

# Native cytokines do not bind to uromodulin (Tamm-Horsfall glycoprotein)

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Uromodulin bound with high affinity to human tumour necrosis factor (TNF) coated on microtitre plates. This interaction was not competitively inhibited by native TNF in solution. No interaction was observed between immobilized uromodulin and TNF in the liquid phase unless conditions were chosen which denatured the latter protein. Recombinant interleukin-1 $\alpha$  adsorbed on microtitre plates also interacted with uromodulin. However, gel filtration experiments demonstrated no interaction between the proteins in the liquid phase. These and additional results indicate that uromodulin interacts with denatured cytokines, but not with native, soluble cytokines.

Uromodulin; Tamm-Horsfall glycoprotein; Interleukin-1; Tumour necrosis factor

## 1. INTRODUCTION

Uromodulin isolated from the urine of pregnant women, has been described as a unique 85 kDa immunosuppressive glycoprotein [1]. The N-linked oligosaccharides were proposed to be the immunosuppressive component [2,3]. TH-glycoprotein is the most abundant protein of renal origin in normal urine but a physiological role for this protein has not been established [4]. The primary structure of uromodulin, recently determined from the cDNA clone [5,6], may be identical to that of TH-glycoprotein [5]. Both proteins

are isolated as high molecular mass aggregates with molecular masses  $>1 \times 10^6$  Da [1,7].

Brown et al. [8] suggested that the immunosuppressive properties of uromodulin are related to its ability to act as an inhibitor of IL-1. As we have discussed elsewhere [9], the apparent inhibition of the IL-1 lymphocyte activating factor assay by uromodulin [8] is probably an artefact unrelated to specific effects on IL-1. It has been reported using a solid-phase binding assay [6,10,11] that uromodulin binds IL-1 $\alpha$  and TNF with high affinity. Here we have characterized this assay in more detail and provide evidence that uromodulin and TH-glycoprotein do not interact with soluble native cytokines.

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**Abbreviations:** TH-glycoprotein, Tamm-Horsfall glycoprotein; TNF, tumour necrosis factor; IL-1, interleukin-1; GM-CSF, granulocyte-macrophage colony stimulating factor; HBcAg, hepatitis core antigen; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Con A, concanavalin A; PBS, phosphate-buffered saline

## 2. MATERIALS AND METHODS

### 2.1. *Proteins*

TH-glycoprotein was purified from urine pools of two males and uromodulin from urine pools of two pregnant females (second and third trimester). The procedure described below was used for both proteins. Urine (2 l) was adjusted to pH 6.0 and NaCl (33.6 g/l) was added. The cloudy solution

was left overnight at 4°C and then centrifuged at  $15000 \times g$  for 60 min. The pellet was resuspended with phosphate-buffered saline (PBS), pH 7.5, containing additional 0.58 M NaCl and centrifuged. The pellet was dissolved in 50 mM Tris-maleate, pH 7.2 (buffer A), containing 8 M urea, dialyzed against buffer A containing 10% (w/v) ethyleneglycol and applied to a Fractogel TSK-65 (s) (Merck, Darmstadt) column 60 cm  $\times$  5 cm diameter, equilibrated in buffer A containing 10% ethyleneglycol. Uromodulin and TH-glycoprotein eluted in the void volume. Proteins were concentrated by ultrafiltration and dialyzed against 3-fold diluted PBS. About 25 mg uromodulin or TH-glycoprotein were obtained.

Recombinant human IL-1 $\alpha$  was purified from *E. coli* as described [12]. Recombinant human TNF, recombinant human GM-CSF and HBcAg were from Biogen (Cambridge, USA). Aldolase was obtained from Sigma.

## 2.2. Solid-phase binding assay

The procedure described by Muchmore and Decker [10,11] was followed with minor modifications. Briefly, flat bottom micro-ELISA plates made of Immulon (Dynatech, Plochingen, FRG) were coated by overnight incubation at 4°C with protein solutions (100  $\mu$ l/well) 1–20  $\mu$ g/ml in 0.1 M carbonate buffer, pH 9.6 (unless otherwise stated). Control wells were incubated with buffer alone. Plates after protein coating, and other described incubations, were washed with PBS containing 0.05% (w/v) Tween 20 (PBS/Tween). Coating of the plates was checked with antibody against the coated protein. Uromodulin or TH-glycoprotein (0.003–3  $\mu$ g/ml in PBS/Tween) were added in duplicate (100  $\mu$ l/well) to coated and non-coated wells and the plates incubated at 37°C for 2 h. In competition experiments, a solution of the protein used to coat the plates was also added. The plates were washed and anti-uromodulin (100  $\mu$ l/well of a 500-fold dilution) added. After incubation for 1 h at 37°C and washing, peroxidase-labelled goat anti-rabbit IgG (Kirkegaard and Perry, Gaithersburg, MD) diluted 1000-fold in PBS/Tween was added (100  $\mu$ l/well) and the plates incubated for a further 1 h at 37°C. Colour development was made by the addition of 0.1% (w/v) 2,2'-amino-di-[3-ethyl-benzthiozoline sulfonate] (Boehringer, Mannheim) in 50 mM sodi-

um citrate, pH 4.8, containing 0.1% (v/v) of 30% H<sub>2</sub>O<sub>2</sub>. Absorbances at 630 nm were measured using a Dynatech MR 580 micro-ELISA autoreader. The absorbances of control non-coated wells were subtracted from corresponding coated wells.

## 2.3. Other methods

IL-1 $\alpha$  was iodinated with mono-iodo-Bolton Hunter reagent as previously described [13]. The radiolabelled IL-1 $\alpha$  had a specific radioactivity of 1500 Ci/mmol and normal activity in an in vitro IL-1 receptor binding assay [14]. For gel filtration experiments, uromodulin or TH-glycoprotein (20  $\mu$ g) in buffer B (PBS/Tween containing 0.01% (w/v) bovine serum albumin and 0.02% (w/v) sodium azide), were premixed with [<sup>125</sup>I]IL-1 $\alpha$  (10<sup>5</sup> cpm) in a volume of 0.5 ml for 2 h at 37°C and applied to a 28 cm  $\times$  1 cm diameter column of TSK-65 (s) equilibrated with buffer B. For some experiments, buffer B also contained [<sup>125</sup>I]IL-1 $\alpha$  (25000 cpm/ml).

Antibodies against proteins used were obtained from New Zealand white rabbits after immunization at multiple sites intradermally. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described [15]. Sedimentation velocity and fluorescence measurements were performed as previously described [16].

# 3. RESULTS AND DISCUSSION

## 3.1. Characterization of uromodulin (TH-glycoprotein)

Uromodulin and TH-glycoprotein gave single bands ( $M_r$  = 85000) when analysed by SDS-PAGE followed by Coomassie blue staining. N-terminal sequence analysis of TH-glycoprotein indicated two N-termini: Asp (75%) and Thr (25%). The results of 14 cycles of Edman degradation indicated a major sequence starting with Asp which was in exact agreement with that predicted by the cDNA sequence for mature uromodulin [5,6]. The N-terminal Thr gave rise to a sequence plus one residue out of phase with the main sequence.

## 3.2. Interaction of uromodulin (TH-glycoprotein) with TNF

The interaction of uromodulin with TNF was studied by the solid-phase binding assay. A strong

interaction was observed between TNF coated on plastic and uromodulin added in solution (fig.1); half-maximal binding of uromodulin occurred at about 0.1–0.075  $\mu\text{g/ml}$ . Hession et al. [6] have recently reported a similar finding. The interaction was highly dependent on the type of microtitre plate used. For example, only a weak interaction was observed when Nunc-Immo microtitre plates (Nunc, Raskilde, DK) were used instead of the Immulon plates (fig.1). The coating of TNF to both types of plate was similar as estimated with anti-TNF. Contrary to the above results, TNF in solution at neutral pH did not bind to uromodulin immobilized on Immulon microtitre plates (fig.2). However, at acidic pH a strong interaction was observed with half-maximal binding occurring at about pH 4.75–4.50 (fig.2). TNF at acid pH undergoes limited protein unfolding and aggregation which can be monitored by changes in tryptophan fluorescence and the sedimentation coefficient ( $s_{20,w}$ ) [16]. Under acid conditions, the fluorescence peak emission wavelength of TNF red-shifts from 320 nm to 345 nm with a concomitant decrease in fluorescence intensity. This change in fluorescence, which titrates with an apparent  $pK_a$  of 4.5 (fig.2), is due to solvent exposure of buried tryptophan residues accompanying protein unfolding. Protein aggregation at acid pH was indicated by large increases in the  $s_{20,w}$  value (fig.2).

Mouse TNF which shares a high degree of sequence similarity with human TNF [17] rapidly loses its in vitro biological activity under acid conditions [18]. This fact, together with the above findings suggest that uromodulin interacts more strongly with aggregated/denatured, and presumably inactivated, TNF than with native protein. The strong interaction between surface-adsorbed TNF and uromodulin in solution (fig.1) is consistent with this conclusion since adsorption of proteins to plastic surfaces can, depending on the stability of the protein, lead to denaturation and aggregation [19]. The nature of the plastic surface might be expected to influence this process (fig.1).

Further support for the non-specific nature of the uromodulin-TNF interaction was obtained by competition studies in which uromodulin and TNF at neutral pH (0–10  $\mu\text{g/well}$ ) were added sequentially to microtitre plates coated with TNF. The result that TNF, in the liquid phase, failed at all

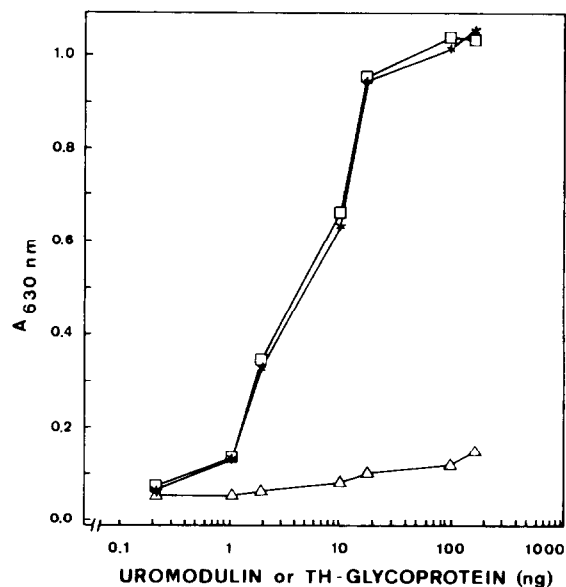


Fig.1. Binding of uromodulin to TNF-coated Immulon (□) or Nunc-Immuno (Δ) micro-ELISA plates and of Tamm-Horsfall glycoprotein to TNF-coated Immulon plates (\*). The plates were coated by overnight incubation with 100 ng TNF/well in buffer, washed and processed as described in section 2.

concentrations tested to inhibit the binding of uromodulin to immobilized TNF again indicates that native TNF does not bind to uromodulin.

### 3.3. Interaction of uromodulin (TH-glycoprotein) with IL-1 $\alpha$

Muchmore and Decker [10] found that uromodulin binds with high affinity ( $K_d = 3 \times 10^{-10}$  M) to surface coated recombinant murine IL-1 $\alpha$  (the characteristics of the IL-1 $\alpha$  preparation used were not reported). Using a well characterized preparation of human IL-1 $\alpha$  [12] we have been unable to reproduce this result although we did observe some interaction ( $K_d > 10^{-9}$  M). On the other hand, when uromodulin was immobilized, no interaction with IL-1 $\alpha$  in solution was observed over a wide range of protein concentration (not shown). There was also no interaction between IL-1 $\alpha$  and uromodulin when both proteins were in the liquid phase. Thus, preincubated mixtures of uromodulin and IL-1 $\alpha$  subjected to size exclusion chromatography (see section 2) resolved into protein eluted in the void volume (uromodulin) and protein eluted with an apparent  $M_r$  of 18000

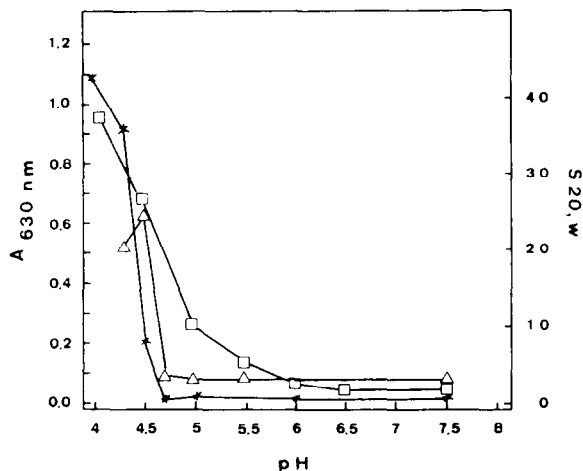


Fig. 2. The pH-dependent interaction of soluble TNF with uromodulin-coated Immulon-plates ( $\square$ ). The changes in the sedimentation coefficient,  $S_{20,w}$  ( $\Delta$ ) and fluorescence emission intensity at 317 nm ( $*$ ) as functions of pH are also indicated. The microtitre plate was coated with 250 ng uromodulin/well, washed with PBS/Tween and then incubated with 100 ng TNF/well in PBS (pH 7.5) or in 0.1 M sodium acetate buffers with pH ranging from 4.0 to 6.5 for 2 h at 37°C.

(IL-1 $\alpha$ ). SDS-PAGE and Western blot analysis of column fractions, indicated no co-elution of IL-1 $\alpha$  and uromodulin. Also, the elution volumes of the separated proteins were the same as when they were chromatographed individually. To measure possible weak interactions, a mixture of uromodulin and IL-1 $\alpha$  were applied to a column pre-equilibrated with buffer containing radio-labelled IL-1 $\alpha$  (see section 2). Column fractions containing uromodulin had the same specific radioactivity as the starting column buffer indicating that no binding of IL-1 $\alpha$  to uromodulin had occurred. These results indicate that uromodulin interacts with surface-bound IL-1 $\alpha$  but not with the native protein in solution.

### 3.4. Interaction of uromodulin (TH-glycoprotein) with other proteins

Rabbit muscle aldolase is a tetrameric protein which, analogous to trimeric TNF, rapidly inactivates at pH values below 5 due to protein unfolding [20]. Microtitre plates were coated with aldolase at pH 9.6 (native state) and at pH 3.5 (unfolded state). Protein coated at either pH showed an equivalent strong interaction with uromodulin

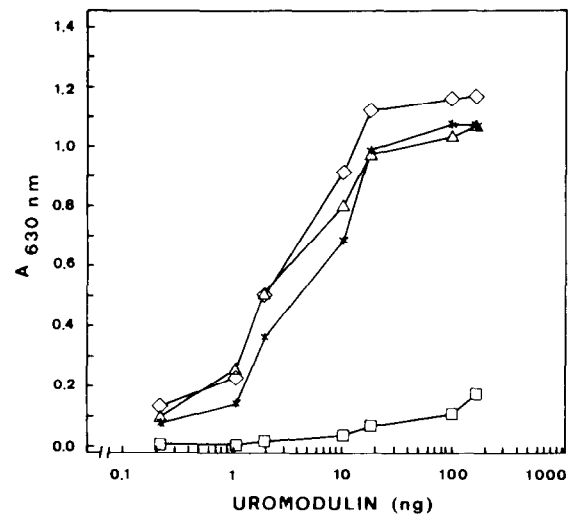


Fig. 3. Binding of uromodulin to plates coated with various proteins. HBcAg ( $\square$ ) and TNF ( $\Delta$ ) were coated at 100 ng and 2  $\mu$ g/well, respectively. Aldolase was coated at 300 ng/well in pH 7.0 ( $*$ ) and pH 4.0 ( $\diamond$ ) buffer.

(fig. 3). The binding affinity was similar to that obtained with immobilized TNF (fig. 1).

Uromodulin bound with a 100-fold lower affinity to immobilized hepatitis core antigen (HBcAg) (fig. 3). HBcAg is a multimeric complex which is extremely resistant to pH and protein chaotrope-induced denaturation (unpublished, P.W.) and is, therefore, less likely to denature upon adsorption to plastic surfaces than, for example, TNF. Recombinant GM-CSF purified from *E. coli* is a monomeric protein containing two intramolecular disulphide bonds [21]. Reduction of disulphide bonds with dithiothreitol leads to protein unfolding and aggregation (unpublished, P.W.). When native and reduced GM-CSF were separately coated on microtitre plates, only the latter bound uromodulin; the binding affinity was similar to that observed with TNF (fig. 1).

## 4. CONCLUSIONS

Uromodulin and TH-glycoprotein behaved similarly to each other in all the binding assays described here, adding further support for the identity of these two proteins. (Uromodulin supplied by Dr A.V. Muchmore [1] behaved similarly to the uromodulin used here.) Both proteins did

not bind in solution to the cytokines tested namely, IL-1 $\alpha$  and TNF, but did interact with these proteins when they were adsorbed on plastic, that is, under conditions where they are likely to be unfolded and aggregated. The 'assay-specificity' of monoclonal antibodies has also been shown to be related to whether proteins are coated on solid surfaces or are in solution [22]. The high affinity binding of uromodulin to proteins unrelated to the above mentioned cytokines namely, aldolase and to a lesser degree HbcAg, also point to the non-specific nature of this interaction.

The lack of affinity in solution between uromodulin/TH-glycoprotein and IL-1 $\alpha$  is also consistent with the following observations: (a) the glycoproteins have no inhibitory effect on co-stimulus independent bioassays for IL-1 (unpublished, P.M.); (b) uromodulin has no effect on the receptor-binding affinity of IL-1 $\alpha$  (Winger, L., personal communication); and (c) IL-1 $\beta$  immobilized under mild conditions to Sepharose 4B (using Tressyl activated Sepharose) fails to bind uromodulin from solution (Muchmore, A.V., personal communication). These observations, together with the findings reported here, strongly suggest that native cytokines namely, IL-1 $\alpha$ , TNF and GM-CSF, do not bind to uromodulin (TH-glycoprotein). Uromodulin is, therefore, unlikely to be an inhibitor of soluble IL-1.

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