The rat Pkd2 protein assumes distinct subcellular distributions in different organs

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1Institute for Anatomy and Cell Biology I, University of Heidelberg, 69120 Heidelberg, Germany; 2Section of Nephrology, Yale University School of Medicine, New Haven, Connecticut 06520-8029; and 4Department of Pathology, University of Heidelberg, 69120 Heidelberg, Germany

Obermüller, Nicholas, A. Rachel Gallagher, Yiqiang Cai, Nikolaus Gassler, Norbert Gretz, Stefan Somlo, and Ralph Witzgall. The rat Pkd2 protein assumes distinct subcellular distributions in different organs. Am. J. Physiol. 277 (Renal Physiol. 46): F914–F925, 1999.—Mutations in the PKD2 gene account for ~15% of all cases of autosomal-dominant polycystic kidney disease. In the present study the cellular distribution of the Pkd2 protein was investigated by immunohistochemistry in different rat organs. Although the Pkd2 protein showed a widespread expression, a strikingly different distribution of the protein was observed between individual organs. Whereas in renal distal tubules and in striated ducts of salivary glands a basal-to-basolateral distribution of Pkd2 was found, a punctate cytoplasmic location was detected in the adrenal gland, ovary, cornea, and smooth muscle cells of blood vessels. Interestingly, in the adrenal gland and ovary, the rat Pkd2 protein was more heavily N-glycosylated than in the kidney and salivary gland. These results suggest that Pkd2 accomplishes its functions by interacting with proteins located in different cellular compartments. The extrarenal expression pattern of the Pkd2 protein hints at other candidate sites of disease manifestations in patients carrying PKD2 mutations.

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Four-micrometer-thick paraffin sections were prepared for histological examination by immunohistochemistry and hematoxylin and eosin (H & E) staining.

Immunohistochemistry. Deparaffinized sections were equilibrated in PBS and incubated with blocking solution (2% BSA in PBS) for 30 min at room temperature. The rabbit polyclonal anti-PKD2 antibody YCC2, raised against a glutathione S-transferase (GST)-PKD2 fusion protein containing amino acids 687–962 of human PKD2 (27), was applied at a dilution of 1:400 in blocking solution for 2 h at room temperature and subsequently overnight at 4°C. The next morning, sections were rinsed twice for 10 min each in PBS and incubated with a Cy3-coupled secondary antibody (Dianova, Hamburg, Germany) for 1 h at room temperature. After washing in PBS, sections were mounted in bicarbonate-buffered glycerol pH 8.6. For double-labeling experiments, the anti-PKD2 antibody was applied together with one of the following antibodies: mouse monoclonal anti-calbindin D28k (catalog no. C 8666; Sigma, Deisenhofen, Germany) diluted 1:500, or sheep polyclonal anti-Tamm-Horsfall glycoprotein (catalog no. 8595–0054; Biotrend, Cologne, Germany) diluted 1:300. Signal detection was carried out using FITC- and Cy3-labeled secondary antibodies (Dianova and Sigma). To facilitate the detailed morphological analysis of PKD2 expression in various cellular structures, slides were subjected to H & E staining after photographing the immunofluorescence results.

In control experiments, primary antibodies were replaced by PBS; otherwise, sections were processed as described. No specific staining was obtained under these conditions. As a further control, the primary antibody was preabsorbed for 30 min with either GST (2 ng/µl) or the GST-PKD2 fusion protein (4 ng/µl) used to generate the polyclonal antibody (these concentrations correspond to approximately equimolar amounts of both proteins). After the preabsorption step, the antibody was applied to the sections; otherwise, the staining protocol was followed as described in the previous paragraph.

Preparation of membrane proteins from rat organs and PNGase treatment. Membrane proteins were prepared from whole organs (adrenal glands, kidneys, ovaries, and salivary glands) of adult female Sprague-Dawley rats. Fresh tissues were homogenized with a motor-driven Teflon pestle (Dounce homogenizer). The homogenized tissue samples were first centrifuged for 15 min at 6,000 g and 4°C. After the second centrifugation step, the supernatant was transferred to a fresh tube and centrifuged for 15 min at 10,000 g and 4°C. After the second centrifugation step, the supernatant was centrifuged for 60 min at 100,000 g and 4°C. The resulting pellet represented the membrane fraction and was resuspended in the homogenization buffer. The protein concentration was determined according to the method of Bradford (3) using the Protein Assay kit from Bio-Rad (Munich, Germany) and BSA as a standard. Fifty micrograms of membrane protein were digested with 500 U of peptide:N-glycosidase F (PNGase F) (New England Biolabs, Schwalbach, Germany) for 1 h. Antigen-antibody complexes were visualized using a chemiluminescence detection kit (NEL Life Science, Cologne, Germany).

Processing of images. Black-and-white photographs were taken both from immunofluorescent- and H & E-stained specimens. Photographs and X-ray films from Western blots were scanned with a Nikon Coolscan LS-2000 using the Silverfast 4.1 software (LaserSoft, Kiel, Germany) and then processed with Photoshop 4.0 (Adobe Systems, San Jose, CA).

RESULTS

Immunohistochemical localization of the Pkd2 protein in the kidney. The distribution of the Pkd2 protein in the rat kidney is shown at low-power magnification in Fig. 1. Pkd2 is expressed in tubular profiles from the cortex through the inner stripe of the outer medulla. In the cortex, labeling was found in the medullary rays as well as in the cortical labyrinth. In addition to profiles with a continuous labeling, some cortical profiles displayed a discontinuous staining pattern. To more precisely determine the distribution of the Pkd2 protein along the nephron, double immunofluorescence labeling with specific markers as well as structural characteristics were used. Pkd2 expression in thick ascending limb cells of the outer stripe was demonstrated using an antibody against the Tamm-Horsfall glycoprotein as a marker for this nephron segment (10). Double immunostaining with both antibodies revealed expression of Pkd2 in thick ascending limb profiles. Neighboring proximal tubules did not exhibit any detectable immunostaining for Pkd2 (Fig. 2, A and B). To assess the expression of Pkd2 in distal convoluted tubules and connecting tubules, double immunohistochemistry with antibodies against Pkd2 and calbindin D28k was performed (Fig. 2, C and D). Calbindin, a Ca2+-binding protein, is strongly expressed in connecting tubule cells, whereas its expression in distal convoluted tubule cells is only weak (13). Figure 2, C and D, shows many tubular profiles intensely stained for Pkd2 and calbindin D28k, indicating coexpression in connecting tubule cells, whereas intermingling intercalated cells, known to be unreactive for calbindin D28k, were also unreactive with the anti-PKD2 antibody. Other profiles were stained homogeneously for Pkd2, but not for calbindin D28k, therefore probably representing distal convoluted tubule profiles (Fig. 2, C and D). To confirm the expression of the Pkd2 protein in distal convoluted tubule profiles and to see whether macula densa cells express this protein, the end portion of the thick ascending limb and its transition to the distal convoluted tubule were investigated more closely. Cells of the macula densa, which lie within the end portion of the cortical thick ascending limb, were consistently unreactive with the anti-PKD2 antibody, whereas surround-
ing thick ascending limb cells were heavily stained (Figs. 2C and 3, A and C). Expression of the Pkd2 protein was also demonstrated in profiles of the distal convolute, which were identified by their onset shortly beyond the macula densa, the increased height of the cells, and their apical nuclei. Figure 3, C and D, shows the Pkd2-negative macula densa cells, followed by continuous labeling of tubular cells reaching far into the distal convoluted tubule. In all Pkd2-expressing cells, a basal staining pattern was observed. This is emphasized by a high-power micrograph of a thick ascending limb profile in the inner stripe (Fig. 4, A and B), where the Pkd2 immunoreactivity appears to be associated with the lateral interdigitations of a cell. Pkd2 immunoreactivity was also detected as a thin basal stripe in collecting duct profiles of the cortex and outer stripe (Fig. 4, C and D), but the signal in collecting ducts was much more difficult to detect. Although we cannot present direct evidence for expression of Pkd2 in principal cells of collecting ducts because of the faint signal, we are inclined to assume that intercalated cells do not express Pkd2, since they were already unreactive with the anti-PKD2 antibody in the connecting tubule. In kidneys of neonatal rats, the Pkd2 protein again was found on the basal side of distal tubules, whereas the subcapsular region, containing the structures of the nephrogenic zone, was unreactive (data not shown).

The expression pattern of the Pkd2 protein in the rat kidney was then compared with the distribution in human and mouse kidneys. A low-power magnification of the border between the inner stripe and the inner medulla illustrates that the immunofluorescence pattern seen in both species is strikingly similar to that obtained in the rat kidney (Fig. 5, A and B). Accordingly, in the cortex of all three species, distal tubules are heavily labeled by the anti-PKD2 antibody (data not shown). Moreover, the distinct basal distribution is virtually identical in all three species (compare Fig. 5, C and D, with Fig. 4A). As in the rat, a faint basal staining for PKD2 was noted in individual collecting duct cells of the human kidney (Fig. 5F).

In addition to the kidney, the following organs were examined: adrenal gland, aorta, bladder, brain, epididymis, esophagus, eye, heart, small and large intestine, liver, lung, lymph nodes, mammary gland, ovary, pancreas, prostate, salivary glands, seminal vesicle, skeletal muscle, skin, spinal cord, spleen, stomach, testis, thymus, trachea, and uterus. Consistent specific staining could only be detected in the adrenal gland, blood vessels, the eye, ovary, and salivary gland and will be described in the following paragraphs.

Localization of the Pkd2 protein in salivary glands. Immunohistochemical analysis revealed that the Pkd2 protein is abundantly expressed in striated ducts of the rat submandibular and sublingual glands. As seen in Fig. 6, Pkd2 displays a basal-to-basolateral distribution within striated duct cells. Heavy staining was detected in intra- as well as in interlobular striated ducts of both glands; acinar cells and intercalated ducts were not stained with the anti-PKD2 antibody. Immunohistochemical staining of paraffin sections from human submandibular glands confirmed the abundance of the PKD2 protein in cells of smaller and larger striated ducts as seen in the overview of Fig. 7. In addition to the basal staining in the kidney, a lateral distribution of PKD2 was noticed (Fig. 7, B and D). In the human parotid gland, a similar cellular and subcellular distribution was found (data not shown).
Localization of the Pkd2 protein in the adrenal gland. Pkd2 protein-containing cells were found in different layers of the adrenal cortex as shown in Fig. 8. The Pkd2 protein was detectable in the outermost parenchymal cells of the zona glomerulosa. Intensive staining with the anti-PKD2 antibody was also observed in cells of the zona fasciculata, and again the labeling was confined to only a subpopulation of cells. Although most heavily labeled cells could be detected in the outer part of this layer, Pkd2-positive cells were also found closer to the zona reticularis (Fig. 8A and B). No labeling was found in the adrenal medulla. The cellular distribution of the Pkd2 protein is demonstrated by high-power magnification in Fig. 8C, where a fine punctate staining within the entire cytoplasm of zona fasciculata cells can be seen.

Localization of the Pkd2 protein in the ovary. Prominent immunoreactivity for Pkd2 could be detected in granulosa cells of corpora lutea (Fig. 9). As in the adrenal cortex, a punctate cytoplasmic staining pattern was noticed. The portion of Pkd2-expressing granulosa cells varied when individual corpora lutea of a section were examined, which probably reflects the different developmental stages of the corpora lutea. No Pkd2 immunoreactivity was found in granulosa cells of developing follicles.

Localization of the Pkd2 protein in vascular structures. Arteries of the muscular type revealed labeling of the smooth muscle cell layers as demonstrated in Fig. 10, where several arteries branching into a salivary gland are shown. A high-power view shows a punctate to striped staining pattern. Although clearly detectable, it should be emphasized that the staining intensity was less pronounced than in the striated duct profiles. Arteries of the elastic type such as the aorta were unreactive with the anti-Pkd2 antibody.

Localization of the Pkd2 protein in the eye. Sagittal paraffin sections of the rat eye were subjected to immunohistochemical analysis. Intense labeling occurred exclusively in the basal cell layer of the corneal epithelium, demonstrating again a punctate cytoplasmic distribution of the protein (Fig. 11). The staining intensity for Pkd2 appeared to vary between different basal cells. Other structures, notably the retina, were not found to express Pkd2.

Western blot analysis of Pkd2 protein expression. Because the subcellular distribution of the Pkd2 protein varied strikingly between different organs (basal/basolateral in kidney and salivary gland vs. punctate cytoplasmic in adrenal gland, ovary, smooth muscle cells, and the eye), we wanted to rule out a nonspecific staining in the different organs. Preabsorption of the anti-PKD2 antibody with the GST-PKD2 fusion protein used to generate the antibody abolished both the basolateral staining in the kidney and salivary gland and the punctate staining in the adrenal gland, ovary, and blood vessels, whereas after preabsorbing the antibody with GST alone, the respective staining patterns were still present (data not shown). Furthermore, membrane proteins were prepared from the adrenal

Fig. 2. Expression of the Pkd2 protein in distal segments of the nephron in rat kidney. Paraffin sections of rat kidneys were stained by double immunofluorescence with the anti-PKD2 antibody YCC2 (A and C) and an anti-Tamm-Horsfall protein antibody (B) or anti-calbindin D28k antibody (D). In A, Pkd2 protein expression corresponds exactly to the Tamm-Horsfall protein-expressing thick ascending limb profiles (B) of the outer stripe. Connecting tubule profiles, recognized in D by the expression of calbindin D28k, also express the Pkd2 protein (C). Coexpression occurs in connecting tubule cells, but not in intercalated cells (the latter are unreactive with both antibodies, arrowheads in C and D). Asterisks in C and D mark putative distal convoluted tubule profiles, which also express Pkd2 protein but are nearly unreactive with the anti-calbindin D28k antibody on paraffin sections. Arrows in C and D delimit the Pkd2-negative macula densa in an otherwise stained thick ascending limb profile. G, glomerulus. Magnifications, ×220 (A and B) and ×175 (C and D).
gland, kidney, ovary, and salivary gland and subjected to Western blot analysis with the anti-PKD2 antibody. Somewhat surprisingly, the Pkd2 protein from the rat adrenal gland and ovary displayed a slower mobility than the Pkd2 protein from the rat kidney and salivary gland (Fig. 12, left). To examine whether a different degree of N-glycosylation accounted for that difference, the membrane protein preparation was digested with PNGase F, a glycosidase that cleaves between the asparagine of the peptide backbone and the first sugar residue. Treatment with PNGase F resulted in an equal mobility of the rat Pkd2 protein from all four organs (Fig. 12, right).

**DISCUSSION**

In the present study, we were able to determine the distribution of the Pkd2 protein in different rat and human organs using immunohistochemistry and Western blot analysis. In the rat kidney, Pkd2 is strongly expressed in cells of the distal nephron but at much lower levels in collecting duct cells. A comparable expression of the Pkd2 protein was noted in human and mouse kidneys. The pronounced Pkd2 staining in the thick ascending limb, distal convoluted tubule, and connecting tubule contrasted with the faint labeling in the collecting duct, where lateral interdigitations are absent and basal infoldings are scarce (11). Interestingly, macula densa cells lack Pkd2 expression. These cells, which serve as a sensor of tubular function, do not interdigitate with each other, nor do they participate in electrolyte absorption. Basal infoldings, however, are also present in the macula densa (11). The observations made in the kidney were significantly extended, since we could also document the expression of Pkd2 in striated ducts of rat and human salivary glands. This is of particular interest since the salivary glands share some important functional properties with the kidney (26). The composition of the isosmotic saliva produced by the acinar
cells is later modified by the electrolyte-transporting epithelium of the striated ducts. These cells, which for example reabsorb sodium and secrete potassium, show obvious structural similarities to renal thick ascending limb cells. As in the thick ascending limb, the basal labyrinth with numerous mitochondria is highly developed in striated duct cells. Comparable to the kidney, a countercurrent exchange exists between saliva and blood. Therefore, the comparable staining pattern for Pkd2 in the thick ascending limb of the kidney and in striated ducts of the salivary gland suggests that this protein accomplishes a similar function in both organs.

The basal-to-basolateral distribution of Pkd2 in the kidney and salivary gland strikingly differed from that seen in the adrenal cortex. There, the Pkd2 protein was located in fine cytoplasmic structures often occupying the entire cytoplasm. It is known that cells of the adrenal cortex have a well-developed smooth endoplasmic reticulum and contain numerous mitochondria, which represent the compartments of the early and late stages of steroid biosynthesis. The distribution of Pkd2 in the adrenal cortex, where its expression was most prominent in the outer zona glomerulosa and the outer zona fasciculata, corresponds well with the notion that these subregions exhibit very strong steroidogenic activity for mineralocorticoids and glucocorticoids, respectively (26). The assumption that Pkd2 is located in the endoplasmic reticulum is supported by the distribution of Pkd2 in the ovary and by in vitro findings. First, the subcellular distribution pattern of Pkd2 in the adrenal gland was virtually identical to that seen in granulosa cells of corpora lutea in the ovary. These cells show ultrastructural and functional characteristics similar to cells in the adrenal cortex (both produce steroid hormones) (26), thus suggesting an analogous subcellular location and function for Pkd2 in both endocrine cell types. Second, in all permanent cell lines examined so far, both the endogenous protein and stably expressed human PKD2 protein were located in the endoplasmic reticulum, regardless of the tissue from which the cell lines were derived (3a; and unpublished observations of A. Cedzich, N. Obermüller, and R. Witzgall). Therefore, an additional signal probably exists in vivo that is responsible for the basal-to-basolateral distribution of Pkd2 in the kidney and salivary gland. Interestingly, it had been shown previously that the endoplasmic reticulum extends deep down into the lateral interdigitations of renal distal tubular cells, where it appears to wrap around the mitochondria and comes to lie between these and the plasma membrane (1, 2). Furthermore, there also is evidence for an extensive intercellular fibrillar network that connects the lateral interdigitations and may be responsible for the very regular spacing of neighboring distal tubular cells (30). If Pkd2 indeed is a subunit of a channel protein, then it should also be considered that in the adrenal cortex this protein is part of a structure that functionally links the endoplasmic reticulum and the mitochondrion. Such a structure-function relationship has been proposed for the regulation of aldosterone secretion in bovine adrenal glomerulosa cells (19).

The different distribution patterns of Pkd2 in the kidney and salivary gland on the one hand and in the adrenal gland and ovary on the other hand also appear to be reflected in the distinct glycosylation patterns of Pkd2 in the respective organs. In the adrenal gland and the ovary, two organs with a punctate distribution of Pkd2, the Pkd2 protein was more heavily N-glycosylated than in the kidney and salivary gland, two organs with a basal-to-basolateral distribution of Pkd2. We do not believe that the immunohistochemical signals and the signals on the Western blot arise from different proteins, because after a digest with PNGase F, the detected protein in all four organs displayed the same mobility. Cross-reactivity with Pkd2L/PkdL also is unlikely, because the human proteins are only 38%
identical in the region of PKD2 used to generate the antibody [the comparison was done with the Gap program of the Genetics Computer Group of Wisconsin (5) using a gap creation penalty of 8 and a gap extension penalty of 2].

Our results may help to recognize other extrarenal symptoms in patients with mutations in the PKD2 gene. Although several reports of polycystic disease in the salivary glands have been published (e.g., Refs. 6 and 25), a connection to polycystic disease of the kidney has not been demonstrated yet. It will be interesting to see whether patients with PKD2 mutations develop cysts in their salivary glands or present with other dysfunctions in this organ. It might also be relevant to find out whether PKD2 mutations affect the endocrine status of patients because of the prominent expression of the protein in the adrenal gland and in the ovary. Interestingly, a possible association between abnormal steroid synthesis and cyst formation has been suggested for the Ke 6 gene in several murine models of PKD (Ref. 8 and references therein).

Our observation that the Pkd2 protein is also present in the cornea of the eye is of particular interest, since mutations in a variety of genes can cause abnormalities in both the eye and the kidney. Somewhat surprisingly, Pkd2 expression was confined to the basal cell layer of the corneal epithelium. These cells, which show dis-

Fig. 5. Expression of the Pkd2 protein in mouse (A, C, and E) and human (B, D, and F) kidneys. Immunofluorescence labeling of thick ascending limb profiles at border between outer and inner medulla are seen both in mouse (A) and human (B) kidneys. High-power magnification from thick ascending limb profiles of the inner stripe again reveals a basal staining pattern (C, D, and F) (also compare corresponding phase-contrast view of C in E). In F, a collecting duct (CD), located in vicinity of thick ascending limb profiles, is also weakly stained. Magnifications, ×118 (A), ×47 (B), ×480 (C–E), and ×275 (F).
crete infoldings on their lateral sides, are sitting on a prominent basement membrane which demarcates the epithelium from the stromal compartment of the cornea (26). However, since we could not detect any expression of the Pkd2 protein in the epidermal layer of the skin, which also contains a stratified squamous epithelium, we consider it unlikely that Pkd2 is in-

Fig. 6. Expression of the Pkd2 protein in rat salivary glands. Sections were first stained by immunofluorescence with anti-PKD2 antibody (A, C, and E) and subsequently with H & E (B, D, and F). Pkd2 immunoreactivity is seen in a basal-to-basolateral pattern in intralobular striated ducts of both the serous submandibular gland (A and B) and the mucous sublingual gland (C and D). In E and F, a Pkd2-expressing interlobular striated duct of sublingual gland is shown. Magnifications, ×285 (A and B) and ×180 (C-F).

Fig. 7. Expression of PKD2 protein in human submandibular gland. Sections were first stained by immunofluorescence with anti-PKD2 antibody (A, B, and D) and subsequently with H & E (C and E). Strong immunoreactivity is exclusively found in numerous striated duct profiles (A–C). In D, basal and lateral distribution of PKD2 is shown at a high magnification, and arrows point to lateral cell borders. Magnifications, ×37 (A), ×150 (B and C), and ×460 (D and E).
involved in cell-matrix interactions in the cornea. This assumption is also supported by the punctate cytoplasmic distribution of the Pkd2 protein in the corneal epithelium. In contrast to PKD2, the PKD2L mRNA is expressed in the retina, but not in the cornea (28), suggesting distinct functions for both proteins in different cells.

Apart from the expression in epithelial cells, our data provide clear evidence for Pkd2 expression in smooth muscle cells of the vasculature, thus confirming observations by other investigators (16, 23), although in the article by Ong et al. (16) expression of the human PKD2 protein was also described in the endothelium of arteries. The expression of the Pkd2 protein in vascular smooth muscle cells provides a clue about the pathogenesis of intracranial aneurysms in ADPKD patients (24). It is tempting to speculate that a mutated PKD2 protein expressed in smooth muscle cells affects the vascular compliance, especially under conditions of higher blood pressure levels.

Although by Northern blot analysis the human PKD2 mRNA and its murine homolog could also be detected in other organs such as the brain, heart, liver, and pancreas (14, 29), we could not find convincing evidence for
the expression of the rat Pkd2 protein in those organs. The reason for this discrepancy is not clear, but it may result from species differences, lack of sensitivity of the immunofluorescence protocol, and/or posttranscriptional regulation of Pkd2 expression in those organs, i.e., lack of translation of the mRNA. In contrast to the well-documented occurrence of cysts in the livers of Pkd2 mutant mice (27) and in the liver and pancreas of ADPKD patients (for review, see Refs. 7 and 9), we failed to detect Pkd2 protein expression in bile ducts and pancreatic ducts of the rat. We cannot rule out that in the pancreas and the liver Pkd2 is expressed at a level below the detection limit; we were able, however, to detect Pkd2 in intrapancreatic vessels. In a recent article, expression of the human PKD2 protein was demonstrated in the liver and pancreas (16). The same
article also describes that in the adult kidney the distal convoluted tubules and the collecting ducts were predominantly stained, and an even more widespread distribution in the fetal kidney was shown (16). This contrasts with our observations of the strongest expression of the Pkd2 protein in the distal tubules and connecting tubules and a much weaker expression in the collecting ducts. Furthermore, we only detect the rat Pkd2 protein at an advanced stage of nephron development (unpublished observations of N. Obermüller and R. Witzgall).

In summary, our demonstration of Pkd2 protein expression in different structures underlines the widespread distribution and potentially very distinct function of Pkd2 in various organ systems. The basal-to-basolateral staining pattern observed on the one hand...
(kidney and the salivary glands) and the punctate cytoplasmic distribution on the other hand (adrenal gland, ovary, cornea, blood vessels) raises the possibility that the Pkd2 protein accomplishes its function in concert with different proteins. These proteins may be located in the plasma membrane or in cytoplasmic compartments. Clearly, highly specific antibodies and immuno-electron microscopy will help to resolve the issue of the subcellular location of Pkd2 and Pkd1.

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