Human prostasomes express glycolytic enzymes with capacity for ATP production

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Prostasomes are prostate-derived, exosome-like microvesicles that transmit signaling complexes between the acinar epithelial cells of the prostate and sperm cells. The vast majority of prostasomes have a diameter of 30–200 nm, and they are generally surrounded by a classical membrane bilayer. Using a selected proteomic approach, it became increasingly clear that prostasomes harbor distinct subsets of proteins that may be linked to adenine triphosphate (ATP) metabolic turnover that in turn might be of importance in the role of prostasomes as auxiliary instruments in the fertilization process. Among the 21 proteins identified, most of the enzymes of anaerobic glycolysis were represented, and three of the glycolytic enzymes present are among the top 10 proteins found in most exosomes, once again linking prostasomes to the exosome family. Other prostasomal enzymes involved in ATP turnover were adenylate kinase, ATPase, 5′-nucleotidase, and hexose transporters. The identified enzymes in their prostasomal context were operational for ATP formation when supplied with substrates. The net ATP production was low due to a high prostasomal ATPase activity that could be partially inhibited by vanadate that was utilized to profile the ATP-forming ability of prostasomes. Glucose and fructose were equivalent as glycolytic substrates not being membrane permeable (glyceraldehyde 3-phosphate). Prostasomes are prostate-derived, exosome-like microvesicles that transmit signaling complexes between the acinar epithelial cells of the prostate and sperm cells. Their traffic to different cellular locations (45). Lipid rafts are enriched in cholesterol and saturated sphingolipids and phospholipids and characterized by tight lipid packing and reduced molecular diffusion (3, 27). Hence, raft formation may be involved in reendoctysis and trafficking of membrane-bound glycoproteins to endosomes, from where they can be transported to lysosomes or recycled back to the plasma membrane (9). Intracellular endosomes may themselves undergo budding inward, giving rise to small intraluminal vesicles. This budding is determined apparently by raft-based microdomains being enriched in sphingolipids from which ceramide is formed through the action of neutral sphingomyelinase (53), giving rise to multivesicular bodies and, in the case of prostasomes, to storage vesicles (36). Indeed, cholesterol enrichment has been reported to occur in multivesicular bodies (24), and ceramide was reported recently to be present in highly purified prostasomes.

Prostasomes are exosome-like, membrane-surrounded microvesicles, a vast majority of which have a diameter of 30–200 nm (34), and their extracellular occurrence is the result of a fusion process between the membrane surrounding the storage vesicle (containing the intraluminal vesicles) and the plasma membrane of the prostate epithelial cell (exocytosis) (36, 41). Prostasomes contain an array of different proteins (30, 55) and are believed to act as intercellular messengers between the epithelial secretory cells of the prostate gland and sperm cells (34). Herewith, they are able to transfer molecules propitious for fertilization by influencing, e.g., sperm motility (11) and to exert antibacterial, complement inhibitory, antioxidant, and immunosuppressive activities (8, 39, 40, 49). Prostasomes are also important components of capacitation and acrosome reactions (34), manifesting their unambiguous role in the fertilization process.

The control of cell proliferation, differentiation, and signal transduction pathways is mediated generally by protein kinases and phosphatases (1, 6, 10) whose actions are modified by hormones, growth factors, and mitogens (1, 14). Adenosine triphosphate (ATP) functions as phosphoryl group donor in reactions catalyzed by protein kinase. Approximately one-half of the protein kinase activity in human prostatic fluid was found to be associated with prostasomes (50, 57), and coinoculation of spermatozoa and prostasomes was shown to result in a 10-fold increase in total protein phosphorylation compared with the level of phosphorylation achieved through incubation with either component alone (50). These findings support the interactive relationship between prostasomes and spermatozoa.

In the present study, we carried out selected proteomic examinations of purified human prostasomes combined with a database search for identification of any prostasomal enzyme involved in ATP metabolic turnover that might be of impor-
tance in the role of prostasomes as auxiliary instruments in the fertilization process.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the University of Uppsala and has been performed according to the Declaration of Helsinki.

Chemicals

Adenosine 5’-diphosphate sodium salt, adenosine triphosphate (ATP), D(−)-fructose, D(+)-glucose, DL-glyceraldehyde 3-phosphate, potassium sodium tartrate tetrahydrate, levamisole hydrochloride, sodium ioacetate, magnesium chloride hexahydrate, potassium chloride, β-nicotinamide adenine dinucleotide hydrate, and sodium 5,5-diethyl barbiturate were from Sigma-Aldrich (St. Louis, MO), and glucose, potassium chloride, and magnesium chloride were from Merck (Darmstadt, Germany). The ATP determination kit, including dihiotretiol (DTT), was from InVitrogen (Paisley, UK).

Microtiter plates, polystyrene 96-well PM; were purchased from Sigma-Aldrich, and the analysis was performed by a Victor™ 1420 Multilabel counter (Perkin-Elmer, Santa Clara, CA).

Phosphate-buffered saline (PBS), pH 7.6, was freshly prepared and autoclaved to exclude any bacterial interference. Small volumes of the autoclaved PBS were frozen for use in each experiment.

Liquid Chromatography-Tandem Mass Spectrometry Analyses of Prostasomal Peptides

Prostasomes in PBS suspension corresponding to 50 μg of protein were removed for digestion. SDS (1%) and ammonium bicarbonate (100 mmol/l) were added, followed by reduction (DTT, 10 mmol/l) for 10 min at 58°C and alkylation (iodoacetamide, 20 mmol/l) for 30 min at 20°C under dark conditions. Material was then transferred to spin tubes (Pall, 3-kDa cutoff) for “filter-aided sample preparation” (58), and urea (4 mol/l) was included. Four exchanges with at least tenfold dilution each time were done, and retained volumes were estimated with a pipette. Two volumes of 100 mmol/l of ammonium bicarbonate were added and 1 mmol/l calcium chloride thereafter. Digestion commenced with the addition of 0.5 mg/ml of trypsin (seq grade; Roche) and proceeded overnight at 20°C. The digestes were mixed with 5% acetic acid and collected by centrifugation. The tubes with their content were washed with 100 μl of urea and 4 mmol/l acetic acid, 5% of which after centrifugation was added to the primary filtrate. The filtrates were passed through STAGE tips (31) twice, eluted, and dried. Resolubilization was in 20 μl of 0.1% trifluoroacetic acid. Mass analysis was carried out on a Thermo LTQ Ultra coupled with an Agilent nanoflow 1100 HPLC. The gradient was 2–4% B 0–10 min flow at 0.5 μl/min, 4–35% B 10–80 min flow at 0.2 μl/min, 35–50% B 80–98 min flow at 0.2 μl/min, 50–100% B for 98–103 min, and 100% B 103–124 min flow ramped up to 0.5 μl/min. The column was made in-house with ReproSil Pur C18AQ 3-μm beads in a fused silica capillary, −120 × 0.3 mm. The mass spectrometer was run in FT mode with a resolution range of 5–0.08 μmol/l and 10 μl of the prostasome-buffer mixture (now with doubled concentration of constituents to compensate for dilution) to six separate Eppendorf tubes for incubation at 37°C for 10 min. The ADP-enriched prostasome-buffer mixture was generally preincubated for 10 min at 37°C to reduce the ATP contamination of the ADP batch (Sigma) by prostasomal ATPase. One-hundred microliters of preincubated prostasomes was transferred separately to Eppendorf tubes containing selected effectors for 10-min incubations at 37°C before retention of an ATPase inhibitory experiments.

Preparation of Prostasomes

All prostasomal preparations were carried out at 4°C, if not otherwise stated, in accord with previous reports (37, 38). Briefly, semen samples of 30–40 attendants, referred to the fertility clinic at Uppsala University Hospital for infertility investigations, were centrifuged for 20 min at 1,000 g and 20°C to remove spermatozoa and potential cells from the seminal plasma that was again centrifuged at 10,000 g for 30 min to remove possible cell debris. The supernatant was subsequently subjected to preparative ultracentrifugation for 2 h at 100,000 g to pellet prostasomes. The prostasomes were resuspended in PBS, pH 7.6. This suspension was further purified on a Superdex gel column (GE Healthcare, Uppsala, Sweden) and equilibrated with the PBS to separate prostasomes from amorphous substance (51). PBS was used as eluant at a speed of 4 ml/h, and the eluates (~1.5-ml fractions) were monitored at 260 and 280 nm photometrically. High molecular fractions with elevated absorbances at 260/280 nm were collected, pooled, and ultracentrifuged at 100,000 g for 2 h. The pellet representing a first purification step of prostasomes was resuspended in PBS and loaded on the top of a sucrose cushion produced by 1.5 mol/l sucrose overlaid by 1 mol/l sucrose. A band of purified prostasomes appeared in between the layers after 17 h of ultracentrifugation at 85,000 g (cf.; Ref. 36). The band was collected and PBS added before ultracentrifugation at 100,000 g for 2 h to pellet the prostasomes. The pellet was resuspended in PBS, adjusted to a protein concentration of 2 mg/ml using a BCA protein assay kit (Merck), and kept frozen at −70°C.

General Framework for ATP Determination Involving Prostasomes

A buffer mixture was prepared by adding 10 μl each of MgCl2 (0.1 mol/l), KCl (0.1 mol/l), NAD⁺ (0.1 mol/l), and DTT (0.1 mol/l) to 750 μl of PBS (step 1). Two-hundred microliters of purified prostasomes (2 mg/ml) were added to the buffer mixture, resulting in a prostasome-buffer mixture (step 2). ADP was then added, giving a final concentration of 0.05 mmol/l (step 3). The ADP-enriched prostasome-buffer mixture was generally preincubated for 10 min at 37°C to reduce the ATP contamination of the ADP batch (Sigma) by prostasomal ATPase. One-hundred microliters of preincubated prostasomes was transferred separately to Eppendorf tubes containing selected effectors for 10-min incubations at 37°C to examine ATP formation under various conditions (see Incubations). The 10-min incubations were then transferred in 10-μl portions into separate wells (column-wise) of a 96-well plate (10 μl/well). Ninety microliters of luciferase solution was then added to each well, and the plate was incubated at 37°C for 15 min for ATP determination. The whole procedure was repeated at least twice for each experiment.

Prostasomal ATPase Activity Evaluated on ATP Standard

Seven stem solutions of ATP standard dilutions with concentrations ranging from 5 to 0.08 μmol/l (diluted in steps of 1:2) were produced, containing either the buffer mixture or prostasome-buffer mixture with or without 3.3 mmol/l vanadate (final concentration). Regarding the buffer mixture, an ATP standard ladder was produced by transferring 20 μl of each of the six stem solutions in the concentration range of 2.5–0.08 μmol/l into separate Eppendorf tubes for incubation at 37°C for 10 min. Regarding the prostasome-buffer mixture on the other hand, an ATP standard ladder was produced by transferring 10 μl of each of the six stem solutions in the concentration range of 5–0.16 μmol/l and 10 μl of the prostasome-buffer mixture (now with doubled concentration of constituents to compensate for dilution) to six separate Eppendorf tubes for preincubation at 37°C for 10 min.

Incubations

ATPase inhibitory experiments. The 10-min-preincubated prostasomes (100 μl) were transferred into four Eppendorf tubes containing 1 μl each of fructose (0.5 mol/l), fructose plus vanadate (V; 0.083 mol/l); fructose plus V (0.165 mol/l), and fructose + V (0.33 mol/l). The tubes were incubated at 37°C for 10 min, and ATP determination was accomplished as described in the general framework for ATP determination.

Glycolytic ATP formation in the presence of different effectors. The 10-min-preincubated prostasomes (100 μl) were transferred into six Eppendorf tubes containing 1 μl each of glucose (0.5 mol/l) plus V...
(0.33 mol/l), glucose (0.5 mol/l) plus sodium fluoride (NaF: 0.1 mol/l) plus iodoacetate (IA: 0.1 mol/l) plus V (0.33 mol/l), glucose (0.5 mol/l) plus NaF (0.1 mol/l) plus IA (0.1 mol/l) plus V (0.33 mol/l) plus barbiturate (BARB; 0.05 mol/l), fructose (0.5 mol/l) plus V (0.33 mol/l), fructose (0.5 mol/l) plus NaF (0.1 mol/l) plus IA (0.1 mol/l) plus V (0.33 mol/l) plus BARB (0.05 mol/l). The remaining preincubated prostasomes were saved as control. The tubes were incubated at 37°C for 10 min, and ATP determination was accomplished as described in the general framework for ATP determination.

 Glycolytic ATP formation with an alternative substrate. The 10-min-preincubated prostasomes (100 µl) were transferred into two Eppendorf tubes containing 1 µl each of glyceraldehyde 3-phosphate (GAP) (1 mol/l) and GAP (1 mol/l) plus NaF (0.1 mol/l) plus IA (0.1 mol/l). The remaining preincubated prostasomes were saved as controls. The tubes were incubated at 37°C for 10 min, and ATP determination was accomplished as described in the general framework for ATP determination.

 Adenylate kinase-catalyzed ATP formation in the presence and absence of tartrate and levamisole. ADP-buffer mixture was prepared by adding 3 µl each of MgCl2 (0.1 mol/l), KCl (0.1 mol/l), NAD+ (0.1 mol/l), DTT (0.1 mol/l), and ADP (5 mmol/l) to 285 µl of PBS in an Eppendorf tube (control 1). Measurement of adenylate kinase activity was started by transferring prostasomes suspended in buffer mixture (200 µl) into three Eppendorf tubes already containing 0.05 mmol/l ADP (final concentration). Two of the tubes contained either 3.3 mmol/l of V alone or 3.3 mmol/l V together with 10 mmol/l tartrate (T) and 10 mmol/l of levamisole (L) (final concentrations). The third tube containing ADP and the prostasome-buffer mixture served as control 2. All tubes were incubated at 37°C for 10 min, and ATP determination was accomplished as described in the general framework for ATP determination.

 Calculation of ATP formation and standard deviation. In all calculations, the wells in the top and bottom rows were not included because of possible deviant values. Samples with high luminescence were separated with one empty column in between to avoid luminescence spillover that occurred between wells. Graphs were prepared and standard deviations (SDs) were calculated with Matlab.

Statistical Analyses

Statistical P values were calculated with Student’s t-test, using the luminescence values in rows 2–7 (wells in top and bottom rows were excluded; see above). Since one of the mean values could always be accounted as higher than the other, we used the one-sided t-test. P values were foremost calculated in cases where the significance was not obvious.

**RESULTS**

Selected proteomic studies were utilized to gain knowledge of enzymes involved in ATP metabolic turnover of prostasomes purified by differential centrifugation steps, including preparative ultracentrifugation and gel chromatography. Integrity, purity, and reproducibility of preparatory procedures of relatively homogeneous prostasomes have been documented in previous reports (37, 38). Enzymes involved in ATP metabolic turnover and hexose transporters from purified prostasomes are shown in Table 1. It is noticeable that fructokinase is absent, although fructose is the physiological hexose of seminal fluid, and glucose and fructose were equivalent substrates in generating ATP (see Fig. 4). Apparently, hexokinase phosphorylated not only glucose but also fructose in position 6. It could also be annotated that aldolase A (1 of 3 isoenzymes) is present in prostasomes, being found normally in developing embryo and produced in even greater amounts in adult muscle, whereas it is repressed in adult liver, kidney, and intestine.

Aldolase A catalyses a reversible aldol reaction, and the substrate is fructose 1,6-bisphosphate, generating GAP and dihydroxyacetone phosphate. Hence, glycolytic enzymes are present in prostasomes (Fig. 1A). Other enzymes in ATP metabolic turnover are adenylate kinase and 5'-nucleotidase (Fig. 1B), and they are associated with prostasome as well (Table 1). It could be mentioned that GAP dehydrogenase, enolase-1α, and pyruvate kinase are among the top 10 proteins found in most exosomes (22), once again linking prostasomes to the exosome family (38).

We confirm previous findings of a strong ATPase activity in seminal prostasomes (32, 33) (Table 1 and Fig. 2) and of V as an efficient inhibitor of this ATPase (Fig. 3) (33). Net ATP production by seminal prostasomes in absence of the ATPase inhibitor was very low. Glucose and fructose were both valid substrates for ATP formation in the presence of V, and this ATP generation was distinctly inhibited by two well-known glycolytic inhibitors, IA and NaF (Fig. 4). IA and NaF demonstrated an additive inhibitory effect (not shown in the figure) emphasizing the involvement of the glycolytic chain, since IA is inhibitory of GAP dehydrogenase (the “upper part” of glycolysis) by reacting with and blocking the essential thiol group of the enzyme. On the other hand, fluoride ions, in presence of phosphate ions, form fluorophosphate, which in turn give rise to a complex with magnesium ions at the active center of enolase (the “lower part” of glycolysis). Thus, although we did not establish the formation of lactate due to too
small amounts of prostasomes in our assay system, we believe that there is no doubt about the relevance of glycolysis for ATP prostasomal production. The effect of the two inhibitors was somewhat attenuated by BARB, an inhibitor of the hexose transporter (17). This suggests that some of the hexoses might have been trapped by their transporters and therewith being inaccessible to surface-located glycolytic enzymes of prostasomes. The surface localization of glycolytic enzymes was established by the validity of GAP (which was not membrane permeable due to its polarity) as a substrate for ATP formation. Again, a distinct augmentation of the ATP pool was noticed in presence of V, and this pool was clearly reduced in presence of IA and NaF (Fig. 5). It should be kept in mind regarding “sideness” that the membrane surrounding the prostasome is “right side out” in relation to the plasma membrane. There are reports, as already mentioned, corroborating the view that glycolytic enzymes are present at the plasma membrane surface (46, 56, 59). Since the prostasomes were subjected to extensive isolation and purification procedures, it is suggested

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**Fig. 1.** Schematic diagrams showing anaerobic glycolysis (A) and reactions involving ATP turnover and detection (B).

**Fig. 2.** Detection of different amounts of ATP suspended in either buffer mixture or buffer mixture containing prostasomes (P) using the luciferase assay method. Prostasomes exhibited a distinct ATPase activity, reducing the amount of ATP detected. The ATPase activity was inhibited partly by 3.3 mmol/l of vanadate (V). AU, arbitrary units.

**Fig. 3.** Effect of increasing concentrations of vanadate on ATP formation by prostasomes in the presence of fructose. Prostasomes were preincubated with ADP (0.05 mmol/l) to reduce the ATP contamination of added ADP before the addition of vanadate (3.3 mmol/l) and fructose (5 mmol/l). Vertical bar indicates SD (all P values <0.001).
that the glycolytic enzymes involved were bound tightly to the prostasomal membrane.

Additionally, ATP was produced readily by prostosomal adenylate kinase (Fig. 6). The presence of this enzymatic activity could not be proven by the classical adenylate kinase inhibitor diadenosinepentaphosphate due to too much contamination of this enzymatic activity by prostasomal membrane.

ATP in this inhibitor (Sigma). Since adenylate kinase catalyzes a reversible dismutation reaction of two ADP, giving rise to one ATP and one AMP, we tested two modest inhibitors (T and L) of prostosomal 5'-nucleotidase (13). By influence on 5'-nucleotidase, the equilibrium constant (which is close to 1) of the dismutation reaction would be affected, since removal of AMP is compromised by the two modest inhibitors being unfavorable for ATP formation. A reducing effect on ATP formation was noticed in the presence of T and L provided that V contributed with its inhibitory effect on ongoing ATPase activity (Fig. 6).

**DISCUSSION**

Selected proteomic examinations of purified prostasomes revealed the presence of glycolytic enzymes that were operational for ATP formation when supplied with substrates. These findings on prostasomes are in line with previous conclusions that three of the glycolytic enzymes demonstrated in the present study on prostasomes (glyceraldehyde 3-phosphate dehydrogenase, enolase, and pyruvate kinase) belong to the top 10 proteins found in most exosomes (22), which is indicative of an association between prostasomes and exosomes. Glycolysable substrates are indeed essential for sperm motility (12, 25), protein tyrosine phosphorylation (54), and fertilization (5). We used both glucose and fructose as glycolysable substrates in the present study. It should be kept in mind, however, that fructose is the physiological substrate in human semen, whereas glucose is present in only small amounts. Still, glucose was as efficient as fructose in substrate in human semen, whereas glucose is present in only small amounts. Still, glucose was as efficient as fructose in

**Fig. 4.** Profiles of ATP-producing capacity of prostasomes with either glucose (5 mmol/l) or fructose (5 mmol/l) as substrate under various incubation conditions. Column 1 displays background luminescence in prostasome-buffer mixture, columns 2–4 represent incubation with glucose, and columns 5–7 represent incubations with fructose. Vanadate (3.3 mmol/l) was included in columns 2–7. Vertical bar indicates SD (P value between columns 3 and 4 = 0.001 and between columns 6 and 7 = 0.017). Prostasome-buffer mixture containing ADP (PBMA: 0.05 mmol/l). Column 1: PBMA; column 2: PBMA + glucose and vanadate; column 3: PBMA + glucose, sodium fluoride (NaF), iodoacetate, and vanadate; column 4: PBMA + glucose, NaF, iodoacetate, vanadate, and barbiturate; column 5: PBMA + fructose and vanadate; column 6: PBMA + fructose, NaF, iodoacetate, and vanadate; column 7: PBMA + fructose, NaF, iodoacetate, vanadate, and barbiturate.

**Fig. 5.** Prostasomal ATP formation with alternative substrate [glyceraldehyde 3-phosphate (GAP), 5 mmol/l] with and without glycolytic inhibitors. Vertical bar indicates SD (P < 0.001, GAP vs. no substrate (P); P = 0.003, GAP vs. GAP + NaF + iodoacetate (IA)). Prostasomes in buffer mixture preincubated with ADP for 10 min and then incubated with either P, GAP, or GAP and inhibitors (GAP + NaF + IA).

**Fig. 6.** ATP formation by prostosomal adenylate kinase (in the absence of any glycolytic substrate), with immediate addition (no preincubation) of 0.05 mmol/l of ADP under various conditions. Duplicate columns at left represent ATP contamination of ADP added (control 1), and duplicate columns at right represent remaining ATP after incubation of prostasome-buffer mixture containing ADP in absence of any ATPase inhibitor (control 2). Vertical bar indicates SD. B, buffer mixture with ADP incubated at 37°C for 10 min; P, prostasome-buffer mixture with ADP incubated at 37°C for 10 min; P + V = prostasome-buffer mixture with ADP and vanadate incubated at 37°C for 10 min; LT, prostasome-buffer mixture with ADP, vanadate, and levamisole + tartrate incubated at 37°C for 10 min.
confirmatory of a previous investigation (26). Accordingly, anaerobic glycolysis ends up in production of lactate. In a cellular context, lactate is transported out of the cell by a family of proton-coupled monocarboxylate transporters, and basigin/CD147 is a cell surface glycoprotein that functions as an monocarboxylate transporter subunit (44). It is noteworthy that basigin/CD147 is one of the 21 prostasomal proteins found in the present study (Table 1).

The expression of different isoforms of glucose transporters (GLUTs) is probably an adaptation mechanism to a differentiated requirement of substrates for cellular energy metabolism in different cells. GLUT1 and GLUT3 share many structural and functional properties (4, 19), but they are distributed differently on a cellular level. In polarized epithelial cells, like the secretory epithelial cells of the prostate gland, GLUT1 is expressed on the basolateral surface, whereas GLUT3 is sorted to the apical surface (16, 29). Therefore, it is not surprising that we identified GLUT3 in prostasomes. Additionally, GLUT3 has been observed in intracellular vesicles (52). We speculate that GLUT3 might have been operational in our experimental system, since barbiturate being an inhibitor to GLUT3 (17) was able to recruit extra glucose, otherwise being trapped by GLUT3, for glycolytic ATP formation (Fig. 4), and the same tendency was apparent when fructose was the substrate (Fig. 4).

The physiological function of the enzymes on prostasomes with capacity to form ATP is not known. As mentioned above, approximately one-half of the protein kinase activity in human prostatic fluid is associated with prostasomes, and coinubcation of sperm cells and prostasomes resulted in a 10-fold increase in total protein phosphorylation compared with the level of phosphorylation achieved through incubation with either component alone (50). Hence, extracellularly formed ATP by prostasomes could be a substrate for prostosomal protein kinases in possible transphosphorylation reactions with other cells, preferentially sperm cells. We also hypothesize that prostasomes may constitute a pool of enzymes/proteins from which energetically sperm cells. We also hypothesize that prostasomes could be a substrate for prostosomal protein kinases in possible transphosphorylation reactions with other cells, preferentially sperm cells. We also hypothesize that prostasomes may constitute a pool of enzymes/proteins from which energetically sperm cells.

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