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ORIGINAL PAPER

# Glycosylation of prothrombin fragment 1 governs calcium oxalate crystal nucleation and aggregation, but not crystal growth

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**Abstract** Urinary glycoproteins play an important role in the modulation of calcium oxalate crystallisation. In several cases, this has been attributed to glycosylation of the proteins as evidenced by urinary prothrombin fragment 1 where there is a correlation between sialylation and calcium oxalate kidney stone disease. In the present study, plasma-derived prothrombin fragment 1 (PTF1) was enzymatically modified in order to generate its asialo and aglyco forms. The parent glycoprotein and its two glycoforms were used in calcium oxalate crystallisation studies to assess the role of the carbohydrate moeity in PTF1's potent inhibitory activity. The glycans inhibited crystal aggregation and promoted crystal nucleation, but had no effect on crystal growth. The terminal sialic acid residues had a small effect on inhibition of crystal aggregation whereas they contributed significantly to promotion of nucleation. These results indicate that glycosylation of PTF1 governs calcium oxalate crystal nucleation and aggregation but it does not affect the protein's role in inhibiting crystal growth. Since promotion of nucleation and inhibition of aggregation are both regarded as protective mechanisms against calcium oxalate urinary stone formation, the kringle domain on which the glycans are located is implicated in PTF1's inhibitory activity. It is speculated that modifications in the glycosylation of urinary PTF1 in stone-formers may regulate its capacity to protect against calcium urolithiasis.

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**Keywords** Calcium oxalate · Crystallization · Glycosylation · Kidney stones · Protein · Prothrombin fragment 1

**Abbreviations**

| | |
|---|---|
| agPTF1 | Aglyco prothrombin fragment 1 |
| asPTF1 | Asialo prothrombin fragment 1 |
| CaOx | Calcium oxalate |
| Gla | $\gamma$-carboxyglutamic acid |
| gPTF1 | Glyco prothrombin fragment 1 |
| $I_N$ | Inhibition of nucleation |
| $I_G$ | Inhibition of growth |
| MALDI-TOF | Matrix-assisted laser-desorption-ionisation time-of-flight |
| PT | Prothrombin |
| PTF1 | Prothrombin fragment 1 |
| SEM | Standard error of mean |
| SU | Synthetic urine |
| UPTF1 | Urinary prothrombin fragment 1 |

## Introduction

Urinary proteins are of considerable interest in view of their reported role in the inhibition of physical processes, which direct kidney stone formation [1, 2]. Recent technological advancements in the fields of protein biochemistry and molecular biology have greatly increased scientists' ability to elucidate the detailed structures of these proteins and to explore how these influence functionality.

One of the main roles proposed for urinary proteins is to retard calcium oxalate (CaOx) crystallisation. Structure function studies of Tamm-Horsfall protein [3, 4], osteopontin [5–7], bikunin [8] and nephrocalcin [9] have demonstrated

the importance of post-translational modifications to their inhibitory activities.

The protein urinary prothrombin fragment 1 (UPTF1) has received much attention and has attracted considerable interest. One result which distinguishes it from many other proteins is its inhibitory potency towards in vitro CaOx crystallisation [10]. Independent studies have demonstrated that UPTF1 promotes the nucleation of CaOx crystals [11], but inhibits their growth and aggregation [11, 12] in urine. The $\gamma$-carboxyglutamic acid (Gla) residues of plasma-derived PTF1, which is structurally similar to the urinary form, are speculated to impart this activity in urine [13] as well as in an inorganic milieu [14]. In addition to UPTF1's Gla domain, the protein's carbohydrate moiety is likely to influence its functionality. Indeed, our own studies have identified differences in the glycosylation of UPTF1 from controls and stone-formers [15], which provide motivation for further structure–function studies of this protein.

In the present study, we modified plasma-derived prothrombin fragment 1 (PTF1) in order to generate its asialo and aglyco forms. The inhibitory activity of these two modified forms of PTF1 towards urinary CaOx crystallisation processes were then compared to that of the fully glycosylated protein in order to assess the role of the carbohydrate moiety in determining the protein's proven inhibitory potency.

## Methods

### Protein deglycosylation

Prothrombin fragment 1 purified from human plasma was purchased from Haematologic Technologies (Vermont, USA) and is henceforth referred to as "gPTF1" (i.e. glycosylated PTF1). The commercial preparation was resolved on a 12.5% polyacrylamide gel and stained using Coomassie Brilliant Blue. SDS-PAGE showed the protein was highly pure with a minor component (~2%) of partly deglycosylated PTF1 (Fig. 1).

The gPTF1 was treated in one of three ways in order to either generate a control sample (**1**), remove sialic acid residues only (**2**), or remove all of the glycans (**3**):

**1**: incubated overnight at 37°C in a 5 × concentrated buffer containing 0.25 M $NaH_2PO_4$, pH 6.0;

**2**: the same as (**1**) but with the addition of *Clostridium perfringens* neuraminidase (EC 3.2.1.18, 40 mU/mg) (Sigma, Schnelldorf, Germany) which removes α2-3, α2-6 and α2-8 linked sialic acids;

**3**: the same as (**1**) but with the addition of *Clostridium perfringens* neuraminidase (EC 3.2.1.18, 40 mU/mg) (Sigma), Chryseobacterium (Flavobacterium) meningosepticum endoglycosidase F2 recombinant in *E. coli* (EC 3.2.1.96, 100 mU/mg) (Sigma) which cleaves between the two *N*-acetylglucosamine residues in the diacetylchitobiose core of the oligosaccharide, and *Diplococcus pneumoniae O*-glycosidase (EC 3.2.1.97, 25 mU/mg) (Roche Diagnostics, East Sussex, UK) which removes Gal$\beta$1–3GalNAc linked chains.

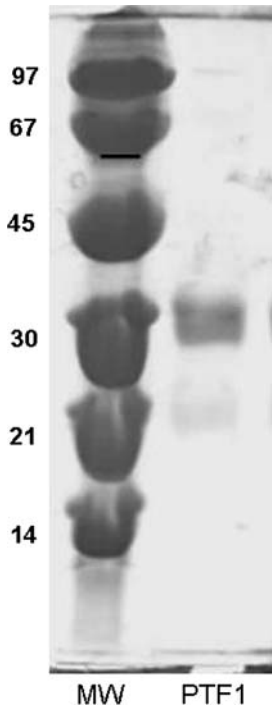


**Fig. 1** SDS-PAGE of human plasma-derived PTF1. MW denotes a low molecular weight marker

Following digestion, samples were applied to a Microcon centrifugal filtration device (10 kDa) (Millipore, Watford, UK) and washed thoroughly with double distilled water to desalt the samples and to separate the released glycans from the remaining protein. The digested proteins were made up in PBS (8.21 mM $K_2HPO_4$, 1.84 mM $KH_2PO_4$, pH 7.3, containing 0.145 M NaCl) and the concentrations were determined using the Bio-Rad protein assay with BSA as the standard protein. The protein stock solutions were adjusted to 1 mg/ml using PBS for crystallisation studies. The proteins obtained from the three treatments described above are henceforth referred to as gPTF1, asPTF1 and agPTF1, respectively.

### Mass spectrometry

Matrix-assisted laser-desorption-ionisation time-of-flight (MALDI-TOF) mass spectra of the three preparations described above as well as the untreated commercial protein were recorded using a PerSeptive Biosystems Voyager DE-PRO Biospectrometry Workstation (PerSeptive Biosystems, Framingham, Massachusetts) in order to determine their glycosylation status. A saturated solution of sinapinic acid in 50% acetonitrile and 0.3% trifluoroacetic

acid was used as the matrix. Mass measurements (equivalent to mass/charge or *m*/*z*) based on previously determined glycan masses of gPTF1 [15] were compared to calculated masses of its aglyco form.

## Crystallisation studies

All solutions were prepared using distilled water and filtered (0.22 μm) before use. Methods were chosen that provide information about the three mechanisms of crystallisation independently of one another. Therefore inorganic solutions were used as test media and not real urine. The results cannot be extrapolated to physiological conditions; however, this strategy facilitated direct comparison of the PTF1 glycoforms without interference from the many other substances present in urine.

The in vivo concentration of urinary PTF1 has been reported in the range 0.03–1.1 mg/l [16]. However, we elected to use the concentration 1.25 mg/l since this is consistent with previous urinary PTF1 studies [11, 12] and also to ensure our successful observation of any effects on CaOx crystallisation.

### *Crystal nucleation*

Crystal nucleation was induced and monitored according to the procedure described by Hess et al. [17]. Briefly, 1 ml of a calcium chloride solution (8.5 mM $CaCl_2$, 10 mM NaAc, pH 5.70, containing 200 mM NaCl) was transferred to a glass cuvette, which was placed in a UV–VIS Specord 40 (AnalytikJena, Germany) fitted with a thermostatted bath and stirrer. The appropriate protein solution was added at a final concentration of 1.25 mg/l and the mixture was stirred at 500 rpm. For the control sample, an equal volume of distilled water was added instead of protein. Nucleation was induced by the addition of 1 ml of a sodium oxalate solution (1.0 mM $Na_2Ox$, 10 mM NaAc, pH 5.70, containing 200 mM NaCl) and the absorbance was monitored at 620 nm and 37°C for 600 s. Inhibition of nucleation ($I_N$) in the presence of protein was calculated using the expression $[1 - (Sp/Sc)] \times 100$, where Sp and Sc denote the maximum slope of increase of the protein-dosed and control samples, respectively. The following number of experiments was performed: control, $n = 8$; gPTF1, $n = 7$; asPTF1, $n = 6$; agPTF1, $n = 8$.

### *Crystal growth*

A modification of seeded crystal growth systems reported variously [9, 18, 19] was used in the present study. A metastable solution of CaOx containing 0.5 mM $CaCl_2$ and 0.5 mM $Na_2Ox$ was prepared in a 10 mM tris(hydroxymethyl)-aminomethane (Tris) buffer, pH 7.2, containing 90 mM NaCl. The solution was maintained at room temperature with fast stirring throughout the experimental period. A seed crystal slurry was prepared by suspending CaOx monohydrate crystals, prepared according to Pak et al. [20], in the same Tris buffer (16 mg/ml) and it was equilibrated overnight with fast stirring. The following day, 2 ml of the metastable CaOx solution was transferred to a quartz cuvette, which was placed in a UV–VIS Specord 40. The appropriate protein solution was added at a final concentration of 1.25 mg/l and the mixture was stirred at 500 rpm. For the control sample, distilled water was added instead of protein. Growth was induced by the addition of the seed crystal slurry (50 μl) and the absorbance was monitored at 214 nm and 37°C for 400 s. Inhibition of growth ($I_G$) was calculated using the equation $[(Ac - Ap)/Ac] \times 100$, where Ac and Ap denote the change in absorbance of the control and protein-dosed samples, respectively, after 400 s. The following number of experiments was performed: control, $n = 7$; gPTF1, $n = 6$; asPTF1, $n = 7$; agPTF1, $n = 7$.

### *Particle size distributions*

Synthetic urine (SU) was prepared according to Walton et al. [21]. The final calcium and oxalate concentrations were 4.38 and 2.32 mM, respectively. SU was adjusted to pH 6.0 using NaOH, heated to 37°C and filtered (0.22 μm) immediately before use. Aliquots (10 ml) were dosed with gPTF1, asPTF1 or agPTF1 at final concentrations of 1.25 mg/l and incubated in soda-lime glass bottles at 37°C in a shaking water bath (Labmark, Johannesburg; 100 rpm) for 2 h. In the case of the control sample, distilled water was added instead of protein. After 2 h, the particle volume–particle size distributions were measured using a Coulter Multisizer II (Coulter Electronics Ltd, Luton, UK) fitted with a 140 μm orifice (2.8–50.0 μm particle size range). The experiment was performed in triplicate.

Following the completion of the study, it came to our attention that PBS should have been added to the control samples in the crystallisation studies rather than distilled water. Nonetheless, an independent study found that there was no significant difference between the two treatments in any of the afore-mentioned experiments. Consequently, it is highly unlikely that the use of distilled water rather than PBS impacted on the outcomes reported for the present study.

### *Scanning electron microscopy*

After measuring particle size distributions, 2 ml of the remaining SU/protein mixtures were filtered (0.22 μm) in order to retrieve the deposited crystals. These filter papers were glued onto aluminium stubs, coated with a double

layer of Au/Pd and the crystals were examined using a Leica S440 scanning electron microscope (Leica Cambridge Ltd., Cambridge, England). The microscope settings were as follows: accelerating voltage 10 kV, working distance 23 mm, probe current 20 pA, magnification 4,000×.

### Statistical analysis

For the nucleation and growth assays, mean values and standard errors of the mean (SEM) are reported. Single factor analysis of variance and a post hoc Tukey HSD test (for unequal *N*) was performed using Statistica 7.0 to compare all possible pairs of samples. For these analyses, *P* values are reported and values $\leq 0.05$ are regarded as significant. Mean particle size distributions of SU and the protein-dosed samples are reported. Skew-normal distributions were fitted to each mean data set using the software package *R* and parameter values for the location, scale and shape were determined. From these parameter values, theoretical moments and particle modes were also computed.

## Results

### Protein deglycosylation

A detailed analysis of the *N*- and *O*-linked glycans of UPTF1 implicated the sialic acid residues as a factor influencing CaOx kidney stone formation [15]. In order to investigate the contribution of the glycans to crystallisation, MALDI-TOF mass spectrometry data was collected at various stages of deglycosylation (Fig. 2) and the mass loss after each treatment was compared to calculated values based on previously published mass data of the glycans [15]. The mass spectrum of gPTF1 (i.e. treatment 1, Fig. 2a) exhibited a single, broad peak (*m/z* 20,223–22,662). The mass spectrum of gPTF1 before incubation in the digest buffer was identical to that of treatment 1, thus confirming the former's suitability as a control sample (data not shown). After treatment with neuraminidase (i.e. treatment 2, Fig. 2b), a sharp peak was observed at *m/z* 20,292 which corresponded to a mass loss of 69-2370. The calculated mass of sialic acid residues on PTF1 is 1,536 and falls within this range. Digestion with a 1.5-fold higher concentration of neuraminidase did not result in further mass loss (data not shown), thereby confirming that the protein generated after neuraminidase treatment corresponded to asialo PTF1. After treatment with neuraminidase as well as exoglycosidases targeting the *N*- and *O*-linked glycans of PTF1 (i.e. treatment 3, Fig. 2c), a broad peak was observed with *m/z* 11,400–14,070, which indicated a mass loss of 7,338–10,008. Comparison with the calculated mass loss of ca. 3,500–10,000 verified that the latter treatment had generated the aglyco form of PTF1.

### Crystallisation studies

The rates of crystal nucleation in the presence of glycosylated, asialo and aglyco PTF1 were determined using a spectrophotometric assay. The rate of nucleation increased in the order: control ≅ agPTF1 < asPTF1 < gPTF1 (Fig. 3). While gPTF1 increased the rate of nucleation significantly, the rates declined steadily as the glycans were trimmed. After removal of all of the glycans from PTF1, the nucleation rate returned to that of the control. The percentage inhibition of nucleation was calculated from these nucleation rates (Table 1). Both gPTF1 and asPTF1 promoted crystal nucleation, as indicated by the negative inhibition of nucleation percentages. Removal of the sialic acid residues accounted for an almost twofold decrease in this tendency to promote nucleation (−117 vs. −65.5%). Removal of all the sugars reduced this tendency even further.

The effect of the glycosylated and modified glycoforms of PTF1 on crystal growth rate is shown in Fig. 4. The rates of crystal growth followed the trend: agPTF1 ≅ asPTF1 ≅ gPTF1 < control. While gPTF1 decreased the rate of crystal growth, there was no change in this rate as the glycans were removed sequentially. Glycosylated PTF1 and its two modified forms all inhibited crystal growth and the amounts of inhibition ranged from 22.3 to 33.8% (Table 1).

The effect of PTF1 and its glycoforms on particle size was also investigated. The mean particle volume–particle size distribution of synthetic urine dosed with each protein sample is shown in Fig. 5. The removal of the *N*- and *O*-linked glycans resulted in the largest mode of the PTF1-dosed urine distributions, i.e. the largest average particle size. Statistical analysis of these distributions showed that the particle modes increased in the order: control ≅ gPTF1 < asPTF1 < agPTF1 (Table 2). Removal of sialic acid residues resulted in a 1.8 μm increase in particle size relative to the glycosylated protein, and removal of the remaining glycans further increased the particle size by 6.2 μm. Thus, removal of the carbohydrate moiety resulted in a 35% increase in particle size.

All size data were fitted to a skew-normal probability distribution and analysis of their respective functions generated data regarding the asymmetry (i.e. skewness) and peakedness (i.e. kurtosis) of the distributions. The control and gPTF1-dosed urines were similar with respect to their coefficients of skewness and kurtosis. The asPTF1-dosed urine tended towards a more negatively skewed distribution and a more peaked distribution. The latter trend was greatly enhanced by the removal of the remaining glycans; the agPTF1-dosed urine exhibited the distribution with the sharpest peak and the longest left tail (indicated by the smallest skewness coefficient and the largest kurtosis coefficient, respectively).

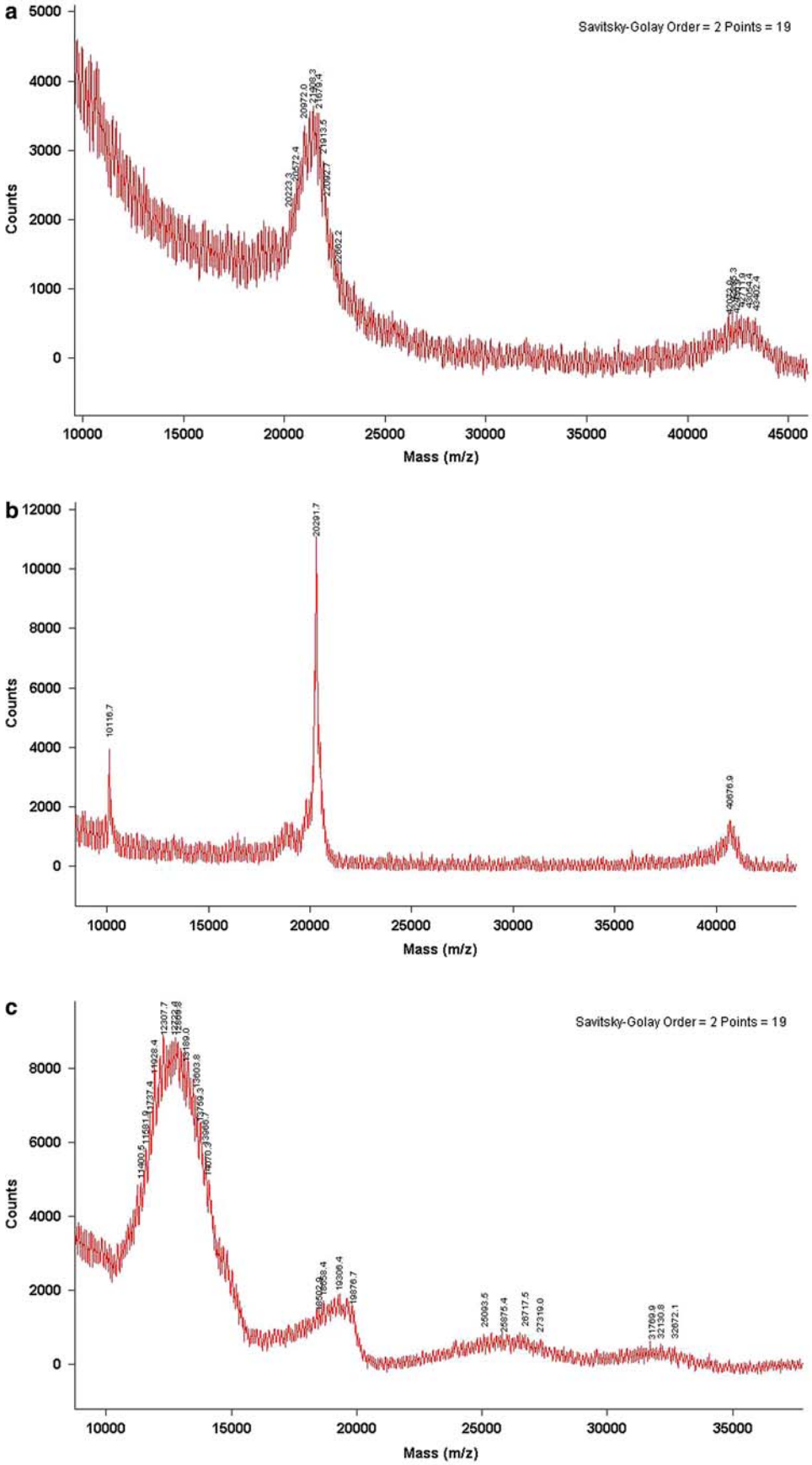


**Fig. 2** MALDI-TOF mass spectrometry chromatograms of gPTF1 after treatment 1, incubation in the digest buffer alone (**a**); treatment 2, incubation with neuraminidase (**b**); and treatment 3, incubation with neuraminidase, endoglycosidase F2 and *O*-glycosidase (**c**)

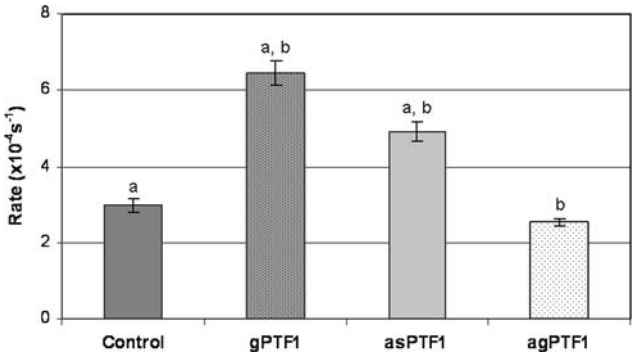


**Fig. 3** Effect of the glycosylation status of PTF1 on calcium oxalate crystal nucleation. Pairwise comparisons of histograms which are labelled with the same symbol are significantly different ($P < 0.001$). Error bars indicate SEM. *gPTF1* glycosylated PTF1; *asPTF1* asialo PTF1; *agPTF1* aglyco PTF1

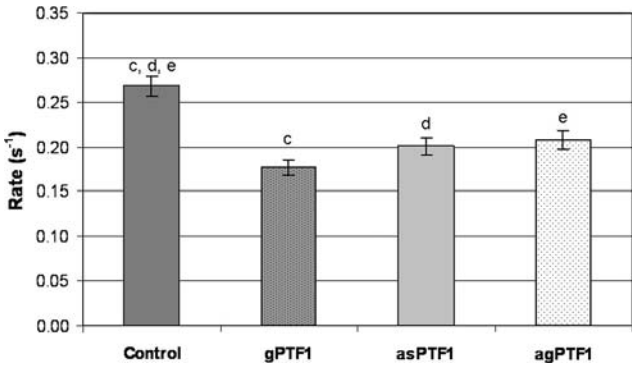


**Fig. 4** Effect of the glycosylation status of PTF1 on calcium oxalate crystal growth. Pairwise comparisons of histograms which are labelled with the same symbol are significantly different ($P < 0.01$). Error bars indicate SEM. Refer to Fig. 3 legend for abbreviations

**Table 1** Effect of the glycosylation status of PTF1 on calcium oxalate crystal nucleation and growth

| | Inhibition of nucleation, $I_N$ (%) (SEM) | Inhibition of growth, $I_G$ (%) (SEM) |
|---|---|---|
| gPTF1 | −117 (15.6) | 33.8 (3.8) |
| asPTF1 | −65.5 (11.8) | 25.0 (4.3) |
| agPTF1 | 14.7 (5.6) | 22.3 (4.7) |

Refer to Fig. 1 legend for abbreviations. A negative percentage indicates promotion

Scanning electron micrographs of CaOx crystals deposited in the synthetic urine and protein-dosed urines (Fig. 6) confirmed the differences in the average particle sizes demonstrated by size distribution analysis and representative micrographs of numerous fields ($n > 10$) are shown. A mixture of small, oval-shaped CaOx monohydrate and larger, bipyramidal CaOx dihydrate crystals were observed in all four samples. Crystal aggregates were deposited in each

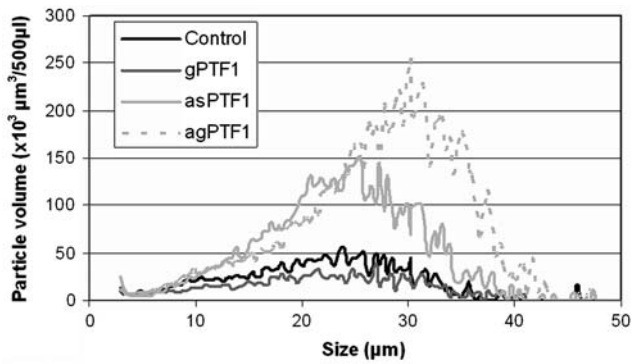


**Fig. 5** Effect of the glycosylation status of PTF1 on the particle size distribution of synthetic urine. Refer to Fig. 3 legend for abbreviations

**Table 2** Data derived from particle size distributions of synthetic urine (SU) dosed with PTF1 at various degrees of glycosylation

| | Particle mode (µm) | Theoretical skewness | Theoretical kurtosis |
|---|---|---|---|
| SU | 22.3 | −0.12 | 0.052 |
| SU + gPTF1 | 22.6 | −0.10 | 0.041 |
| SU + asPTF1 | 24.4 | −0.18 | 0.091 |
| SU + agPTF1 | 30.6 | −0.63 | 0.477 |

Refer to Fig. 1 legend for abbreviations

urine but their sizes varied. The synthetic urine (Fig. 6a), gPTF1-dosed (Fig. 6b) and asPTF1-dosed urines (Fig. 6c) appeared to contain similarly sized aggregates (mean cross-section: ~20 µm). However, the agPTF1-dosed urine typically contained noticeably larger aggregates of CaOx crystals (Fig. 6d) (mean cross-section: 20–30 µm). Several CaOx dihydrate crystals resembling twinned crystals with fourfold rotational symmetry were observed typically in the urine dosed with asPTF1 (Fig. 6c).

## Discussion

Modifications in glycosylation are indicators of many diseased states [22–24]. The presence of several glycoproteins in urine [1], therefore, provides the promise of possible markers for kidney stone disease. Indeed, we have shown a difference between levels of PTF1 sialylation in controls and stone-formers [15].

The present study addressed the hypothesis that the glycosylation of PTF1, and in particular its high proportion of sialic acids, could serve a protective function by reducing CaOx crystallisation. Unlike other studies, techniques were employed that provided information about the three mechanisms of crystallisation independently of one another, thereby providing a higher degree of credibility to the interpretations than was previously possible.

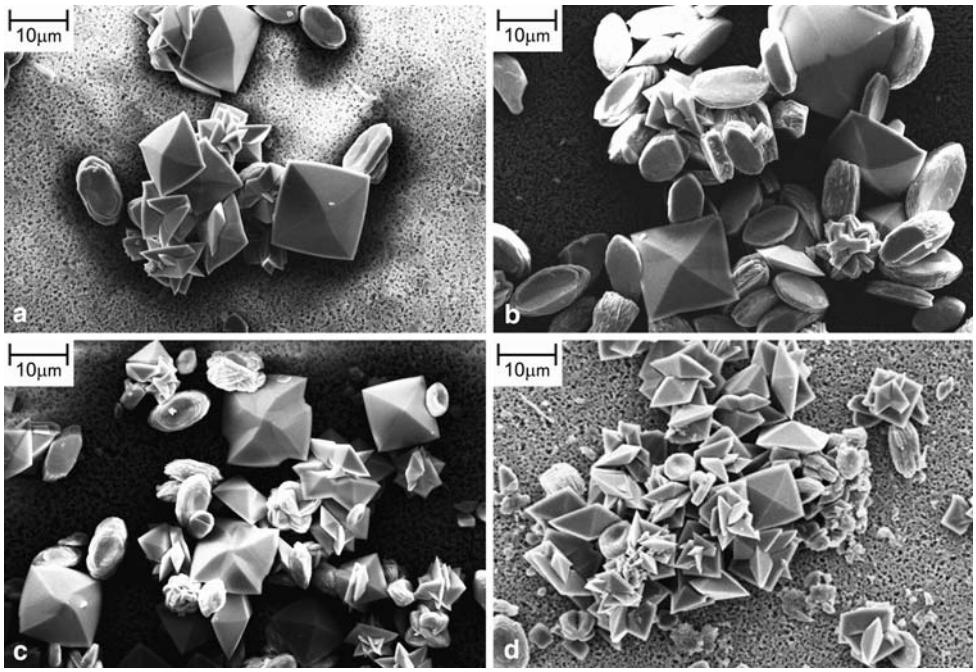


**Fig. 6** Scanning electron micrographs of calcium oxalate crystals deposited in synthetic urine. The crystals were precipitated from synthetic urine (**a**) and synthetic urine dosed with glyco PTF1 (**b**), asialo PTF1 (**c**) and aglyco PTF1 (**d**). All magnifications are ×4,000

## Inhibitory activity studies

Crystallisation studies showed that the fully glycosylated form of PTF1 is a promoter of CaOx crystal nucleation and an inhibitor of CaOx growth in inorganic solutions. The same effects on nucleation [11] and growth [11, 12] have been demonstrated previously in urine, although the nucleation effect was shown using an indirect measurement rather than the direct method used in the present study. When the glycans were removed from PTF1, its ability to promote crystal nucleation was abrogated and particle sizes increased significantly. Since the glycans did not affect PTF1's capacity to inhibit crystal growth, it is concluded that the increase in particle size is due to promotion of aggregation and not from an increase in the size of individual crystals. Scanning electron microscopy provided further evidence in support of this by demonstrating the presence of larger crystal aggregates after dosing with aglyco PTF1 than with the glycoprotein. It is, therefore, evident that glycosylation directs crystal nucleation and aggregation, but not crystal growth.

Five independent studies have investigated the inhibitory activity of the glycoprotein PTF1 [10–14]. Although the test media (ultrafiltered urine and seeded inorganic system), protein source (plasma and urine) and protein concentrations (0.5–2.5 mg/l) varied, they all reported inhibition of crystal aggregation and all but one [13] detected inhibition of crystal growth. In the present study, gPTF1 inhibited crystal growth to a similar extent; however, no activity towards crystal aggregation was detected. This may be due to the use of synthetic urine rather than the seeded inorganic system used in the other "non-urine" studies. However, it is important to note that unlike all of the previous studies, our assay measured crystal nucleation directly. Thus, for the first time our results clearly demonstrate that gPTF1 promotes crystal nucleation. This is interpreted as decreasing the risk of stone formation as it rapidly reduces the supersaturation of CaOx [25].

## Mechanism of inhibition by glycans

While the present study confirmed gPTF1's previously reported effect on CaOx crystallisation, it also provided novel information about the relationship between the protein's molecular structure and its inhibitory activity. The influence of PTF1's glycans on the different mechanisms of crystallisation indicates several features regarding their functionality. Moreover, the fact that the glycans selectively affect the three crystallisation mechanisms suggests that these processes are regulated differently. Firstly, it is possible that the glycans prevent the protein's chelation of calcium and/or oxalate ions. Either of these would inhibit a decrease in the supersaturation of CaOx, culminating in a possible increase in the probability of nucleation and ultimate precipitation of CaOx crystals. Secondly, it is likely that gPTF1 inhibits crystal growth by binding to the surface of CaOx crystals and thereby blocking growth sites [26]. Since the glycans do not affect this mechanism, it is probable that gPTF1 binds via its Gla domain and not the kringle domain on which the glycans are located. Thirdly, it is likely that the glycans aid in PTF1's inhibition of crystal aggregation via the protein's adsorption onto the CaOx crystal surface by means of viscous binding [27]. Another possible mechanism by which agents reduce aggregation is

by altering the surface charge of crystals [26], which could be influenced by the presence or absence of the sugars.

### Sialylation of gPTF1

The function of the glycoprotein's sialic acid residues is of particular interest since there is a difference between levels of urinary PTF1 sialylation in controls and stone-formers [15]. Studies involving other proteins have demonstrated the importance of sialic acids in the context of kidney stone formation. In these studies, partial or complete removal of the sialic acid moieties from Tamm-Horsfall protein [3, 4], osteopontin [6] and bikunin [8] resulted in a decrease in the respective protein's inhibitory activity.

In the present study, sialic acids contributed largely to PTF1's promotory effect on nucleation but had only a small effect on the size of crystal aggregates (as evidenced by changes of 44 and 8% in inhibitory activity after desialylation, respectively); crystal growth was unaffected. Thus the dominant effect of the sialic acid residues on PTF1 is to promote crystal nucleation, which in turn reduces supersaturation, as explained earlier. A recent report suggested that control of supersaturation might be the key to stone prophylaxis [28] and thus we propose that sialylation of PTF1 is a key factor in its protective function against calcium urolithiasis. This hypothesis remains to be tested in an in vitro study. In light of the twinned CaOx dihydrate crystals observed in the asialo PTF1-dosed urine, we speculate that the asialo protein directs the formation of a modified morphology of CaOx dihydrate.

### Kringle and Gla domains of PTF1

PTF1 has a two-domain primary structure. Previous structure–function studies have focused on the N-terminal Gla domain, which contains ten Gla residues that bind strongly to calcium atoms. Activity studies have been carried out using the intact parent protein prothrombin (PT) as well as several of its activation products [14]. When tested in an inorganic solution (at 0.50 mg/l), PTF1 was the most effective inhibitor of crystal growth and aggregation, followed by PT. The two fragments that lacked the PT Gla domain, namely PT fragment 2 and thrombin, were the least effective inhibitors, suggesting that the inhibitory effects of PT and PTF1 could be attributed in part to their Gla domain [14]. This structure–function relationship was later confirmed in (ultrafiltered) urine [13]. The present results have demonstrated the importance of the kringle domain and have thus drawn attention to the fact that both PTF1 domains contribute towards the protein's inhibitory potency. Collectively, the present and previous studies have shown that both protein domains direct crystal aggregation, whereas nucleation and growth are influenced by the kringle and Gla domains, respectively. The Gla domain has yet to be investigated with regard to crystal nucleation.

## Conclusion

The glycans on PTF1 play a pivotal role in the protein's ability to retard CaOx crystallisation. Of the different crystallisation mechanisms, aggregation (and nucleation to a certain extent) is regarded as the most critical in stone formation [29]. Growth is considered pathologically insignificant [30]. Thus, the finding in the present study that the glycans on gPTF1 influence aggregation (and nucleation) is of importance. Indeed, this finding compliments our previous study in which we reported differences in the glycosylation of UPTF1 from controls and stone-formers [15]. The results of the present study suggest that modifications in the glycosylation of urinary PTF1 in stone-formers may compromise its capacity to protect against calcium urolithiasis. Thus, it is clear that further evaluation of the role of glycosylation in kidney stone formation is indicated.

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