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Protein Reabsorption in the Amphibian Kidney: Comparative and Evolutionary Aspects

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Abstract

Protein reabsorption in the renal proximal tubule (PT) is a vitally important process which prevents the loss of filtered proteins and provides their participation in subsequent metabolism. Despite considerable changes in renal structure and function in the process of evolution, very little is known about the functional similarities or specifics of tubular protein reabsorption in the kidney of lower vertebrates compared with the mammalian and human kidney. This article presents an overview of our recent studies on protein reabsorption in the kidney of amphibians, which are used as one of the main animal models for current biological and biomedical research. In frogs, newts, and rats, the absorption capacity of epithelial PT cells was studied after the introduction of green fluorescent protein (GFP), yellow fluorescent protein (YFP), and lysozyme. Molecular mechanisms of receptor-mediated protein endocytosis were also investigated by immunohisto- and immunocytochemistry, electron, fluorescent, and laser scanning confocal microscopy.

Keywords: amphibians, cubilin, comparative physiology, endocytosis, evolution, frog, kidney, megalin, protein reabsorption, proximal tubule

1. Introduction

Renal protein reabsorption is a process which reduces urine protein excretion and allows the absorbed proteins to participate in subsequent metabolism. It also provides the retrieval of other specific substances including the conservation of carrier-bound vitamins. Detailed investigations of this process are of great importance for understanding renal physiology, tubular disorders, and homeostatic control mechanisms. Reabsorption of filtered proteins occurs in the epithelium of proximal tubule (PT). Despite the high absorption capacity of mammalian PTs, increasing protein uptake and prolonged overload situation may lead to tubular proteinuria

and tubule injury and subsequently may induce tubular interstitial damage [1, 2]. Obviously, extensive studies of tubular protein reabsorption are of great importance for renal physiology and pathology of proteinuric diseases in humans and mammals. Probably for this reason, such studies are limited by primarily clinical investigations and use of theoretical and mammalian animal models, although a novel model was also proposed using the axolotl [3]. To date, the process and mechanisms of protein reabsorption in mammalian PTs have been rather well explored at the cellular and molecular level. At the same time, very little is known about tubular reabsorption and endocytic transport of proteins in the non-mammalian kidney. Despite considerable and progressive transformation of vertebrate renal structure and function in the process of evolution (e.g., see [4]), the structure and function of PTs do not appear to have undergone major evolutionary changes [5]. It can be assumed that PT functions are mostly evolutionarily conserved, but this assumption does not have sufficient experimental foundations, at least in relation to protein reabsorption. There is no sufficient information about the degree of the similarities or differences in tubular protein uptake, molecular mechanisms, and regulation of this process in the ascending series of the vertebrates or during ontogeny. Our interest in the study of renal protein reabsorption in the amphibian kidney is due to several reasons. Amphibians occupy a key position in the evolution of terrestrial vertebrates and bridge the gap between the aquatic fishes and the terrestrial vertebrates. The basic renal physiology of these poikilothermic tetrapods is relatively well understood, primarily with regard to water and ion transport but not to tubular protein uptake. Along with that, amphibians as animal models are one of the main objects of current biological and biomedical research. In this chapter, we present a brief survey of available information about tubular protein reabsorption and molecular mechanisms of protein endocytosis in the kidney of amphibians, based on our research within the context of existing literature and current ideas about molecular and cellular mechanisms of endocytosis. Some comparative and evolutionary aspects of the issues involved are also considered.

2. Structural and functional basis of glomerular filtration and tubular protein reabsorption in the amphibians

Vertebrate kidneys develop via three successive stages of formation in the process of evolution or during ontogeny—pronephros, mesonephros, and metanephros. Pronephros constitutes the mature kidney in most primitive vertebrates (cyclostomes); it is the earliest stage in fishes and tetrapods and the functional embryonic kidney in amphibians. Mesonephros is the permanent kidney of amphibians and most fish, replacing the pronephros of the embryonic and larval stages. It serves as the main excretory organ of aquatic vertebrates and as a temporary kidney in reptiles, birds, and mammals. During embryogenesis in amniotes, pronephros is succeeded by the mesonephros, which gradually degenerates, and a more complex metanephros arises caudal to the mesonephros and develops as functional adult kidney of higher vertebrates. The nephron is the basic structural and functional unit of the kidney. Glomeruli, proximal, and distal segments as major parts of the kidney nephrons are present in nearly all vertebrates. It is known that the filtration properties of the glomeruli are determined by the pore size of the filtration barrier and depend on the physical–chemical properties of plasma

proteins. The cut-off molecular mass for filtration of plasma proteins in renal glomeruli during normal conditions has generally been assumed to be lower than the molecular mass of serum albumin and some other large proteins (in the range of 60–85 kDa). Structure of the filtration barriers within the glomeruli of studied amphibians and mammals is very similar [6, 7]. It concerns the ultrastructure of the glomerular wall, in particular capillary endothelium, basement membrane, endothelial cell layer, and slit diaphragm, limiting permeability.

According to our morphological studies, the glomerular filtration barrier in the kidney of *Rana temporaria* showed the classic three-component structure (**Figure 1A**), as described in other anuran and urodel species [8–10]. It is composed of a layer of capillary endothelial cells facing the blood, a heterogeneous glomerular basement membrane, and a visceral epithelial cell layer which faces the urinary space of Bowman's capsule. Mesangial cells are distributed between the capillary loops. The ultrastructure of the PT has also typical features of these parts of the nephron [8, 9, 11, 12]. Well-preserved endocytic apparatus, including vesicles, dense apical tubules, endosomes, and lysosomes (**Figure 1B**), indicates the active uptake capacity of the PT cells [8, 13].

Studies of proximo-distal patterning of the nephrons in the frog, *Xenopus laevis*, showed the presence of early physiological specialization of PTs at the stage of pronephros [14]. In particular, it was found that pronephric PT has an early and a late segment and different transporters are expressed within unique subdomains similar to those in mammalian metanephric PT. The ability of tadpole pronephros to filter and reabsorb fluorescently tagged proteins (serum albumin, codfish parvalbumin) was also revealed after cardiac injections.

In our morphophysiological studies, we investigated the mechanisms of protein reabsorption in the amphibian mesonephros by the methods of fluorescent and confocal microscopy, immunohisto-, and immunocytochemistry. Experiments were performed on common frogs (*Rana temporaria*), newts (*Triturus vulgaris*), and also Wistar rats (*Rattus norvegicus*) for some comparisons.

Figure 1. The ultrastructure of glomerular filtration barrier (A) and apical area of proximal tubular cell in the kidney (B) of the frog *Rana temporaria*. Arrows point to the foot processes of podocytes covering thin lamina rara externa; asterisks show the processes of mesangial cells, arrowheads demonstrate numerous fenestrae of the endothelial cells. En, endosome; DT, dense apical tubules; Ld, lamina densa; M, mitochondria; Mv, microvilli; N, nucleus; P, podocyte; Tj, tight junction; V, vesicle. Scale bar 1 μ m. Author's drawings.

Figure 2. Protein reabsorption in the proximal tubules of the amphibian and rat kidney. In panels: The uptake pattern of GFP, YFP, and lysozyme; 30 min (A–D, F, G), 5 min (E), and 20 min (H) after protein introduction. Scale bars: 100 μ m (A, B), 25 μ m (C–H). Author's drawings.

We used recombinant fluorescent proteins, green fluorescent protein (GFP), and yellow fluorescent protein (YFP), which turned out to be freely filtered in glomeruli and reabsorbed in epithelial cells of PTs after intravenous injections in frogs, as in rats [15]. Earlier, intestinal absorption and the resulting accumulation of these fluorescent proteins in renal PTs after their intragastric administration were established [16]. Later, the uptake of lysozyme in the amphibian kidney was also demonstrated [17]. Some examples of tubular reabsorption of abovementioned proteins in amphibians and rats are shown below (**Figure 2**).

3. Protein uptake pattern in the amphibian and rat kidney and quantification of protein reabsorption

In our studies, protein uptake was analyzed after intravenous (i.v.) protein introduction in immobilized (double-pithed) frogs and anesthetized rats. Intraperitoneal (i.p.) injection and subcutaneous (s.c.) introduction (into dorsal lymph sac) were applied to mobile amphibians. Before and during experiments, amphibians were in terms of optimal hydration. Absorbed GFP or YFP was detected in fixed kidney slices by fluorescent or laser scanning confocal microscopy. In frogs, these proteins were revealed in epithelial layer of PT profiles situated in the dorsolateral part of the kidney including supraglomerular zone and superficial areas (**Figure 2A** and **B**). Initially diffuse, a specific signal was visualized in endocytic vesicles of PT cells 10–30 min after protein injections. Bright fluorescent vesicles were located predominantly in apical cytoplasm near brush border and also in perinuclear areas (**Figure 2C** and **D**). In rats, the fluorescent PT profiles were revealed in periglomerular areas of the rat kidney cortex

(Figure 2E); the distribution of the protein-containing vesicles was similar to that seen in frogs (Figure 2F). Renal uptake of lysozyme in frogs and newts (after i.v. and i.p. injection, accordingly) was proved by immunohistochemistry using rabbit anti-hen lysozyme (as primary antibody) and Alexa Fluor 488 conjugate (secondary goat anti-rabbit IgG conjugated with Alexa Fluor 488). Intracellular distribution of labeled lysozyme in a vesicular compartment of PT cells does not differ fundamentally from the uptake pattern of GFP and YFP (Figure 2G and H). To discover regularities in protein reabsorption we used a variety of approaches for quantification of protein uptake [15]. After GFP introduction at the doses 0.034–34 $\mu\text{g}/100\text{ g}$ body weight, reabsorption of this protein in the kidney was dose-dependent in both frogs and rats [15]. The specific fluorescence intensity, maximum fluorescence, and fluorescence density increased in response to increasing doses of GFP and a high positive correlation was revealed. Reabsorption of fluorescent proteins was also time dependent [15, 18]. With increasing time after injection, there was an accumulation of vesicles with GFP or YFP and a movement of some fluorescent endocytic vesicles from the apical cytoplasm to perinuclear and basal areas. As shown in our recent studies, the number of the formed fluorescent endocytic vesicles is the most suitable and rather adequate parameter for quantitative morphological analysis of the protein absorption rate over a fixed period of time [19]. The dynamics of the accumulation of various proteins in renal PT cells within 30 min after i.v. injections were generally similar (Figure 3A) and prolonged for fluorescent proteins in frogs (Figure 3B).

Figure 3. Time-dependent protein uptake pattern in the frog and rat kidney. Ordinate: The average number of protein-contained vesicles (per five neighboring epithelial cells). (A) The uptake of GFP and lysozyme in rats and frogs; (B) the absorption dynamics of YFP in frogs; R^2 , approximation confidence. Inverted confocal images of tubule profiles show the accumulation fluorescent endocytic vesicles with increasing time after YFP injection. Scale bars: 10 μm . Author's drawings.

4. Comparative analysis of renal protein uptake in the rat and frog kidneys

Known stability of the molecular structure of GFP and YFP and their resistance to lysosomal degradation give an advantage in detection of completely absorbed proteins, allowing us to estimate the uptake and intracellular transport of intact protein molecules for quite a long time. Using these proteins, we focused our research on functional differences in the kidneys of mammals and amphibians based on more detailed comparative analysis of protein uptake. Despite the similarity of the basic patterns of tubular protein absorption in rats and hydrated frogs after injections of equal doses of different proteins, it is obvious that tubular handling of GFP is quicker in rats compared to frogs (**Figure 3A**). Granular fluorescence in rat PT cells appeared 2–5 min after GFP introduction (**Figure 2E**). In 30 min, the number of vesicles with internalized protein significantly increased and fluorescent vesicles scattered over the epithelial cell cytoplasm (**Figure 2F**). However, in the subsequent period, the number of GFP-containing vesicles in PT cells and the means of maximum fluorescence have dramatically decreased [15, 20]. In 1–2 h, green fluorescent vesicles were absent in the vast majority of PTs. Since the disappearance of GFP signal signifies the destruction of its molecular structure, it is reasonable to suggest that in the rat kidney GFP is metabolized in some fashion and at least a partial lysosomal degradation of this protein occurs.

In contrast to rats, in the frog PT cells intracellular transport GFP and YFP and the gradual accumulation of fluorescent vesicles took place for a long time and had a similar character, regardless of the way of protein introduction [15, 20, 21]. It means that in frogs these proteins are filtered at a relatively slow rate and remain in circulation for quite a long time. Absorbed fluorescent proteins migrated from apical cytoplasm to perinuclear zone only in 40–60 min. Process of absorption and accumulation of injected protein lasted for 1.5 h and then ended (**Figure 3B**), without reduction in the number of fluorescent vesicles as a sign of protein degradation.

As shown in our comparative physiological study of renal functions, in hydrated frogs an intense water diuresis occurs, in contrast to rats whose values of osmotic free water clearance indicate antidiuresis [20]. Despite the active fluid filtration, the protein absorption rate in the frog kidney was substantially slower than that in the rat kidney. This can be due to a slower glomerular filtration rate (GFR), resulting in a longer period of protein circulation in blood and in prolonged tubular protein reabsorption. According to our research, creatinine clearance-measured GRF in frogs is 0.028 ml/min, that is about 8 times slower than GRF in rats. Generally, GFR in the kidney of *Rana temporaria* corresponds to the range of this parameter measured for a number of tailless and tailed amphibian species. Specifically, it most closely approximates the GFR values in such amphibian species as the Chilean toad (*Calyptocephalella gayi*), clawed frog (*Xenopus laevis*), and northern leopard frog (*Rana pipiens*)—0.031, 0.05, and 0.056 ml/min, respectively (see [22]). Thus, the peculiarities of the protein uptake revealed in the frog kidney, as compared with the rat kidney, consisted of a lower protein reabsorption rate, intracellular distribution of internalized protein in cytoplasmic compartments, and protein degradation rate.

5. Hormonal modulation of protein uptake in the frog kidney: effect of arginine vasotocin (AVT)

The contribution of the glomerular activity to the process of renal protein reabsorption may be very important for amphibians because in their mesonephros, unlike the mammalian metanephros, the degree of diuresis highly depends on the blood flow through the glomerular capillaries. The role of glomerular filtration in controlling the volume of extracellular fluid differs markedly in lower and higher vertebrates [23–25]. For instance, in fish, amphibians, and reptiles, GFR is not constant, and diuresis depends on variable or intermittent glomerular filtration, in contrast to birds and mammals [24]. In fish and poikilothermic tetrapods, tubular water reabsorption is far less variable compared with that of higher vertebrates [23]. In semiaquatic frogs, urine flow is the greatest in hydrated animals and reduced during dehydration [23, 24], and GFR is hormone dependent [25]. Arginine vasotocin (AVT) causes a reduction of GFR by constricting the preglomerular arteries [26–28]. The glomerular action of AVT is supported by the location of vasotocin receptors subtype 1 over the glomeruli in the amphibian kidney [27, 29].

We suppose that AVT-induced decline in GRF and following reduction of tubular fluid flow can hinder the transfer of proteins to their binding sites on the luminal membrane of frog PT cells and reduce the rate of protein reabsorption. To study the effect of AVT on tubular protein reabsorption in hydrated frogs, we estimated the pattern of GFP uptake after preliminary injections of this hormone [15, 30]. When AVT (0.1 fmol–1 nmol) was introduced 20 min before GFP, reabsorption of injected protein decreased in a dose-dependent manner. At the dose over 1 pmol, AVT provoked irregular GFP uptake pattern and the clusters of fluorescent PTs were observed in only some dorsolateral parts of the kidney. Absence of differences in GFP reabsorption between frogs after injections of low AVT doses and control animals suggests that in hydrated frogs, at room temperature and without osmotic stimulus, most of the glomeruli are continually active. Uneven distribution of fluorescent PT profiles may be a consequence of a decrease or complete cessation of filtration in individual glomeruli. The data suggests that not all of the glomeruli or preglomerular vessels are equally responsive to AVT. To insure whether AVT-induced reduction of GFP uptake is a consequence of the hormone effect on the vascular tone, a V1a receptor antagonist was applied [15, 30]. Administration of V1 antagonist (0.01–1 nmol) 10 min before AVT significantly increased GFP uptake reduced by the action of AVT. Thus, in *Rana temporaria*, AVT may indirectly modulate the tubular protein transport and its effect is mediated by V1a-like receptors.

6. Molecular and cellular mechanisms of endocytosis in the amphibian kidney

In mammalian and human kidneys, the filtered proteins are reabsorbed in PT cells by receptor-mediated endocytosis, then are transferred into endosomes, and finally to lysosomes for degradation. According to modern concepts, this process involves two main membrane receptors, megalin (megalin/lrp2) and cubilin, and also amnionless, and their coordinated action-mediated

internalization of different proteins [1, 31]. Existence of genes for megalin, cubilin, and amni-onless in *Xenopus* genome was established and the expression of these receptors in *Xenopus* tadpole pronephros was discovered [32], suggesting their participation in endocytic protein uptake in amphibians. In other lower vertebrates, megalin- and cubilin-dependent endocytosis was shown for the zebrafish pronephros [33].

In our studies, the expression of endocytic receptors in PT cells of the frog mesonephros was revealed after injections of YFP [18] and lysozyme [17] using polyclonal antibodies against megalin and cubilin. In 15–30 min, absorbed YFP was colocalized with immunolabeled megalin or cubilin in apical endocytic vesicles (**Figure 4A–D**).

In the process of time-dependent lysozyme absorption during 10–30 min, similar internalization of megalin, cubilin, and lysozyme was revealed in frogs and also in newts. After protein injections, receptor-specific signals were initially distributed diffusely, along the base of the brush border (**Figure 4E**), and then became more intensive and punctate, in the subapical area of PT cells (**Figure 4F**). So, the involving endocytic receptors in the tubular uptake and vesicular protein transport in the amphibian kidney were proved. No detectable receptor signal was found in PTs of control animals. This indicated to a ligand-induced process of endocytosis with participation of megalin and cubilin, as also noted for zebrafishes [33]. In order to identify the early step of lysozyme internalization, antibody against clathrin was used. This adaptor protein was detected in most PT profiles (**Figure 4G**). The availability of clathrin in most of the PTs of both control and lysozyme-injected frogs confirms the data about constitutive expression of clathrin and its involvement in the continuous uptake of essential nutrients in mammalian cells [34]. Initial colocalization of clathrin and lysozyme and following divergence of both signals were detected (**Figure 4H and I**), pointing the movement of lysozyme to the early and

Figure 4. Demonstration of YFP and lysozyme internalization rate in the frog proximal tubular cells by immunohistochemistry and confocal microscopy. In panels: absorbed YFP (A), immunolabeled megalin (B), colocalization of their red and green signals (yellow) on merged image (C), and the same for YFP and cubilin (D); immunodetection of megalin (E), cubilin (F), and clathrin (G) after lysozyme introduction; colocalization (yellow) of lysozyme and clathrin (H) and following divergence of their green and red signals (I), merged; double-labeling of lysozyme (green) and LAMP1 (red), merged (J); the combined uptake of YFP (red) and GFP (green) and their colocalization (yellow) after injection of GFP 1 h before YFP (K), merged. Scale bars: 10 (A–F, H–K) and 40 μ m (G). Author's drawings.

late endocytic compartment within 20–30 min. When lysozyme and lysosomal marker LAMP1 antibodies were used, there was no convergence of immune signals (**Figure 4J**). So, lysozyme was retained within endosomal compartment during this time, in contrast to the faster protein traffic in the mammalian kidney. It may be connected with lower metabolic rate and following inhibition of the intracellular transport in hibernating frogs [17].

The results of our immunohistochemical studies of the mechanisms of protein endocytosis were confirmed by immunocytochemistry. Immunoelectron microscopy revealed more detailed intracellular localization of GFP, lysozyme, endocytic receptors, and clathrin 10–30 min after protein injections (**Figure 5**).

As shown by the distribution of gold particles, absorbed proteins can be detected in the apical cytoplasm underneath the brush including intermicrovillar space, in small apical vesicles and large endosomes (**Figure 5A and B**). Similar label distribution including intermicrovillar invaginations of luminal membrane and vesicular structures was typical for cubilin, megalin, and clathrin (**Figure 5C–E**). Immunodetection of clathrin proved the internalization of lysozyme via clathrin-coated vesicles. Overall, we provided the evidence that protein reabsorption in the frog mesonephros occurs by receptor-mediated clathrin-dependent endocytosis.

Figure 5. Electron micrographs of the apical region of proximal tubule cells from protein-injected frogs. In panels: immunostaining of GFP (A), lysozyme (B), cubilin (C), megalin (D), and clathrin (E). En, endosome; Mv, microvilli; V, vesicle. Arrows shows the distribution of gold particles. Scale bar: 1 μ m. Author's drawings.

7. Effects of combined protein injections and previous protein loading

According to existing data, the results of *in vivo* and *in vitro* studies concerning the selectivity and competition of tubular reabsorption of proteins in mammals do not always have a clear explanation and are not well understood. As YFP and GFP are filtered and absorbed in the kidney in the same way, these proteins may be competitive in the absorptive process. We examined the uptake and intracellular traffic of both GFP and YFP under different conditions for competitive absorption *in vivo* after simultaneous and sequential introduction of equal amounts of these proteins [18, 19]. After simultaneous introduction of GFP and YFP, predominantly colocalized fluorescent signals indicated accumulation of both proteins in the same endocytic vesicles. When two proteins were injected in sequence, one before the other or vice versa, the second protein can be colocalized with the first protein but also located in individual endosomes (**Figure 4K**) because most of the vesicles containing the first protein moved from the apical cytoplasm to other cell areas. Effect of combined injections did not depend on the order of GFP and YFP introduction [18]. Therefore, the total result is shown below (**Figure 6A**). With increasing time interval between injections, a progressive accumulation of the first protein was viewed in 60 and 120 min compared with control (30 min after injection of this protein alone). So, the second protein should be more competitive in the process of the

Figure 6. Changes in absorption capacity of frog renal proximal tubule epithelial cells. (A) The effect of combined introduction of two fluorescent proteins: pre-injected protein (1, 3) and the other protein (2, 4) introduced after 30 and 90 min, respectively; (B) the result of immunofluorescence detection of megalin (1), cubilin (2), and lysozyme (3) on the fifth day after cessation of lysozyme loading. Significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control (A) or the third day after stopping the load (B); ### $p < 0.001$, compared with 1 and 2, respectively (one-way ANOVA followed by a Newman–Keuls test). Author's drawings.

absorption because significant amount of the first protein have been already absorbed in PT cells. However, the second protein uptake decreased.

The results demonstrate the availability of the mechanism capable to limit in vivo the absorption capacity of renal PT cells in frogs. The physiological implication of this downregulation is unknown. In mammals, it may be due to deficiency of endocytic receptors on the apical plasma membrane of PT cells and linked to changes in the initial steps of endocytosis, as due to inhibition of protein hydrolysis in the lysosomes and subsequent recycling of receptors [31, 35]. Not all proteins used in various experimental models inhibited bovine serum albumin endocytosis in mammalian PT cells [35–37]. When lysozyme was used instead of GFP in our frog experiments, it did not change the uptake of pre-injected YFP [19]. At the same time, 4-day lysozyme loading reduced YFP reabsorption and expression of endocytic receptors [19, 38]. Absorption capacity of PT cells was restored on the fifth day after cessation of loading and the number of YFP-associated profiles reached the control level [38]. Recovery of tubular YFP reabsorption occurred with a simultaneous increase in the number of internalized endocytic receptors and decrease in accumulation of lysozyme within PT cells (**Figure 6B**).

Thus, the results suggest the dependence of receptor-mediated endocytosis in the frog kidney on the molecular nature of absorbable ligands, conditions of their competitive absorption, and lysosomal accumulation in PT cells.

8. Conclusions

In general, morphophysiological study of the capacity for protein reabsorption in PT of the amphibian kidney was performed. Dose- and time-dependent tubular protein uptake and the existence of mechanisms limiting the protein absorption in epithelial PT cells were shown in frogs. Subcellular localization of endocytic receptors, megalin and cubilin, was revealed in amphibian PT cells after protein treatment. Intracellular trafficking of injected proteins was coincided with the distribution of megalin and cubilin. Specific marking of endocytic pathways revealed clathrin-dependent internalization of lysozymes and its subsequent transfer to endosomes. Thus, the protein uptake in the amphibian mesonephros is mediated by megalin and cubilin that confirms a critical role of endocytic receptors in the renal reabsorption of proteins in amphibians as in mammals. Based on our data, a frog model can be successfully used for investigating molecular mechanisms involved in the process of renal protein reabsorption and its comparative aspects.

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