Mutagenesis of the HMGB (high-mobility group B) protein Cmb1 (cytosine-mismatch binding 1) of *Schizosaccharomyces pombe*: effects on recognition of DNA mismatches and damage

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Cmb1 (cytosine-mismatch binding 1) is a high-mobility group (HMG) protein of *Schizosaccharomyces pombe*, which consists of 223 amino acids and has a single HMG domain at the C-terminal end. We have created several mutant and deletion forms of the Cmb1 protein and studied the effects on general DNA binding and specific binding to DNA mismatches and damaged DNA. Cmb1 Δ 41 (i.e. Cmb1 from which the 41 N-terminal amino acids have been deleted) bound specifically to cytosine-containing mismatches, to the cisplatin-induced intrastrand cross-links *cis*-GG and *cis*-AG and to an *O*⁶-methylguanine lesion. DNA binding was not affected when the 45 N-terminal amino acids were deleted, but was abolished in the absence of the 50 N-terminal amino acids, and was reduced when Cmb1 was truncated

by between five and eleven C-terminal amino acids. Cmb1, both with and without the C-terminal truncations, retained its DNA binding affinity after heating at 95 °C. The *cmb1* gene was induced when *S. pombe* cells were treated with cisplatin. Mitotic mutation rates were increased in a *S. pombe cmb1* null mutant and in a *cmb1*-(1–212) mutant, which encodes a Cmb1 protein lacking the 11 C-terminal amino acids. We conclude that mutation avoidance by Cmb1 is distinct from Msh2-dependent mismatch repair, but related to nucleotide excision repair.

Key words: cisplatin, DNA repair, high-mobility group, mismatch, mutation avoidance.

INTRODUCTION

Several pathways have evolved that remove mismatches or damage from DNA. Base–base mismatches and small loops, which can arise due to replication errors, by spontaneous base alterations or during recombination, are corrected efficiently by the long-patch mismatch repair (MMR) system, which requires, among others, the factors Msh2 and Mlh1 (reviewed in [1,2]). The nucleotide excision repair (NER) pathway removes a wide variety of DNA adducts, such as cyclobutane pyrimidine dimers and 6–4 photoproducts induced by UV radiation, as well as intrastrand cross-links caused by the anti-tumour agent *cis*-diammine-dichloroplatinum(II) (cisplatin) (reviewed in [3,4]). To some extent, even base–base mismatches are processed by human NER [3]. Factors involved in NER in *Schizosaccharomyces pombe* have been shown to act in MMR-independent mismatch correction [5–7]. This pathway is able to correct C/C mismatches, which are not a substrate of MMR [8].

Two mismatch binding activities have been identified in *S. pombe* [9]. One binds to most types of base–base mismatches, but not to C/C, and is dependent on functional Msh2 [10]. The second activity, showing specific affinity for C/C and other cytosine-containing mismatches, has been identified as Cmb1 (cytosine-mismatch binding) [11]. Cmb1 contains a single high-mobility group (HMG) domain in the C-terminal part, and thus belongs to the family of HMGB proteins (designation according to the new nomenclature for HMG proteins described in [12]). HMGB proteins are members of a superfamily of non-histone chromatin proteins in eukaryotes. The HMGB family can be divided into two subgroups of DNA binding proteins. One consists

of proteins containing one HMG domain, such as the transcription factors LEF-1 [13], SRY [14] and TCF1 [15], which bind to specific nucleotide sequences. The second subgroup includes proteins with one or more HMG domains, such as HMGB1, HMGB2 [16], hUBF1 [17] and ABF2 [18]. These proteins bind to DNA with no or low sequence specificity, and have been termed non-sequence-specific HMGB proteins [19]. They are believed to act in the regulation of chromatin structure and to be involved in DNA transcription, replication or repair [20]. Several HMGB proteins are known to bind specific DNA structures, such as four-way junctions [21], intermediates of V-(D)-J recombination [22] or cisplatin-modified DNA (reviewed in [23]). Cmb1 of *S. pombe* is able to bind to cisplatin-induced intrastrand cross-links, to *O*⁶-methylguanine (*O*⁶meG) lesions and to cytosine-containing mismatches [11], indicating a role in some aspects of DNA repair.

In the present study, we constructed derivatives of Cmb1 with deletions of N-terminal or C-terminal amino acids, or with amino acid substitutions. These Cmb1 proteins were tested for their ability to bind to DNA mismatches and to damaged DNA using a band-shift assay. In addition, an *S. pombe cmb1*-disruption strain and a mutant that encodes a Cmb1 derivative lacking the C-terminal HMG domain were analysed for a defect in mitotic mutation avoidance.

MATERIALS AND METHODS

Yeast media and genetic methods

The yeast media YEA (yeast extract agar; full medium), YEL (yeast extract liquid; full medium) and MMA (minimal medium

Abbreviations used: cisplatin, *cis*-diammine-dichloroplatinum(II) (*cis*- denotes a cisplatin adduct); Cmb, cytosine-mismatch binding; Cmb1 Δ 41 (etc.), Cmb1 from which the 41 N-terminal amino acids have been deleted (etc.); HMG, high-mobility group; MMA, minimal medium agar; MMR, long-patch mismatch repair; NER, nucleotide excision repair; *O*⁶meG, *O*⁶-methylguanine; YEA, yeast extract agar; YEL, yeast extract liquid.

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agar) and general genetic methods were used as described in [24]. Mutation rates were determined as reversions of *ade6-72291* [8] to *Ade*⁺. Nine colonies derived from YEA plates were each inoculated in 3 ml of YEL, grown at 30 °C to stationary phase and plated on MMA. Appropriate dilutions were plated on MMA supplemented with adenine for determination of cell titres. Mutation rates per cell division were calculated by the method of the median [25] or by the zero method [26]. Reversion assays were performed at least three times for each strain.

PCR

PCR was performed in a Perkin Elmer DNA thermal cycler. Standard reactions of 50 µl contained 10–100 pmol of each of two primers, 0.1 mM each dNTP, and either 1 unit of *Taq* polymerase (Amersham Pharmacia Biotech) or 2.5 units of *Pfu* polymerase (Stratagene) in standard PCR buffers. As template, 2–20 ng of plasmid DNA was used. Reactions included an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s annealing at a temperature depending on the composition of the primers and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Differences from this standard protocol are specified.

Construction of Cmb1-expressing plasmids by PCR cloning

Plasmids with DNA sequences expressing the various Cmb1 proteins, shown in Figure 2, were obtained by PCR cloning or site-directed mutagenesis. Primers are listed in Table 1. Construction of plasmids expressing full-length Cmb1 or Cmb1Δ41 (Cmb1 from which the 41 N-terminal amino acids have been deleted) has been described [11]. The plasmids expressing Cmb1 mutant proteins with N-terminal or C-terminal deletions were obtained by insertion of PCR fragments via *Nde*I and *Bam*HI restriction sites into pT7-7. The forward primers contain an *Nde*I site and the reverse primers a *Bam*HI site. pOL64 (pT7-7 carrying the wild-type *cmb1* gene) was used as template. Correct clones were identified by restriction analysis and checked for mutations by DNA sequencing. For expression of the proteins, plasmids were transformed into the *Escherichia coli* strain BL21/DE3.

Site-directed mutagenesis

Reactions of 50 µl were performed, containing 20 pmol of each of two complementary primers carrying the desired mutation (see Table 1), 20 ng of pCK1 (pT7-7 containing *cmb1* without the 5' sequence coding for amino acids 2–41) as template, 0.2 mM each dNTP and 2.5 units of *Pfu* polymerase (Stratagene) in a standard reaction buffer. The reactions started with an initial denaturation step of 5 min at 95 °C, followed by 14 cycles of 30 s at 95 °C, 1 min at 55 °C and 6 min at 69 °C, and a final elongation step of 10 min at 72 °C. The template DNA was digested with 10 units of *Dpn*I and subsequently transformed into *E. coli* DH5α. Correct integration of the mutation was determined by DNA sequencing. For protein expression, plasmids were transformed into BL21/DE3.

Preparation of *E. coli* crude protein extracts

A 50 ml volume of Luria–Bertani broth containing ampicillin (100 µg/ml) was inoculated with 2 ml of a stationary-phase culture of *E. coli* BL21/DE3 transformed with a plasmid expressing one of the Cmb1 derivatives. Cultures were incubated at 37 °C

until an *D*₆₀₀ of approx. 0.7 was reached. For induction, isopropyl β-D-thiogalactoside was added to a final concentration of 1 mM. After 4 h, cells were harvested at 4000 g for 10 min at 4 °C, and then suspended in 5 ml of buffer A (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 mM spermidine, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol and 0.1 mM PMSF). Subsequently, cells were frozen in liquid nitrogen and stored at –70 °C until use. Cells were thawed on ice and disrupted by six 30 s intervals of sonication, with at least 30 s of cooling on ice between each round. When Cmb1 derivatives had to be purified, cells were sonicated twelve times, and cooled on ice for 1 min between each round. Soluble proteins were separated from insoluble proteins by a 20 min centrifugation at 10 000 g and 4 °C. The degree of overexpression and solubility was checked on SDS/14 % polyacrylamide gels.

Purification of Cmb1 proteins

Purification of the various Cmb1 proteins from *E. coli* crude extracts was performed as described in [11], with some modifications. Crude extracts were prepared in buffer B150 [25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 % (v/v) glycerol, 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 0.1 mM PMSF] and loaded on to a heparin–agarose column (type I; Sigma; 1.8 cm² × 11 cm) previously equilibrated with buffer B150. After washing with buffer B300 (same as buffer B150, but with 300 mM NaCl), bound proteins were eluted by a 100 ml gradient from 300 to 800 mM NaCl. The purest fractions were collected and dialysed. In the case of Cmb1Δ41 and Cmb1Δ45, which showed strong DNA binding in crude extracts, dialysis was performed against buffer B300. Fractions containing Cmb1Δ50, Cmb1-(42–213) or Cmb1-(42–212), which showed no or reduced DNA binding activity, were dialysed against buffer B150. The dialysed fractions were loaded on to a double-stranded-DNA–cellulose column (Sigma; 0.64 cm² × 5 cm) previously equilibrated with buffer B300 or B150. Bound proteins were eluted either by a 40 ml gradient from 300 to 600 mM NaCl or by a 60 ml gradient from 150 to 600 mM NaCl. Cmb1Δ41 and Cmb1Δ45 peaked at ~400 mM NaCl, whereas Cmb1Δ50, Cmb1-(42–212) and Cmb1-(42–213) peaked at ~260 mM NaCl. The purest fractions were pooled, dialysed against buffer B300 and loaded on to a P11 phosphocellulose column (Whatman; 0.64 cm² × 4 cm) previously equilibrated with buffer B300. Bound proteins were eluted with a 40 ml gradient from 300 to 600 mM NaCl. Elution of all Cmb1 derivatives peaked at ~480 mM NaCl. No proteins other than Cmb1 could be detected on polyacrylamide gels stained with Coomassie Brilliant Blue, with the exception of Cmb1Δ50, where two additional faint bands were present. Concentrations of purified proteins were determined according to the Bradford method [27].

Preparation of *S. pombe* crude extracts

A 500 ml volume of YEL was inoculated with 5 ml of a preculture and grown overnight to stationary phase at 30 °C. Cells were harvested by centrifugation and resuspended in buffer B150 to a concentration of 0.5 g of cells/ml. Aliquots of 1.3 ml were frozen in liquid nitrogen and stored at –70 °C until use. After thawing on ice and addition of an equal volume of glass beads, cells were disrupted in a Fastprep FP 120 machine (Savant Instruments Inc.) by 15 × 30 s intervals at speed 4, with at least 1 min cooling between each round. Soluble proteins were separated from cell debris by centrifugation at 10 000 g for 1 h at 4 °C.

Table 1 Primers used for construction of Cmb1-expressing plasmids and *S. pombe* *cmb1* mutants

Forward primers are indicated by f and reverse primers by r. Nucleotides deviating from the wild-type sequence to introduce specific amino acid substitutions in Cmb1 are given in lower-case letters.

Name	Sequence	Purpose
ON-Δ41n-f	5'-CTTCATATGAAAAGAATGGATTACAGAAG-3'	Forward primer for C-terminal deletions
ON-c-r	5'-GTAGGATCCTCATCATCGAAATCCGGCTTC-3'	Reverse primer for N-terminal deletions
ON-Δ45n-f	5'-CTTCATATGTTACAGAAGTTGATCCCCC-3'	Cmb1Δ45
ON-Δ50n-f	5'-CTTCATATGCCCCCTAGGCTGAAAACCATTTGG-3'	Cmb1Δ50
ON-Δ124n-f	5'-CCTCATATGCGTCGTCGCTCGAAGGAGGCTC-3'	Cmb1Δ124
ON-Δ11c-r	5'-GTAGGATCCTCATCATTTGCTCTTTAAGTGCTTGC-3'	Cmb1-(42–212)
ON-Δ10c-r	5'-GTAGGATCCTCATCAGTATTGCTCTTTAAGTG-3'	Cmb1-(42–213)
ON-Δ5c-r	5'-GTAAGGTCCTCATCATTTTCATAAACTTATCGTATTGCTC	Cmb1-(42–218)
ON-W191R-f	5'-CCTATGCATCAAAGGCGcGtAAGGAATTGGCGGAAG-3'	Cmb1W191R
ON-W191P-f	5'-CCTATGCATCAAAGGCGcGcGAAGGAATTGGCGGAAG-3'	Cmb1W191P
ON-K192P-f	5'-CCTATGCATCAAAGGCTGGcGAATGGCGGAAG-3'	Cmb1K192P
ON-Δ41n-m5-f	5'-CTTCATATGAAAAGAATGGAAgTgCAtcgtTgTaCCCCCTAGGCTGAAAACC-3'	Cmb1Δ41-mut46–50
ON-Δ41n-m2-f	5'-CTTCATATGAAAAGAATGGATTACAtcgtTTGATCCCCCTAGGCTG-3'	Cmb1Δ41 mut47–48
ON-P147A-f	5'-CATCCAGATGTTgCTAAAAACCTTC-3'	Cmb1Δ41-P147A
ON-P147A-r	5'-GAAGGTTTTTTAGcAACATCTGGATG-3'	Cmb1Δ41-P147A
ON-Q47N-f	5'-GAATGGATTAAcAAGTTGATCC-3'	Cmb1Δ41-Q47N
ON-Q47N-r	5'-GGATCAACTTgTtTAATCCATTC-3'	Cmb1Δ41-Q47N
ON-Q47H-f	5'-GAATGGATTACaAAGTTGATCCC-3'	Cmb1Δ41-Q47H
ON-Q47H-r	5'-GGGATCAACTTgTgTAATCCATTC-3'	Cmb1Δ41-Q47H
ON-K48R-f	5'-GGATTACAGAgGTTGATCCCC-3'	Cmb1Δ41-K48R
ON-K48R-r	5'-GGGGATCAACtCTGTATCC-3'	Cmb1Δ41-K48R
ON-K48A-f	5'-GGATTACAGgcGTTGATCCCC-3'	Cmb1Δ41-K48A
ON-K48A-r	5'-GGGGATCAACGcCTGTATCC-3'	Cmb1Δ41-K48A
ON-I50T-f	5'-CAGAAGTTGAcCCCCCTAGG-3'	Cmb1Δ41-I50T
ON-I50T-r	5'-CCTAGGGGGgTCAACTTCTG-3'	Cmb1Δ41-I50T
ON-DC1ura-f	5'-CTTCACTTAACCTTACCAAGCACCATTATCTTTGATCATTTTACAAAATGGTA- TTTGAATAGAAATACGCCAGTTTCACACTACTCTGTTTCGCCAAGCTTAGCTACAAATCCCA-3'	<i>S. pombe</i> disruption strain <i>cmb1::ura4</i>
ON-DC1ura-r	5'-TGCATCATCGAAATCCGGCTTCTTTCATAAACTTATCGTATTGCTCTTTAAGTG- CTTTGCTTTTATCAATAAATGGCTTTTCTGTCTTCGCCCAATTCAGCTTGTGATATTGACGAA-3'	<i>S. pombe</i> disruption strain <i>cmb1::ura4</i>
ON-i-f	5'-ATAGTCTATGGTGAGTAATG-3'	<i>S. pombe</i> <i>cmb1</i> -(1–212) mutant
ON-iΔ11c-r	5'-GGCAAGTAGAGTGTGCATCATTTGCTCTTTAAGTGCTTGC-3'	<i>S. pombe</i> <i>cmb1</i> -(1–212) mutant
ON-i-3'-f	5'-TGATGCACACTCTACTTGCC-3'	<i>S. pombe</i> <i>cmb1</i> -(1–212) mutant
ON-i-r	5'-TTAGTCCAACAAGCTTTGTG-3'	<i>S. pombe</i> <i>cmb1</i> -(1–212) mutant

DNA binding studies

DNA binding of wild-type and mutant Cmb1 proteins was tested by a band-shift assay. Oligonucleotides were 5'-end-labelled by T4 polynucleotide kinase (MBI Fermentas) in the presence of [γ - 32 P]ATP and annealed with their complementary strands as described in [9]. Substrates and competitor DNA used to analyse binding to mismatches were nucleotides 1380–1411 of the *ade6* sequence [28]. Oligonucleotide 3ade6 + C, 5'-AAGCACTTGGTGATCGTCCGCTTTATGTTGAA-3', was annealed with complementary strands, resulting in double-stranded substrates containing C:G, C/C or C/Δ at the position underlined in 3ade6 + C. The sequence contexts of substrates containing either cisplatin adducts or an O⁶meG lesion were described previously [11]. Proteins were allowed to react with 40 fmol of radiolabelled substrate for 20 min at 4 °C in 25 mM Tris/HCl (pH 7.5), 25 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 4 mM spermidine, 0.01 mM ZnCl₂, 10% (v/v) glycerol and 60–240 mM NaCl. The amounts of protein and of unlabelled homoduplex DNA used as competitor that were added to the reactions are given in the text. Reactions were loaded on to non-denaturing 6% (w/v) polyacrylamide gels. After electrophoresis, which was carried out for 3 h at 110 V and 4 °C in 40 mM Tris/HCl (pH 7.5), 20 mM sodium acetate and 1 mM EDTA, gels were subjected to autoradiography. For quantification, gels were dried, exposed to a PhosphorImager screen and analysed using ImageQuant software (Molecular Dynamics).

S. pombe strains

The *S. pombe* strains were derived from Ru39 (*h⁻ msh2::his3 his3-D1*) [10], OL455 (*h⁻ swi10::kanMX his3-D1 leu1-32 ura4-D18*) [7], OL142 (*h⁺ cmb1::his3 his3-D1*) [11] or CK1 (*h⁻ cmb1::ura4 ura4-D18*; the present study). The *ade6* allele T2291, used for fluctuation tests, was described by Schär and Kohli [8]. In the *cmb1::ura4* disruption strain CK1, the *ura4* gene replaced nucleotides 123–576 of *cmb1*. CK1 was obtained by transformation of strain *h⁻ ura4-D18* [29] with a PCR fragment containing *ura4* flanked by sequences identical to the sequences 5' of nucleotide 123 and 3' of nucleotide 576 of *cmb1*. The fragment was amplified with primers ON-DC1ura-f and ON-DC1ura-r (Table 1). The reaction comprised an initial step of 5 min at 94 °C, then three cycles of 1 min at 94 °C, 1 min at 49 °C and 2 min at 72 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, and a final elongation step at 72 °C for 10 min.

The strain KZ1 [*h⁻ cmb1*-(1–212) *ura4-D18*] was constructed by transformation of CK1 with a PCR fragment that contained a truncated *cmb1* gene, which encodes a Cmb1 derivative lacking the 11 C-terminal amino acids. The DNA fragment was obtained by fusion PCR. In a first step, two fragments were amplified that had a 20 bp overlap. The 5' fragment was obtained with primers ON-i-f and ON-iΔ11c-r, and the 3' fragment with primers ON-i-3'-f and ON-i-r (Table 1). PCR reactions were performed as described above, except that pOL56 (pUC18 containing *cmb1*

and its flanking genomic sequences) [11] served as template. Reactions comprised an initial step of 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C, and a final elongation step of 15 min at 72 °C. The remaining primers were removed using a NucleoSpin Extract kit (Macherey-Nagel). For fusion of the two fragments, a second round of PCR was applied. PCR was performed in 50 µl containing 10 pmol each of primers ON-i-f and ON-i-r, 2.5 units of *Pfu* polymerase and 0.2 mM each dNTP in a standard reaction buffer. Reactions comprised an initial step of 5 min at 94 °C, then five cycles of 1 min at 94 °C, 1 min at 58 °C and 2.5 min at 72 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 49 °C and 2.5 min at 72 °C, and a final elongation step of 15 min at 72 °C. Replacement of the *ura4* disruption cassette by the transformed DNA fragments results in uracil auxotrophic cells. Therefore *ura4*[−] transformants were selected on plates containing 5-fluororotic acid (1 g/l) [29]. Correct integration was checked by PCR and DNA sequencing.

Northern blot analysis

To test the effect of cisplatin on the expression of *cmb1*, a 200 ml culture of the *S. pombe* wild-type strain *h*[−] 972 was grown at 30 °C in YEL to an *D*₆₀₀ of approx. 1.0. Then 50 ml of this culture was centrifuged and suspended either in 0.5 × YEL or in 0.5 × YEL containing 0.6 mM cisplatin. After a 90 min incubation at 30 °C, cells were harvested by centrifugation and RNA was prepared as described [30]. Samples containing 20 µg of RNA were loaded on a 1 % (w/v) agarose gel and electrophoresis was carried out for 3 h in 25 mM sodium phosphate buffer (pH 6.5) at 70 V and 4 °C. The RNA was transferred to a GeneScreen Plus nylon membrane (NEN) using a vacuum blotter (Amersham Pharmacia Biotech). Hybridization was performed with a radiolabelled 486 bp PCR fragment of *cmb1* in ULTRAhyb hybridization buffer (Ambion) according to the manufacturer's protocol. After exposure to a PhosphorImager screen, the membrane was stripped, hybridized with a 413 bp fragment of *act1* [31] and again exposed to a PhosphorImager screen. Levels of Cmb1 and Act1 RNAs were analysed using ImageQuant software (Molecular Dynamics). For comparison of RNA levels in samples from cisplatin-treated cells and untreated cells, the determined Cmb1 RNA levels were normalized against Act1 RNA levels.

RESULTS

Mutagenesis of Cmb1

We created a series of mutant Cmb1 proteins in order to obtain information on the parts or amino acids that are required for specific binding to DNA mismatches and damaged DNA. The mutant proteins were overexpressed in *E. coli* BL21/DE3 and crude extracts were tested for binding to mismatched DNA by band-shift analysis. As a control, crude extracts from BL21/DE3 harbouring the empty expression vector pT7-7 were tested. Originally, Cmb1 was partially purified from *S. pombe* as a proteolytic peptide, Cmb1Δ41, lacking the 41 N-terminal amino acids [11]. Cmb1Δ41 either derived from crude extracts of *S. pombe* or *E. coli* or in purified form from either organism showed the same specificity for mismatches as the full-length Cmb1 protein. However, we realized in the previous work that deletion of the 41 N-terminal amino acids resulted in a remarkable increase in overexpression when compared with the full-length protein [11]. Therefore most of the mutant clones were constructed without the nucleotides encoding the 41 N-terminal

Figure 1 Binding of Cmb1 derivatives to mismatched and homoduplex DNA

DNA binding was tested with *E. coli* crude protein extracts expressing the indicated Cmb1 derivatives (see Figure 2) by band-shift analysis. As a negative control, extracts from *E. coli* cells harbouring the empty pT7-7 vector were used. Reactions were performed in the absence of competitor DNA and contained 3 µl of crude extract (~5 µg of total protein), 40 fmol of radiolabelled substrate as indicated and 60 mM NaCl. The position of the Cmb1–DNA complex is indicated. Other shifted bands originated from the binding of *E. coli* proteins.

Protein	50100150200	Solubility	Mismatch binding
Cmb1			
Cmb1	..P...WK..	+	++
Cmb1W191P	..P...K..	−	N/A
Cmb1W191R	..P...K..	−	N/A
Cmb1K192P	..P...W..	+/-	+
Cmb1Δ41-P147A	..A...WK..	+	++
Cmb1Δ41			
Cmb1Δ41 / Cmb1	..KEQYDKFMKEAGFR		
Cmb1-42-212	..KEQ	+	+
Cmb1-42-213	..KEQY	+	+
Cmb1-42-218	..KEQYDKFMK	+	+
Cmb1Δ41			
Cmb1Δ45			
Cmb1Δ50			
Cmb1Δ124		+	−
Cmb1Δ41	MEKNGLQKLIPP..	+	++
Cmb1Δ45	MLQKLIPP..	+	++
Cmb1Δ50	MPP..	+	−
Cmb1Δ41-Q47N	MEKNGLQKLIPP..	+	++
Cmb1Δ41-Q47H	MEKNGLQKLIPP..	+	++
Cmb1Δ41-K48R	MEKNGLQKLIPP..	+	++
Cmb1Δ41-K48A	MEKNGLQKLIPP..	+	++
Cmb1Δ41-I50T	MEKNGLQKLIPP..	+	++
Cmb1Δ41-mut47-48	MEKNGLQKLIPP..	+	++
Cmb1Δ41-mut46-50	MEKNGLQKLIPP..	+	++

Figure 2 Binding ability of wild-type and mutant Cmb1 proteins

Wild-type Cmb1 contains 223 amino acids. Positions of deletions and substitutions are indicated. The Cmb1 proteins were overexpressed in *E. coli*, and crude extracts were tested by band-shift analysis for binding to DNA containing one unpaired cytosine, DNA containing a C/C mismatch and homoduplex DNA. The different degrees of solubility are indicated as +, +/- and −. Binding efficiency to mismatches is expressed as ++ (not significantly different from Cmb1Δ41), + (reduced) and − (not detectable). The C-terminal HMG domain is depicted as a hatched box. Amino acid substitutions are highlighted. N/A, not applicable.

amino acids. Some of the band shift gels are shown as examples in Figure 1. The results of the binding studies are summarized in Figure 2.

In order to investigate the role of the HMG domain of Cmb1 in binding to mismatches, highly conserved amino acids within the HMG motif were substituted by other amino acids. In Cmb1W191R and Cmb1W191P, Trp¹⁹¹ was substituted by arginine and proline respectively (numbering of amino acids according to full-length Cmb1). On SDS/polyacrylamide gels containing fractions of soluble and insoluble proteins, Cmb1W191R and Cmb1W191P could be detected only in the insoluble fractions (Figure 2). Even an attempt to enrich traces of soluble Cmb1W191R by chromatography failed (results not shown). Although substitution of Lys¹⁹² by proline in Cmb1K192P caused reduced solubility, some DNA binding capacity was retained. Binding was not affected in Cmb1 Δ 41-P147A, in which the conserved Pro¹⁴⁷ was replaced by an alanine (Figure 1). Cmb1-(42–212), with deletion of the 11 C-terminal amino acids, including the conserved Tyr²¹³, showed a remarkable decrease in DNA binding. However, a decrease was also observed with Cmb1-(42–213) and Cmb1-(42–218), in which Tyr²¹³ is present (Figure 2).

No DNA binding was detectable with Cmb1 Δ 124 or Cmb1 Δ 50, in which the 124 and 50 N-terminal amino acids respectively were deleted. In contrast, Cmb1 Δ 45, with deletion of the 45 N-terminal amino acids, bound mismatches with the same efficiency as Cmb1 Δ 41 (Figure 1). Thus amino acids between positions 46 and 50 play a role in DNA binding. In order to identify specific amino acid residues in this region that are required for DNA binding, the amino acids between positions 46 and 50 were substituted. However, Cmb1 with either one or two amino acid substitutions showed the same DNA binding capacity as Cmb1 Δ 41, and even changing all five amino acids (Cmb1 Δ 41-mut46–50) did not impair DNA binding (Figures 1 and 2).

Based on the results obtained with crude extracts, we decided to purify Cmb1 Δ 41, Cmb1 Δ 45, Cmb1 Δ 50, Cmb1-(42–213) and Cmb1-(42–212) as representative examples of truncated Cmb1 derivatives (see the Materials and methods section). These purified derivatives of Cmb1 were used for more detailed binding studies, and to exclude the possibility that factors present in the crude extracts affect binding of DNA by Cmb1.

Cmb1-(42–212) shows lowered affinity for DNA

DNA binding studies were initiated by titration experiments with purified Cmb1 Δ 41 and Cmb1-(42–212). Homoduplex DNA and DNA containing one unpaired cytosine were incubated with increasing amounts of protein (Figure 3). Quantification of the binding data allowed us to determine the K_D values of both proteins for the mismatched substrate. The K_D of Cmb1-(42–212) for homoduplex DNA represents only an estimation, due to the low affinity for this substrate. Cmb1 Δ 41 showed a 3.6 times higher affinity for the mismatched substrate (K_D 9.5×10^{-8} M) than for the homoduplex substrate (K_D 3.4×10^{-7} M). Compared with Cmb1 Δ 41, binding of Cmb1-(42–212) was reduced by 2.3-fold for the mismatched substrate (K_D 2.2×10^{-7} M) and by approx. 7-fold for homoduplex DNA (K_D 2.4×10^{-6} M).

Influence of N-terminal and C-terminal deletions on the affinity of Cmb1 for damaged DNA

We tested the affinity of purified Cmb1 Δ 41, Cmb1 Δ 45, Cmb1 Δ 50, Cmb1-(42–212) and Cmb1-(42–213) for the intrastrand cross-links *cis*-GG and *cis*-AG, the two major cisplatin adducts [32]. Aliquots of 3.4 pmol of proteins were used, since reasonable binding was obtained in the titration experiment with

Figure 3 Efficiency of binding of Cmb1 Δ 41 and Cmb1-(42–212) to a C/Δ mismatch

Reactions contained 0.85–6.8 pmol of the proteins, 40 fmol of radiolabelled homoduplex DNA (A) or C/Δ-containing substrate (B), a 120-fold excess of unlabelled homoduplex DNA and 240 mM NaCl in a standard reaction buffer. (C) DNA binding of Cmb1 Δ 41 (□, ■) and Cmb1-(42–212) (○, ●) to homoduplex (□, ○) and C/Δ (■, ●) was quantified as a percentage of the total substrate added to the reaction. Shown are mean values $\pm$ S.D. of three to four experiments.

this amount (Figure 3). We found that 34% of the *cis*-GG-containing substrate and 15% of homoduplex DNA was bound by Cmb1 Δ 41 (Figure 4). Since Cmb1 Δ 45 showed comparable affinities, deletion of four more amino acids at the N-terminus did not alter binding to a *cis*-GG cross-link or to undamaged DNA. Binding of cisplatinated DNA by Cmb1-(42–212) or Cmb1-(42–213) was approx. 3 times reduced when compared with Cmb1 Δ 41. The affinities of Cmb1 Δ 41 and Cmb1-(42–212) for the *cis*-AG cross-link (Figure 5) were comparable with those observed with *cis*-GG (Figure 4). In contrast with the other Cmb1 mutant proteins, no binding to either type of cisplatin adduct was detected with Cmb1 Δ 50 (Figures 4 and 5).

Since Cmb1 is a cytosine-mismatch binding protein [11], it was interesting to test whether binding is influenced by the presence of a mismatch in cisplatinated DNA. Therefore the thymine opposite the adenine in the *cis*-AG:CT intrastrand cross-link was replaced by a cytosine, creating a compound lesion with an A/C mismatch

Figure 4 Influence of N-terminal and C-terminal deletions on the affinity of Cmb1 for a 1,2 *cis*-GG:CC intrastrand cross-link

(A) Binding to cisplatinated DNA by Cmb1 proteins with N-terminal and C-terminal deletions (see Figure 2). Reactions contained 3.4 pmol of the protein, 40 fmol of the indicated substrate, a 200-fold excess of unlabelled homoduplex DNA and 60 mM NaCl in a standard reaction buffer. (B) Quantitative expression of binding of the Cmb1 derivatives. Columns represent mean percentages of substrate bound, and were calculated from three band-shift gels.

Figure 5 Binding of Cmb1 derivatives to *cis*-AG intrastrand cross-links

Reactions contained 3.4 pmol of Cmb1Δ41, Cmb1Δ50 or Cmb1-(42–212). The proteins were incubated with 40 fmol of radiolabelled oligonucleotides in the presence of a 200-fold excess of unlabelled homoduplex and 60 mM NaCl in a standard reaction buffer. Substrates were homoduplex DNA (AG:CT) and oligonucleotides containing an A/C mismatch (AG/CC), a *cis*-AG:CT lesion or the compound lesion *cis*-AG/CC.

(*cis*-AG/CC). Interestingly, introduction of this mismatch reduced binding by Cmb1, and an A/C mismatch in an undamaged context was not bound to a greater extent than homoduplex DNA (Figure 5).

Our previous study has shown that an O⁶meG lesion, which can be produced by methylating agents, is bound specifically by Cmb1Δ41 [11]. Cmb1Δ45 bound as strongly to O⁶meG:C as Cmb1Δ41. Weak binding was observed with Cmb1-(42–212)

Figure 6 Effect of cisplatin on *cmb1* expression

Wild-type *S. pombe* cells were incubated in the absence (–) or presence (+) of 0.6 mM cisplatin before preparation of RNA. For normalization, the same RNA samples were probed with an *act1*-specific DNA fragment.

and Cmb1-(42–213), and no binding could be detected with Cmb1Δ50 (results not shown).

Cisplatin induces expression of the *cmb1* gene in *S. pombe*

We tested whether the level of expression of the *cmb1* wild-type gene was altered when cells were treated with cisplatin. Indeed, we found that cells treated with 0.6 mM cisplatin showed a 3-fold induction of *cmb1* expression when compared with untreated cells (Figure 6).

Cmb1 is heat resistant

During our DNA binding analysis, we observed that binding by Cmb1Δ41 still occurred in a control reaction that had been heated to 95 °C. To study this phenomenon in more detail, a crude extract containing overexpressed Cmb1Δ41 was incubated at 95 °C. At several time points, aliquots were taken and immediately chilled on ice, and denatured proteins were separated from native proteins by centrifugation. Aliquots of each fraction were loaded on to an SDS/polyacrylamide gel to check whether Cmb1Δ41 remained in the native fraction (Figure 7A). Heating denatured most of the *E. coli* proteins, whereas a significant portion of Cmb1Δ41 was present in the native fraction even after 2 h at 95 °C. The same observation was made with purified Cmb1Δ41 (Figure 7A), indicating that heat resistance is not mediated by stabilizing factors present in the crude extract. To test whether mismatch binding is affected by heat, we investigated binding to homoduplex DNA and to DNA containing a C/C mismatch (Figure 7B). Neither general DNA binding nor specificity for mismatched DNA was abolished by heating of Cmb1Δ41 (either derived from crude extracts or purified). In contrast, no binding to DNA was detected when the crude extract had been treated with Proteinase K. This is consistent with the finding that Cmb1Δ41 was not detectable in the Proteinase K-treated sample (Figure 7A).

When testing *S. pombe* protein extracts, a heat-stable activity that showed preferential binding to a C/Δ-containing substrate could be also detected in a wild-type strain, but not in a *cmb1* disruption strain (Figure 8). Weak binding to C/Δ was observed with a heat-treated Cmb1-(1–212) extract. The constructed *cmb1*-(1–212) gene encodes a Cmb1 protein without the 11 C-terminal amino acids. Thus DNA binding was reduced, similar to recombinant Cmb1 proteins with a truncated C-terminus, while heat resistance was retained.

Role of Cmb1 in mitotic mutation avoidance

Because Cmb1 is able to bind to cytosine-containing mismatches, we addressed the question of whether Cmb1 is involved in

Figure 7 Cmb1 is heat resistant

(A) SDS/polyacrylamide gel with samples from an *E. coli* protein extract containing Cmb1 Δ 41 and with purified Cmb1 Δ 41. The crude extract was incubated at 95 °C for the times indicated. Native (S, supernatant) and denatured (P, pellet) fractions were separated by centrifugation. For each time point and fraction, equal volumes were loaded on to the gel. Input, sample of the crude extract before heating; Proteinase K, sample of the crude extract treated with Proteinase K; Cmb1 Δ 41, supernatant of purified Cmb1 Δ 41 (17 pmol) after a 60 min incubation at 95 °C. (B) Band-shift analysis to test mismatch binding of the heat-treated supernatants shown in (A). One-third of the volume that was loaded on to the SDS gel was used. The samples were incubated with 40 fmol of radiolabelled substrate as indicated, a 120-fold excess of unlabelled homoduplex and 240 mM NaCl in a standard reaction buffer.

Figure 8 Mismatch binding in *S. pombe* *cmb1* mutants and wild type

Reactions contained 3 μ l of extract [$\sim$ 50 μ g of total protein; either heated (+) or not (–)], 40 fmol of substrate as indicated and 100 mM NaCl in a standard reaction buffer.

Table 2 Mitotic mutation rates in *S. pombe* *msh2*, *swi10* and *cmb1* mutants

Values are means $\pm$ S.D. obtained from three to six independent experiments. Fold increase is given relative to the wild-type rate.

Relevant genotype	<i>ade6-T2291</i>	
	Mutation rate	Increase (fold)
Wild type	$(2.9 \pm 1.2) \times 10^{-10}$	1
<i>msh2</i>	$(1.8 \pm 0.9) \times 10^{-9}$	6
<i>swi10</i>	$(3.0 \pm 1.4) \times 10^{-9}$	10
<i>cmb1</i>	$(1.5 \pm 0.8) \times 10^{-9}$	5
<i>cmb1</i> -(1–212)	$(1.8 \pm 1.2) \times 10^{-9}$	6
<i>msh2 swi10</i>	$(4.5 \pm 0.5) \times 10^{-9}$	16
<i>msh2 cmb1</i>	$(5.7 \pm 2.6) \times 10^{-9}$	20
<i>msh2 cmb1</i> -(1–212)	$(4.6 \pm 1.5) \times 10^{-9}$	16
<i>swi10 cmb1</i>	$(3.6 \pm 1.7) \times 10^{-9}$	12

mutation avoidance *in vivo*. We measured mitotic reversion rates of the *ade6* allele T2291, which is a G $\rightarrow$ T transversion [8]. We tested the *cmb1* disruption strain and the *cmb1*-(1–212) mutant, and performed epistasis analysis with the MMR mutant *msh2* and the NER mutant *swi10*, which are defective in distinct mutation avoidance pathways [5,10]. Compared with the wild type, reversion rates were increased in the *msh2*, *swi10* and *cmb1* mutants (Table 2). A further increase was observed in the double mutant *msh2 cmb1*, suggesting that Cmb1 acts in a pathway that is distinct from MMR. The mutation rate was also elevated in the *cmb1*-(1–212) mutant and further increased in the *msh2 cmb1*-(1–212) double mutant when compared with either single mutant. Thus deletion of C-terminal amino acids affects Cmb1-dependent mutation avoidance *in vivo*. The mutation rate was only slightly higher in *swi10 cmb1*, but not significantly different from *swi10*. These data indicate that Cmb1 is involved in NER-mediated mutation avoidance.

DISCUSSION

We have analysed the effects of various deletions and amino acid substitutions in Cmb1 on binding of this protein to mismatched and damaged DNA. Based on results obtained with *E. coli* crude extracts (Figure 2), we purified several Cmb1 derivatives and compared their binding efficiency with that of Cmb1 Δ 41, which lacks the 41 N-terminal amino acids. Cmb1 was originally enriched as a proteolytic peptide from *S. pombe* cells in this form [11]. Cmb1 Δ 41 bound specifically to the guanine adducts *cis*-GG and O⁶meG, and to the mismatches C/C and C/ Δ , as reported previously [11]. We could further demonstrate that a substrate containing a single *cis*-AG cross-link was bound efficiently (Figure 5). Interestingly, replacing the thymine opposite the cross-linked adenine by a cytosine reduced binding, and an A/C mismatch in the same sequence context was not bound better than homoduplex DNA. Since, in another sequence context, Cmb1 bound A/C as efficiently as other cytosine-containing mismatches [9,11], recognition of mismatches by Cmb1 is likely to be influenced by flanking nucleotides.

Effects of alterations in the HMG domain of Cmb1

Trp¹⁹¹ and Lys¹⁹² are conserved amino acids of HMG domains. In addition, the subfamily of non-sequence-specific HMGB proteins

is characterized by the presence of Pro¹⁴⁷ and Tyr²¹³ [19]. We tested the effects of substitutions of Pro¹⁴⁷, Trp¹⁹¹ and Lys¹⁹² on Cmb1 function, and also investigated Cmb1 derivatives with C-terminal deletions in which Tyr²¹³ was either present or absent.

Cmb1 derivatives in which Trp¹⁹¹ was substituted by either arginine or proline could not be detected in the fraction of soluble proteins. Consistently, substitution of the tryptophan by arginine in the HMG domain A of HMGB1 results in a largely unfolded protein [33]. The tryptophan has been located to an aromatic cluster stabilizing the L-shaped form of the HMG domain [34,35]. Thus a structural function is attributed to Trp¹⁹¹ of Cmb1. The protein structure seems to be also destabilized in Cmb1K192P, which might be due to the rigid nature of proline, which generally induces fixed kinks in polypeptide chains. However, the exchange of Lys¹⁹² does not have the same dramatic effect as substitution of Trp¹⁹¹, since some DNA binding activity was detected in *E. coli* crude extracts containing Cmb1K192P. Substitution of Pro¹⁴⁷ by alanine in Cmb1Δ41-P147A affected neither solubility nor DNA binding (Figures 1 and 2). Thus, although it is highly conserved among non-sequence-specific HMGB proteins, an important function in DNA binding cannot be attributed to Pro¹⁴⁷ of Cmb1.

The proteins Cmb1-(42–212), Cmb1-(42–213) and Cmb1-(42–218) showed reduced binding to mismatched and damaged DNA (Figures 1–5), suggesting that a complete HMG domain must be present for efficient DNA–protein interaction. A decrease in binding is not dependent on the absence of the conserved Tyr²¹³, since no difference could be detected between proteins with or without the tyrosine.

All types of substrates were bound less efficiently by purified Cmb1-(42–212) than by Cmb1Δ41, including unmodified oligonucleotides. On the other hand, Cmb1-(42–212) apparently showed a higher specific affinity for a C/Δ-containing substrate (11 times higher than the affinity for homoduplex DNA) than did Cmb1Δ41 (3.6 times higher). The relative high specificity of Cmb1-(42–212) might be due primarily to a decrease in general DNA binding. A reduction in binding to C/Δ is rather an indirect consequence, and not as pronounced as the reduction of general DNA binding. We propose that the HMG domain mediates general DNA binding, while regions that are located outside the HMG domain of Cmb1 are required for specific recognition of mismatches. An exception could be binding to cisplatinated DNA, which forms a structure that is bound by most, if not all, HMGB proteins and even by isolated HMG domains (reviewed in [36]).

Effects of alterations in the N-terminal region of Cmb1

The N-terminal two-thirds of Cmb1 shows no significant identity with any other known protein. To determine the minimal requirements for DNA binding, several proteins with deletions of N-terminal amino acids were studied. The HMG domain alone seems to be not sufficient for stable DNA binding, since Cmb1Δ124, a protein consisting of the HMG domain and 22 additional amino acids, was not able to bind in the band-shift assay (Figure 2). Although we cannot exclude the possibility that the additional amino acids affect DNA binding by the HMG domain, our findings indicate a difference from other HMGB proteins. DNA binding in a band-shift assay has been observed with the single HMG domains A and B of the non-sequence-specific proteins HMGB1 and HMGB2 [33,37,38] and with the HMG domain of the sequence-specific transcription factors hLEF-1 and Rox1 [39,40].

In the band-shift assay, Cmb1Δ45 showed the same affinity and specificity for mismatched and damaged DNA as Cmb1Δ41, whereas no binding could be detected with Cmb1Δ50, which lacks the 50 N-terminal amino acids. However, Cmb1Δ50 could bind to double-stranded-DNA–cellulose, although the protein eluted at a much lower salt concentration than Cmb1Δ41 and Cmb1Δ45 (see the Materials and methods section). Thus Cmb1Δ50 retained some affinity for DNA, which was not detectable with the band-shift assay. In a recent study, binding of Cmb1 to a C/Δ mismatch was analysed with the resonant mirror biosensor technique [41]. The isolated HMG domain exhibited reduced binding to both homoduplex and mismatched DNA, while the binding and specificity of Cmb1Δ108, which lacks the 108 N-terminal amino acids, were not affected. Although a band-shift assay is generally considered to be less sensitive, our studies revealed that deletion of only 50 N-terminal amino acids was sufficient to prevent binding of Cmb1. To detect binding of a protein to DNA, tight association is probably required in a band-shift assay, but not with a resonant mirror biosensor or on a double-stranded-DNA–cellulose resin.

Our data show that the amino acids between positions 46 and 50 of Cmb1 are required for efficient DNA binding. However, none of the conservative or non-conservative amino acid substitutions that were introduced in Cmb1 affected DNA binding (Figures 1 and 2). Thus the stretch of five amino acids, rather than the presence of specific amino acids, is required for the binding activity of Cmb1. The stretch of amino acids might ensure proper embedding of amino acids, located C-terminal to Ile⁵⁰, in the peptide structure and thereby preserve important functions of such amino acids for DNA binding.

Cmb1 remains active after heating

After incubation at 95 °C, a substantial amount of Cmb1Δ41 remained native and active in DNA binding. This phenomenon could be observed with various Cmb1 derivatives present in crude extracts of either *E. coli* or *S. pombe*, as well as with purified Cmb1Δ41 (Figures 7 and 8). Interestingly, Cmb1-(1–212) and Cmb1-(42–213), which contain a truncated HMG domain, were also resistant to heat (Figure 8 and results not shown). Like Cmb1, mammalian HMGB1 also retains the capacity to bind to DNA after heating, which has been proposed to be due to rapid refolding of the protein [42]. Thus heat resistance may be a general feature of HMGB proteins, and could allow the rapid and elegant purification of such proteins.

Role of Cmb1 in the cellular response to DNA damage

The *cmb1* disruption strain showed an increased reversion rate of the *ade6* allele T2291 (Table 2). Epistasis analysis with the MMR mutant *msh2* and the NER mutant *swi10* revealed that mutation avoidance mediated by Cmb1 is probably independent of MMR, but not of NER. Our current analysis has revealed that increased mutation rates in NER mutants of *S. pombe* depend on active polymerase Z (T. M. Marti, C. Kunz and O. Fleck, unpublished work), which has a function in error-prone translesion synthesis [43,44]. Thus, based on the epistasis analysis with the NER mutant *swi10* (Table 2), increased mutation rates in *cmb1*-defective cells are likely to be due to reduced repair of spontaneous lesions.

A recent study using DNA microarrays identified 140 genes of *S. pombe*, including *cmb1*, that are induced under various cellular stress conditions [45]. Relative to unstressed cells, the

RNA level of *cmb1* was 3.3 times higher in cells treated with hydrogen peroxide, which causes oxidative damage. *cmb1* was induced 2.4-fold in response to heat, and to lesser extent in cells treated with cadmium, sorbitol or methyl methanesulphonate (http://www.sanger.ac.uk/PostGenomics/S_pombe/projects/stress/). Similarly, we observed a 3-fold induction of *cmb1* when cells were exposed to cisplatin (Figure 6). Remarkably, with the exception of the SPBC23G7.11 gene, which encodes a putative DNA 3-methyladenine glycosylase with a function in base-excision repair, no other DNA repair genes were induced by the environmental factors tested in that study [45]. TG/TACGT is an ATF (activating transcription factor)/CRE (cAMP-response element)-related motif that has been found in the promoter regions of a number of stress-induced genes in *S. pombe* [45]. This motif is a putative binding site for transcription factors of the Atf1 family and is also present 30 bp upstream from the start codon of *cmb1*. Thus, in response to various types of stress, *cmb1* appears to be induced by Atf1 or a related transcription factor.

The recognition and repair spectrum of Cmb1 is similar to that of NER [3,5]. However, the situation is probably not as simple as Cmb1 being an integrated component of NER. Inactivation of *swi10* causes cellular sensitivity to UV radiation, while *cmb1*-null cells are as resistant to UV as wild-type cells (results not shown). With the data available, we favour the idea that Cmb1 serves as a sensor of DNA damage induced by genotoxic stress. Cmb1 may bind and mark alterations in DNA, and thereby facilitate the recruitment of DNA repair proteins.

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