Elevation of serum and urine levels of TIMP-1 and tenascin in patients with renal disease

Jan Henrik Hörstrup1, Mathias Gehrmann2, Birgit Schneider1, Angela Plöger3, Peter Froese1, Thea Schirop1, Dieter Kampf1, Ulrich Frei1, Rainer Neumann4 and Kai-Uwe Eckardt1

1Department of Nephrology and Medical Intensive Care, Charité, Campus Virchow-Klinikum, Berlin, 2Bayer Research Centre, Uerdingen, 3Department of Nephrology, Gilead II Hospital, Bielefeld and 4Bayer Vital GmbH, Leverkusen, Germany

Abstract

Background. Chronic kidney disease is characterized by increased synthesis and inhibited destruction of collagenous and non-collagenous matrix proteins. Elevation of collagen fragments has been demonstrated in the serum and urine of patients with renal disease, but the dynamics of renal matrix deposition remain difficult to determine.

Methods. To obtain a further insight into renal matrix metabolism we have assessed whether serum and urine concentrations of the non-collagenous protein, tenascin, and of the tissue inhibitor of metalloproteinases 1 (TIMP-1) are altered in association with renal disease. Serum and urine concentrations of both proteins were determined using a newly developed magnetic particle enzyme immunoassay and were compared with levels of N-terminal procollagen III-peptide (PIIINP) and related to the degree of renal failure and proteinuria.

Results. Circulating levels of tenascin and TIMP-1 were moderately, but significantly, higher in patients with chronic renal disease (n = 54; mean creatinine clearance, 62 ml/min) than in healthy controls (n = 176). Urine concentrations per mg creatinine of tenascin and TIMP-1 were significantly lower than serum levels, but were on average six- and 18-fold higher, respectively, in patients with renal disease than in controls. Urinary concentrations increased with progressive reduction in renal function, but were unrelated to proteinuria. TIMP-1 concentrations in urine correlated with tenascin, which is compatible with the impact of TIMP-1 on the accumulation of matrix proteins. The concentrations of proteins measured did not differ depending on the aetiology of renal disease.

Conclusion. Urinary concentrations of tenascin and TIMP-1 are elevated in association with renal disease and may reflect specific aspects of renal fibrosis.

Keywords: procollagen III-peptide; renal fibrosis; serum; tenascin; tissue inhibitor of metalloproteinases-1; urine

Introduction

 Independently of the specific cause of renal disease development, the progression of glomerular and/or tubulointerstitial fibrosis is an important determinant of the loss of renal function [1]. Limited possibilities exist, however, for monitoring the process of extracellular matrix (ECM) accumulation in the kidney. The invasive nature of kidney biopsies precludes frequent follow-up examinations, and sampling errors may occur if the fibrosis is focal. For prognostic assessment, and to aid the search of antifibrotic therapies, markers in serum or urine that reflect different aspects of renal fibrosis would be highly desirable.

In patients with liver fibrosis, serum levels of ECM compounds, including fragments of different collagens and non-collagenous proteins, are elevated. Serum concentrations of these molecules have been found to correlate with histological scores of fibrosis and expression of their mRNAs, indicating that they reflect the extent of fibrogenesis [2–4]. Due to the size of the liver and its large sinusoidal surface area, levels of ECM components are generally higher in liver diseases than in fibrosis of other organs. Nevertheless, in patients with renal disease it has also been shown that serum and/or urine levels of the N-terminal procollagen III peptide (PIIINP) of type IV collagen, and levels of the C-terminal, non-collagenous domain of type IV collagen may be elevated [5–8]. In addition,
evidence has been provided that increased serum levels of these ECM components are not due to the loss of renal function [5,6,9].

Serum and urine concentrations of non-collagenous matrix proteins have so far not been studied in renal disease. Moreover, it is increasingly recognized that fibrosis results from an imbalance of fibrogenesis and fibrolysis, and that inhibition of matrix degradation plays an important role in renal disease [10]. In the present study we have, therefore, used two newly developed ELISAs for the non-collagenous matrix protein, tenascin, and for the tissue inhibitor of metalloproteinases 1 (TIMP-1) to compare the serum and urine concentrations of both proteins in patients having different types and severities of renal disease with those in healthy controls. Tenascin has emerged as one of the most significant ECM components. It seems to play an important role not only in nephrogenesis but also in many pathological processes in the glomerulus and the renal interstitium [11–14]. TIMP-1 is a physiological inhibitor of the matrix-degrading enzymes, collagenase, gelatinase and stromelysin [15,16]. Upregulation of TIMP-1 mRNA and protein has been demonstrated in different models of renal disease [17–23] and human sclerotic glomeruli [24] and is thought to play a major role in the inhibition of matrix degradation.

**Subjects and methods**

**Patients and healthy blood and urine donors**

Fifty-four outpatients with a defined range of nephropathies were evaluated prospectively after their informed written consent was obtained both for their participation in the investigation, and for donating three additional blood samples and a fresh urine specimen during a regular visit to the outpatient clinic (Table 1). All patients had been well known to the attending physician for at least 6 months. They were only included in the study if a specific renal disease had been proven by previous renal biopsy or was highly likely on clinical grounds, and if their nephropathy could be assigned to one of five diagnostic categories: (1) biopsy-proven primary glomerulopathy (n = 21); (2) strong suspicion of primary glomerulopathy on clinical grounds in patients who had not been biopsied (n = 8); (3) renal involvement in systemic diseases, such as Wegner’s granulomatosis, Schoenlein Hennoch disease or systemic lupus erythematoses (n = 5); (4) diabetic nephopathy (n = 14); or (5) nephrosclerosis (n = 6). The patients had different degrees of renal failure, but patients requiring renal replacement therapy were deliberately not included. Patients were only included if they were in the age range of 18–65 years. In each patient, the following comorbid conditions, potentially affecting matrix metabolism, were also excluded on the basis of thorough historical and clinical examinations and laboratory tests: liver parenchymal disease, alcoholism, chronic respiratory disease, chronic dermal disease, retroperitoneal fibrosis, malignancy, haematological disease other than renal anaemia and acute bouts of infection or inflammation.

To determine the values of TIMP-1 and tenascin in a healthy population, serum samples were collected from 111 male and 65 female blood donors at a blood bank and 67 urine samples were collected from attendees of a regular routine check-up examination in an outpatient department. Urine samples were excluded if the outpatient’s check-up revealed any evidence for acute or chronic disease, such as those mentioned above, or if an elevated level of urinary albumin was measured by radioimmunoassay.

**Blood and urine sampling**

Blood samples were kept on ice for a maximum of 1 h after collection. Then serum and citrate plasma were separated by centrifugation at 2000 g for 10 min, aliquotted and frozen at −80°C until analysis.

Since 24 h urine collections were only possible for 31 patients, urine measurements of the markers investigated were always performed in freshly voided midstream-samples and the values were expressed relative to the urinary creatinine concentrations. Creatinine clearances in patients

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**Table 1. Patient characteristics (mean ± SD)**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Sex (male/female)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Creatinine clearance (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Glomerulonephritis</td>
<td>29</td>
<td>15/14</td>
<td>1.94 ± 1.55</td>
<td>64.4 ± 34.7</td>
</tr>
<tr>
<td>Minimal change disease</td>
<td>3</td>
<td>1/2</td>
<td>0.73 ± 0.17</td>
<td>128 ± 24.7</td>
</tr>
<tr>
<td>Focal glomerulosclerosis</td>
<td>5</td>
<td>1/4</td>
<td>2.1 ± 1.88</td>
<td>60.9 ± 29.3</td>
</tr>
<tr>
<td>IgA nephropathy</td>
<td>7</td>
<td>5/2</td>
<td>1.86 ± 0.1</td>
<td>63.5 ± 32.7</td>
</tr>
<tr>
<td>Membranous GN</td>
<td>3</td>
<td>2/1</td>
<td>1.1 ± 0.22</td>
<td>74 ± 11.8</td>
</tr>
<tr>
<td>Membrano-proliferative GN</td>
<td>3</td>
<td>1/2</td>
<td>3 ± 2.34</td>
<td>43.3 ± 23.5</td>
</tr>
<tr>
<td>Clinical diagnosis of glomerulopathy without biopsy</td>
<td>8</td>
<td>5/3</td>
<td>2.29 ± 1.49</td>
<td>47.8 ± 19.9</td>
</tr>
<tr>
<td>II. Systemic disease</td>
<td>5</td>
<td>1/4</td>
<td>1.7 ± 0.77</td>
<td>59.4 ± 23</td>
</tr>
<tr>
<td>Wegner’s granulomatosis</td>
<td>1</td>
<td>0/1</td>
<td>1.1</td>
<td>95</td>
</tr>
<tr>
<td>Systemic lupus erythematoses</td>
<td>3</td>
<td>0.3</td>
<td>1.4 ± 0.16</td>
<td>58 ± 11.3</td>
</tr>
<tr>
<td>Schoenlein-Henoch nephritis</td>
<td>1</td>
<td>1.0</td>
<td>3.2</td>
<td>28</td>
</tr>
<tr>
<td>III. Nephrosclerosis</td>
<td>6</td>
<td>3/3</td>
<td>3.38 ± 2.15</td>
<td>41.1 ± 30.3</td>
</tr>
<tr>
<td>IV. Diabetic nephropathy</td>
<td>14</td>
<td>8/6</td>
<td>1.54 ± 0.93</td>
<td>67.2 ± 25.4</td>
</tr>
<tr>
<td>IDDM</td>
<td>7</td>
<td>3/4</td>
<td>1.6 ± 1.01</td>
<td>61.8 ± 22.3</td>
</tr>
<tr>
<td>NIDDM</td>
<td>7</td>
<td>5/2</td>
<td>1.49 ± 1.54</td>
<td>72.6 ± 27.1</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>27/27</td>
<td>1.98 ± 1.54</td>
<td>62.1 ± 32</td>
</tr>
</tbody>
</table>

GN, glomerulonephritis; IDDM, insulin dependent diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus.
who had not collected urine for 24 h were estimated in accordance with Cockcroft and Gault [25].

ELISA for tenasin

For quantitative determination of tenasin in serum and urine a newly developed, fully automated, magnetic particle enzyme immunoassay (Bayer AG, Leverkusen, Germany) was used [26]. The heterogeneous sandwich tenasin assay consisted of a fluorescinated capture mouse-antibody (monoclonal), an alkaline phosphatase-labelled detector mouse-antibody (monoclonal) and a magnetic particle solid phase. Enzymatically produced para-nitrophenoxide was measured at 405 nm.

The detection limit was 2 ng/ml. The assay demonstrated within-run and between-run CVs of 3.6–4.0% and 6.7–8.4%, respectively. No cross-reactivities with fibronectin, fibrinogen, epidermal growth factor, vitronectin and laminin were detectable.

The normal value of tenasin in serum was 438 ± 167 ng/ml, and in urine 0.3 ± 1 ng/mg creatinine.

ELISA for TIMP-1

For the measurement of TIMP-1 an identical immunoassay with two specific monoclonal mouse-antibodies against TIMP-1 was used [27].

The detection limit was also 2 ng/ml. The intra-assay variation ranged between 2.8–3.0% and the inter-assay variation between 3.2–4.7%. There was no cross-reactivity detectable either in the absence of the analyte or in the presence of 0.6 μg/ml TIMP-1 with TIMP-2 (4 μg/ml), activated or latent MPP-2 (5 μg/ml), MPP-3 (5 μg/ml) or active MMP-9 (5 μg/ml).

The mean and standard deviation of TIMP-1 in the serum and the urine, normalized to creatinine concentration, of the healthy population was 613 ± 112 ng/ml and 8.8 ± 13.6 ng/mg creatinine, respectively.

For both assays, dilutions of sera were linear and parallel with the standard curve and had recoveries ranging from 96 to 105%. There were no interferences with haemoglobin (1 g/dl), bilirubin (25 mg/dl), triglycerides (1 g/dl), albumin (6.5 g/dl), heparin (0.5 mg/ml = 65 IU/ml) and EDTA (1 g/dl).

Radioimmunoassay for PIIINP

The amino terminal propeptide of type III procollagen was measured by the frequently used commercial radioimmunoassay kit of Orion Diagnostica (Espoo, Finland) [28]. This is an equilibrium assay based on polyclonal antibodies, which primarily detects the intact propeptide and its higher molecular weight forms.

The sensitivity was 1 ng/ml, the intra-assay variation ranged from 1.7 to 4.3% and the inter-assay variation from 3.2 to 5.3%. The use of haemolysed, lipaemic or icteric samples was reported not to interfere with assay results. According to the description of the producer, the normal range in plasma is 1.53–4.2 ng/ml.

Statistics

Statistical evaluation was performed using SPSS software. Comparisons of patients and control groups were performed using Student’s t-test, and analysis of variance (ANOVA) was used to determine the significance of linear regressions. A probability of <0.05 was considered significant.

Results

Figure 1 illustrates the levels of TIMP-1 in serum (upper panel) and urine (lower panel) of patients with different nephropathies. The mean serum levels for all patients and for the subgroups of patients with glomerulonephritis, nephrosclerosis and diabetic nephropathy was significantly elevated. Eleven of the 54 patients had values more than two standard deviations higher than the mean value in controls.

Also, the concentrations of TIMP-1 in urine, normalized to creatinine concentrations, were significantly elevated in the whole patient group and different subgroups compared with controls, the only exception being values for the small subset of patients with systemic diseases. On average urinary TIMP-1 concentrations were sixfold higher in patients with renal disease than in controls. In those 31 patients for whom 24 h urine collections were available, urinary TIMP-1 levels, normalized for creatinine, correlated closely with the 24 h TIMP-1 excretion ($r = 0.95; P < 0.0005$).

Of those 15 patients who had elevated urine levels of TIMP-1, eight patients (53%) also had an elevated serum level. For the whole group of patients there was a significant positive correlation between TIMP-1 levels in serum and urine ($r = 0.55; P < 0.0005$). Furthermore, as shown in Figure 2, TIMP-1 levels in serum and urine correlated inversely with creatinine clearance ($r = 0.53$ and $r = 0.33; P < 0.0005$ and $P < 0.05$, respectively). The renal clearance for TIMP-1 was 0.058 ± 0.1 ml/min (mean ± SD; $n = 31$).

Serum and urine concentrations of tenasin are shown in Figure 3. Consistent with the observed TIMP-1 levels, the mean values for the whole patient group and for three of the four subgroups were higher than the levels in healthy controls (upper panel). Urinary tenasin levels were only detectable in 16% of the control individuals. In contrast, measurable concentrations of tenasin in urine were found in 80% of the patients with nephropathies. Urine concentrations in patients with renal disease were on average 18-fold, and in some individual patients more than 100-fold, higher than in controls. Only four of the 23 patients with elevated urinary tenasin excretion also had elevated tenasin levels in their serum. Tenasin values, related to urinary creatinine concentrations, in freshly voided urine samples correlated strongly with the total amount of tenasin excreted in 24 h urine collections ($r = 0.89; P < 0.0005$). In contrast to TIMP-1 levels, serum tenasin concentrations did not increase with progressive reduction in renal function (Figure 4). Tenasin levels in the urine, however, correlated inversely with creatinine clearance ($r = 0.28; P < 0.05$). The renal clearance for tenasin was 0.0082 ± 0.013 ml/min (mean ± SD; $n = 31$).
For comparison, concentrations of PIIINP were determined in the same urine samples and simultaneously collected plasma samples (Table 2). In accordance with previous investigations, plasma levels of PIIINP were on average significantly increased, and up to about twofold elevated in patients with renal disease compared with controls [5,7–9]. Plasma and urine concentrations of PIIINP were not correlated with each other and neither of them correlated with glomerular filtration rate (data not shown). The renal clearance of PIIINP was 1.71 ± 2.01 ml/min and, therefore, very similar to previous reports of individuals with and without renal disease [5].

To further assess whether the urine concentrations of the markers under investigation reflect an increased glomerular permeability, the values were related to urinary protein concentrations. As shown in Figure 5, there were no significant correlations between proteinuria and urinary tenascin or TIMP-1, but there was a tendency towards higher excretion in patients with less severe proteinuria. Also urinary levels of PIIINP were not related to protein excretion (data not shown).

In order to investigate whether the measured parameters reflect similar or potentially different aspects of renal pathology, the correlations between serum and urinary levels of TIMP-1, tenascin and PIIINP were determined. As shown in Table 3, there was a significant positive correlation between TIMP-1 and PIIINP in serum and plasma, whereas circulating levels of TIMP-1 and tenascin, or PIIINP and tenascin, did not correlate with each other. There was a positive correlation between TIMP-1 levels and tenascin concentrations in urine.

Discussion

This study demonstrates that nephropathies of diverse aetiology are associated with measurable increases in serum and urine levels of the non-collagenous matrix protein, tenascin, and of the enzyme inhibitor, TIMP-1. Tenascin is known to accumulate in diseased kidneys [11–14], and TIMP-1 is probably upregulated in many types of kidney disease and appears to play an
important role in fibrogenesis by inhibiting naturally occurring matrix-degrading enzymes \([17–24]\). By measuring the levels of the N-terminal portion of collagen III we have confirmed previous studies, showing that circulating and urinary fragments of collagen may be elevated in renal disease \([5–9]\).

Many aspects of the process of renal fibrosis remain incompletely understood. The evidence available and studies on fibrosis in other organs clearly indicate that mechanisms of ECM accumulation in general are neither disease- nor organ-specific. Measurements of circulating matrix components and modulators of matrix metabolism can therefore only be interpreted with respect to a pathology of a certain organ if additional evidence suggests an organ-limited disease. Consequently, in the present study we have taken care to select patients for whom significant alterations of matrix metabolism at extrarenal sites were unlikely. Several lines of evidence indicate that the observed changes in circulating and urinary levels of tenascin, TIMP-1 and PIIINP are not just a secondary phenomenon of reduced renal function or increased glomerular permeability, but are more likely to reflect an increase in the renal expression of the three proteins. Firstly, the circulating levels of tenascin and PIIINP were not related to GFR, which suggests that both substances do not simply accumulate with progressive kidney failure (Figure 4). Secondly, although for TIMP-1 a significant inverse relationship was found between its serum levels and creatinine clearance, there was also a significant inverse relationship between TIMP-1 excretion in the urine and creatinine clearance (Figure 2), which argues against a retention with increasing loss of renal function. Thirdly, the urinary excretion of tenascin, TIMP and PIIINP was unrelated to the degree of proteinuria (Figure 5), which makes it unlikely that increased urinary excretion is mainly due to enhanced glomerular permeability. In a study

Fig. 2. Correlation between TIMP-1 levels (a) in serum and (b) in urine with glomerular filtration rate. ○, glomerulonephritis; ●, systemic disease; ●, nephrosclerosis; ○, diabetic nephropathy. Regression for serum levels: \(y = 923.2 - 3.58x, r = -0.528, P < 0.0005\); and for urine levels: \(y = 123.4 - 1.14x, r = -0.33, P < 0.05\).
by Soylemezoglu et al. [9] a strong correlation was shown between serum and urine levels of collagen fragments and the extent of collagen immunoreactivity in renal biopsies, supporting the renal origin of these matrix components.

Since fibrosis at any site occurs against a background of physiological matrix turnover in virtually any organ, it may not be surprising that a considerable overlap was found between serum/plasma values in an apparently healthy population and patients with renal disease. This has similarly been reported for levels of TIMP-1 in patients with cancer [29], rheumatoid arthritis [29–31] or systemic sclerosis [31] and for tenascin levels in patients with cancer [32,33]. Even in patients with histologically proven liver fibrosis circulating levels of TIMP-1 and tenascin in many patients do not exceed a ‘normal’ range, despite the approximately fivefold greater organ size as compared with the kidneys [4,34]. Moreover, although recent kidney biopsies were not available for most of our patients, it can be assumed that the extent and activity of renal fibrosis was variable. In many of our patients renal scarring may not have been of sufficient severity to raise circulating levels of ECM markers.

Interestingly, more frequent and more marked (up to more than 100-fold) elevations of the proteins investigated were found in urine samples, as compared with serum levels. Although urinary excretion of TIMP-1 and tenascin has not been systematically investigated in fibrotic diseases of other organs, the molecular size of TIMP (28.5 kD) and of tenascin subunits (180–320 kD) is likely to preclude them from glomerular filtration. Although the absolute levels of these proteins were several-fold lower in urine than in serum (Figures 1 and 2), their measurement in urine may therefore reflect aspects of renal fibrosis more specifically. Notably, in few patients for whom a second set of serum and urine samples was available (collected 3–9 months after the initial investigation) all three parameters were fairly stable within individual patients (data not shown). This might indicate that any changes during the natural course of disease or following intervention may be meaningful even if they occur within the ‘normal’ range.

**Fig. 3.** Levels of tenascin in (a) serum and (b) urine of patients with different nephropathies. TP, total patients; GN, glomerulonephritis; SD, systemic disease; NS, nephrosclerosis; DN, diabetic nephropathy; *P < 0.05; **P < 0.005; ***P < 0.0005 vs controls.
The present study also suggests that it is worthwhile to perform parallel determinations of various ECM markers because the circulating and urinary levels of tenascin, TIMP-1 and PIIINP fragments were not generally related to each other, suggesting that they may reflect different aspects of pathology. A positive correlation was found, however, between the circulating levels of TIMP-1 and PIIINP, as previously reported in patients with chronic liver disease and between urinary concentrations of TIMP-1 and tenascin (Table 3). These correlations are in accordance with experimental data, suggesting that the cellular mechanisms inducing fibrosis, such as the overexpression of transforming growth factor \( \beta \), stimulate simultaneously increased expression and reduced degradation of ECM.

Irrespective of diagnostic considerations the question arises as to whether the increase in circulating levels of TIMP-1 that was observed with increasing severity of renal disease (Figure 2) may have any functional relevance. Although we have not measured the levels of matrix-degrading enzymes that are

![Fig. 4. Correlation between tenascin levels in (a) serum and (b) urine with glomerular filtration rate.](image)

**Table 2. Plasma and urine levels of PIIINP**

<table>
<thead>
<tr>
<th></th>
<th>Plasma PIIINP (ng/ml)</th>
<th>Urine PIIINP/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Glomerulonephritis</td>
<td>29 3.21 ± 0.8***</td>
<td>5.23 ± 4.09</td>
</tr>
<tr>
<td>II. Systemic disease</td>
<td>5 3.16 ± 0.67</td>
<td>5.45 ± 0.7</td>
</tr>
<tr>
<td>III. Nephrosclerosis</td>
<td>6 3.1 ± 0.72**</td>
<td>5.1 ± 5.88</td>
</tr>
<tr>
<td>IV. Diabetic nephropathy</td>
<td>14 2.91 ± 1.1*</td>
<td>4.53 ± 6.08</td>
</tr>
<tr>
<td>Total</td>
<td>54 3.1 ± 0.89***</td>
<td>5.06 ± 5.12</td>
</tr>
</tbody>
</table>

Values are mean ± SD; *P < 0.05, **P < 0.005, ***P < 0.0005 vs control values as given by the supplier of the PIIINP–RIA (2.5 ± 0.6 ng/ml).
complexed by TIMP-1, previous studies in healthy individuals and patients with different diseases have found that TIMP-1 normally occurs in plasma in molar excess of its substrates and that TIMP-1 complexes matrix-degrading enzymes with a high affinity constant of $10^{-10}$ M. It is unlikely, therefore, that a further increase in TIMP-1 affects the collagenolytic activity of plasma. However, any increase of TIMP-1 in the bloodstream might also raise its concentration in vessel walls, where the enzyme appears to promote atherosclerosis. It is also noteworthy that, apart from its inhibitory effect on metalloproteinases, purified TIMP-1 and TIMP-1 in serum have growth promoting activities for a range of different cells. Whether the moderate changes in serum levels observed in the present study are relevant in this respect, requires further investigation.

In conclusion, we have provided the first evidence that different kidney diseases can lead to detectable increments in the circulation and, probably more specific and relevant, in urine, of not only collagen fragments but also of non-collagenous matrix proteins and enzymes involved in the pathogenesis of scarring. We believe that the present investigation warrants further studies to assess the relationship between fibrosis and histological scores, and to determine the value of the currently selected, and possibly other, ECM markers during long term follow-ups.

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References
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