Osmotic Stress Regulates Mammalian Target of Rapamycin (mTOR) Complex 1 via c-Jun N-terminal Kinase (JNK)-mediated Raptor Protein Phosphorylation

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Background: mTORC1 integrates diverse signals including stress to control cell growth.

Results: JNK phosphorylates Raptor, a component of mTORC1, and activates mTORC1 kinase upon osmotic stress.

Conclusion: mTORC1 is regulated by JNK during osmotic stress.

Significance: Our findings provide the JNK-Raptor relationship as a potential mechanism by which stress activates mTORC1 signaling pathway.

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved protein kinase that has key roles in several fundamental cellular processes (1, 2). In cells, mTOR exists as two functionally distinct multiprotein complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2) (1). These two complexes have different sensitivities to the allosteric inhibitor rapamycin and distinct roles in cells. Rapamycin strongly and specifically inhibits mTORC1, which acts as a central controller of cell growth by regulating several biosynthetic pathways, including protein synthesis, ribosome biogenesis, and lipid biosynthesis (1, 3). Genetic and pharmacological studies have demonstrated that abnormal hyperactivation of mTORC1 induces cell growth and proliferation, and aberrantly elevated mTORC1 activity has consistently been found in various human diseases such as cancer and diabetes (4, 5).

mTORC1 needs to be tightly regulated by a variety of intracellular and extracellular signals to control cell growth accurately. Indeed, mTORC1 senses and integrates diverse signals, including growth factors, nutrient availability, energy status, and stress (6). Signaling by growth factors such as insulin and epidermal growth factor (EGF) activates mTORC1; consequently, complex metazoans are able to maintain homeostasis of organ and organism size (6). Simultaneously, mTORC1 activity is highly sensitive to nutrient and energy levels because mTORC1-induced biosynthetic pathways consume much energy and nutrients (6). Consequently, uncontrolled mTORC1 activation during energy or nutrient deprivation causes cellular apoptosis (7, 8). mTORC1 is also regulated by several stresses, including hypoxia, DNA damage, and oxidative stress, enabling cells to adapt in these stresses (9). At the molecular level, many of these signals converge on TSC1/TSC2 and small GTPase Rheb to regulate mTORC1 (10). Rheb directly binds to and enhances mTORC1 kinase activity (11, 12). TSC1/TSC2 complex negatively regulates Rheb via its GTPase-accelerating protein activity and thereby inhibits mTORC1 (13). Several kinases including Akt, Erk, and 5'-AMP-activated protein kinase (AMPK) were reported to phosphorylate and thereby modulate the GTPase-accelerating protein (GAP) activity of TSC1/TSC2 to regulate mTORC1 (10). TSC1/TSC2 and Rheb are essential for the regulation of mTORC1; however, recent studies have shown that components of mTORC1...
including Raptor can function as molecular sensors for mTORC1 (12, 14–18).

Raptor, a defining component of mTORC1, is essential for mTORC1 activity (19, 20). It functions as a scaffold protein that recruits mTORC1-specific substrates (21). Recently, several studies have suggested Raptor as an important signal acceptor of mTORC1. Energy deprivation causes Raptor phosphorylation via 5′-AMP-activated protein kinase, thereby inhibiting mTORC1 (14). The Ras-mediated oncogenic signal also regulates mTORC1 via Raptor phosphorylation by p90 ribosomal S6 kinase (RSK) and Erk (15, 16). Recent findings also revealed that mTORC1 is regulated via cdc2-dependent Raptor phosphorylation during mitosis (17, 18). Therefore, mTORC1 activity is closely related with the status of Raptor phosphorylation.

In this study, we demonstrate that osmotic stress also regulates mTORC1 via Raptor phosphorylation. We found that Raptor is phosphorylated at several proline-directed sites upon osmotic stress and identified JNK as being responsible for the phosphorylation and activation of mTORC1 under osmotic stress. Our findings reveal a novel mechanism of mTORC1 regulation by osmotic stress and suggest that the molecular link between JNK and Raptor has a potential role in the stress regulation of mTORC1.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—All chemicals were purchased from Sigma unless stated otherwise. SP600125, U0126, SB203580, roscovitine, rapamycin, and calyculin A were obtained from Merck (Darmstadt, Germany). Antibodies for mTOR, Rictor, phospho-mTOR (Ser-2448), phospho-S6K (Thr-389), phospho-S6 (Ser-240/244), phospho-TSC2 (Thr-1462), phospho-Akt (Ser-473), phospho-RSK (Thr-573), phospho-JNK (Thr-183/185) and phospho-c-Jun (Ser-63) were acquired from Cell Signaling Technology (Beverly, MA). Anti-phospho-Raptor (Ser-863) and anti-phospho-c-Jun (Ser-63) were acquired from Cell Signaling Technology (Beverly, MA). Anti-phospho-Raptor (Ser-863) and anti-phospho-Raptor (Thr-706) were purchased from Millipore (Billerica, MA). Anti-phospho-Raptor (Ser-696) was kindly provided by Dr. Diane C. Finger (University of Michigan Medical School, Ann Arbor, MI).

**Constructs**—HA-Raptor and GST-S6K1 were kindly provided by Dr. David M. Sabatini (Massachusetts Institute of Technology (MIT), Cambridge, MA). Full-length Raptor cDNA obtained by PCR was subcloned into pFLAG-CMV2 (Sigma). FLAG–Raptor S696A/T706A/S863A mutant and FLAG–Raptor S863A were generated by site-directed mutagenesis using Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan). FLAG–Raptor WT, S696A/T706A/S863A mutant, or S863A mutant were serum-starved for 24 h and then lysed from Triton X-100 buffer (40 mM HEPS, pH 7.5, 1% Triton X-100, 120 mM NaCl, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/ml leupeptin). The soluble fractions of the cell lysates were isolated by centrifugation at 14,000 rpm for 15 min. For immunoprecipitation, anti-FLAG M2 beads (Sigma) were added to the lysates and incubated at 4 °C with gentle agitation. The immunoprecipitates were washed four times with lysis buffer. Whole-cell lysates or immunoprecipitates were subjected to SDS-PAGE and immunoblotting. All immunoblots were detected by enhanced chemiluminescence (ECL System; Amersham Biosciences).

**In Vitro Kinase Assay**—The mTORC1 kinase assay was performed as described by Sancak et al. (12). Briefly, mTORC1 was immunoprecipitated with anti-FLAG M2 beads from FLAG-Raptor-transfected cells. The immunoprecipitates were washed twice in 25 mM HEPS (pH 7.5), 20 mM KCl. The kinase assay was performed for 30 min at 30 °C in mTORC1 kinase assay buffer (25 mM HEPS, pH 7.5, 50 mM KCl, 10 mM MgCl2, 250 µM ATP) and 150 ng of 4E-BP1 (Stratagene, La Jolla, CA) or GST–S6K1. The reactions were stopped by boiling in gel loading buffer and then analyzed by SDS-PAGE and immunoblotting.

For the JNK assay, HEK293 cells transfected with FLAG-Raptor WT, S696A/T706A/S863A mutant, or S863A mutant were serum-starved for 24 h and then lysed with Triton X-100 lysis buffer (40 mM HEPS, pH 7.5, 1% Triton X-100, 120 mM NaCl, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM Na3VO4, 1 mM PMSF, and 10 µg/ml leupeptin). Then, the FLAG–Raptor proteins were immobilized with anti-FLAG–M2 beads. To purify activated JNK1, FLAG–JNK1–transfected HEK293 cells were serum-starved for 24 h and then stimulated with 0.5 M sorbitol for 1 h to activate JNK. The cells were lysed with Triton X-100 lysis buffer. FLAG–JNK1 was isolated from the lysate with anti-FLAG M2 beads and eluted with 100 µg/ml 3×FLAG peptide (Sigma). The JNK assay was performed by incubating purified active JNK1 and immunoprecipitated FLAG–Raptor proteins in JNK assay buffer (20 mM HEPS, pH 7.4, 10 mM MgCl2, 0.5 mM DTT, 100 µM ATP) at 30 °C for 60 min.

**Protein Purification and in Vitro Binding Analysis**—GST–S6K1 expression construct was transfected into HEK293 cells, and after 24 h, serum-starved for 24 h. The cells were treated with 20 nM rapamycin for 1 h prior to cell lysis. GST–S6K1 was purified using glutathione-Sepharose bead (GE Healthcare) and eluted with 50 mM reduced glutathione (GSH). GST and GST–tagged JNK1 proteins were purified from *Escherichia coli* strain BL21 containing the appropriate constructs. Expression was induced by adding 0.1 mM isopropyl- β-
Osmotic Stress Induces Raptor Phosphorylation—To explore the stresses affecting the posttranslational modification of Raptor, we examined the mobility of Raptor in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Among several stressors, we found that osmotic stress induced by high osmolarity sorbitol treatment retarded the mobility of Raptor (Fig. 1A). An upshift of Raptor was clearly observed after 30 min of sorbitol treatment (Fig. 1B). Then, we wanted to determine which modification causes the mobility retardation.

FIGURE 1. Osmotic stress induces Raptor phosphorylation. A, HEK293 cells were serum-starved overnight and exposed for 60 min to 25 mM 2-deoxyglucose (2DG) for glucose starvation, 45 min to Dulbecco’s PBS (DPBS) for amino acid withdrawal, 60 min to 0.5 M sorbitol to induce osmotic stress, or 30 min to 20 mM rapamycin (Rapa). The lysate was resolved by SDS-PAGE and subjected to immunoblotting with the indicated antibody. NT, not treated. B, HEK293 cells were serum-starved overnight and exposed to 0.5 M sorbitol for 60 min. The immunoprecipitates (IP) were incubated with or without calf intestinal phosphatase (CIP) and then subjected to immunoblotting with Raptor antibody. WCL, whole-cell lysates.

RESULTS

Osmotic Stress Induces Raptor Phosphorylation—To explore the stresses affecting the posttranslational modification of Raptor, we examined the mobility of Raptor in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Among several stressors, we found that osmotic stress induced by high osmolarity sorbitol treatment retarded the mobility of Raptor (Fig. 1A). An upshift of Raptor was clearly observed after 30 min of sorbitol treatment (Fig. 1B). Then, we wanted to determine which modification causes the mobility retardation.

First, we suspected phosphorylation because several studies have reported on mobility retardation of phosphorylated Raptor (17, 18, 22). To examine this issue, the modified Raptor was...
incubated with calf intestinal phosphatase in vitro. We found that calf intestinal phosphatase treatment reversed the osmotic stress-induced mobility shift of Raptor, indicating that the upshift is due to the phosphorylation of Raptor (Fig. 1C). Therefore, we concluded that Raptor is strongly phosphorylated in response to osmotic stress.

**Multiple Sites of Raptor Are Phosphorylated in Response to Osmotic Stress**—To identify the residues of Raptor phosphorylated by osmotic stress, Raptor immunoprecipitated from HEK293 cells was challenged to osmotic stress, digested with trypsin, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Several phosphorylation sites were identified, including Ser-696, Thr-706, Ser-863, Ser-859, and Ser-877, from both samples and controls (Fig. 2, A and B, and data not shown). To determine the residues phosphorylated by osmotic stress, we compared the MS/MS spectral count of each phosphopeptide. We found that the spectra of NYALP(pS)PATTEGGSL(pT)PVR for Ser(P)-696 and Thr(P)-706 and VLDTSSTQAPA(pS)PPTKN (where pS and pT are phospho-Ser and phospho-Thr, respectively) for Ser(P)-863 were more identified in the sorbitol-treated sample than in the control sample, indicating that osmotic stress induces the phosphorylation at Ser-696, Thr-706, and Ser-863. All of the identified phosphorylation sites are located between HEAT repeats and WD40 domains (Fig. 2C). To confirm that these sites are responsible for the osmotic stress-induced phosphorylation, we examined whether mutation of the identified sites affects the mobility shift of Raptor induced by sorbitol treatment. Mutation of Ser-696/Thr-706/Ser-863 to nonphosphorylatable alanine (S696A/T706A/S863A) did not show the band shift with
sorbitol treatment, whereas WT Raptor did (Fig. 2D). We also examined the phosphorylation level with phospho-specific antibodies against Ser-696, Thr-706, and Ser-863. The phospho-specific antibodies recognized only WT Raptor and not S696A/T706A/S863A mutant Raptor, and osmotic stress increased the phosphorylation of WT Raptor at Ser-696, Thr-706, and Ser-863 (Fig. 2D). In addition, we also tried to examine the phosphorylation of endogenous Raptor with phospho-specific antibodies. Although the phosphorylation at Ser-696 was unable to be detected because the antibody is too weak to detect the phosphorylation of endogenous Raptor, the phosphorylation of Raptor at Ser-863 and Thr-706 was detected beginning 30 min after sorbitol treatment when the upshift of Raptor began to appear (Fig. 2E). Taken together, we concluded that osmotic stress induces the Raptor phosphorylation at Ser-696, Thr-706, and Ser-863.

**JNK Pathway Mediates Osmotic Stress-induced Raptor Phosphorylation**—Next, we tried to identify the upstream kinase responsible for Raptor phosphorylation upon osmotic stress. We selected several kinases as candidates, including kinases known to phosphorylate Raptor (14–18). Proline-directed kinases were also selected because the phosphorylated residues that we identified are followed by proline. Then, we examined whether the inhibition of each kinase affected the osmotic stress-induced mobility shift of Raptor. Of the inhibitors, SP600125, which inhibits JNK, was found to totally block the mobility retardation of Raptor in osmotic stress. Consistently, SP600125 resulted in the inhibition of Raptor phospho-Ser-863 and phospho-Thr-706 (Fig. 3A). To confirm that JNK is the responsible kinase, we also examined the effect of JNK1/2 knockdown on Raptor phosphorylation. We found that JNK1/2 knockdown also inhibited the Raptor mobility shift and phosphorylation induced by osmotic stress (Fig. 3B). As JNK is known to be activated by osmotic stress (23), we concluded that JNK is activated upon osmotic stress and subsequently induces Raptor phosphorylation.

**JNK Physically Associates with and Phosphorylates Raptor**—The fact that inhibition of the JNK pathway blocks the osmotic stress-induced Raptor phosphorylation suggests that JNK phosphorylates Raptor directly. First, we examined whether JNK interacts with Raptor. HA-tagged Raptor was co-immunoprecipitated with FLAG-tagged JNK1 (Fig. 4A). Endogenous JNK was also co-immunoprecipitated with endogenous Raptor (Fig. 4B). In addition, we readily detected the interaction between Raptor from HEK293 cell lysate and bacterially purified GST-JNK1 in a GST pulldown experiment (Fig. 4C). Because we prepared the HEK293 cell lysate with lysis buffer containing Triton X-100 detergent, which disrupts the interaction between Raptor and mTOR during lysis, the result indicates that Raptor interacted with JNK directly, not via the other mTORC1 components. Finally, we performed an in vitro JNK kinase assay to examine whether JNK could phosphorylate Raptor directly. Activated JNK1 was found to induce the phosphorylation of WT Raptor at Ser-696, Thr-706, and Ser-863 in vitro, whereas JNK could not phosphorylate S696A/T706A/S863A mutant Raptor (Fig. 4D). Taken together, we concluded that JNK interacts with and phosphorylates Raptor directly.

**JNK Alone Sufficiently Activates mTORC1 Signaling Pathway**—Next, we wanted to determine the role of JNK-mediated Raptor phosphorylation in osmotic stress, which perturbs several signaling pathways, as well as JNK. Therefore, to examine the JNK-specific effect on mTORC1, we used a constitutively active JNK1 construct. Constitutively active JNK1 (called active JNK1) is fused to an upstream kinase MKK7. Therefore, MKK7 can phosphorylate JNK1 constitutively; consequently, JNK1 can be activated without any signal (24, 25). As a negative control, we used an MKK7-JNK1 fusion construct containing JNK1 mutant in which the dual TPY phosphorylation site was replaced with APF (called inactive JNK1) (25). We found that the expression of active JNK1 brings about a phosphorylation-induced mobility shift of Raptor and phosphorylation at Ser-696, Thr-706, and Ser-863, confirming that JNK1 can phosphorylate these sites in cells (Fig. 5A). We monitored the phosphorylation status of several signaling proteins that are upstream regulators or downstream effectors of mTORC1. Notably, the expression of active JNK1 dramatically increased the phosphorylation of S6K, 4E-BP1, and S6, which are markers of mTORC1 activity, whereas inactive JNK1 did not affect their phosphorylation (Fig. 5B), indicating that JNK activation leads...
to up-regulated mTORC1 activity in cells. Rapamycin treatment completely blocked the JNK-enhanced S6K phosphorylation, consistent with JNK functioning upstream from mTORC1 (Fig. 5C). The expression of active JNK1 had no effect on known upstream regulators, such as the Akt, Erk, and Thr-706, and Ser-863, along with mTORC1 activation in cells.

... We speculated that the possibility exists that the dephosphorylation of S6K is not due to the inactivation of mTORC1. Calyculin A-sensitive phosphatase is reportedly responsible for the dephosphorylation of S6K upon osmotic stress (26). It has been reported that 4E-BP1 is also dephosphorylated by calyculin A-sensitive phosphatase (27). Therefore, we reasoned that upon osmotic stress, S6K and 4E-BP1 are dephosphorylated by the strong phosphatase activity despite the activation of mTORC1. To examine this possibility, we pretreated cells with calyculin A to exclude the effect of phosphatase. Surprisingly, under the calyculin A-treated condition, the phosphorylation of S6K and 4E-BP1 was strongly induced by sorbitol treatment, whereas sorbitol treatment strongly decreased the phosphorylation of S6K and 4E-BP1 without calyculin A, supporting our hypothesis (Fig. 6, C and D).

**DISCUSSION**

Understanding how cell growth is controlled with diverse environmental perturbations is important because many diseases are caused by the uncontrolled regulation of cell growth under such circumstances (4). As a critical integrator of environmental inputs into cell growth, mTORC1 has been studied intensively in numerous environmental contexts. However, how osmotic stress, one such environmental perturbation, affected the mTORC1 signaling pathway at a molecular level...
remains unclear. Here, we demonstrated that osmotic stress regulates mTORC1 kinase activity via JNK-mediated Raptor phosphorylation. We found that osmotic stress induces strong Raptor phosphorylation and determined that Raptor Ser-696, Thr-706, and Ser-863 are phosphorylated. We also found that JNK interacts with Raptor directly and phosphorylates these sites. Finally, we found that the kinase activity of mTORC1 is up-regulated in osmotic stress.

JNK-mediated Raptor Phosphorylation during Osmotic Stress

We demonstrated that osmotic stress induces Raptor phosphorylation at three sites, Ser-696, Thr-706, and Ser-863, as demonstrated in previous studies. mTOR phosphorylates Raptor at Ser-696, Thr-706, and Ser-863 with the insulin signal (22). Erk can also phosphorylate Raptor at Ser-696 and Ser-863 in Ras-dependent mTORC1 activation (16). In addition, cdc2 phosphorylates Raptor at Ser-696 and Thr-706 during mitosis (17, 18). Recently, arsenite was shown to induce Raptor phosphorylation at Ser-863 mediated by p38 (28). Here, we found that upon osmotic stress, JNK is the predominant kinase inducing Raptor phosphorylation. This was demonstrated by blocking mTOR, Erk, p38, and cdc2 with specific inhibitors that did not affect or partially affected osmotic stress-induced Raptor phosphorylation and the electrophoretic mobility shift, whereas JNK inhibition using SP600125 fully blocked the Raptor phosphorylation upon osmotic stress. Consistently, JNK knockdown reduced Raptor phosphorylation, confirming that the JNK pathway is responsible for Raptor phosphorylation in osmotic stress (Fig. 3). Furthermore, we demonstrated that JNK directly phosphorylates Raptor at three sites. During the preparation of this manuscript, Fujishita et al. (29) also reported that JNK can phosphorylate the Ser-863 site of Raptor in intestinal tumorigenesis. Here, we claim that JNK also phosphorylates Raptor Ser-696 and Thr-706 as well as Ser-863. We found that constitutively active JNK still induced the mobility shift of Raptor that had its Ser-863 site mutated to alanine, indicating the existence of additional phosphorylation (supplemental Fig. 2A). In agreement, the expression of active JNK1 resulted in increased phosphorylation of three sites in Raptor (Fig. 5A). Finally, we demonstrated that JNK could phosphorylate Raptor at three sites in vitro (Fig. 4E, supplemental Fig. 2B).

In this study, we also demonstrated that mTORC1 kinase activity was enhanced under osmotic stress. The Raptor phos-
phorylation sites that we identified are reported to have a positive role in mTORC1. Therefore, our results suggest that mTORC1 is in an active state under osmotic stress. Consistently, we demonstrated that mTORC1 kinase activity increased when measured with an in vitro kinase assay (Fig. 6).

Contrary to our results, however, the notion that osmotic stress suppresses mTORC1 has been widely accepted because S6K, an established effector of mTORC1, is completely inhibited upon osmotic stress. Calyculin A-sensitive phosphatase has been suggested to mediate the inhibition of S6K upon osmotic stress (26). However, no evidence exists that mTORC1 is required for the inhibition of S6K. Therefore, to resolve this discrepancy, we hypothesized that S6K inhibition during osmotic stress is independent of mTORC1. To test this hypothesis, we excluded the effect of phosphatase by using calyculin A. We found that osmotic stress induces S6K phosphorylation in the absence of phosphatase activity, indicating the activation of mTORC1.

Evidence supporting our conclusion has appeared in previous studies. First, Inoki et al. (13) showed that the expression of the constitutively active mutant of Rheb, which is a potent activator of mTORC1, does not rescue the inhibition of S6K by osmotic stress, whereas it restores the phosphorylation of S6K under other stress conditions that inactivate mTORC1, implying that the dephosphorylation of S6K is not dependent on mTORC1 inhibition under osmotic stress. Second, Kim et al. (19) showed that mTORC1 kinase activity in vitro does not change with a 10-min sorbitol treatment, although cellular S6K phosphorylation disappears completely under that condition. The inconsistency between Kim et al. (19) and our study regarding the in vitro mTORC1 activity may be due to the difference in the length of the sorbitol treatment. A 10-min sorbitol treatment did not induce Raptor phosphorylation (Fig. 2E); therefore, the mTORC1 activity may not be affected at that time. Third, the osmotic stress in 3T3-L1 adipocytes induces IRS-1 Thr-307 phosphorylation in a rapamycin-sensitive manner, indicating that mTORC1 activity increases with osmotic stress in adipocytes (30).

Here, we also showed that JNK is responsible for the activation of mTORC1 upon osmotic stress (Figs. 5 and 6B). JNK kinase is activated by a variety of cellular stresses, especially including UV and reactive oxygen species (31). Interestingly, several studies have shown that UV and reactive oxygen species are stresses that induce the activation of mTORC1 (32, 33). Therefore, this raises several interesting possibilities. First,
the possibility exists that JNK-activating stressors activate mTORC1. Therefore, one must determine whether JNK mediates mTORC1 activation under those stress conditions. Second, mTORC1 may also have active roles in apoptosis because JNK activation induces apoptosis. Unregulated mTORC1 activation has been reported to induce apoptosis under nutrient-starved conditions (7, 8). Constitutive mTORC1 activity has been shown to induce p53 activation by stimulating p53 translation in response to energy starvation (8). Therefore, JNK may utilize mTORC1 activity to induce apoptosis efficiently. Further investigation is required to better understand the role of mTORC1 in stress and apoptosis.

Although most mammalian cells maintained in an internal environment are exposed to very limited changes in osmolality, increasing evidence supports the importance of the osmotic stress response in various tissues, including kidney and lymphoid tissues (34). The renal medulla is exposed to extremely hyperosmotic stress in the process of reabsorbing water in the kidneys to concentrate urine. Lymphoid tissues, including the thymus, spleen, and liver, can also be hyperosmolar relative to blood (35). Therefore, the role of osmotic stress regulation of mTORC1 in these tissues remains to be investigated.

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