Mapping the Binding Domain of Immunoglobulin Light Chains for Tamm-Horsfall Protein

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Cast nephropathy, or myeloma kidney, is a potentially reversible cause of chronic renal failure. In this condition, filtered light chains bind to a common site on Tamm-Horsfall protein (THP), which is produced by cells of the thick ascending limb of the loop of Henle. Subsequent aggregation of these proteins produces casts that obstruct tubule fluid flow and results in renal failure. In the present study, we used the yeast two-hybrid system to determine the site of interaction of light chains with THP. The third complementarity-determining region (CDR3) of both κ and λ light chains interacted with THP. These findings were confirmed in a series of competition studies using a synthetic peptide that corresponded to the CDR3 region and purified THP and light chains. Variations in the CDR3 sequence of the light chain affected binding. Thus, the current studies increase our understanding of the process of cast formation and provide an opportunity to develop strategies that may inhibit this interaction and prevent the clinical manifestations of myeloma kidney. (*Am J Pathol* 2001, 158:1859–1866)

More than 150 years ago, an astute clinician, Dr. William Macintyre, found an abnormal substance in the urine of one of his patients. He sent the material to Dr. Henry Bence Jones, who reported the newly described protein and the association with multiple myeloma.¹ Bence Jones proteins were subsequently identified as immunoglobulin light chains.² Light chains are typically filtered from the blood by the kidney and metabolized similarly to other low molecular weight proteins.³ However, these proteins can be nephrotoxic.⁴ During the process of absorption and catabolism, light chains have been shown to cause proximal tubular epithelial cell injury.^{4,5} When the reabsorptive capacity of the proximal tubular cells is saturated, light chains are presented to the distal nephron, where they form casts that obstruct flow of tubular fluid. The resultant renal failure is known clinically as cast nephropathy, or myeloma kidney.⁶

Cast nephropathy represents the most common cause of renal failure in multiple myeloma.⁷ To initiate cast formation, light chains bind to a specific peptide domain on Tamm-Horsfall protein (THP),^{8–12} which is synthesized exclusively by cells of the thick ascending limb of the loop of Henle.^{13,14} Co-aggregation of light chains with THP produces the intraluminal casts that are the prominent feature of myeloma kidney.⁸ The electrolyte composition of the tubule fluid as well as tubule fluid flow rates and amount of THP^{8–12} modulate binding. The structure of the light chain plays an important role in association with THP¹⁰ and may also promote homotypic aggregation.¹⁵ Although myeloma kidney is potentially reversible, prevention of cast formation is the key to controlling the problem. Understanding the protein interactions involved in cast formation represents the initial advance in development of potential treatment strategies designed to prevent myeloma kidney. The current study determined the domain on the light chain involved in binding THP.

Materials and Methods

Yeast Two-Hybrid Studies

The yeast two-hybrid system (Matchmaker LexA Two-Hybrid System; Clontech Lab. Inc., Palo Alto, CA) was used initially to detect binding interactions between THP and immunoglobulin light chains. This approach was similar to the original description of the yeast two-hybrid assay,¹⁶ but was a LexA-based interaction trap system.¹⁷ The host strain in these experiments was *Saccharomyces cerevisiae* EGY48[p8op-lacZ]. The bait consisted of two fragments of human THP that were obtained by polymerase chain reaction using cDNA that was provided by Genentech, Inc. (South San Francisco, CA). Description of the cloning and characterization of THP has been published.¹⁸ All primers used in this study were obtained commercially (Operon Tech. Inc., Alameda, CA). Because there is a single binding domain (amino acid residues 225 to 233) for light chains on THP,^{10,12} the present

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Table 1. Primers Used to Generate the κ Light Chain Constructs

Light chain construct, κ	Primer name	Forward and reverse primers
ITPBL5	LB62	5'- <u>CCGGAATTCGAAATTGTGTTGACGCAGTCTCCA</u> -3'
	H170	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'
ITPBL11	LB62	5'- <u>CCGGAATTCGAAATTGTGTTGACGCAGTCTCCA</u> -3'
	H170	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'
BC Syn9	LB62	5'- <u>CCGGAATTCGAAATTGTGTTGACGCAGTCTCCA</u> -3'
	H170	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'
SSH23	LB62	5'- <u>CCGGAATTCGAAATTGTGTTGACGCAGTCTCCA</u> -3'
	LSK19	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'
SSH23a	LB62	5'- <u>CCGGAATTCGAAATTGTGTTGACGCAGTCTCCA</u> -3'
	SSH23a	5'- <u>ACGCTCGAGTGGAGATTGGCCTGGCCT</u> -3'
SSH23b	SSH23b	5'- <u>CCGGAATTCAGGCGCCTAATTATAAG</u> -3'
	H170	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'
SSH23c	SSH23c	5'- <u>CCGGAATTCATTACTGCATGCAA</u> -3'
	H170	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'
SSH23 Δ	LB62	5'- <u>CCGGAATTCGAAATTGTGTTGACGCAGTCTCCA</u> -3'
	LSK19	5'- <u>ACGCTCGAGAACTGCTCATCAGATGGCGGGAAG</u> -3'

Underlined sequences contained EcoRI and Xho I restriction sites on the forward and reverse primers, respectively.

study used two fragments of THP that contained this domain. A 787-bp fragment (encoding amino acid residues 148 to 410, termed THP787) was polymerase chain reaction-amplified using 5'-CCGGAATTCCAATGTGGTGGGCAGCTACTT-G-3' as the forward primer and 5'-ACGCTCGAGCTCCACGGAGCTGGGGTCTGTGC-3' as the reverse primer. Underlined sequences in the upstream and downstream primers contained an EcoRI and XhoI site, respectively. A 248-bp fragment (encoding amino acid residues 188 to 270, termed THP248) was created using 5'-CCGGAATTCCGCATGGCCGAGACCTGCGTGC as the forward primer and 5'-ACGCTCGAGCTCCACGGAGCTGGGGTCTGTGC-3' as the reverse primer. THP787 and THP248 were each ligated into pBluescript II SK(-) vector (Stratagene, La Jolla, CA) using EcoRI and XhoI and then cloned. Sequences of both products were confirmed using a kit (T7 Sequenase DNA sequencing kit; Amersham Life Science, Inc., Cleveland,

OH). THP787 and THP248 were then cloned into the pLexA expression vector using EcoRI and XhoI.

Representative cDNAs encoding immunoglobulin κ and λ light chains were generous gifts from Dr. S. Louis Bridges, Jr., at the University of Alabama at Birmingham, Birmingham, AL. A total of four unique κ and seven λ light chains were used. Descriptions of these molecules have been published.¹⁹⁻²¹ Each cDNA was used as a template in polymerase chain reactions to generate fragments that were initially cloned into pBluescript II SK(-) for sequencing and then inserted in-frame into pB42AD expression vector, using EcoRI and XhoI. The expressed fusion proteins consisted of the light chain of interest and the LexA transcriptional activation domain. Primers and light chains used in these reactions were shown in Tables 1 and 2. A series of truncation and deletion mutants of the κ light chain, SSH23, and the λ light chain, ITPBLL1, were

Table 2. Primers Used to Generate the λ Light Chain Constructs

Light chain construct, λ	Primer name	Forward and reverse primers
ITPBL1	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL1a	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	PBL1a	5'- <u>ACGCTCGAGT</u> CGCTCAGGGATCTCTGA-3'
ITPBL1b	PBL1b	5'- <u>CCGGAATTC</u> AGCGACCGGCCCTCAGAG-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL1c	PBL1b	5'- <u>CCGGAATTC</u> AGCGACCGGCCCTCAGAG-3'
	PBL1c	5'- <u>ACGCTCGAGT</u> AGGACGGTGACCTT-3'
ITPBL1d	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	PBL1d	5'- <u>ACGCTCGAGAT</u> AGACGACAGCAC-3'
LKPBL12	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL11	LB76	5'- <u>CCGGAATTC</u> TCCGAATTCTCTGCACAGTCTCTGAGGCC-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL22	LB77	5'- <u>CCGGAATTC</u> CCCTGAATTCTCGGCGTCTTGCTTACTGCA-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL68	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL7	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'
ITPBL75	LB75	5'- <u>CCGGAATTC</u> TCCGAATTCTCCTCTCTCACTGCACAG-3'
	LB69	5'- <u>ACGCTCGAGGGGAATTCGCTCCCGGGTAGAAGTCTCT</u> -3'

Underlined sequences contained Eco RI and Xho I restriction sites on the forward and reverse primers, respectively.

Figure 1. To determine the THP-binding domain on light chains, a series of mutants of κ (SSH23) and λ (ITPBL1) light chains was examined. The four framework regions (FR) and three CDRs of the variable subunit of the light chains are shown. The lengths (in bp) of the constructs and interaction of the fusion proteins with THP are displayed.

also created (Figure 1). Restricting the 506-bp fragment with *Bsi*ZI that cut the insert in two unique sites, and subsequently purifying and intramolecularly re-ligating the product created a truncation mutant of SSH23. To generate the SSH23pep construct, two 5'-phosphorylated synthetic oligonucleotides, 5'-CCGGAATTCATGCAAGGTACACACTGGCCTCCGCTCACTCTCGAGCGT-3' and 5'-ACGCTCGAGAGTGAGCGGAGGCCAGTGTGTACCTTGCATGAATTCCGG-3', were annealed and ligated into pBluescript II SK(-) for sequencing and then into pLexA, using *Eco*RI and *Xho*I. This sequence encoded a 10-amino acid peptide (MQGTHWPPLT) that corresponded to the amino acid residues from 94 to 103 on SSH23. ITPBL1pep, which encoded a 10 amino acid peptide (QVWDSTSDHY) that corresponded to amino acid residues 88 to 97 of ITPBL1, was prepared in similar manner using 5'-phosphorylated 5'-CCGGAATTCAGGTATGGGATAGTACTAGTGATCATTATCTCGAGCGT-3' and 5'-ACGCTCGAGATAATGATCACTAGTACATCCCATACCTGGAATTCCGG-3'. Both SSH23pep and ITPBL1pep corresponded to the complementarity-determining region (CDR)3 of light chains. Automated

DNA sequencing confirmed proper construction of all plasmids and authenticity of the sequences.

EGY48[p8op-lacZ] yeast were co-transformed with pLexA-THP787 (or pLexA-THP248) and pB42AD-light chain construct, using the lithium acetate method (Alkali-Cation yeast transformation kit; BIO101 Inc., La Jolla, CA). EGY48[p8op-lacZ] yeast were also co-transformed with plasmids (pLexA-Pos alone and pLexA-53/pB42AD-T) that served as positive control experiments and with plasmids (pLexA-Lam/pB42AD-T) that served as a negative control. The pLexA-Pos plasmid encoded a fusion of the DNA-binding domain (LexA) with the GAL4-activating domain, allowing activation of genes under control of LexA operators (*LEU2* and *lacZ*). pLexA-53 and pB42AD-T provided another positive control experiments by encoding proteins known to interact (murine p53 and SV40 large T-antigen, respectively). pLexA-Lam encoded a fusion of the DNA-binding domain with human lamin C that does not interact with SV40 large T antigen and thus served as a negative control. Double transformants were selected in a standard manner using medium

that lacked histidine and tryptophan. The transformants were incubated at 30°C for 4 days. Interactions between the two hybrid proteins were then tested by growth in galactose-containing, leucine-deficient medium and by β -galactosidase plate and liquid culture assays. In the *in vivo* plate assay, 200 to 400 cfu of transformed yeast were dispersed on to 100-mm agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, 80 mg/L), $1\times$ BU salts (26 mmol/L Na_2HPO_4 , 25 mmol/L Na_2HPO_4 , pH 7), and either 2% galactose or 2% dextrose. The plates were incubated at 30°C for 3 to 6 days to generate the blue color. The interactions were further quantified by liquid culture assay of β -galactosidase activity using *o*-nitrophenyl β -D-galactopyranoside (Amersham Life Science, Inc., Cleveland, OH) as the substrate. Using other proteins, relative affinities detected with this reaction have been shown to correlate with interactions detected using other biochemical methods.²² Five separate transformants were each examined in triplicate. Reactions were performed at 30°C in solutions containing 60 mmol/L Na_2HPO_4 , 40 mmol/L NaH_2PO_4 , 10 mmol/L KCl, 1 mmol/L MgSO_4 , and 50 mmol/L β -mercaptoethanol. Timing of the reaction began as *o*-nitrophenyl β -D-galactopyranoside was added and the reaction continued until a yellow color was observed. The reaction mixture was then centrifuged at 14,000 rpm for 10 minutes to pellet cell debris. Absorbance of the supernatant at 420 and 600 nm was determined. Units of β -galactosidase activity were calculated as follows: units = $[1,000 \times \text{OD}_{400}] / [(\text{elapsed time}) \times (0.1 \times \text{concentration factor}) \times \text{OD}_{600}]$.

To verify expression of the fusion proteins, selected samples were examined using Western blotting. Yeast cells were pelleted, then resuspended in cracking buffer, 8 mol/L urea, 5% sodium dodecyl sulfate, 40 mmol/L Tris-HCl, 0.1 mmol/L ethylenediaminetetraacetic acid, 0.08% β -mercaptoethanol and a combination of protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany) was included. The yeast were disrupted by vigorous shaking for 3 minutes at 5,000 rpm (MiniBeadbeater; Biospec Products Co., Bartlesville, OK) after addition of 425 to 600 $\mu\text{mol/L}$ glass beads (Sigma Chemical Co., St. Louis, MO). Cell debris was removed by centrifugation at 14,000 rpm for 5 minutes at 4°C. Western blot detection of THP and light chains then proceeded in standard manner.^{23,24} Briefly, the supernatant fractions were boiled briefly, then separated using 8 or 12% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were then probed using rabbit anti-human THP antiserum (Biomedical Technologies, Inc., Stoughton, MA) or rabbit anti-human Ig/L-chain type lambda and type kappa (Behringwerke AG, Marburg, Germany).

Purification of Human THP and Immunoglobulin Light Chains

Human THP and light chains were obtained from urine in standard manner.⁸⁻¹² THP was purified from urine of a healthy adult male by precipitation in 0.64 mol/L NaCl, followed by dialysis and lyophilization. Purified human

THP was biotinylated as described previously,²⁵ using sulfo-*N*-hydroxysuccinimide biotin (ImmunoPure Sulfo-NHS-Biotin; Pierce Chemical Co., Rockford, IL), followed by dialysis against phosphate-buffered saline (PBS) at 4°C for 24 hours to remove free biotin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis identified a single band at ~ 100 kd (data not shown). Six different light chains (three κ and three λ) were also used in this study and were obtained from patients who had light chain proteinuria and clinical renal failure. The patients who donated $\lambda 2$ and $\lambda 5$ had biopsy-proven cast nephropathy. Patients who donated $\lambda 5$, $\kappa 3$, and $\kappa 7$ had clinical presentations that were compatible with cast nephropathy, but did not undergo kidney biopsy. The patient who donated $\kappa 6$ had Waldenström's macroglobulinemia and progressive renal failure and was also not biopsied. These light chains were purified from urine by precipitation using 70% ammonium sulfate, followed by ion-exchange chromatography.^{4,5,8,9} Purified light chains were dialyzed and lyophilized. A single band at ~ 22 kd was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Proteins were kept at -20°C until use.

Peptide Competition Experiments

A synthetic peptide was obtained commercially (Research Genetics Inc., Huntsville, AL). MQGTHWPPLT corresponded to the CDR3 region of SSH23. Peptides were purified by high-pressure liquid chromatography; the molecular masses were confirmed by mass spectrometry.

Competition experiments were performed using solid-phase enzyme-linked immunosorbent assay and Western and dot blotting. To perform enzyme-linked immunosorbent assay, wells of microtiter plates were coated with each of the six purified light chains, 0.2 mmol/L in PBS, and incubated overnight at room temperature. Biotinylated human THP, 0.2 $\mu\text{mol/L}$, was incubated overnight at 4°C alone or with the CDR3 peptide. The concentration of the peptides ranged from 0 to 4 mmol/L. After washing the wells with 0.05% Tween 20 in PBS and blocking with 1% bovine serum albumin in PBS, the pre-incubated, THP/peptide mixtures were added to the wells and incubated at 37°C for 2 hours. After washing, samples were incubated with avidin conjugated with horseradish peroxidase (ImmunoPure avidin, horseradish peroxidase conjugated; Pierce Chemical Co., Rockford, IL), 1:2,000 dilution in PBS. After additional washes, wells were developed using Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) as substrate in citrate-phosphate buffer, pH 4.2. Optical density was determined at 405 nm using a microplate reader (VERSAmax; Molecular Devices Corp., Menlo Park, CA).

In other competition experiments, 10 μg samples of each of the six light chains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12% polyacrylamide gels and then transferred onto a nitrocellulose membrane. Twenty μg of yeast extract containing the fusion proteins were dot-blotted directly onto a

Table 3. Summary of the Interactions between the Two THP Fusion Proteins with the Light Chain Proteins

Light chain	Isotype	Size (bp)	Interaction with THP β -galactosidase activity (units)	
			248-bp fragment	787-bp fragment
κ				
ITPBL5	κ I	480	4.0 ± 0.1	4.3 ± 0.4
ITPBL11	κ II	465	3.4 ± 0.5	5.4 ± 0.6
BC Syn9	κ IV	462	3.2 ± 0.2	4.4 ± 0.3
SSH23	κ II	506	5.4 ± 0.2	5.6 ± 0.1
SSH23a		147	NI	NI
SSH23b		392	5.8 ± 0.2	5.8 ± 0.2
SSH23c		123	5.7 ± 0.2	5.4 ± 0.1
SSH23 Δ		372	NI	NI
SSH23pep		30	5.8 ± 0.4	5.8 ± 0.8
λ				
ITPBLL1	λ IIIa	434	7.1 ± 0.1	7.5 ± 0.8
ITPBLL1a		180	NI	NI
ITPBLL1b		283	9.2 ± 0.5	14.1 ± 1.1
ITPLL1c		173	5.1 ± 0.3	6.7 ± 0.3
ITPBLL1d		146	NI	NI
ITPBLL1pep		30	7.5 ± 0.6	6.2 ± 1.0
LKPBLL2	λ I	442	4.0 ± 0.2	3.5 ± 0.5
ITPBLL11	λ IIIb	430	4.8 ± 0.7	4.1 ± 0.5
ITPBLL22	λ IIIc	426	5.1 ± 0.6	6.8 ± 1.3
ITPBLL68	λ IV	441	4.5 ± 0.4	3.5 ± 0.6
ITPBLL7	λ V	454	3.6 ± 0.5	3.9 ± 0.4
ITPBLL75	λ VI	431	2.2 ± 0.2	2.5 ± 0.1

All interactions were galactose-specific. NI, no interaction.

nitrocellulose membrane. After blocking and washing, the blots were probed with 0.2 μ mol/L biotinylated THP or 0.2 μ mol/L biotinylated THP that had been pre-incubated with CDR3 peptide, 4 mmol/L, overnight at 4°C. After additional washes, the membranes were incubated with streptavidin-conjugated HRP, 1:10,000 dilution in Tris-buffered saline. The membranes were developed using ECL Western blotting system and Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical Analysis

All data were presented as mean $\pm$ SE. Significant differences were determined using one-way analysis of variance with standard post hoc testing (Statview, version 5.0; SAS Institute, Inc., Cary, NC). A *P* value of < 0.05 assigned statistical significance.

Results

Human THP Interacted with Human Light Chains *in Vivo*

Initial experiments ensured that the hybrid proteins that consisted of the pLexA DNA-binding domain and the two fragments of human THP did not, by themselves, *trans*-activate the reporter genes. Both peptide segments of THP contained the known single binding site for light chains.^{10,12} The light chain constructs that were tested are shown in Tables 1 and 2. Western analysis of extracts of representative co-transformed yeast confirmed expression of fusion proteins that contained THP and light chain (Figure 1). When co-expressed with pLexA-

THP248 and pLexA-THP787, fusion proteins that consisted of the LexA activation domain and both κ and λ light chains activated both *LEU2* and *lacZ* reporter genes. Reporter gene activity was strictly galactose-dependent. There were no interactions among any of the light chain constructs and pLexA-Lam, which encoded human lamin C protein. Also, transformation of yeast with pB42AD-T, which encoded the SSV40 large T-antigen, and either pLexA-THP248 or pLexA-THP787 did not activate either reporter gene. Interactions were further quantified using a liquid culture assay of β -galactosidase activity (Table 3). Although all of the light chains interacted with THP, the relative strength of the interactions differed among the 11 light chains examined. The λ VI light chain, ITPBLL75, showed a low-affinity interaction: yeast transformed with this construct grew slowly in leucine-deficient medium and possessed low β -galactosidase activity. The λ IIIa light chain, ITPBLL1, demonstrated the highest binding affinity among the light chains tested.

The CDR3 Domain of the Variable Region of the Light Chain Interacted with THP

To identify the domain on the light chain that interacted with THP, a series of truncated constructs of SSH23 and ITPBLL1 (Figure 2) were tested for binding to the THP fragments *in vivo*. Deletion of the N-terminal portions of the variable regions of both SSH23 and ITPBLL1 did not abolish interaction with THP. Removal of the constant homology domains of the light chains also did not alter binding. In contrast, SSH23 Δ , which lacked a portion of the third and fourth framework regions and the CDR3 region, did not interact with THP. The two 10-amino acid

Figure 2. Western blots of cytoplasmic extracts of yeast co-expressing fusion proteins that consisted of the pLexA DNA-binding domain and a fragment of human THP and the LexA activation domain fused to κ light chains. **Top:** The yeast expressed the κ light chain/LexA activation domain proteins (ITPBL5, ITPBL11, BC Syn 9, and SSH23). **Bottom:** Expression of the THP/pLexA DNA-binding domain fusion protein. The expected contribution of the κ light chain and THP fragments to each of the fusion proteins was ~ 23 kd and 29 kd, respectively.

peptide sequences that represented the CDR3 portions of both molecules interacted with THP *in vivo*.

A Synthetic Peptide Corresponding to the CDR3 Domain Inhibited Binding of Light Chains to THP

Biotinylated human THP bound to six different light chains, three κ and three λ , which had been purified from patients who had renal failure and light chain proteinuria. Pre-incubation of the THP with a synthetic 10-amino acid peptide that represented the CDR3 region of SSH23 effectively inhibited interaction of THP with the bound light chains (Figure 3). Using enzyme-linked immunosorbent assay, this peptide inhibited, in a dose-response manner, binding of THP to these light chains (Figure 3). Extracts from yeast that had been transformed with the various light chain constructs were dot blotted and then incubated with biotinylated THP to show binding (Figure 4). As anticipated from the *in vivo* studies, THP bound to samples that contained SSH23, SSH23b, SSH23c, and SSH23pep, but did not interact with samples containing SSH23a and SSH23 Δ . THP also bound to samples that contained ITPBLL1, ITPBLL1b, and ITPBLL1c, but not extracts from yeast that expressed ITPBLL1a and ITPBLL1d. These findings confirmed the *in vivo* studies described above. Pre-incubation of THP with the CDR3 peptide resulted in inhibition of binding to these samples.

Figure 3. **Top:** Biotinylated human THP binds to six different human light chains purified from the urine of patients with renal failure and multiple myeloma. Pre-incubation of THP (0.2 $\mu\text{mol/L}$) with the CDR3 peptide, MQGTHWPPLT (4 mmol/L), which corresponded to the CDR3 region of SSH23, inhibited binding to the light chains. **Bottom:** The dose-response effect of this peptide on inhibiting binding of THP to these same light chains bound to microtiter wells. An inhibitory effect was observed even at the lowest concentration (4 $\mu\text{mol/L}$) of peptide.

Discussion

Cast nephropathy is a form of progressive renal failure that occurs in the setting of multiple myeloma. Although it was

THP alone			THP + CDR3 peptide	
SSH23	—	IT PBL1	SSH23	IT PBL1
SSH23a	—	IT PBL1a	SSH23a	IT PBL1a
SSH23b	—	IT PBL1b	SSH23b	IT PBL1b
SSH23c	—	IT PBL1c	SSH23c	IT PBL1c
SSH23del	—	IT PBL1d	SSH23del	IT PBL1d
SSH23pep	—	pB42AD	SSH23pep	pB42AD

Figure 4. Biotinylated human THP binds to samples of extracts from yeast expressing the various light chain fusion proteins (**left**). As anticipated from the *in vivo* studies, biotinylated THP bound to samples containing SSH23, SSH23b, SSH23c, SSH23pep, ITPBLL1, ITPBLL1b, and ITPBLL1c. Binding was inhibited by pre-incubation of the THP, 0.2 $\mu\text{mol/L}$, with the CDR3 peptide, MQGTHWPPLT, 4 mmol/L (**right**). Both gels were produced simultaneously and developed using the same film and developing solution.

well known that immunoglobulin light chains participate integrally in the process,^{26–30} dissecting the pathogenesis of this complex problem proved difficult. More recently, evidence demonstrated an important role of THP in cast nephropathy.^{8–12} Using immunofluorescence microscopy, THP has been shown to be a component of the casts.³¹ THP, a protein that is synthesized by cells of the thick ascending limb of the loop of Henle,^{13,14} exists in the kidney both in soluble form and attached to the outer leaflet of the apical plasma membrane by a phosphatidylinositol (GPI) anchor.^{18,32} In an experimental model of cast nephropathy, this area of the nephron was also the initial site of cast formation. By altering production of THP, intranephronal obstruction from light chain precipitation was prevented.⁹ Subsequent analysis of the THP interaction with light chains demonstrated a single binding site on THP.¹² The resultant aggregation of these proteins produced intraluminal obstruction from cast formation.⁹ The present studies used the yeast two-hybrid system, which has been successfully used to identify biologically important protein-protein interactions,^{17,22,33–37} to clarify the interaction of THP with light chains. The THP-binding site on both κ and λ light chains was the CDR3 region in the variable domain of the molecules. The yeast two-hybrid findings were confirmed by showing a synthetic peptide that corresponded to the CDR3 region of SSH23 inhibited binding of THP to six different light chains from patients who had light chain proteinuria and clinical manifestations of renal failure. This peptide also inhibited binding of purified THP to the fusion proteins that were present in the yeast extracts (Figure 4).

Every light chain contains two globular subunits that are termed variable and constant homology domains.³⁸ The variable domain consists of a series of four framework regions that form irregular β -pleated sheets that surround a tightly packed hydrophobic interior.^{39,40} Three hypervariable segments, termed CDRs, configure part of the antigen-binding site on the immunoglobulin molecule.^{38,41} The CDRs form loop structures and represent the regions of sequence variability among light chains.^{42–44} Thus, although possessing similar structures, no two light chains are identical. The CDR3 region is perhaps the most variable portion of the molecule, in part because of V-J recombination and because of the capability of terminal deoxynucleotidyl transferase to insert nongerm-line-encoded nucleotides in this region.^{19,20,45} It is therefore interesting that CDR3 binds THP, although there are similarities in this region among many κ and λ light chains. Kabat and colleagues,⁴⁶ first identified the appearance of certain amino acid residues in CDRs of heavy and light chains. Mian and associates⁴⁷ suggested that amphipathic amino acids, especially tryptophan and tyrosine, are frequently present in CDR regions, because they permit flexibility to interact with a wide range of antigens. Thus, although required to promote antigen binding, these residues also allow cross-reactivity with other proteins. However, the relative affinities of the light chains for THP varied, with one of the λ light chains, ITPBLL75, in the present study showing very low affinity. The Kyte-Doolittle hydrophathy plot of the CDR3 region of ITPBLL75 differed from the plots of SSH23 and ITPBLL1, which were light chains that

showed higher binding affinities for THP. Thus, the entire domain appeared to modulate the interaction.

In summary, the identification of the CDR3 region as the single binding site of light chains for THP provides new insights into the pathogenesis of cast nephropathy related to multiple myeloma. Differences in the CDR3 region accounts for the variable affinity of light chains for THP, when this process is examined under controlled conditions. Our previous studies, using a rodent model of *in vivo* cast formation, showed that binding of light chain to THP was required for cast formation.^{8,9,48} However, other factors modulate this interaction and determine the clinical expression of the disease.^{9–11} For example, a light chain from a patient who had no clinically evident renal dysfunction bound THP *in vitro*, albeit at lower affinity. This same light chain did not obstruct the lumen of perfused nephrons of euvoletic rats, but did obstruct nephrons of hydropenic rats.⁹ Thus, although there is only a general correlation between binding affinity and clinical cast nephropathy, all tested light chains that potentially form casts *in vivo* also bind THP.^{8–12,48} Certainly, not all light chains are nephrotoxic. Some patients excrete grams of light chains in the urine and yet do not manifest renal injury clinically.⁴⁹ The present study demonstrated that the amino acid sequence of the CDR3 region, along with other factors previously reported,^{8–12} modulates the binding of THP to light chains and subsequent development of clinical manifestations of renal failure. Finally, because the basic mechanism of this process was elucidated, these studies have provided an opportunity to pursue strategies that inhibit this interaction and potentially prevent the severe renal failure that occurs in this setting.

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