Biochemical characterization of *Caenorhabditis elegans* UBC-1: self-association and auto-ubiquitination of a RAD6-like ubiquitin-conjugating enzyme *in vitro*

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The *Caenorhabditis elegans* ubiquitin-conjugating enzyme UBC-1 is distinct from other RAD6 homologues in possessing a C-terminal tail 40 amino acid residues long [Leggett, Jones and Candido (1995) DNA Cell Biol. 14, 883–891]. Such extensions from the core catalytic domain have been found in a subset of known conjugating enzymes, where they have been shown to have diverse roles including target recognition, membrane attachment and sporulation. In the present study we used mutagenesis *in vitro* to examine the role of the tail in specific aspects of UBC-1 structure and activity. Cross-linking experiments with purified recombinant UBC-1 reveal that it forms dimers and

probably tetramers. The acidic tail of UBC-1 has an important role in this interaction because deletions of the tail significantly decrease, but do not abolish, this self-association. Ubiquitin conjugation assays show that, in addition to accepting a thiol-bound ubiquitin at its active site, UBC-1 is stably mono-ubiquitinated. Deletion analysis and site-directed mutagenesis localize the site of ubiquitination to Lys-162 in the tail. These findings demonstrate that the C-terminal tail of UBC-1 is important both for its quaternary structure and post-translational modification *in vitro*.

INTRODUCTION

The ubiquitin–proteasome pathway has emerged as the central system of ATP-dependent protein degradation in eukaryotic cells. This pathway has been clearly implicated in such basic cellular processes as DNA repair, cell cycle control, antigen presentation, peroxisomal biogenesis, the stress response, neuronal pathfinding, programmed cell death and cell differentiation [1,2]. Specific proteins known to be ubiquitinated include NF- κ B and I κ B [3], p53 [1,4], mitotic and G1 cyclins [1,5,6], CDK inhibitors [7] and G-proteins [8]. The ubiquitin system also has an important role in the down-regulation of both cell-surface receptors [9] and STAT-family transcription factors [10]. In addition, many oncogene products including c-Myc, c-Fos and c-Jun are specifically recognized and degraded by ubiquitin [1].

Ubiquitin-conjugating enzymes, or E2s, are a family of proteins that catalyse the attachment of ubiquitin via an isopeptide bond to a lysine residue on target proteins [4]. This reaction can be performed by the E2 alone or in conjunction with a ubiquitin: protein ligase (E3), which aids in target recognition. E2s can be separated into two classes based on their structure: class I E2s consist of an evolutionarily conserved catalytic domain, whereas Class II E2s contain the catalytic domain and a C-terminal extension or tail. The tails of type II E2s have diverse functions. Initially it was suggested that these extensions might be involved in target recognition, on the basis of the demonstration that the tail of the yeast E2, RAD6, is required for the ubiquitination of histones H2A and H2B [11]. Other work supporting this view has shown that a chimaeric RAD6, containing the tail of the yeast E2 CDC34, can complement the cell cycle defect in a *cdc34* mutant [12]. Recently it has been shown that E2s can be engineered to ubiquitinate selected proteins by fusing the appropriate protein-binding region to the C-terminus of the E2 [13]. In addition to target recognition, the C-terminal tail might also have a role in

interactions with other components of the ubiquitin system: work with RAD6 has shown that its tail is involved in interactions with UBR1, the E3 involved in the N-end rule pathway [14]. Furthermore the tail of UBC6 is required for its attachment to the membrane of the endoplasmic reticulum in yeast [15], and self-association of CDC34 is dependent on its C-terminal extension [16].

As we have shown previously, *Caenorhabditis elegans* UBC-1 differs from other RAD6 homologues in containing a 40-residue C-terminal tail [17]. Although able to complement the DNA repair functions of a *rad6*-null yeast mutant, *ubc-1* was unable to complement the sporulation deficiency, which is a function of the RAD6 tail. The conservation of similar tails in the nematodes *Ascaris suum* and *Caenorhabditis briggsae* suggested that this might be an important feature of the nematode proteins. In this study we show that the tail of UBC-1 is mono-ubiquitinated at Lys-162. We also demonstrate that UBC-1 self-associates to form dimers and tetramers *in vitro* and that the tail of UBC-1 greatly facilitates this interaction.

MATERIALS AND METHODS

Culture of *C. elegans*

C. elegans Bristol strain was maintained in Basal S medium as previously described [18].

Plasmid constructs

PCR was performed with *ubc-1* cDNA as a template to introduce restriction sites to facilitate cloning. The 5' end of the *ubc-1* coding sequence was hybridized to the oligonucleotide DL3, 5'-GAGGATCCATGACGACGCCAGCCGT-3'. The *Bam*HI site is shown in bold and the initiator methionine codon is

Abbreviations used: BS³, bis(sulphosuccinimidyl) suberate; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin:protein ligase.

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underlined. The 3' end of the coding sequence hybridized to the oligonucleotide DL5, 5'-**AAGCTTTT**AAGCATTCGATCC-3'. The termination codon is underlined and the *Hind*III site is shown in bold. After amplification, the product was purified by agarose gel electrophoresis, digested with *Bam*HI and *Hind*III, and inserted into the *Bam*HI and *Hind*III sites of the bacterial expression vector pRSET A (Invitrogen) to yield plasmid pHis-UBC-1, which expresses an N-terminal histidine-tagged UBC-1 (HisUBC-1). This plasmid was then digested with *Nde*I and *Bam*HI, blunted and re-ligated to remove the histidine affinity tag contained in the pRSET A vector, to produce plasmid pUBC-1, which expresses wild-type UBC-1. The deletions *ubc-1Δ169* and *ubc-1Δ152* were made in the same way, with the 3' oligonucleotides Δ152 (5'-GGAAGCTTTCAGAAATTGAGC-CAGGACTGCTC-3') and Δ169 (5'-GGAAGCTTTCATTCT-TCAATTTCCACGTCATCC-3') to generate pUBC-1Δ169 and pUBC-1Δ152. The termination codons are underlined and the *Hind*III sites are shown in bold.

The *ubc-1* mutant *ubc-1R162* was generated by site-directed mutagenesis [19] of wild-type *ubc-1*. The mutation was made with the oligonucleotide R162, 5'-AATTTCCACGTCATCCCT-GAGCACTGCGTCGCC-3'. The nucleotide causing the mutation is underlined. The mutant clone was cut with *Acc*I and *Hind*III and the fragment was used to replace wild-type *ubc-1* in the UBC-1 expression vector. All constructs were verified by DNA sequencing.

Protein expression

All expression vectors were transformed into the *Escherichia coli* strain BL21(DE3). To express unlabelled proteins, cells were grown at 37 °C in Luria broth containing 50 µg/ml ampicillin to a D_{600} of 0.6. The cultures were then induced with 1 mM isopropyl β-D-thiogalactoside and allowed to grow for 2 h. The cells were harvested by centrifugation and stored at -20 °C.

[³⁵S]Methionine-labelled proteins were produced essentially as described above with the following modifications. Cells were grown at 37 °C in 10 ml of M9 medium containing 1 mM MgSO₄, 0.1 mM CaCl₂, 50 µg/ml ampicillin, 18 µg/ml thiamine and 40 µg/ml of each of the amino acids except methionine and cysteine, to a D_{600} of 0.6. The cells were induced with 1 mM isopropyl β-D-thiogalactoside for 20 min followed by the addition of 300 µg/ml rifampicin. The cells were then shifted to 42 °C for 10 min and returned to 37 °C for 1 h. Tran³⁵S-methionine label (ICN) was added to a final concentration of 25 µCi/ml and the cells were incubated for a further 10 min. The cells were harvested by centrifugation and stored at -20 °C until needed.

Protein purification

E. coli cells containing UBC-1 or its derivatives were lysed by sonication in buffer A [50 mM Tris/HCl (pH 7.5)/1 mM EDTA/100 mM NaCl/2 mM dithiothreitol]. The lysate was clarified by centrifugation and loaded on a DEAE-Sephacel (Pharmacia) column. The column was washed with 3 vol. of buffer A and the expressed protein was eluted with a 0.1–0.8 M NaCl gradient in buffer A. Fractions containing the expressed protein were pooled and concentrated by ultrafiltration (Amicon cell). The concentrated protein was then loaded on a G100 superfine (Pharmacia) gel exclusion column in PBS containing 2 mM dithiothreitol, and eluted in the same buffer. Fractions containing the expressed protein were pooled and stored at -70 °C in the presence of 10 % (v/v) glycerol.

Protein cross-linking

All cross-linking reactions were performed in cross-linking buffer [50 mM Hepes (pH 7.5)/150 mM NaCl/2 mM dithiothreitol]. Proteins to be cross-linked were preincubated in cross-linking buffer for 3 min on ice. Appropriate volumes of 100 mM bis(sulphosuccinimidyl) suberate (BS³; Pierce) were then added and the reaction was allowed to proceed on ice for 30 min. The reactions were terminated by the addition of 1 M Tris/HCl, pH 6.8, to a final concentration of 60 mM. The reactions were separated by SDS/PAGE [12.5 % (w/v) gel] and detected by autoradiography.

Ubiquitin conjugation

Clarified *C. elegans* extract was used as the source of ubiquitin-activating enzyme (E1) in all assays. *C. elegans* embryos were prepared as described elsewhere [18]. The embryos were resuspended in lysis buffer [50 mM Tris/HCl (pH 7.5)/0.75 mM EDTA/1.5 mM dithiothreitol/2.5 mM PMSF/15 µg/ml aprotinin/15 µg/ml leupeptin/15 µg/ml pepstatin A] and homogenized with 30 strokes in a stainless steel Dounce homogenizer. The extract was clarified by centrifugation and stored at -70 °C. [¹²⁵I]-Ubiquitin was prepared by the Chloramine-T method as described previously [20].

Reactions were performed in 50 mM Tris/HCl (pH 7.5)/1.5 mM ATP/14 µM bovine ubiquitin (Sigma) containing 35 µg of *C. elegans* lysate at 20 °C for 15 minutes. Unless otherwise stated, UBC-1 or its derivatives were used at 6 µM in the reaction. Reactions were stopped by the addition of SDS sample buffer with or without 360 mM 2-mercaptoethanol as indicated. The samples were subjected to SDS/PAGE [15 % (w/v) gel] and transferred to Immobilon-P (Millipore) membrane for Western blotting [21]. For Figure 3 the samples were subjected to SDS/PAGE [15 % (w/v) gel] and detected by autoradiography.

Immunochemical techniques

Anti-(UBC-1) antibody was prepared in Balb/C mice with bacterially expressed UBC-1 as antigen. UBC-1 was prepared by SDS/PAGE followed by electroelution. Mice were injected subcutaneously with 25 µg of the purified UBC-1 in TitreMax adjuvant (Vaxcel), and boosted every 2 weeks. Serum obtained after the third boost was the source of the antibodies. Western blots were developed with peroxidase-conjugated anti-mouse secondary antibodies (Promega) and enhanced chemiluminescence (ECL; Amersham).

RESULTS

UBC-1 self-associates *in vitro*

When bacterially expressed UBC-1 was run on a G100 superfine gel exclusion column, it eluted at a molecular mass of 42 kDa instead of the expected 21.5 kDa. One possible explanation for this is that UBC-1 might have an abnormally large Stokes radius owing to its 40-residue C-terminal tail. This seems to be true of the UBC-1 yeast homologue, RAD6: the yeast RAD6 tail extends from a globular core and results in an aberrantly large Stokes radius [11]. In contrast, because the observed molecular mass of UBC-1 by gel exclusion chromatography was exactly twice its predicted molecular mass, a second possibility was that UBC-1 exists as a dimer.

To examine whether UBC-1 forms a dimer, chemical cross-linking was performed with the lysine cross-linker BS³ on ³⁵S-labelled UBC-1. The addition of 1 mM BS³ generated a cross-linked dimer of 47 kDa as well as higher-molecular-mass products

Figure 1 UBC-1 self-associates *in vitro*

Cross-linking reactions containing either 0 or 1 mM BS³ cross-linking reagent were performed with 200 nM radiolabelled UBC-1 (30 000 c.p.m.) in the presence or absence of a 10-fold molar excess of BSA. The reaction products were separated by SDS/PAGE [12.5% (w/v) gel] and detected by autoradiography. The faint dimeric band seen in the absence of cross-linker represents disulphide-linked dimer. The positions of molecular mass standards are shown at the left.

Figure 2 The tail of UBC-1 is involved in self-association

Cross-linking reaction products of purified radiolabelled UBC-1, UBC-1Δ169 and UBC-1Δ152 (30 000 c.p.m.) were separated by SDS/PAGE [12.5% (w/v) gel] and detected by autoradiography. Reactions contained either 0 or 1 mM BS³ cross-linking reagent as indicated. The positions of molecular mass standards are shown at the left.

(Figure 1). These persisted even when cross-linking was performed in the presence of a 10-fold molar excess of BSA (Figure 1), indicating that the interaction is specific. Because UBC-1 has a molecular mass of 24 kDa on SDS/PAGE minigels, the cross-linked band at 47 kDa is probably a dimer. The next cross-linked product has an apparent molecular mass of 93 kDa, based on more accurate sizing on a 16 cm SDS/PAGE gel (results not shown), and is probably a tetramer. Yeast CDC34 also displays bands in a molecular mass range consistent with dimers and tetramers after cross-linking [16], although the results for this enzyme were less clear owing to the existence of a complex banding pattern. Thus UBC-1 and CDC34 might form similar quaternary structures. Although this demonstrates that UBC-1 self-associates to form at least a dimer, it does not rule out the possibility that its elution behaviour on the size-exclusion column is due to an abnormal Stokes radius, i.e. that the bulk of the protein under these conditions might be monomeric.

The tail of UBC-1 is involved in self-association:

As mentioned above, studies on the *Saccharomyces cerevisiae* ubiquitin-conjugating enzyme UBC3 (CDC34) have shown that it also self-associates *in vitro*. Like UBC-1, CDC-34 is a type II conjugating enzyme and its C-terminal tail has been shown to affect its ability to self-associate [16]. To determine whether the tail of UBC-1 has a similar role, two deletions of this region were constructed and tested for their ability to form cross-linked products *in vitro*. UBC-1Δ152 lacks all of the tail to residue 152, whereas UBC-1Δ169 lacks the tail to residue 169 but retains the first 18 residues, which were shown to be strongly conserved between the tails of UBC-1 and its homologues from *A. suum* and *C. briggsae* [17].

As can be seen in Figure 2, deletion of the tail of UBC-1 severely affected its ability to self-associate. Complete removal of the tail of UBC-1, as seen in the UBC-1Δ152 deletion, caused a decrease to approximately one-eighth in the ability of UBC-1 to self-associate, whereas with UBC-1Δ169 a decrease in self-association to approximately one-half was seen compared with wild-type (when compared by densitometry and normalized for protein concentration). Therefore the conserved 18 residues of the tail do not seem to have a pivotal role in self-association, because progressive deletion of the tail decreased the efficiency of self-association in a roughly linear fashion. Interestingly, the larger complex generated in the UBC-1Δ169 cross-linking had a molecular mass of approx. 65 kDa, closer to that of a trimer than to the tetramer seen with wild-type UBC-1. This suggests that the tail might affect not only the efficiency of UBC-1 self-association but also the quaternary structure formed.

The above results suggest that the E2 core domain of UBC-1 is able to associate weakly with itself and that the tail enhances this ability. This conclusion is further supported by the observation that when the concentration of UBC-1Δ152 is increased from 350 to 700 nM, the equilibrium shifts further towards dimer formation (results not shown). As with UBC-1Δ169, the larger complex generated by cross-linking 700 nM UBC-1Δ152 had a molecular mass of approx. 60 kDa, approximating the size of a trimer.

UBC-1 forms both thiol-sensitive and thiol-insensitive ubiquitin-UBC-1 adducts

Previously, we have shown that N-terminally histidine-tagged UBC-1 is capable of forming an *S*-ester bond with ubiquitin, a prerequisite for E2 function, in a reaction with bacterially expressed wheat E1 [17]. When this experiment was repeated with non-histidine-tagged UBC-1 and *C. elegans* extract as a source of E1, in addition to the expected ubiquitin *S*-ester, UBC-1 also formed a thiol-insensitive ubiquitin adduct. The 30 kDa band in Figure 3 represents the *S*-ester adduct, on the basis of its sensitivity to 2-mercaptoethanol. The molecular mass of this protein is consistent with that of a single ubiquitin bound to UBC-1. In addition to this 30 kDa band, an additional band with an apparent molecular mass of 34 kDa was seen. The latter was resistant to 2-mercaptoethanol, suggesting the linkage of a ubiquitin moiety to a lysyl residue in UBC-1. The relative intensity of the 34 kDa band in the non-reducing lane compared with the reducing lanes suggests that the band in the non-reducing lane might contain a thiol-linked ubiquitin as well. The difference in the observed molecular masses of the 30 and 34 kDa adducts might be due to steric effects of stable ubiquitin binding on the migration of the protein. On re-examination of the data, the histidine-tagged UBC-1 assays with wheat E1 also contained a thiol-insensitive ubiquitin adduct, but at much lower levels

Figure 3 UBC-1 forms thiol-sensitive and thiol-insensitive adducts with ubiquitin

Ubiquitin conjugation reactions with ^{125}I -ubiquitin were performed as described in the Materials and methods section. The samples were then subjected to SDS/PAGE [15% (w/v) gel] with or without treatment with 360 mM 2-mercaptoethanol (β -Merc) as indicated. The positions of molecular mass standards are shown at the left.

Figure 4 Mapping of the auto-ubiquitination site to the tail of UBC-1

Ubiquitin conjugation assays were performed with purified UBC-1, UBC-1 Δ 169 and UBC-1 Δ 152. The reactions were treated with or without 2-mercaptoethanol (β -Merc) as indicated, separated by SDS/PAGE and blotted as described in the Materials and methods section. The blot was probed with anti-UBC-1 antibody and developed by enhanced chemiluminescence. The positions of molecular mass standards are shown at the left.

(results not shown). A third ubiquitin-conjugated component of 38 kDa was seen in the non-reducing lane; this had a molecular mass consistent with two ubiquitin moieties bound to UBC-1 and was labile to reducing agents, suggesting that the linkage to UBC-1 is via its cysteinyl residue. This suggests that UBC-1 might possess the ability to form multi-ubiquitin chains on its active site cysteine residue, although this remains to be verified with purified *C. elegans* E1.

Determination of the ubiquitination site of UBC-1

UBC-1 contains eight lysyl residues that could potentially form isopeptide bonds with ubiquitin. Seven of these residues lie within the core of UBC-1; one is located in the tail. Ubiquitination assays performed on UBC-1 Δ 169 and UBC-1 Δ 152 showed that although both formed the Ub-UBC-1 *S*-ester, UBC-1 Δ 152 did not form the thiol-stable ubiquitin adduct (Figure 4). The latter observation focused our attention on Lys-162, which is located within the tail. Accordingly, Lys-162 was

A

B

Figure 5 Lys-162 is involved in stable ubiquitination of UBC-1

(A) Immunoblot of an SDS/15% polyacrylamide gel of ubiquitin conjugation assays performed on bacterial lysates containing expressed UBC-1 or UBC-1R162. The samples were run with or without 360 mM 2-mercaptoethanol (β -Merc) as indicated. The blot was probed with anti-UBC-1 antibody. The positions of UBC-1, as well as the thiol-insensitive (UBC-1-Ub) and thiol-sensitive (UBC-1-S~Ub) ubiquitin conjugates are shown. (B) Protein alignment of the C-terminal tails of UBC-1 and its homologues from the nematodes *C. briggsae* and *A. suum*. The location of Lys-162 is indicated.

mutated to arginine and the altered protein was expressed in *E. coli*. Ubiquitin conjugation assays on UBC-1R162 showed that it could form an *S*-ester with ubiquitin, but failed to form a thiol-stable adduct (Figure 5A), thus demonstrating that Lys-162 is the residue that is stably ubiquitinated in UBC-1. Interestingly, Lys-162 has been conserved in the UBC-1 homologues from the nematodes *A. suum* and *C. briggsae* (Figure 5B), suggesting that these proteins might also form stable ubiquitin adducts at this site.

DISCUSSION

Cross-linking experiments performed on purified recombinant UBC-1 demonstrate that it self-associates *in vitro*. These results, together with earlier work, suggest that a quaternary structure consisting of both homo-interactions and hetero-interactions is an important feature of E2s. Early work *in vitro* showed that some E2s can be purified as dimers by gel exclusion chromatography [22,23]. Other evidence *in vivo* has shown that yeast CDC34 interacts with both RAD6 and itself and that its homo-interaction is required for yeast viability [12]. Furthermore the yeast E2s UBC6 and UBC7 also interact with each other *in vivo* to mediate the degradation of MAT α 2 [24]. Owing to the high degree of similarity between E2s, it seems likely that E2-E2 interactions will prove to be a general structural theme among these enzymes.

The ability of UBC-1 lacking the C-terminal tail to self-associate suggests that at least two separate binding regions are involved in this interaction. The first region is found within the catalytic core of UBC-1 and is responsible for the self-association seen in UBC-1 Δ 152. The likelihood of the existence of such an interacting region in the E2 core domain is strengthened by the observation that yeast UBC4, a type I E2 lacking a tail, has been shown by cross-linking to self-associate *in vivo* [25]. The second binding site is found within the tail, and our results show that this region is able to strengthen the self-association of UBC-1 by

approx. 8-fold. The tail of yeast CDC34 has also been shown to influence strongly the ability of this E2 to self-associate [16]. Thus although they are not essential for such homo-interactions [22,23,25], the tail regions of several E2s seem to facilitate the formation of homodimers, and possibly of higher multimers. The removal of the tails of both UBC-1 and CDC34 [16] greatly decreases their ability to associate as seen by cross-linking *in vitro*. The presence of a tail might also have a role in hetero-associations. Silver et al. [12] have shown that RAD6 lacking its tail can interact with CDC34 *in vivo*. This interaction is dependent on the tail of CDC34. In an analogous situation, yeast UBC6, which has a tail, interacts *in vivo* with UBC7, which does not [24].

The appearance of tetrameric complexes in chemical cross-linking experiments suggests that the quaternary structure of UBC-1 might consist of structures larger than dimers. Chemical cross-linking of both CDC34 and UBC4 yielded complexes larger than dimers [16,25], indicating that higher-order quaternary structures in E2 interactions might be of general significance. Both UBC-1 and CDC34, which are type II E2s, form tetrameric complexes as revealed by cross-linking experiments, whereas UBC4, a type I E2, forms trimers. It is interesting to note that, on removal of its tail, UBC-1 apparently forms trimers, as does wild-type UBC4. Whether E2 tail sequences have a role in the formation of quaternary structures beyond dimers remains to be seen.

Together with previous results, our results on the mono-ubiquitination of UBC-1 at Lys-162 suggests that auto-ubiquitination of E2s can be divided into two distinct classes that probably have different roles in the cell. In the first class, which includes yeast CDC34 [26] and human E2_{EPF} [27], are E2s that conjugate multi-ubiquitin chains to themselves. The fact that these E2s multi-ubiquitinate themselves suggests that they may be targeting their own degradation, thereby regulating their own levels within cells. The second class of auto-ubiquitination involves the mono-ubiquitination of E2s. The only previous example of this reaction was that of yeast UBC4, which was shown to be mono-ubiquitinated at Lys-144 [25]. Our observation that *C. elegans* UBC-1 is also mono-ubiquitinated suggests that this process might be as prevalent among E2s as multi-ubiquitination. Mono-ubiquitinated proteins are not degraded by the 26 S proteasome, so this probably represents a stable modification of the E2 that might be involved in regulating some aspect of E2 function. In contrast, mutation of Lys-144 in yeast UBC4 produced no adverse effects [25], suggesting that, at least for this protein, mono-ubiquitination does not have an essential role. The localization of the site of ubiquitination to the tail, which is involved in dimerization, suggests a possible role for ubiquitin in controlling self-association. Ptak et al. [16] have suggested that the interaction between ubiquitins bound at the active sites of E2s might assist in stabilizing E2 dimers, and ubiquitination of the tail of UBC-1 might have such a role.

In summary, this work demonstrates that the C-terminal tail of *C. elegans* UBC-1 is involved in both the post-translational modification and the quaternary structure of the protein. Given that the properties of the UBC-1 tail sequence (i.e. length, acidity and mono-ubiquitination) differ significantly from those of other known RAD6 homologues, the elucidation of the function of this region in the *C. elegans* protein should provide insights into the general properties of ubiquitin-conjugating enzymes.

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