.99	−4.68	−8.32	−13.45	−19.68	−27.61
	Diabetic	−0.23	−0.81	−1.83	−3.51	−5.77	−8.90
	Nondiabetic	−1.04	−2.47	−4.43	−7.23	−10.72	−15.28
KLK3b	All families	−1.30	−3.23	−6.00	−10.11	−15.29	−22.08
	Diabetic	−0.20	−0.74	−1.69	−3.27	−5.39	−8.28
	Nondiabetic	−0.73	−1.81	−3.36	−5.70	−8.69	−12.68
KLK3c	All families	−1.32	−3.27	−6.08	−10.24	−15.52	−22.43
	Diabetic	−0.21	−0.76	−1.74	−3.34	−5.49	−8.36
	Nondiabetic	−0.72	−1.80	−3.35	−5.69	−8.71	−12.75

*The distance between KLK3b and KLK3c was set at 0 (KLK3b/c) and 0.34 cM (KLK3b and KLK3c) in analyses.

TABLE 6. Analysis of Allele Association of Marker KLK3b to ESRD Using Relative Predispositional Effect Technique in GAS Program

Sibling Group	Family	Individuals, n	Allele	Allele *P*	Total *P*
Proband	All	113	7	.00411	.00053
			9	.00160	.00523
	Nondiabetic	69	9	.00322	.00419
First sibling	All	115	7	.00014	3.58×10^{-5}
			9	.00867	.00439
	Nondiabetic	74	7	.000663	.00405

However, their information content was increased to over 0.72 when they were combined (data not shown).

We mapped the KLK3 gene onto the Stanford G3 radiation map using the STS marker SHGC4-1080, derived directly from the KLK3 cDNA sequence. In addition, we also mapped one of the newly developed markers, KLK3c. The proximity of KLK3c to SHGC4-1080 (6 cR or 180 kb by two-point analysis and 6.6 cR or 198 kb by multipoint analysis, Table 3) suggests that KLK3c is at the same chromosomal locus as KLK3 and can be used as a marker for KLK3. Although we could not map KLK3b onto the G3 radiation map, we did map KLK3b using KLK3a (amplifying the same sequence) to chromosome 4 by somatic hybrid mapping. In addition, both KLK3b and KLK3c were in the same YAC clone of 340-kb length as the original KLK3 marker SHGC4-1080. Thus, KLK3b and KLK3c should be in close proximity. Radiation hybrid mapping is increasingly used and has proven to be a useful alternative to the traditional family-based linkage mapping. This is especially true when the markers are nonpolymorphic STSs. A hindrance to the candidate gene approach used in the studies of human diseases has been the lack of associated polymorphic markers for some genes. One strategy of finding associated polymorphisms is to design STSs from cDNAs, map them onto a radiation map, and use the nearby polymorphic markers on the map. For example, in our KLK3 radiation hybrid map region, the polymorphic marker D4S426 (heterozygosity, 0.77) is 68.4 cR or 2 Mb away from KLK3 (Table 2) and could be useful as a marker for KLK3.

Linkage analysis using SIBPAL did not indicate any evidence of linkage of the KLK3 gene to ESRD, either in the total ESRD family population or in subsets of families based on the etiologies of ESRD. Multipoint exclusion analysis using MAPMAKER/SIBS[25] suggested that KLK3 could be excluded from contributing a maximum relative risk of 3 to ESRD. In fact, it could be excluded at a relative risk of 1.5 in the total population and 1.5 or 2 in the nondiabetic families. The relative risk of developing ESRD in African American siblings of ESRD probands is 9 in comparison to the general population.[26] Therefore, on the basis of the linkage analysis, we could find no evidence that the KLK3 gene was linked to ESRD.

An alternative method to detect linkage is to determine whether individual alleles of a marker locus are associated with ESRD. This association, if it exists, may be due to linkage disequilibrium, which can signal linkage between a marker and a disease. In this study, we found that alleles 7 and 9 of KLK3b were consistently associated with ESRD. The nearly twofold increase in the frequency of these two alleles in ESRD patients compared with control subjects suggests that KLK3 may play a role in ESRD susceptibility. Linkage may not have been detected because of the relatively small size of our family collection.[22] The relevance of our findings would be increased had we been able to demonstrate alterations in plasma kallikrein levels based on the allele detected. Unfortunately, our study patients had end-stage renal failure requiring either hemodialysis, peritoneal dialysis, or kidney transplantation. Therefore, their circulating kallikrein levels are not interpretable. Further linkage and association studies between KLK3 and ESRD should be performed in additional family sets to confirm and extend our findings.

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