Urine Proteome Analysis in Murine Nephrotoxic Serum Nephritis

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Key Words

Glomerulonephritis • Hepcidin • IP-10 • iTRAQ • Nephrotoxic serum • Proteomics

Hepcidin is identified as a potential urinary marker of nephritis and its role in disease pathogenesis warrants further study.

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Abstract

Background: Urine contains serum proteins filtered by the glomerulus or secreted by the renal tubules and proteins produced locally by the urinary tract. Proteomic analysis of urine holds the potential as a noninvasive means of studying or monitoring disease activity. In mice, large concentrations of albumin and lipocalins have complicated the ability to identify urinary biomarkers in disease models. **Methods:** Passive nephrotoxic serum nephritis was induced in mice. Urine proteins were identified and quantified by iTRAQ and MALDI-TOF mass spectrometry. Results were compared to Western blotting and multiplex immunoassays. **Results:** Large concentrations of major urinary proteins dominate the urine proteome of mice even in the context of acute nephritis. Increased proteinuria caused by nephrotoxic serum nephritis is transient and includes increased albumin excretion. There were no alterations in chemokine excretion. Altered hepcidin excretion was identified, most likely reflecting local production and renal retention. **Conclusion:** Proteomic analysis of mouse urine remains challenging due to the abundance of a limited subset of proteins. iTRAQ analysis does not circumvent these challenges, but can provide information on post-translational processing of some proteins.

Introduction

The nephrotoxic serum (NTS) nephritis model is a well-characterized model for the study of immune complex kidney diseases [1–4]. NTS is generated by immunizing sheep with kidney glomerular extracts. In the passive model, NTS is administered to nonsensitized mice. Heterologous sheep antibodies in NTS bind to target antigens in the recipient glomerulus, causing proteinuria and transient kidney injury. Damage in this ‘heterologous’ phase is dependent upon infiltrating neutrophils [1, 5, 6], Fc-receptor [2, 3, 7] and complement activation [8–12], and proinflammatory cytokines [13, 14]. Proteinuria is independent of complement and leukocyte infiltration, and is thought to involve alterations in the capillary loop ultrastructure from direct binding of antibodies in NTS to specific glomerular antigens, such as aminopeptidase A [12, 15–17]. After a few days, mice generate an immune response to the heterologous serum. If sheep antibodies persist in the glomeruli, a distinct ‘autologous’ phase of kidney injury results, mediated by the adaptive immune system [18].

Previous proteomic analyses of urine from mice with NTS nephritis have been limited to the autologous phase. Wu et al. [18] used a selective immunoproteome array bearing 62 cytokines, chemokines and soluble cytokine receptors to assess the responses of mice to rabbit anti-GBM (glomerular basement membrane) serum. Susceptible mice exhibited significantly increased urinary levels of vascular cell adhesion molecule 1, P-selectin, tumor necrosis factor receptor I, and CXCL16. Confirmatory testing showed that both intrinsic renal cells and infiltrating leukocytes were capable of producing these proteins.

Urine proteomic studies have also been performed in other rodent models. An LC/MS/MS approach with quantification by ICAT isotopic labeling was used to study the complement factor H knockout mouse model of MPGN [19]. Two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry was used to study the adriamycin nephropathy and puromycin aminonucleoside models of FSGS [20, 21]. However, mouse urine proteomic analyses have not been widely performed, mainly due to technical challenges involving the massive abundance of a limited number of urinary lipocalins.

This report presents a proteomic analysis of the urine from mice using the passive model of NTS nephritis. Urine was collected in the early heterologous phase and proteins were identified and quantified using the mass spectrometry-based iTRAQ system.

Methods

Nephrotoxic Sheep Sera

GBM-reactive NTS was generated by Rockland Immunochemicals (Gilbertsville, Pa., USA). Sheep were immunized with GBM preparations prepared as previously described [22, 23]. By indirect immunofluorescence, NTS stained C57BL/6 mouse glomeruli strongly, without staining tubules. Prior to injection into mice, the serum was heated to 56°C for 30 min (to heat inactivate the sheep complement) and sterile filtered.

Mice and NTS Nephritis

C57BL/6 mice were maintained in a pathogen-free colony at the University of Texas Health Science Center at Houston. Six- to 8-week-old females were used for all studies, which were approved by the University of Texas Health Science Center at Houston Animal Welfare Committee.

A passive NTS nephritis model was employed. Nonsensitized mice were injected with 200 μ l of either NTS or preimmune sheep serum via the tail vein. A relatively low dose was selected to induce a transient low-grade nephritis. Four-hour urine collections were obtained from 8–12, 20–24 and 44–48 h, using metabolic cages with free access to drinking water. Urinary creatinine excretion was determined by alkaline picrate assay (R&D Systems) and was

similar to baseline at all time points. Urinary protein concentration was determined by Albustix (Bayer). All animals were sacrificed on day 2, and the kidneys were processed for permanent and paraffin sectioning.

Histopathology

Four-micron sections of formalin-fixed, paraffin-embedded kidney tissues were cut and stained with periodic acid-Schiff stain. These sections were examined in a blinded fashion for any evidence of pathology in the glomeruli, tubules or interstitial areas, as previously described [24]. The glomeruli were screened for evidence of cellular infiltration, thrombosis or basement membrane thickening. Sections of OCP-embedded frozen kidneys were cut, fixed in ice-cold acetone and stained for mouse and sheep IgG by direct immunofluorescent antibody staining. Rhodamine-conjugated donkey F(ab')₂ anti-mouse and donkey F(ab')₂ anti-sheep IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pa., USA). Staining was performed at a 1:500 dilution (1 μ g/ml) of either antibody in 1% BSA.

Protein Purification and iTRAQ Labeling

iTRAQ labeling and mass spectrometry analysis was performed in the University of Texas Health Science Center at Houston Proteomics Core. Urine was collected over 4-hour intervals from each set of mice (4 mice per set) into a tube on ice containing protease inhibitors (Complete-Tab, Roche). The use of protease inhibitors is compatible with iTRAQ labeling. Urine samples were immediately spun at 12,000 g for 10 min to remove particulate matter, and the supernatants were removed and frozen. Protein concentrations were then determined in triplicate using the BCA assay (Pierce, Rockford, Ill., USA). Briefly, 100 μ g of protein mixture from each pool was precipitated with 6 volumes of –20°C acetone, which removes the protease inhibitors and the urinary amines that would otherwise quench the iTRAQ reagents. The precipitate was taken up in dissolution buffer and denatured, with the disulfides reduced by incubation in the presence of 0.1% SDS and 5 mM TCEP [tris-(2-carboxyethyl)phosphine]. Cysteine residues were blocked with MMTS (methyl methane-thiosulfonate) and trypsin was added to the mixture to make a protein-trypsin ratio of 10:1. The mixture was incubated overnight at 37°C. The protein digests were labeled by mixing with the appropriate iTRAQ reagent. Following labeling, the individual reaction mixtures were combined and fractionated by stepwise elution from the cation exchange column and desalted on a C18 column (for a more detailed description, see [20]).

Chromatography and Mass Spectrometry

The desalted and concentrated peptide mixtures were identified and quantified essentially as previously described [20] by nano-LC/MS/MS on a QSTAR Elite mass spectrometer (ABS Sciex Instruments, Concord, Ont., Canada) equipped with a Dionex LC-Packings Ultimate Nano-HPLC and FAMOS Autosampler. Using information-dependent acquisition, peptides were selected for collision-induced dissociation by alternating between an MS (1-second) survey scan and MS/MS (3-second) scans.

Data and Statistical Analysis

The accumulated MS/MS spectra were analyzed by ProteinPilot software (Applied Biosystems) using the *Mus musculus* Swiss-

Fig. 1. Histopathology. Representative sections from female C57BL/6 mouse kidneys harvested 36 h after injection of 200 μ l of NTS, derived from sheep. Formalin-fixed tissue was processed for paraffin sectioning and sections were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Frozen tissue was sectioned in OCT and fixed in acetone for direct immunofluorescence staining with rhodamine-conjugated donkey anti-sheep IgG and FITC-conjugated goat anti-mouse IgG. Images shown are 200 $\times$ (PAS) and 400 $\times$ magnification (sheep and mouse IgG). $n = 5$ mice.

Prot database for protein quantification and identification. The ProGroup reports were generated with a 95% confidence level for protein identification. Supporting data were analyzed using Sigma Stat Version 3.0 (Jandel Scientific, San Rafael, Calif., USA). Comparisons between each time point and baseline were performed using a t test. $p < 0.05$ was assumed to be statistically significant. Repeat analysis comparing quantitative data normalized for urine creatinine had no effect on the findings.

Immunoassays

Urine samples collected for proteomic analysis were also used for Western blots and fluorescent multiplex assays. All 4 sets of urine samples were tested. For immunoblotting, equal volumes of urine were resolved on denaturing SDS-PAGE gels under reducing conditions and then proteins were transferred to nitrocellulose. A polyclonal antibody for epidermal growth factor (EGF; Upstate, Lake Placid, N.Y., USA) and a monoclonal antibody for E-cadherin (clone ECCD-2, Invitrogen) were used to measure candidate protein expression. For multiplex immunoassays, a Luminex instrument was used to detect labeled fluorescent antibodies specific for a panel of 13 cytokines and chemokines (Millipore, Billerica, Mass., USA). Standard curves for each cytokine were run in parallel to quantify urinary cytokine concentrations.

Results

Disease Model

After the NTS injections, the mice developed transient nephritis with localization of heterologous sheep IgG to the glomerulus (fig. 1). Histology at 36 h revealed hypercellularity of the glomeruli with segmental necrosis. There were small cellular infiltrates at the vascular pole

of all glomeruli, without signs of large vessel vasculitis or tubulointerstitial inflammation. Histologically, there was resolution of the acute injury by 72 h. Immunofluorescent antibody staining indicated that large amounts of sheep IgG accumulated globally in a linear pattern in all glomerular capillary loops. At this early time point, only minor amounts of mouse IgG were present within the glomeruli and were localized more to the mesangial areas. There was no change in glomerular C3 staining and no change in urine creatinine concentration from baseline (data not shown).

Proteomics

For the proteomic studies, NTS was injected into an additional 16 female mice weighing 16–18 g, which were randomly separated into 4 sets for timed urine collections (4 mice per set). An increase in proteinuria was detected in pooled urine samples from each set of mice at 8–12 h, with levels returning to baseline by 24 and 48 h (fig. 2). Fresh urine from each mouse was assayed by a urine dipstick, and albumin excretion was uniformly trace to 1+ at baseline, 2+ to 3+ at 8–12 h, and 1+ to 2+ at 20–24 and 44–48 h.

Mass spectrometry analysis of the urine samples identified a total of 29 proteins, of which 22 were identified in urine from 2 or more sets of mice, 16 were identified in 3 or more sets, and 10 were identified in all 4 sets (table 1). Consistent with the results of the albu-strip analysis, albumin was found in all samples at all time points (fig. 3).

Fig. 2. Proteinuria. Total protein excretion, as measured by BCA assay. Urine collected over 4-hour intervals from 4 sets of 4 mice at baseline and after injection of NTS. Error bars reflect the SEM. of the 4 sets (* $p < 0.05$ compared to baseline values).

Table 1. Absolute number of peptides in urine from all sets of mice for each protein identified

SwissProt ID	n	%
MUP1	3,418	16.5
MUP2	6,495	31.3
MUP3	1,335	6.4
MUP4	448	2.2
MUP6	6,311	30.4
EGF	294	1.4
Renal kallikrein	209	1.0
Complement factor D/adipsin	188	0.9
Tamm-Horsfall protein/uromodulin	174	0.8
Albumin	173	0.8
Kidney-derived aspartic protease/napsin	126	0.6
E-cadherin	59	0.3
Hepcidin-2	36	0.2
Glycosylation-dependent cell adhesion molecule	35	0.2
Prostaglandin D2 synthase	26	0.1

Quantitation using the iTRAQ system showed a statistically significant 40% increase in albumin excretion at 8–12 h and a return to baseline by 24 and 48 h. There was also an increase in the excretion of the Tamm-Horsfall protein, a protein known to be secreted by the renal tubules in some, but not all, subsets of mice. This increase occurred later at 20–24 h. Five proteins, the major urinary proteins (MUPs 1, 2, 3, 4 and 6), comprised 85% of the total amount of protein isolated (table 1). There were no significant alterations in excretion of these proteins after NTS (fig. 3).

Hepcidin-2

Besides albumin, hepcidin-2 (HepC2) was the only protein with a significant difference in urinary excretion after NTS (fig. 4a). There was a 4-fold decrease in HepC2 excretion at 20–24 h, with a return to baseline by 44–48 h. HepC2 is a small protein containing several disulfide bonds predicted to form a rigid tertiary structure (fig. 5a). As HepC2 undergoes several post-translational processing steps resulting in the production of multiple isoforms, the iTRAQ data was reanalyzed to look at the quantification of each HepC2-derived tryptic-digested peptide detected by mass spectrometry.

Six peptides encoding 60% of the HepC2 sequence were detected (fig. 4b). Two peptides map to the mature 25-aa isoform previously reported in mouse serum and urine (fig. 5). A third overlapping peptide includes an additional 3 N-terminal residues deriving from the propeptide. All 3 peptides were found to be decreased 2- to 3-fold in urine from mice at 8–12 and 20–24 h, with levels equal to baseline by 44–48 h (fig. 4b). Conversely, 2 peptides from the propeptide were identified in the urine after NTS at levels similar to baseline. Moreover, a peptide mapping to the signal sequence was found to be increased over time in the urines of diseased mice: 3-fold at 8–12 and 20–24 h, and 10-fold at 44–48 h. Similar peptide analyses of EGF and other proteins identified in mouse urine did not show significant differences in abundance after NTS (fig. 4c and data not shown).

Human hepcidin was first isolated in urine as LEAP-1 (liver-expressed antimicrobial peptide), and 3 isoforms were isolated (20-, 22- and 25-aa) [25]. iTRAQ analysis failed to identify peptides that were solely part of the 20-aa isoforms. While it did identify overlapping peptides which could potentially identify the 22-aa versus the 25-aa isoforms, both peptides had highly similar relative expression after NTS compared to baseline. Therefore, iTRAQ analysis failed to show differential expression of the 3 hepcidin isoforms in mice treated with NTS.

Mice have 2 hepcidin genes that encode proteins with an overall 68% identity to each other (fig. 5c). However, they share 100% identity in the signal sequence and the prosequence [26]. While the peptides mapping to the signal sequence or prosequence could have originated from either HepC1 or HepC2, all peptides mapping to the mature forms of hepcidin derived from HepC2. Based on the inability to detect mature forms of HepC1, it is likely that all the urinary hepcidin peptides identified by iTRAQ analysis derive from HepC2.

Fig. 3. iTRAQ analysis. Changes in concentration of major urinary proteins after injection of NTS were determined by comparing those measured at each time point with baseline measurements. Urine was collected over 4-hour intervals from sets of 4 mice. Error bars indicate SE between sets (* $p < 0.05$ compared to baseline values). Alb = Albumin; THP = Tamm-Horsfall protein; MUP = major urinary protein.

Urine Immunoassays

In order to confirm the presence of particular proteins in mouse urine, Western blots were performed for the detection of the 2 proteins identified by mass spectrometry. The same urine samples were resolved by resolving SDS-PAGE gels and immunoblotting revealed the presence of EGF and E-cadherin (fig. 6). Equal volumes of urine were loaded for each time point from each set of mice, without regard for the protein concentration or volume collected. There were no obvious differences in concentrations of either protein between sets of mice or between urine samples collected before of after NTS, although the ability of Western blots to detect differences less than 3-fold in expression is limited.

In order to assess the sensitivity of iTRAQ analysis, the same urine samples were also used for chemokine/cyto-

kine analysis by multiplex immunoassay. Specific cytokines and chemokines have been detected in the urine of mice and humans with inflammatory kidney diseases, but none were detected by mass spectrometry. Nine cytokines and 4 chemokines were assessed, but only 1 chemokine, IP-10 (CXCL10), was detected in urine from any of the mice sets. All 4 sets showed a 2-fold decrease in excretion at 8–12 h compared to baseline (29 vs. 58 pg/ml), with a return to baseline by 44–48 h (fig. 7). Interleukin-1 α was detected in 1 set of mice at baseline and 44–48 h, but not at 8–12 or 20–24 h (data not shown). Therefore, with the degree of inflammation seen in this transient nephritis model, the excretion of chemokines and cytokines in urine is minimal.

Fig. 4. iTRAQ analysis. **a** Changes in concentration of urinary proteins after injection of NTS were determined by comparing those measured at each time point with baseline measurements. Error bars indicate the SE between sets. Changes in concentration of specific peptides from HepC2 (**b**) and EGF (**c**). Although peptides were generated artificially during sample preparation, the relative differences detected in the peptides likely reflect changes in post-translational processing or isoform-specific expressional effects. Urine was collected over 4-hour intervals from sets of 4 mice. Statistically significant increases (**) and decreases (*) were noted at specific time points for specific HepC2 peptides ($p < 0.05$ compared to baseline values). CADH = E-cadherin; cFD = complement factor D.

Discussion

This paper describes the proteomic analysis of the 'heterologous' phase of NTS nephritis. Two methodologies were used to identify proteins in the urine of diseased mice. First, mass spectrometry was used along with the iTRAQ system for differential expression. Second, immunoassays were used to identify candidate proteins. In comparing urine from early and late time points to baseline samples, mice subjected to experimental immune nephritis had very few differences in excreted proteins.

Increases were seen early for albumin and later for the Tamm-Horsfall protein. Albuminuria was modest compared to that seen in other publications, but was consistent with the dosage of NTS used. Decreases were seen in only HepC2, although peptide analysis revealed that the prepeptide of HepC2 was actually present at higher levels. All these effects returned to baseline by 44-48 h.

There have been 2 reports on gene expression in kidney tissue from mice in the heterologous phase of NTS nephritis [27, 28]. Ohse et al. [27] harvested kidneys 2 h after administration of NTS. The mRNA preparations

Fig. 5. Mouse hepcidin protein structure. **a** Diagram shows the domain structure of both HepC1 and HepC2. Predicted disulfide bonds and cleavage sites for signal peptide, propeptide, 22-mer and 20-mer are indicated by vertical lines. **b** Peptides identified by mass spectrometry are shown, mapped to their respective locations on

the HepC2 protein sequence. **c** Sequence comparison of HepC1 and HepC2. Vertical bars indicate sequence identity. Horizontal lines indicate sequences of peptides identified by mass spectrometry. The source of 3 peptides could be either HepC1 or HepC2, whereas the other 3 peptides could only have originated from HepC2.

Fig. 6. Western blot analysis. Forty microliters from timed urine collections using mice at baseline (0) and from 8–12 h (8) and 44–48 h (48) after injection of NTS were run on reducing SDS-PAGE gels. Relative levels of epidermal growth factor (EGF; **a**) and E-cadherin (CADH; **b**) were detected by immunoblotting with specific antibodies. Densitometry revealed no differences in excretion of either protein at any time point (data not shown).

Fig. 7. Multiplex immunoassay analysis. Levels of urinary chemokines were measured by immunostaining at baseline and after injection of NTS. Urine was collected over 4-hour intervals from sets of 4 mice. Urinary excretion of IP-10 (CXCL10) is shown. Error bars indicate the SE ($p > 0.5$ for all collections compared to baseline values).

were of whole unperfused kidneys; thus, changes in expression might reflect differences in parenchymal expression or differences in the number of infiltrating neutrophils. The authors reported upregulation of 54 genes

and downregulation of 25 genes. Several upregulated genes were interferon-inducible genes, and 4 were selected for confirmatory studies by RT-PCR: Stat1, Irf1, Igpt and Tgpt. When the microarray studies were repeated on

mice treated with a leukotriene analog that attenuated neutrophil infiltration, expression of most of the upregulated genes was found to be reduced. Therefore, the approach taken by Ohse et al. suggests that there are few changes in parenchymal mRNA expression in the heterologous phase of NTS nephritis.

Kim et al. [28] used microarray analysis, RT-PCR and ELISA to study kidney gene expression in NTS nephritis. Analysis was performed on days 1, 3, 7, 11 and 16 in order to assess the heterologous phase and the autologous phase. Overall, 1,100 genes were modified compared to mRNA from kidneys of unmanipulated mice, including 3 cytokine genes (*Scya2/Ccl2*, *Scya6/Ccl6* and *Il6*) and 12 genes involved in NF- κ B signaling (*Saa1*, *Egr1*, *Birc5*, *C3*, *Crl*, *Junb*, *Myc*, *Trl2*, *Bcl3*, *Vcam1*, *Scay2* and *Bcl2a1c*) that were upregulated >2-fold. Again, nearly all differences in expression were during the autologous phase, with few alterations during the heterologous phase of kidney injury.

MUPs are a subset of lipocalins with numerous isoforms encoded by 5 distinct gene loci (MUPs 1–5). They are produced by the liver and specific glandular cells and enter the urine by glomerular filtration [29]. Their proposed functions are the binding and transportation of volatile substances, including pheromones, through the kidneys [30]. Expression appears to differ by strain and is upregulated by androgens and at the beginning of estrus in females [31]. In our study, they comprised 85% of the mouse urinary proteome. The molecular weight of the MUPs, 20 kDa, presents significant challenges to remove by size fractionation as many of the non-MUP proteins detected in this study also had molecular weights at or below 20 kDa. Attempts at urinary immunodepletion with MUP-specific antibodies have been disappointing (data not shown).

The only protein, other than albumin, with a significant alteration in expression in the NTS model was HepC2. It is encoded by the gene *Hamp2* (hepcidin antimicrobial peptide 2). The intact molecule has a molecular weight of 9.5 kDa, while the processed forms are cationic amphipathic (pI of 8.1) 20- to 25-aa peptides with a molecular weight of 2–3 kDa. It is produced primarily by the liver, but also in the heart and pancreas, and is detectable constitutively in blood and urine [25, 26]. Hepcidin in humans and the HepC1 protein in mice regulate iron metabolism and act by inhibiting the efflux of iron through ferroportin, the sole known iron exporter that is expressed in hepatocytes, macrophages and the small intestine [32]. In humans with lupus nephritis, urine levels of the 20-aa isoform were found to increase 4 months before renal flares, returning to baseline at the initiation of the

flares; however, levels of the 25-aa isoform were lower at the initiation of the flares and returned to baseline within 4 months after the flares [33]. Absolute serum levels vary widely among patients with lupus nephritis or rheumatoid arthritis, as well as in controls [34]. The mouse HepC2 protein has no human ortholog and overexpression in transgenic mice fails to cause anemia, suggesting a divergent function for this gene [35]. The *HepC2* nucleotide sequence is 93% identical to HepC1, and the 1,000-bp promoter region is similarly conserved. Both *HepC1* and *HepC2* genes are upregulated in iron-loaded mice and completely silenced by a cis-mutation in the *Usf2* gene [35]. However, HepC2 expression is unchanged by IL-6 and bone morphogenic protein, unlike HepC1 [36]. Although 20-, 22- and 25-mer isoforms are possibly based on the conservation of cysteines between human hepcidin and mouse HepC2, the physiologic presence of the 3 mouse isoforms is unclear. There are no reagents available to assess HepC2 protein levels, and the evolutionary advantage for the 2 hepcidin proteins in mice remains speculative.

In this study, urinary levels of the mature HepC2 protein were decreased in response to NTS, but returned to baseline levels by 44–48 h. While effects due to diurnal variation are possible (the 8- to 12-hour time point was during the dark cycle, while the other time points were during the light cycle), we believe that the changes in protein excretion were due to injury-related effects. The decrease in the excretion of mature HepC2 peptides were seen at 20–24 h. In contrast, the urinary excretion of the HepC2 signal sequence was shown to be increased in response to NTS, maximally at 44–48 h. One possibility is that HepC2 may be produced locally by the kidney, processed in the urine and retained by the injured parenchyma, with a subsequent loss of signal sequence in the urine. Hepcidin expression in renal tubules has been shown in rats and humans [37]. Testing of this hypothesis will require the development of additional reagents for mouse HepC2.

In conclusion, this study used mass spectrometry and iTRAQ analysis in an attempt to identify urinary biomarkers in mice with acute glomerulonephritis. Although MUPs predominated the urine of sick and control mice, the protocol described here was able to identify a limited group of differentially expressed proteins and provide detailed information on post-translational processing of the proteins of interest. Hepcidin was identified as a potential urinary biomarker of acute glomerular nephritis and its role in disease pathogenesis warrants further study.

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