Volume 336, number 2, 313–316 FEBS 13447 December 1993
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Two Tcp-1-related but highly divergent gene families exist in oat encoding proteins of assumed chaperone function

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Received 5 November 1993; revised version received 12 November 1993

Tcp-1-related sequences have been isolated from a cDNA library of etiolated 6-day-old oat (*Avena sativa*) seedlings. This attempt was made to obtain cDNAs of a recently published 60 kDa plant chaperone that re-folds denatured phytochrome and which was biochemically characterised as a Tcp-1-related protein [(1993) Nature 363, 644–647]. The translation of the putative coding sequence from one full-length cDNA clone displays no specific homologies to amino acid sequences known from peptide sequencing of the oat 60 kDa chaperone. Antibodies raised against the 60 kDa chaperone and over-expressed protein from one full-length coding sequence for Tcp-1 from oat show no cross-reactivity, whereas a monoclonal antibody raised against mouse Tcp-1 protein recognizes both the 60 kDa protein purified from plant extracts and over-expressed protein from Tcp-1-related cDNA sequences.

cDNA sequence; Tcp-1-related gene family; Cytosolic 60 kDa chaperonin; *Avena sativa*

1. INTRODUCTION

The t-complex polypeptide-1 (Tcp-1) is constitutively expressed in almost all cells in mammals and up-regulated in the testes during spermatogenesis. It is a constituent of the Tcp-1 ring complex (TriC) that seems to be involved in tubulin biogenesis [2–4]. Tcp-1 proteins have been suggested to be the cytosolic hsp60 homologs of mitochondrial and plastidic 60 kDa chaperonins that show homologies to prokaryotic proteins such as GroEL in *E. coli* [5–8].

Structure and in vitro activities of TriC are distinct from the prokaryotic GroEL/ES chaperonins. The complex contains at least 6–7 different but related subunits of Tcp-1 proteins as can be concluded from microsequencing of peptides [2] and 2D gel-electrophoresis [3] and forms a double ring structure each composed of 8–9 subunits [2,3] of between 900–970 kDa [2,4]. The apparent molecular weight of the GroEL complex is about 800 kDa and consists essentially of the homomeric double 7-ring of the 60 kDa protein. This complex is associated with a 7-ring of GroES which is positioned at one end of the double-ring cylinder [9].

Tcp-1-related proteins have also been described in thermophilic bacteria such as *Sulfolobus shibatae* [10]. Mutants lacking these proteins are temperature sensitive, thus the Tcp-related peptide is known as thermophilic factor (TF55). TF55 forms rings of 8–9 subunits, that are assembled into a double doughnut structure, thereby ressembling the eukaryotic TriC structure. It was speculated whether TF55 also plays a role in the formation of cytoskeleton-like structures in the archaebacteria *Sulfolobus shibatae* [10].

Here we present the sequence of an additional Tcp-related protein from oat and discuss the relationship to a second possible 'chaperone machine' [11] from the same plant published recently [1].

2. MATERIALS AND METHODS

2.1. *Growth and treatment of plants*

Oat seedlings (*Avena sativa* cv Pewi) were grown and harvested as described [1].

2.2. *RNA extraction, polymerase chain reaction (PCR) and sequencing procedures*

Total RNA was extracted as described [12] and Poly(A) RNA prepared according to Aviv and Leder [13]. Two oligonucleotides were synthesized as fully degenerated primers corresponding to amino acid sequences TITNDGA and MPKRI respectively, that are consensus sequences in a series of Tcp-1-related sequences [10]. The MPKRI oligo was synthesized as a non-coding strand that was used for reverse transcription of oat Poly(A) RNA with AMV reverse transcriptase (Promega) at 42°C for 45 min. PCR was performed according to protocols for reverse transcription PCR [14]. After 30 cycles (cycle temperatures 92°C/47°C/72°C), the complete reaction mixture was separated on a 1% agarose gel and a DNA fragment of the appropriate size (520 bp) was excised and electro-eluted with a Biotrap (Schleicher & Schuell). The fragment was blunt-ended and cloned into pUC19 (Pharmacia) cut with *Sma*I according to standard protocols [15].

Sequencing was carried out by using a sequencing kit (USB) following the manufacturer's manual, analysing both strands of the PCR product obtained.

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The sequence appearing in this paper and a related sequence are stored under accesion numbers X75777 (ASTCP-K19, this paper) and X75778 (ASTCP-K36), respectively, at the EMBL databank, Heidelberg.

2.3. *Construction of cDNA library and screening procedures*

A cDNA/λZAP library from oat was established using a cDNA kit (Stratagene) following the suggestions of manufacturer's protocol supplied. 10^6 pfu were plated for the screening procedure according to the Stratagene protocol and the 520 bp PCR fragment used as a probe. 36 clones were isolated of which 30 were identified as Tcp-1-related by sequence analysis; 4 of them were full-length clones. 3 of the full-length cDNAs were sequenced in both directions using primers hybridizing with external plasmid and internal cDNA sequences. Subclones of cDNA fragments in pUC18, using a unique *Hin*dIII site in all three cDNAs, were also sequenced.

2.4. *Over-expression of PCR and cDNA fragments*

The 520 bp PCR fragment as well as full-length coding sequences synthesized by PCR from three cDNA clones were ligated into pQE plasmids (QIAGEN) for over-expression of proteins that possessed a His^6 tag at the N-termini. Purification of over-expressed peptides was performed by means of the His^6 tag/Ni^+–Agarose system following the manufacturer's manual (QIAGEN).

2.5. *Antibody production and Western blot analysis*

Purified 60 kDa chaperone from oat extracts [1] was taken to raise antibodies in mice. Antibodies against Tcp-1-related protein were raised in rabbit using over-expressed protein from the 520 bp PCR product containing Tcp-1-related sequences. Western blot analysis was performed following the procedure described by Hofmann et al. [16]

3. RESULTS AND DISCUSSION

We used a PCR-based approach to isolate Tcp-1-related sequences in order to screen for cDNAs from oat coding for a 60 kDa protein with a unique chaperone function possibly related to Tcp-1 [1]. From the four full-length cDNA sequences isolated, three of them (ASTCP-K1, ASTCP-K19, ASTCP-K20) encode the identical amino acid sequence with one clone (ASTCP-K19) showing minor differences in the 5′ and 3′ untranslated region of the transcript sequence. The fourth clone, ASTCP-K36, shows 98.7% similarity and 97.9% identity at the amino acid level compared to the other clones. The sequence of ASTCP-K19 (Fig. 1) encodes a protein consisting of 535 amino acids similar to a sequence from cucumber [17] with a calculated molecu-

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                                                CAGAACCACAGCGCCGCCTCACTCTCCCC   30

CCTTCAGATCAGCTCCCCGCGCGATCTGCCCTCCCAGGAGTTTCTTCAGGGGGGAGCAGCGCC   92

AGCGCTTCGGCGGCC ATG GCG CTC GCC TTC GAC GAG TAC GGG CGG CCC TTC   143
                 M  A  L  A  F  D  E  Y  G  R  P  F                12
ATC ATC CTC AGA GAG CAG GAG AAG AAG TCG CGG CTG CAG GGT CTC GAC   191
 I  I  L  R  E  Q  E  K  K  S  R  L  Q  G  L  D                  28
GCG CAC AAG GCC AAC ATC GCC GCC GCC AAG GCT ATC GCG CGC ATC CTC   239
 A  H  K  A  N  I  A  A  A  K  A  I  A  R  I  L                  44
CGC ACG TCC CTT GGG CCC AAG GGC ATG GAC AAG ATG CTC CAG TCC CCC   287
 R  T  S  L  G  P  K  G  M  D  K  M  L  Q  S  P                  60
GAC GGC GAC GTT ACC ATC ACT AAT GAC GGG GCG ACC ATC CTG GAG CTA   335
 D  G  D  V  T  I  T  N  D  G  A  T  I  L  E  L                  76
ATG GAC GTT GAC AAC CAG ATT GCA AAG CTG TTG GTG GAG TTG TCA CGC   383
 M  D  V  D  N  Q  I  A  K  L  L  V  E  L  S  R                  92
AGT CAA GAC TAT GAC ATT GGA GAT GGT ACC ACT GGA GTG GTG GTC ATG   431
 S  Q  D  Y  D  I  G  D  G  T  T  G  V  V  V  M                  108
GCT GGG GCT CTC CTA GAG CAG GCT GAG AAG CTA CTG GAA CGT GGT ATT   479
 A  G  A  L  L  E  Q  A  E  K  L  L  E  R  G  I                  124
CAC CCA ATA AGG GTT GCT GAA GGT TAT GAG ATG GCG TCA AGG ATA GCT   527
 H  P  I  R  V  A  E  G  Y  E  M  A  S  R  I  A                  140
GTT GAT CAC CTT GAA AGC ATC TCC ACG AAA TAT GAG TTC AGT GCC ACA   575
 V  D  H  L  E  S  I  S  T  K  Y  E  F  S  A  T                  156
GAC ATT GAG CCT TTG GTG CAG ACC TGC ATG ACA ACT TTG TCC TCA AAG   623
 D  I  E  P  L  V  Q  T  C  M  T  T  L  S  S  K                  172
ATT GTT AGC CGT TGT AAG CGG GCC CTA GCT GAG ATT TCT GTC AAA GCA   671
 I  V  S  R  C  K  R  A  L  A  E  I  S  V  K  A                  188
GTC CTT GCG GTT GCT GAT TTG GAA AGG AAG GAT GTA AAT CTG GAC TTG   719
 V  L  A  V  A  D  L  E  R  K  D  V  N  L  D  L                  204
ATT AAG GTG GAA GGC AAG GTT GGT GGG AAG CTA GAG GAT ACT GAG CTA   767
 I  K  V  E  G  K  V  G  G  K  L  E  D  T  E  L                  220
GTG GAA GGA ATC ATT GTT GAC AAA GAT ATG AGC CAC CCC CAA ATG CCA   815
 V  E  G  I  I  V  D  K  D  M  S  H  P  Q  M  P                  236
AAG AGA ATC TAT GAT GCT CAC ATT GCC ATT CTG ACC TGC CCA TTT GAG   863
 K  R  I  Y  D  A  H  I  A  I  L  T  C  P  F  E                  252
CCC CCG AAG CCC AAG ACC AAG CAT AAG GTT GAC ATT GAC ACT GTA GAG   911
 P  P  K  P  K  T  K  H  K  V  D  I  D  T  V  E                  268
AAA TTC CAG ACA CTG CGT GGG CAA GAG CAG AAA TAC TTC GAT GAG ATG   959
 K  F  Q  T  L  R  G  Q  E  Q  K  Y  F  D  E  M                  284

GTT CAG AAA TGC AAG GAT GTT GGT GCA ACC CTA GTT ATT TGT CAA TGG 1007
 V  Q  K  C  K  D  V  G  A  T  L  V  I  C  Q  W                  300
GGT TTT GAT GAT GAA GCC AAT CAT TTA CTA ATG CAA AGA GAA CTT CCT 1055
 G  F  D  D  E  A  N  H  L  L  M  Q  R  E  L  P                  316
GCT GTC AGA TGG GTT GGT GGT GTT GAA TTA GAA TTA ATT GCC ATT GCT 1103
 A  V  R  W  V  G  G  V  E  L  E  L  I  A  I  A                  332
ACA GGT GGG AGG ATT GTT CCG AGA TTC CAA GAG TTG AGT ACT GAA AAG 1151
 T  G  G  R  I  V  P  R  F  Q  E  L  S  T  E  K                  348
CTT GGG AAG GCT GGA TTA GTC AGA GAG AAG TCA TTT GGA ACA ACA AAA 1199
 L  G  K  A  G  L  V  R  E  K  S  F  G  T  T  K                  364
GAT CGG ATG CTT TAC ATC GAA AAG TGT GCC AAT TCC AAA GCT GTA ACT 1247
 D  R  M  L  Y  I  E  K  C  A  N  S  K  A  V  T                  380
ATT TTC ATC CGT GGA GGT AAC AAA ATG ATG ATT GAG GAG ACC AAG CGA 1295
 I  F  I  R  G  G  N  K  M  M  I  E  E  T  K  R                  396
AGT ATT CAT GAT GCT CTT TGT GTT GCA AGG AAT CTC ATC ATC AAC AAC 1343
 S  I  H  D  A  L  C  V  A  R  N  L  I  I  N  N                  412
TCA ATC GTG TAT GGT GGT GGT TCA GCA GAG ATA TCT TGC TCG ATT GCT 1391
 S  I  V  Y  G  G  G  S  A  E  I  S  C  S  I  A                  428
GTT GAA GCT GCT GCT GAT CGG CAT CCT GGA GTT GAG CAG TAT GCA ATC 1439
 V  E  A  A  A  D  R  H  P  G  V  E  Q  Y  A  I                  444
AGG GCA TTT GCT GAT GCT TTG GAT GCT ATT CCA CTG GCT TTA GCT GAA 1487
 R  A  F  A  D  A  L  D  A  I  P  L  A  L  A  E                  460
AAC AGT GGT TTG CCA CCT ATT GAT ACT CTG ACA GTG GTA AAA TCC CAA 1535
 N  S  G  L  P  P  I  D  T  L  T  V  V  K  S  Q                  476
CAT GTC AAG GAG AAC AAT TCC CGC TGC GGC ATT GAC TGC AAC GAT GTG 1583
 H  V  K  E  N  N  S  R  C  G  I  D  C  N  D  V                  492
GGT ACC AAT GAC ATG AAA GAG CAG AAT GTT TTT GAA ACT CTG ATT GGC 1631
 G  T  N  D  M  K  E  Q  N  V  F  E  T  L  I  G                  508
AAG CAG CAG CAG ATC TTG CTG GCA ACC CAG GTC GTC AAG ATG ATC CTC 1679
 K  Q  Q  Q  I  L  L  A  T  Q  V  V  K  M  I  L                  524
AAG ATC GAT GAT GTT ATC ACG CCT TCC GAA TAT TGA CGATGCCTGCAGAGC 1730
 K  I  D  D  V  I  T  P  S  E  Y  *                              535
ATGAATCGACTGTGCTAGGTGACATACGCTCCAGTAGGCAGCTGCATGGGTTGATTTGGATTT 1793

GCTCCTAGAATTCTGACACTGAGTGGTCGCTTTGTTTTGTTTGACGAGCTATATGGTTTTGTG 1856

CGCTCGCTTGATCATTATAACATTGGTGTTCACACTCTGTACAATAGGACAAATCCTGTCGAT 1919

ACCAGTTTAGTCCTTTGTTGTATTGTTTGGGATGCTTAATGCTATTGTCTGGATCATTATTTT 1982

GTCTGCTTTGAAGTAAGTGTTACATTTTTTTTAAAAAAAAAAAAAAAAAAAAAAAAA      2039
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Fig. 1. Nucleotide and deduced amino acid sequence of ASTCP-K19. Underlined amino acid sequence indicates a conserved motif within the putative nucleotide binding domain.

Table I

Amino acid sequence comparison of selected Tcp-1-related proteins

	Sulfolobus shibatae TF55	*Arabidopsis* Tcp-1-related protein	Yeast Tcp-1-related protein	Mouse Tcp-1 protein
Avena sativa ASTCP-K19 Tcp-1-related	60.6% Sim. 40.0% Id.	53.5% Sim. 33.1% Id.	54.1% Sim. 33.0% Id.	53.5% Sim. 33.2% Id.
Sulfolobus shibatae TF55		61.2% Sim. 37.4% Id.	59.6% Sim. 36.8% Id.	61.9% Sim. 39.5% Id.
Arabidopsis Tcp-1-related protein			78.4% Sim. 61.0% Id.	80.2% Sim. 65.8% Id.
Yeast Tcp-1-related protein				78.0% Sim. 62.3% Id.

Similarities include identical amino acids and conservative exchanges. The program was run at EMBL Heidelberg. Amino acids were taken from published nucleic acid sequences (ASTCP-K19, this paper; TF55 [10]; *Arabidopsis* [18]; yeast [21]; mouse [22]). Sim, similarity; Id, identity.

lar mass of 58.9 kDa. Another known Tcp-1-related cDNA sequence from a plant, *Arabidopsis thaliana* [18], predicts a deduced amino acid sequence of 545 amino acids.

Table I shows the homologies between selected Tcp-1-related amino acid sequences and ASTCP-K19. The values were calculated by the HUSAR program package at the DKFZ, Heidelberg, connected to the EMBL databank (Heidelberg). While mammalian sequences have more than 96% identities to each other and more than 60% identity with *Drosophila melanogaster* and yeast orthologues [3], the *Avena* sequence shows comparably low homologies, ranging from about 33% identitiy to mammalian, yeast and *Arabidopsis* sequences up to 40% in case of TF55. Therefore the sequence from oat seems to be a distinct type of Tcp-1 and is predominantly related to TF55.

All Tcp-1 sequences encode a putative nucleotide binding site in the N-terminal region, that shows striking homology to the cyclic AMP-dependent protein kinase (Fig. 1; [3,19,]). The hydrolysis of ATP and other nucleotides by TriC from bovine testes containing Tcp-1 has been demonstrated [2] and is a characteristic feature of molecular chaperones. A comparison of Tcp-1-related sequences to GroEL, mitochondrial hsp60 and rubisco-binding proteins – all chaperonins of about 60 kDa – displays this conserved nucleotide binding domain also in the organellar and bacterial amino acid sequences [2], which includes the absolutely conserved amino acid sequence motif, GDGTT (Fig. 1; [2]).

We compared the immunological relationship between the Tcp-1 protein from oat with a 60 kDa protein from the same plant that re-folds phytochrome [1]. Fig. 2 shows the Western blot of crude protein extract from oat seedlings, purified 60 kDa oat chaperone, over-expressed Tcp-1 full-length polypeptide and over-expressed GroEL protein. Antibodies raised against the 60 kDa chaperone does not cross-react with Tcp-1 and GroEL (Fig. 2A), while antibody against oat Tcp-1 protein does not recognize the 60 kDa protein or GroEL (Fig. 2B). In plant crude extracts, Tcp-1 protein is not detectable under the physiological conditions chosen (Fig. 2B), while the 60 kDa chaperone is abundant in etiolated oat seedlings (Fig. 2A). Fig. 2D shows the cross-reaction of monoclonal antibody 91A against mouse Tcp-1 [20] with both oat proteins being recognized, while no signal is detectable in the case of GroEL. The size of over-expressed oat Tcp-1 protein seems to be more than 60 kDa (Fig. 2C), but the lower migration rate might be due to the His^6 tag at the N-terminus of the polypeptide.

Obviously, the two oat proteins are related to each other, as shown by the cross-reaction with monoclonal antibody 91A (Fig. 2D). On the other hand, the amino acid sequences of the oat chaperone published by Mummert et al. [1] show no significant homologies to the Tcp-1-related sequence from the same plant. We assume that the Tcp-1-related protein is not part of the oligomer formed by the 60 kDa polypeptide, at least not in a stoichometric proportion as in mammalian extracts [2]. We suggest, therefore, that different Tcp-1-related cytosolic chaperone complexes exist in oat. Whether TriC-like heterooligomers appear in plants similar to mammalian species has still to be shown.

Acknowledgements: This work was supported by a grant from the German Research Council (DFG; 'Molekulare Zellbiologie der Hitzestressantwort') to E.S. We wish to thank Dr. K. Willison for the kind supply of monoclonal antibody 91A and Dr. A. Gatenby for GroEL protein.

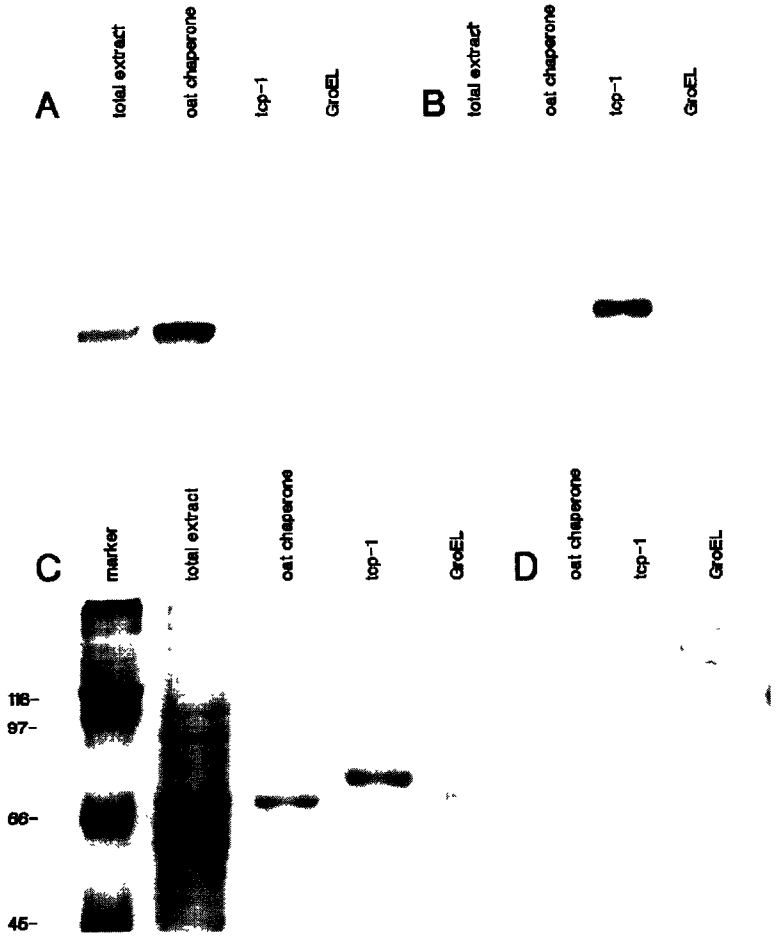


Fig. 2. Western blot analysis using antibodies against 60 kDa chaperone from oat, Tcp-1-related protein from oat and a monoclonal antibody against mouse Tcp-1 (91A; [20]). Gels were run with total protein extract from 6-day-old etiolated oat seedlings (total extract), purified oat chaperone (oat chaperone), over-expressed Tcp-1 protein (Tcp-1) and GroEL. (Fig. 2A–C, respectively; Fig. 2D was without total extract). Blotted filters were incubated with antibodies against oat chaperone (A), oat Tcp-1 protein (B) or monoclonal antibody 91A (D), respectively. Fig. 2C shows the Coomassie-stained gel including size marker (marker).

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