

Brief report

Function of Rad17/Mec3/Ddc1 and its partial complexes in the DNA damage checkpoint

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Abstract

The *Saccharomyces cerevisiae* heterotrimeric checkpoint clamp consisting of the Rad17, Mec3, and Ddc1 subunits (Rad17/3/1, the 9-1-1 complex in humans) is an early response factor to DNA damage in a signal transduction pathway leading to the activation of the checkpoint system and eventually to cell cycle arrest. These subunits show structural similarities with the replication clamp PCNA and indeed, it was demonstrated in vitro that Rad17/3/1 could be loaded onto DNA by checkpoint specific clamp loader Rad24-RFC, analogous to the PCNA-RFC clamp-clamp loader system. We have studied the interactions between the checkpoint clamp subunits and the activity of partial clamp complexes. We find that none of the possible partial complexes makes up a clamp that can be loaded onto DNA by Rad24-RFC. In agreement, overexpression of *DDC1* or *RAD17* in a *MEC3*Δ strain, or of *MEC3* or *RAD17* in a *DDC1*Δ strain shows no rescue of damage sensitivity. © 2005 Elsevier B.V. All rights reserved.

Keywords: DNA damage; Checkpoint; Clamp; Clamp loader

1. Introduction

DNA damage triggers a range of cellular responses including DNA repair, apoptosis, and cell cycle arrest. The DNA damage checkpoint response arrests cells at appropriate points in the cell cycle to allow recovery of the integrity of the DNA prior to reentering the cell cycle [1]. Those steps of the DNA damage checkpoint that result in inhibition of the cell cycle are relatively well understood (recently reviewed in Ref. [2,3]). However, molecular details are still lacking about the damage recognition steps that initiate the checkpoint pathway. While two distinct complexes independently bind to sites of DNA damage in *Saccharomyces cerevisiae*, the presence of both of these complexes is required for proper checkpoint function [4–6]. The first complex, the Mec1/Ddc2 heterodimeric protein kinase functions in DNA damage recognition and in signal transduction [6,7].

The second set of proteins is homologous to the replication clamp PCNA and its clamp loader, replication factor C

(RFC). The functional homolog of PCNA is a heterotrimeric clamp consisting of the *S. cerevisiae* Ddc1, Rad17, and Mec3 subunits, called Rad17/3/1, which are orthologous to the human and *S. pombe* Rad9, Rad1, and Hus1 subunits, respectively, the 9-1-1 complex. In this study, the *S. cerevisiae* clamp subunits will be indicated as Rad17^{Rad1}, Ddc1^{Rad9}, and Mec3^{Hus1}, when practical. Protein threading algorithms have predicted a PCNA-like fold for these subunits, while biochemical studies show its subunit structure to be that of a heterotrimer [8–13]. The five subunit Rad24-RFC clamp loader, consisting of Rad24 (Rad17 in human and *S. pombe*) and the four small subunits of RFC (Rfc2–5) is a specific loader of the checkpoint clamp (reviewed in [14]).

The checkpoint clamp loader has been shown to load the checkpoint clamp onto various DNA substrates, including 3'- and 5'-recessed primer templates and blunt-end double-stranded DNA in an ATP-dependent reaction [13,15–17]. Once loaded, the clamp has the ability to slide across double-stranded DNA similarly to PCNA. However, the presence of the single-stranded DNA binding protein RPA stabilizes both the clamp and its loader at or near the site of loading [13].

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Table 1
Plasmids used in this study

Plasmid	Expressed gene	Selection	Reference
pBL760	<i>DDC1</i>	<i>TRP1</i>	[13]
pBL761	<i>MEC3</i>	<i>URA3</i>	This study
pBL762	<i>GST-MEC3</i>	<i>URA3</i>	This study
pBL764	<i>GST-MEC3, RAD17</i>	<i>URA3</i>	[13]
pBL775	<i>GST-DDC1</i>	<i>TRP1</i>	This study
pBL776	<i>RAD17</i>	<i>URA3</i>	This study
pBL777	<i>GST-RAD17</i>	<i>URA3</i>	This study
pBL778	<i>DDC1</i>	<i>URA3</i>	This study

Although there is ample evidence that the checkpoint clamp functions as a heterotrimeric assembly, there are also several lines of evidence suggesting that alternative forms of this assembly may exist and be functional. *S. cerevisiae* Rad17^{Rad1} is capable of self-interaction suggesting the possible functionality of complexes containing two monomers of Rad17^{Rad1} [18]. Furthermore, the checkpoint defects of a *MEC3* deletion strain can be partially suppressed by overexpression of *DDC1* [19]. For these reasons, we have undertaken a study of the individual subunits of the yeast Rad17/3/1/checkpoint clamp. We find that partial clamp complexes can be isolated and/or reconstituted, however, none of these complexes are proficient in being loaded by the Rad24-RFC clamp loader. In agreement with this conclusion, we are also unable to confirm that a deletion of either *DDC1* or *MEC3* can be partially rescued by overexpression of the other clamp subunit genes.

2. Materials and methods

2.1. Plasmids and DNA

For genetic analysis and for protein purification, all genes were cloned in multicopy yeast shuttle vectors under control of the bi-directional *GAL1-10* promoter for high-level galactose-inducible expression in yeast. When indicated the checkpoint genes were cloned as N-terminal fusions with the *GST* gene, separated by a Prescission protease recognition site [13]. Table 1 shows the plasmids used in this study. The proper identity of the genes inserted in the plasmids was confirmed by DNA sequence analysis.

The oligonucleotides used in the surface plasmon resonance (SPR) analysis were A9 (5'-ccagtgaattcgactcggtagcctaccgctagcgggtagcctcta) and the complementary oligo 3'-20 (5'-tagaggatccccgtagcgg). For attachment to a streptavidin-coated SPR chip, the A9 oligo was biotinylated on the 3' terminus.

2.2. Overexpression and purification of proteins and protein complexes

S. cerevisiae strain BJ2168 (*MATa*: *ura3-52, trp1-298, leu2-3, 112, prb1-1122, prc1-407, pep4-3*) was used as a

host for protein overproduction. All proteins or protein complexes were purified using the same procedure. The yeast cells were transformed with appropriate plasmid or plasmids. For each expression strain one subunit was expressed as a GST-fusion. Cells were grown and induced using conditions and media described previously [13]. Proteins in the crude extract, prepared from 50 g of yeast in 50 ml 2× buffer A (buffer A = 50 mM Hepes 7.5, 125 mM KCl, 175 mM ammonium sulfate, 10% glycerol, 3 mM DTT, 0.1% Tween 20, 0.01% NP-40, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 2 μM pepstatin A, 2 μM leupeptin, 10 mM NaHSO₃, 1 mM benzamidine, 0.2 mM Na₃VO₄, 1 mM α-naphthyl phosphate, 5 mM Na-pyrophosphate, 2 mM β-glycerophosphate) by blending with dry ice, followed by DNA removal with 0.4% polyamin P, and a clearing step at 35,000 rpm for 1 h, were precipitated with ammonium sulfate (55% saturation). The protein pellet was resuspended in 5 ml of buffer B₀ (same as A but lacking ammonium sulfate and KCl), dialyzed against buffer B₀ to a conductivity of B₁₂₅ and loaded onto a 1 ml Glutathione–Sephacrose column. The column was washed with 15 ml of buffer B₁₅₀, 10 ml of buffer C₁₂₅ (as B₁₂₅, but without α-naphthyl phosphate, pepstatin A, leupeptin, PMSF, benzamidine, and NaHSO₃), 10 ml of buffer C₁₂₅ containing 5 mM MgCl₂ and 1 mM ATP, and again 10 ml of buffer C₁₂₅. Proteins were eluted with 8 ml of buffer C₁₂₅ containing 20 mM glutathione (reduced form) and digested overnight at 4 °C with 15 units of Prescission protease (Amersham-Pharmacia). Enzymatic digest was loaded onto a 1 ml heparin–agarose column, washed with 5 ml of buffer C₁₈₀ and eluted with 2 ml of buffer C₅₀₀.

2.3. Analysis of checkpoint proteins and of their interactions

The proteins and complexes (1–5 μg) were analyzed by SDS-10%-PAGE and stained with either Coomassie blue or SYPRO Orange (Molecular Dynamics). Gel filtration analysis was performed at 10 °C by using a SMART chromatography system (Amersham-Pharmacia) with a Superose 12 PC3.2/30 column equilibrated in buffer D (25 mM Hepes 7.8, 100 mM NaCl, 5 mM MgAc₂, 1 mM DTT). The proteins or their mixture (5 μg each) were preincubated for 30 min in buffer D containing 100 μM ATP prior to injection.

2.4. DNA–protein interaction analysis

Surface plasmon resonance was performed in a BIAcoreX apparatus. Buffer D containing 100 μg/ml BSA was the running buffer used in the analysis. The DNA chip was prepared as described before and contained ~50 resonance unit of biotinylated DNA attached via streptavidin to a CM5 chip [13]. The functional interaction between Rad24-RFC (30 nM) and the clamp subunits with DNA was monitored at 20 °C by injecting 80 μl of the factors at the indicated

concentrations in the presence of 100 μ M ATP where indicated, over a DNA chip at flow rate of 30 μ l/min.

2.5. Damage sensitivity analysis

Strains YLL134 (*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 mec3 Δ ::TRP1*) or YLL 244 (*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 ddc1 Δ ::KanMX4*) were transformed with empty vector (pRS426-GAL) or plasmids pBL761 (MEC3), pBL778 (DDC1), or pBL776 (RAD17) overexpression plasmids. Two individual colonies of each transformation were grown to saturation in selective minimal media containing 2% raffinose. Serial 10-fold dilutions (from 10^5 to 10 cells/spot) were spotted on both selective minimal media containing 2% glucose or 2% galactose, and also on the same media containing in addition 0.008% methylmethane sulfonate (MMS). Growth after 3 days at 30 °C was recorded.

3. Results and discussion

The checkpoint clamp Rad17/3/1 consists of three different polypeptides: Rad17^{Rad1}, Mec3^{Hus1} and Ddc1^{Rad9}, in *S. pombe* and human designated as the 9-1-1 complex. Numerous biochemical studies have demonstrated that the checkpoint clamp and clamp loader complexes interact (reviewed in [14]). Previous studies have indicated an interaction between the human or *S. pombe* Rad1 clamp subunit and the clamp loader Rad17 subunit, orthologous to the Rad24 subunit of *S. cerevisiae* Rad24-RFC [20–22]. Modeling studies were in agreement with this assignment and suggested that this subunit should be the only one to bind the clamp loader large subunit [12]. Therefore, we were particularly interested in the possible activity of partial complexes containing the Rad17^{Rad1} clamp subunit.

3.1. Purification of the Rad17/3/1 clamp subunits and clamp reconstitution studies

To test the activity of the individual clamp subunits and two subunit complexes, we overexpressed in yeast all three individual subunits of the checkpoints clamp, and purified them to near homogeneity (Fig. 1, lanes 3–5). We also isolated a Rad17/Mec3 complex (lane 2). The stoichiometry of the Rad17/Mec3 complex, measured by intensity of the bands in a Coomassie stained gel suggested a molar ratio for Rad17^{Rad1}:Mec3^{Hus1} of 1.5:1. However, SYPRO Red staining indicated a 2:1 stoichiometry of Rad17^{Rad1} to Mec3^{Hus1}. This analysis suggests that the Rad17/Mec3 complex might be able to form a clamp-shaped trimer with two Rad17^{Rad1} and one Mec3^{Hus1} subunits. We were unable to isolate either a stable Rad17/Ddc1 or a Mec3/Ddc1 complex from yeast cells, regardless whether the GST-purification tag was fused to the *MEC3*, *DDC1*, or *RAD17* gene (data not shown).

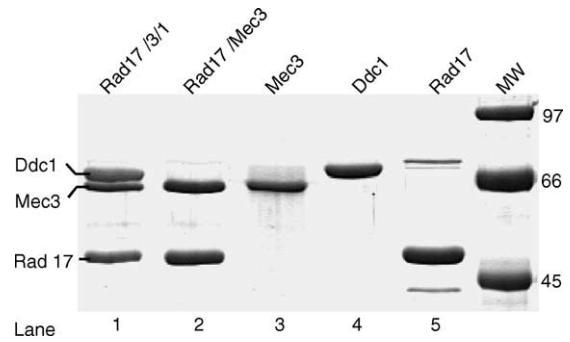


Fig. 1. DNA damage checkpoint clamp subunits. Purified individual clamp subunits and the indicated dimer and trimer were analyzed by 10% SDS-PAGE. Staining was by colloidal Coomassie.

Therefore, we attempted to reconstitute these complexes from individually purified subunits.

To investigate whether the checkpoint clamp subunits interact we carried out a series of gel filtration experiments. While Rad17^{Rad1} migrated as a unique monomeric species through the gel filtration matrix, both Mec3^{Hus1} and Ddc1^{Rad9} eluted throughout a large range of the column indicative of aggregation problems with these two individual subunits (Fig. 2, runs 2–4). However, incubation of a mixture of the

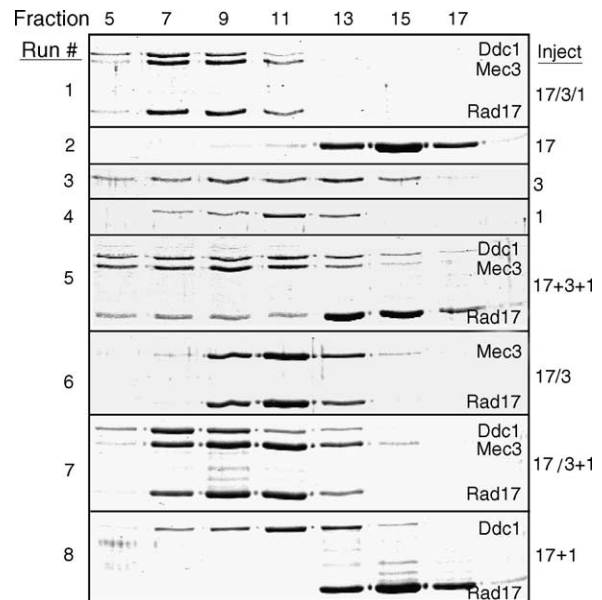


Fig. 2. Gel filtration analysis of clamp subunits. Individual proteins and mixtures were analyzed by superose 12 gel filtration as detailed in Section 2. Protein in the indicated fractions was precipitated with 10% trichloroacetic acid and analyzed by 10% SDS-PAGE. Run 1: Rad17/Mec3/Ddc1 complex (17/3/1). Run 2: Rad17^{Rad1} (17). Run 3: Mec3^{Hus1} (3). Run 4: Ddc1^{Rad9} (1). Run 5: Mixture of Rad17^{Rad1}, Mec3^{Hus1}, and Ddc1^{Rad9} (17 + 3 + 1). Run 6: Rad17/Mec3 complex (17/3). Run 7: Mixture of Rad17/Mec3 complex and Ddc1^{Rad9} (17/3 + 1). Run 8: Mixture of Rad17^{Rad1} and Ddc1^{Rad9} (17 + 1). Mixtures of the individual subunits and/or partial complexes (5 μ g each) were preincubated in running buffer for 30 min prior to injection. Standard proteins eluted in the following fractions: thyroglobulin (669 kDa), fraction 3; apoferritin (440 kDa), fraction 8; yeast alcohol dehydrogenase (150 kDa), fraction 11; BSA (66 kDa), fraction 15.

three individual clamp subunits reconstituted a heterotrimeric Rad17/3/1 complex as deduced from an observed retention time identical to that of the native clamp (compare run 1 with run 5). The elution position of the Rad17/Mec3 complex suggests that if this complex is that of a heterotrimer with two Rad17^{Rad1} and one Mec3^{Hus1} subunits, it must be much more compact than the native Rad17/3/1 complex because of its later elution position from the column (run 6). However, addition of Ddc1^{Rad9} to this Rad17/Mec3 complex appeared to restore the intact Rad17/3/1 clamp (run 7). We did not detect complex formation between Mec3^{Hus1} and Ddc1^{Rad9} (not shown), nor between Rad17^{Rad1} and Ddc1^{Rad9} (run 8). This is in contrast to the human system where these interactions between the orthologous subunits could be demonstrated [23].

3.2. Only a Rad17/Mec3/Ddc1 heterotrimer can be loaded onto DNA

While the direct gel filtration studies only verified the existence of a stable Rad17/Mec3 complex, the possibility exists that transient unstable clamp complexes could be formed between Rad17^{Rad1} and Ddc1^{Rad9} or between Mec3^{Hus1} and Ddc1^{Rad9}. If any of these complexes were competent as a checkpoint clamp, i.e. could be loaded onto DNA by Rad24-RFC, it should be possible to detect these events in a loading assay. Therefore, we carried out a surface plasmon resonance study to detect clamp loading in a real-time assay.

When 30 nM Rad24-RFC and 50 nM Rad17/3/1 were flowed across a chip to which template-primer DNA was immobilized via a biotin–streptavidin linkage, a strong binding signal was detected, but only if ATP was also present (Fig. 3A) [13,24]. A significant signal was also detected if the concentration of Rad17/3/1 was lowered to 1 nM, in agreement with previous studies [24]. Based on these considerations, we reasoned that if an alternative clamp existed with an efficiency of complex formation and loading 1% that of Rad17/3/1, we should be able to detect this species in the SPR assay.

In agreement with our gel filtration studies suggesting successful reconstitution of the clamp from its individual subunits, the mixture of Rad17^{Rad1}, Mec3^{Hus1}, and Ddc1^{Rad9} produced a strong positive signal when flowed across the DNA chip together with Rad24-RFC and ATP (Fig. 3B). In contrast, none of the individual subunits produced a significant signal which would have been indicative of loading. Although some signal increase was observed upon injection of a single subunit, particularly of Ddc1^{Rad9}, together with Rad24-RFC and ATP, the pattern was more suggestive of non-specific binding. First, after injection stopped at $t = 160$ s, material persistently remained on the chip and failed to dissociate indicative of non-specific binding to the chip. This is in agreement with the tendency of these individual subunits to aggregate as shown by the gel filtration studies (Fig. 2). Second, the observed signal was independent of the presence of ATP indicating a lack of specificity.

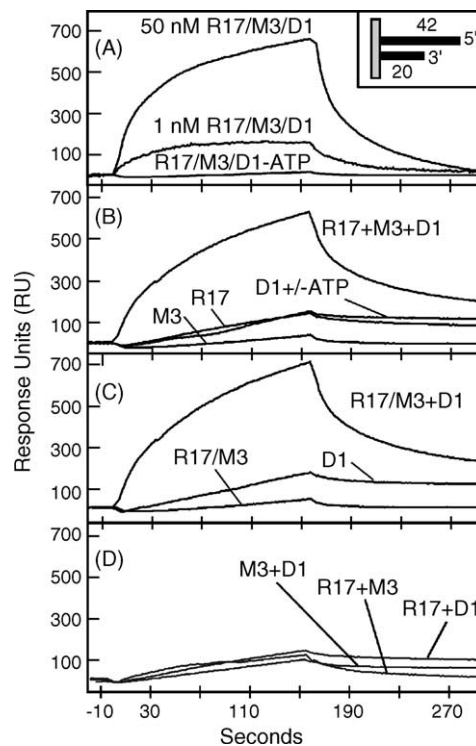


Fig. 3. Partial checkpoint clamps are inactive for loading onto DNA. Rad24-RFC (30 nM) together with the indicated clamp complexes or mixtures and 100 μ M ATP were flowed across a partial duplex DNA chip (inset in A). (A) 1 nM or 50 nM Rad17/3/1 or 50 nM Rad17/3/1 without ATP. (B) 50 nM of Rad17^{Rad1}, 50 nM of Mec3^{Hus1}, or 50 nM Ddc1^{Rad9} with or without ATP, or a mixture of 50 nM each of Rad17^{Rad1}, Mec3^{Hus1}, and Ddc1^{Rad9}. (C) 50 nM Rad17/Mec3 complex, or 50 nM Ddc1^{Rad9}, or a mixture of 50 nM each of Rad17/Mec3 complex and Ddc1^{Rad9}. (D) A mixture of 50 nM each of Rad17^{Rad1} and Mec3^{Hus1}, Rad17^{Rad1} and Ddc1^{Rad9}, or Mec3^{Hus1} and Ddc1^{Rad9}. The mixtures of the individual subunits and partial complexes were preincubated in running buffer for 30 min prior to injection.

We also did not observe a specific signal with the Rad17/Mec3 complex. Again, addition of Ddc1^{Rad9} reconstituted a clamp species that could be efficiently loaded by Rad24-RFC (Fig. 3C). Finally, injection of a mixture of either Rad17^{Rad1} and Ddc1^{Rad9}, or a mixture of Mec3^{Hus1} and Ddc1^{Rad9} also failed to elicit a specific loading signal by SPR (Fig. 3D). Therefore, formation of even a transient two subunit clamp could not be detected by SPR.

Our experiments failed to find any indication that partial complexes of the checkpoint clamp constitute an alternative clamp that can be loaded onto DNA by Rad24-RFC. We were particularly interested in Rad17^{Rad1}-containing alternative clamps because Rad17^{Rad1} may mediate the protein–protein interaction with the Rad24-RFC checkpoint clamp loader [20–22]. In addition, in *S. cerevisiae* Rad17^{Rad1} is capable of self-interaction in response to DNA damage, suggesting the possibility of alternative clamps that, if trimeric, would contain two Rad17^{Rad1} subunits together with Ddc1^{Rad9} or Mec3^{Hus1} [18]. Of the possible two subunit complexes, only Rad17/Mec3 formed a stable complex and could be purified. However, this complex was inactive.

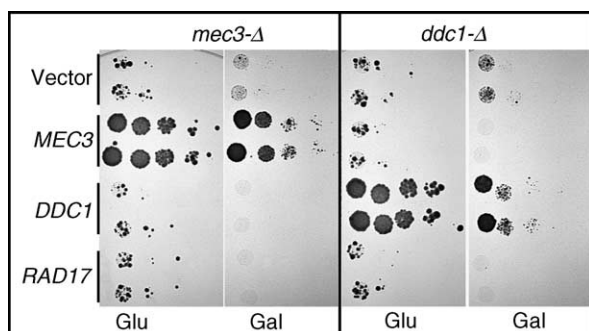


Fig. 4. MMS sensitivity of overexpression strains. See Section 2 for details. All growth media contained 0.008% MMS. Growth of plates lacking MMS was similar for all transformants plated on glucose-containing media, and for all transformants plated on galactose-containing media (data not shown).

3.3. Only a Rad17/Mec3/Ddc1 heterotrimer is an active clamp in yeast

The possible function of a Rad17/Ddc1 complex was of interest because of genetic studies showing that the checkpoint defects of a *MEC3* deletion strain could be partially suppressed by overexpression of *DDC1* [19]. We were not able to purify a Rad17/Ddc1 complex from yeast. We were able to purify the individual subunits and show that they were properly folded because upon simple mixing of the three subunits a functionally active heterotrimeric clamp was reconstituted (Fig. 3B). However, neither a stable nor a transient Rad17/Ddc1 complex could be demonstrated (Figs. 2 (run 8) and 3D).

One possible explanation for the genetic suppression result could be the existence of a bypass mechanism for damage response which does not proceed through the canonical Rad17/3/1 clamp [19]. In order to determine whether this potential bypass proceeded through Ddc1^{Rad9} or through another clamp subunit, we repeated the suppression analysis (Fig. 4). Surprisingly, using the deletion strain from the original study, we failed to detect suppression of MMS sensitivity by overexpression of either *DDC1* or *RAD17* in the *mec3Δ* strain. In fact, we noticed a minor but reproducible enhancement of MMS sensitivity, compared to vector control, when *DDC1* or *RAD17* were overexpressed in the *mec3Δ* strain. Strictly analogous results were obtained when the overexpression experiment was carried out in a *ddc1Δ* strain (Fig. 4). While we cannot offer an explanation for this discrepancy, we are certain that our overexpression plasmids are fully functional, as they were used in the biochemical overproduction studies [13,24]. Moreover, the *MEC3* overexpression plasmid complemented MMS sensitivity of the *mec3Δ* strain while the *DDC1* overexpression plasmid complemented that of the *ddc1Δ* strain (Fig. 4). The enhanced sensitivity observed upon overexpression of the clamp subunit genes in the mutants suggests that increased abundance of these subunits may interfere with the proper function of an alternative repair pathway.

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