Recycling of AQP2 occurs through a temperature- and bafilomycin-sensitive trans-Golgi-associated compartment

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Recycling of AQP2 occurs through a temperature- and bafilomycin-sensitive trans-Golgi-associated compartment. Am. J. Physiol. Renal Physiol. 278: F317–F326, 2000.—The exo- and endocytotic pathway in which aquaporin-2 (AQP2) travels between the plasma membrane and intracellular vesicles is only partially characterized. It is known that the antidiuretic hormone vasopressin induces a translocation of AQP2 from an intracellular to a plasma membrane location, both in kidney collecting duct principal cells and in transfected epithelial cells. Here we provide evidence suggesting that while AQP2 shifts from an intracellular location to the cell surface in response to vasopressin, AQP2 also constitutively recycles through a similar pathway in transfected LLC-PK1 cells even in the absence of hormonal stimulation. This pathway can be blocked by 20°C, which blocks AQP2 recycling in a perinuclear compartment, regardless of whether vasopressin is present. The H^+^-ATPase inhibitor bafilomycin A1 also blocks the recycling pathway of AQP2 in a perinuclear compartment adjacent to the Golgi in the presence and absence of vasopressin stimulation. Incubating cells at 20°C blocks AQP2 recycling in a perinuclear compartment, regardless of whether vasopressin is present. The H^+^-ATPase inhibitor bafilomycin A1 also blocks the recycling pathway of AQP2 in a perinuclear compartment adjacent to the Golgi in the presence and absence of vasopressin stimulation, indicating a role of vesicle acidification in both the constitutive and regulated recycling of AQP2. Colocalization of AQP2 with clathrin, but not with giantin, after both bafilomycin treatment and a 20°C block suggests that the compartment in which recycling AQP2 is blocked may be the trans-Golgi, and not cis- and medial-Golgi cisternae.

The physiological properties of renal epithelia are related to the regulated, polarized insertion of membrane proteins in highly specialized cell types that perform unique transport functions along the urinary tubule (4). The aquaporin-2 (AQP2) water channel is located in renal collecting duct principal cells (16, 34), although we have recently reported AQP2 expression in epithelial cells of the vas deferens (33). The antidiuretic hormone, vasopressin, induces movement of AQP2 from intracellular vesicles to the plasma membrane in rats (12, 16, 44). Through this regulated recycling pathway, the amount of AQP2 at the cell surface determines the final concentrating ability of the collecting duct by allowing the luminal fluid to equilibrate osmotically with the hypertonic medullary interstitium. The physiological importance of AQP2 is evident from studies on nephrogenic diabetes insipidus. Mutations in AQP2 have been identified which result in either a nonfunctional protein or altered trafficking that prevents AQP2 from reaching the cell surface (6, 10, 31, 32). The pathways involved in the delivery and recycling of some membrane proteins such as the transferrin receptor (TR) to and from the cell surface have been widely studied. In polarized epithelia, immediately following endocytosis of the apical or basolateral membrane-located TR in a Rab5-positive "sorting endosome," the TR may be rapidly recycled back to the membrane. Several of the Rab family of proteins, which are monomeric GTPases, have been identified as essential components of membrane trafficking pathways (35, 53, 54). In addition to rapid membrane recycling, TR also follows a more complex pathway of recycling from the sorting endosome to a recycling endosomal compartment. This compartment is characteristically tubulovesicular and is pericentriolar in location. The TR-positive endosomal tubules of this recycling compartment contain clathrin-γ-adaptin-coated domains that are responsible for the repackageing and preferential delivery of TR to the basolateral surface (17, 47).

Recycling endosomes have been reported to be heterogeneous with respect to their functional acidity, biochemical composition, and ion transport properties (49). Although it is known that AQP2 recycles between the cell surface and intracellular vesicles (23), details of this pathway are only partially understood. To facilitate the dissection of intermediate steps in exo- and endocytosis of AQP2, we utilized our previously developed cell culture models in which c-myc- or green fluorescent protein (GFP)-tagged AQP2 cDNA was expressed (19, 25). These stably transfected cells provide a rapid and sensitive system for analyzing intracellular trafficking. The data presented in this report suggest that while the localization of AQP2 shifts from an intracellular site to the cell surface in response to vasopressin stimulation, AQP2 constitutively recycles through the same or a similar pathway even in the absence of hormonal stimulation. This pathway can be...
bafilomycin, an inhibitor of the vacuolar H^+−ATPase.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO) and all cell culture reagents were from BioWhittaker (Walkersville, MD).

Cell culture. Renal epithelial LLC-PK_1 cells, stably transfected with AQP2 constructs (LLC/AQP2) were grown at 37°C in 5% CO_2 as described (25). Cells were cultured in DMEM supplemented with 10% bovine calf serum and 2 mM L-glutamine. Cells were routinely determined to be mycoplasma negative. All experiments were repeated several times using three to six different batches of cultured cells.

AQP2 constructs. The previously constructed AQP2 with a c-myc 10-amino-acid tag at its carboxy terminus (25) was used in some of the experiments described. In addition, to facilitate colocalization studies with monoclonal antibody markers, cells stably expressing AQP2 tagged on the amino terminus with GFP was also used. This construct has been characterized previously and was shown to traffic and function similarly to the AQP2 wild-type protein (19).

LLC-PK1/AQP2 immunofluorescence. LLC-PK1/AQP2 cells were plated on 12 × 12-mm glass coverslips (Fisher Scientific, Pittsburgh, PA) 2 days prior to each experiment. Each experiment was carried out in duplicate with cycloheximide (10 µg/ml) and without cycloheximide present in the medium to determine the potential contribution of newly synthesized vs. recycling AQP2 to the observed effects, as previously described (23). Cells were incubated at 37°C, 20°C, or in medium containing 125 nM bafilomycin A1, 10 nM lysine vasopressin, or 10 µM forskolin (Calbiochem, San Diego, CA) at 37°C. Cells were washed at room temperature for 20 min with 4% paraformaldehyde in PBS containing 5% sucrose. Following fixation, the cells were permeabilized with Triton X-100 (0.1%) for 5 min at room temperature. For the antibodies specified below, permeabilization was followed by 1% SDS treatment for 4 min to enhance antigenicity (3). Non-specific antibody binding sites were blocked by a 10-min incubation with 1% BSA in PBS. The anti-c-myc monoclonal antibody, at a 1:50 dilution (Oncogene Science, Cambridge, MA), was used to detect the AQP2 protein in cells expressing the AQP2-c-myc construct, whereas the inherent fluorescence of the GFP tag was used to detect AQP in double-labeling experiments on cells expressing the AQP2-GFP construct. Primary antibodies used for double labeling were anti-giantin (46) used at 1:50 after SDS treatment of cells to detect Golgi cisternae and anti-daftrin heavy chain (Transduction Laboratories, Lexington KY) used, also after SDS treatment, at a dilution of 1:100 to label the trans-Golgi network.

All primary antibodies were applied for 1 h at room temperature. Secondary antibodies, either Cy3-conjugated goat anti-rabbit IgG (2 µg/ml final concentration; Jackson ImmunoResearch, West Grove, PA) or Cy3-conjugated donkey anti-mouse IgG (0.6 µg/ml final concentration Jackson ImmunoResearch), were applied for 1 h at room temperature. Coverslips were mounted on slides using Vectashield (Vector Laboratories, Burlingame, CA) diluted 1:1 with Tris-HCl, pH 8.9. Cells were examined using a Nikon FXA photomicroscope coupled to an Optronix 3-bit color charge-coupled device camera or by confocal laser-scanning microscopy (model MRC600, Bio-Rad Microscope Division). Some images were recorded by conventional photography on Kodak TMax 400 film push-processed to 1600 ASA. Digital images were stored and analyzed using IP Lab Spectrum imaging software (v3.0.1; Signal Analytics, Fairfax, VA) and Adobe Photoshop, and then printed using a Tektronics Phaser 400 dye sublimation printer.

Electron microscopy. For conventional electron microscopy, LLC-PK_1 cells grown on plastic six-well plates were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, under control conditions or after incubation with 125 nM bafilomycin for 2 h. Cells were dehydrated in graded ethanols, postfixed in 1% aqueous osmium tetroxide, removed from the plastic dishes using propylene oxide, and embedded in Epon. Thin sections were stained using uranyl acetate and lead citrate and then photographed on a Philips model CM10 electron microscope.

cAMP determinations in LLC-PK_1 cells. For measurement of intracellular cAMP levels under different experimental conditions, cells were plated onto 96-well plates and used at confluence. cAMP was measured using the antibody-based BioTak assay system (Amersham Pharmacia Biotech). Cells were used untreated, treated for 10 min with 10 µM forskolin (as a positive control), after incubation at 20°C for 2 h, and finally after incubation for 2 h at 20°C followed by a temperature shift to 37°C for 10 min. Cells were lysed according to the manufacturer’s instructions, and the supernatants were mixed with rabbit anti-cAMP antibody. The solutions were placed into individual 96-well plates (triplicate wells were assayed for each sample) coated with donkey anti-rabbit antibody, and a cAMP-peroxidase conjugate was then added to each well. Substrate was added to each well, and the reaction was stopped by the addition of sulfuric acid. The optical density (OD) of each well was read at 480 nm on an ELISA plate reader. The optical density of each well was compared with a standard curve (0–3,200 fmol/well of cAMP). Because this is a competition assay, the OD of each well is inversely proportional to the amount of cAMP in each sample.

RESULTS

Low temperature induces AQP2 accumulation in the “Golgi” region. AQP2-expressing LLC-PK_1 cells, pre-
treated with cycloheximide to block de novo AQP2 synthesis (23), were cooled to exactly 20°C and incubated for 2 h to block release of proteins from the trans-Golgi and from early endosomes (27). Under baseline conditions, AQP2 was mainly located in perinuclear vesicles, as previously described (25) (Fig. 1A). The 20°C temperature block was carried out for various times, both in the presence and absence of cycloheximide (10 µg/ml). After 2 h at 20°C, a dense accumulation of AQP2 was observed as a bright spot in the perinuclear Golgi region (Fig. 1B). No significant difference in this low-temperature-induced accumulation of AQP2 was observed regardless of whether cycloheximide was present. Thus the accumulation of AQP2 in the Golgi region was probably not a result of newly synthesized protein blocked at the final exit step from the Golgi, but was rather derived from a recycling pool of preexisting protein. The cells shown in Fig. 1 had not been stimulated with vasopressin, indicating that the recycling pathway of these preexisting AQP2 proteins was constitutive as opposed to regulated. Incubation of cells with vasopressin or forskolin did not alter the localization of accumulated AQP2 during the 20°C temperature block (not shown), suggesting that the compartment in which AQP2 accumulates during the block is the same regardless of whether AQP2 is recycling constitutively or is under vasopressin stimulation.

A cohort of membrane proteins can be followed in the exocytotic pathway by incubating cycloheximide-treated cells at 20°C to block protein traffic, followed by a sudden return to 37°C (23, 27). After returning these cells to 37°C for 30 min to release the temperature block, the juxtanuclear patch of staining disappeared and was replaced by a peripheral basolateral membrane pattern of staining (Fig. 1C). However, some vesicular staining was still visible under these conditions, indicating that membrane insertion might not be as complete as after hormonal stimulation or that some reinternalization of AQP2 had already occurred at this time.
time point. Nevertheless, this change in staining pattern occurred in the absence of vasopressin stimulation, supporting the hypothesis that AQP2 can follow a vasopressin-independent recycling pathway between intracellular vesicles and the plasma membrane. After a further 2-h incubation at 37°C, AQP2 had returned to a predominantly intracellular vesicular location (not shown) indistinguishable from the normal baseline distribution of AQP2 illustrated in Fig. 1A.

To determine whether a transient increase in intracellular cAMP occurred in the cells after the 20°C to 37°C temperature shift, intracellular cAMP was measured immediately after this maneuver using an antibody-based cAMP colorimetric assay. The results clearly showed that no increase in cAMP was detectable under these conditions, whereas the same assay on forskolin-treated cells detected the expected large increase in cAMP (Fig. 2).
Low-temperature induces a similar accumulation of AQP2 and clathrin in the “Golgi” region. Clathrin is concentrated on clathrin-coated transport vesicles that are derived from the plasma membrane and from the trans-Golgi network (TGN) (14, 18, 37, 40). It can, therefore, be used as a marker of the TGN (18, 37). LLC-PK₁ cells expressing AQP2-GFP were exposed to 20°C for 2 h, fixed, and stained with anti-clathrin antibodies. As described above, this procedure caused a redistribution of AQP2 (detected by intrinsic GFP-fluorescence) from scattered perinuclear vesicles in control cells (Fig. 3A) into a dense juxtanuclear patch of staining in the majority of cells (Fig. 3E). The merged images show some overlap (yellow) of AQP2 (green) and clathrin (red) at 37°C (Fig. 3C), with a more obvious overlap seen at 20°C (Fig. 3F). This similarity of distribution of the two proteins suggests that AQP2 accumulated, at least partially, in a clathrin-positive trans-Golgi region of the cell at low temperature.

Effect of the H⁺-ATPase inhibitor bafilomycin A₁ on protein trafficking. To further dissect the trafficking pathway of AQP2, we used the fungal metabolite bafilomycin A₁, an H⁺-ATPase-inhibitor that has been reported to interfere with some steps in the intracellular trafficking pathway (1, 7, 41, 51). LLC-PK₁ cells expressing AQP2-GFP were incubated with bafilomycin (125 nM) at 37°C for various times. An accumulation of AQP2 in the Golgi region was evident within 30 min of bafilomycin treatment (not shown). After 2 h, a dense accumulation of AQP2 was seen in a juxtanuclear location (Fig. 3G), similar to the effect of low temperature described above. A dense accumulation of clathrin was also detectable in the same juxtanuclear region of cells after bafilomycin treatment (Fig. 3H). Considerable overlap of the fluorescent signals for AQP2 and clathrin was found in a dense cluster in the Golgi region (Fig. 3I), although clathrin-positive vesicles that did not contain AQP2 were also visible. Because the effects of bafilomycin are irreversible, the response to a release of the trafficking block could not be determined. This effect of bafilomycin was also seen in the presence cycloheximide (not shown), suggesting that the accumulation of AQP2 in a Golgi-related compartment is due to the recycling of preexisting AQP2 protein. No vasopressin was added to the cells shown in Fig. 3 to stimulate AQP2 recycling, suggesting that bafilomycin blocked a constitutive recycling pathway. As reported above for the 20°C block, addition of vasopressin to bafilomycin-treated cells did not induce translocation of AQP2 to the plasma membrane (not shown).

Effect of bafilomycin on the intracellular distribution of FITC-dextran, a fluid-phase marker of endocytosis. To determine whether recycling AQP2 reached the perinuclear region after endocytosis from the cell surface, a fluid-phase marker was used to follow the internalization process. FITC-dextran was added to the medium for 15 min, during which time it accumulated in endosomal vesicles (Fig. 4A). We previously showed by quantitative confocal fluorescence microscopy analysis that the rate of endocytosis of FITC-dextran could be increased sixfold by vasopressin treatment of LLC-PK₁ cells, which we have previously described as “compensatory” endocytosis following a burst of hormone-induced AQP2 exocytosis (25). However, despite a quantitative difference in FITC-dextran uptake, the labeled vesicular pattern was the same whether the cells had been subjected to vasopressin-stimulation or not.

Following FITC-dextran uptake for 15 min (Fig. 4A), cells were washed and incubated in medium alone for 30 min to chase the cohort of FITC-dextran-labeled endosomes through the trafficking pathway. This resulted in a marked reduction in intracellular vesicular labeling (Fig. 4B). However, when the chase was performed in the presence of bafilomycin, a dense accumulation of FITC-dextran was detectable in a perinuclear pattern (Fig. 4C). Thus the FITC-dextran was blocked by bafilomycin in a perinuclear compartment, resembling that in which AQP2 accumulated after a tempera-
that seen with the AQP2-c-myc construct described above (Fig. 5A). The Texas Red-dextran also accumulated in a perinuclear compartment that showed considerable overlap with the AQP2-GFP fluorescence at this level of resolution (Fig. 5B).

AQP2 does not accumulate in Golgi cisternae. The preceding data using clathrin as a marker suggested that AQP2 is located in the TGN after blocking the recycling pathway with bafilomycin or reduced temperature. To determine whether Golgi cisternae might also be an additional site of AQP2 accumulation, giantin was used as a marker of the cis- and medial-Golgi cisternae (46). Bafilomycin treatment for 2 h altered the normal pattern of giantin labeling, suggesting some disruption of the normal cisternal organization (Fig. 6). Electron microscopic analysis of the Golgi morphology following bafilomycin treatment confirmed that bafilomycin caused a disruption of Golgi morphology in a way that is consistent with the altered giantin staining (Fig. 7). Cisternae were diluted, and Golgi stacks were partially fragmented. Giantin staining of bafilomycin-treated LLC-PK1 cells expressing AQP2-GFP clearly showed the distinct localization of these two proteins in different regions of the Golgi apparatus (Fig. 8), indicating that AQP2 does not accumulate in Golgi cisternae under these experimental conditions.

**DISCUSSION**

Movement of AQP2 from intracellular vesicles to the plasma membrane in principal cells of the renal collecting ducts is induced by vasopressin (12, 16, 44). This regulated exocytotic pathway controls the amount of AQP2 at the cell surface, which in turn determines the urine concentrating ability of the kidney collecting duct. The AQP2 protein shares some cellular trafficking characteristics with other proteins that enter a regulated membrane expression pathway, such as the insulin-regulated glucose transporter, GLUT4 (22, 39). Experiments performed in cells expressing the GLUT4 glucose transporter have shown that this protein has a complex pattern of vesicular traffic and undergoes a slow rate of constitutive recycling in addition to regulation by insulin (20, 52). The major finding reported here is that AQP2 also recycles constitutively and that this occurs via a temperature- and bafilomycin-sensitive trans-Golgi network-associated compartment in transfected LLC-PK1 cells.
Incubation of cells at 20°C was performed to block exit of proteins from the trans-Golgi and from early to late endosomes (27). After just 2 h at 20°C in the absence of new protein synthesis, a very dense accumulation of AQP2 was observed in the Golgi region. Partial colocalization with clathrin, which was enhanced by blocking the recycling pathway, suggests that recycling of the AQP2 protein may occur via a TGN-related compartment. Clathrin is an established marker of the TGN, from which many proteins are exported in clathrin-coated vesicles (18, 37). Transporting vesicles that bud from other Golgi cisternae have a coat of different composition and are known as coatamer protein (COP)-coated vesicles (45). The accumulation of the fluid-phase markers FITC- and Texas Red-dextran in a very similar perinuclear compartment following bafilomycin treatment supports the notion that cell surface material can be delivered to a juxtanuclear compartment during membrane recycling. Thus the observed accumulation of AQP2 in this region of the cell probably did not occur by a “retrograde” condensation of preexisting cytoplasmic vesicles (which would not be labeled with endocytosed fluorescent markers). We have previously shown that another fluid-phase marker, horseradish peroxidase (HRP), can be found in the Golgi-region of kidney collecting duct principal cells after in vivo injection and subsequent endocytosis (5). A similar accumulation of HRP in the Golgi was also shown in insulin-secreting B cells of the pancreas (36). These data indicate that the cell-surface to Golgi delivery of fluid phase markers is not restricted to LLC-PK1 cells. Furthermore, the recycling of cell proteins between the plasma membrane and the TGN has been well-documented in other systems (9, 30, 42, 48).

After a cold-induced block of trafficking followed by rewarming the cells to 37°C, a rapid movement of AQP2 to the cell surface occurred. Under these conditions, intracellular cAMP was not elevated above baseline levels, indicating that this movement of AQP2 was cAMP independent. Thus cold treatment followed by a chase at 37°C allowed the accumulation of AQP2 in a constitutive recycling pathway from which a large cohort of protein could be released simultaneously. One explanation for these findings is that after exit from the trans-Golgi (or a recycling endosomal compartment) under normal conditions, the resulting transport vesicles are not static within the cell but can move to the cell surface and subsequently recycle, albeit at a much slower rate than after stimulation by vasopressin or forskolin. After release of the temperature block, the vesicles released from the blocked compartment have properties similar to those produced under “normal” recycling conditions, but because the vesicles are much more numerous during the 37°C chase period, the coordinated movement of AQP2 to the cell surface can be more readily visualized. This explanation has been previously proposed for sorting pathways followed by other proteins after a temperature-induced blockade (27). However, it remains possible that the temperature block induces the formation of a novel type of transport vesicle that is able to move to the cell surface in a constitutive manner and that this pathway may not be active under normal physiological conditions.

Many intracellular compartments have an acidic luminal pH generated by an H^+ -ATPase and a parallel membrane chloride conductance (21, 28, 43). Inhibition of acidification affects a variety of intracellular pathways, including transport from late endosomes to lyso-
patterns. Bar: little or no overlap in the staining (green) are in different locations, with age shows that giantin (red) and AQP2 vesicles (13). Since an H\textsuperscript{+}-ATPase is located on Golgi cisternae (29), it is possible that the effect of bafilomycin on AQP2 exocytosis may be mediated by a block of the coat formation at the exit step from the TGN. Indeed, bafilomycin disrupted Golgi stack morphology, resulting in a change in distribution of the cisternal marker, giantin. More work will be required to determine the mechanism underlying this bafilomycin effect, but it is intriguing that the result of exposure of cells to a reduced (20°C) temperature treatment was very similar. Because the bafilomycin effect is not readily reversible, the fate of blocked AQP2 after sudden release from its site of intracellular accumulation could not be examined. As previously discussed above for the temperature block, the relationship between the trafficking pathway for AQP2 after inhibition of the H\textsuperscript{+}-ATPase and its normal recycling pathway remains to be determined.

Giantin is located primarily in the cis- and medial-Golgi cisternae and did not colocalize with AQP2, suggesting that AQP2 either does not recycle through the cis- or medial-Golgi cisternae or is only transiently present during the recycling process. More probably, AQP2 recycles through and accumulates in the TGN or an associated compartment. The partial colocalization of AQP2 with clathrin, a marker of the TGN, supports this hypothesis. However, a recent study (17) has identified a subpopulation of recycling endosomes that are clathrin coated. It is possible that AQP2 recycles through a similar pathway in LLC-PK\textsubscript{1} cells.

Finally, AQP2 is delivered to the basolateral plasma membrane of transfected LLC-PK\textsubscript{1} cells (25), whereas it is delivered predominantly to the apical membrane of collecting duct principal cells in situ, as well as MDCK cells (11) and transformed rabbit inner medullary collecting duct (IMCD) cells in culture (50). Whether the AQP2 recycling pathway described here is similar to that in principal cells remains to be determined. However, under some experimental conditions, large amounts of AQP2 can be detected on the basolateral membrane of collecting duct principal cells in situ (2), as well as in primary cultures of IMCD cells (26). Previous studies on trafficking in LLC-PK\textsubscript{1} cells as well as other cell types have shown a marked convergence of apical and basolateral endocytic pathways (8, 38), suggesting that after the early endosome stage, apical and basolaterally recycling material may share a common pathway until the exit step from the TGN.

In summary, we provide evidence that in the non-stimulated state, AQP2-containing vesicles follow a constitutive recycling pathway between the basolateral membrane and a TGN-associated compartment that is the same or very similar to that observed in stimulated cells. This result suggests that it is the steady-state localization of AQP2 that is altered upon vasopressin stimulation, not the absolute localization. Indeed, it has been reported that even in Brattleboro homozygous rats, which lack endogenous vasopressin, some AQP2 is detectable at the cell surface in collecting duct principal cells (34, 44, 55). Low levels of AQP2 can also be detected at the cell surface in lines of stably transfected epithelial cells under baseline conditions (see Fig. 2A, for example). These data could reflect the presence of low levels of cAMP in the cytosol of non-stimulated cells, or they could reflect the existence of a cAMP-independent pathway of membrane insertion for AQP2. We and others have previously shown that the shift in AQP2 localization from intracellular vesicles to the plasma membrane is dependent upon protein kinase A-mediated phosphorylation of the serine-256 residue at the AQP2 cytoplasmic COOH terminus (15, 24). The mechanism by which this change in phosphorylation mediates AQP2 redistribution is not known, but either a stimulation of exocytosis, an inhibition of
endocytosis or both would account for the observed cellular redistribution. Our present observations show that this AQP2 recycling pathway in LLC-PK₁ cells is pH sensitive and occurs via a clathrin-positive and giantin-negative compartment. More detailed studies at the ultrastructural level, using additional antibody markers will be required to definitively identify the intracellular compartments through which AQP2 recycles.

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