Drosophila MTN: a metazoan copper-thionein related to fungal forms

M. Valls[a,1], R. Bofill[b,1], N. Romero-Isart[b], R. Gonzàlez-Duarte[a], J. Abián[c], M. Carrascal[c], P. Gonzàlez-Duarte[b], M. Capdevila[b], S. Atrian[a,*]

[a] *Departament de Genètica, Facultat de Biologia, Av Diagonal 645, Universitat de Barcelona, E-08028 Barcelona, Spain*
[b] *Departament de Química Inorgànica, Facultat de Ciències, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain*
[c] *Departament de Bioanàlisis Mèdiques, Institut d'Investigacions Biomèdiques de Barcelona, CSIC, Jordi Girona Salgado 18, E-08034 Barcelona, Spain*

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Abstract Two *Drosophila* metallothioneins (MT) have been reported: MTN, a 40 residue peptide including 10 Cys, and MTO, a 43 residue peptide including 12 Cys. However, neither functional nor evolutionary analyses for either of the *Drosophila* MT are available. Here, heterologous expression of *Mtn* in *Escherichia coli* is reported. The metal binding abilities of the Cu- and Zn-MTN complexes conformed in vivo, as well as the features of the Cd- and Cu-aggregates produced by metal replacement in vitro, have been determined by atomic emission spectrometry, circular dichroism and electrospray ionization mass spectrometry. Primary structure relationships with other MT have been examined. The results indicate a close resemblance of MTN to fungal copper-thioneins.

Key words: Cu(I) binding; Cd(II) binding; Zn(II) binding; Molecular evolution; Recombinant synthesis; *Drosophila* metallothionein

1. Introduction

The metallothionein (MT) superfamily includes a wide range of low molecular mass and cysteine-rich heavy metal binding peptides [1]. Although they were first associated to metal detoxification and homeostasis, their presence in all animal phyla, and in most fungi and plants, supports a housekeeping metabolic function. In fact, protection against oxidative stress and/or free radical tolerance has been claimed recently [2–5].

Three classes of MT have been defined according to their primary structure (Web page, http://www.unizh.ch/–mtpage/classif.html). Class I comprises those polypeptides alignable to horse MT-1B [6]: all vertebrate, crustacea and mollusk forms. Vertebrate MT share a two-domain structure: the N-terminal β domain, with nine Cys in Cys-X-Cys arrays, which binds three divalent metal ions; and the C-terminal α domain, with 11 Cys, some of which are in Cys-Cys doublets, binding four divalent metal ions [7,8]. Crustacean MT are also bidominial peptides, but built from two β-like units [9]. Class II consists of a highly heterogeneous group: MT from most invertebrates, fungi and plants, and comprises sequences with no clear homology to horse MT. Only *Neurospora crassa* and *Saccharomyces cerevisiae* MT have been characterized at the structural and functional level. Both peptides constitute single metal binding domains, in which seven and 10 coordinating Cys bind six and eight Cu(I) ions, respectively [10,11]. They are considered primeval β forms, from which the more evolved β-α mammalian MT arose [12], on the basis of their Cys-X-Cys motifs and their binding capacity, which is restricted to three divalent metal ions. Little is known about MT domain evolution as Class II MT have been poorly characterized, not even reports on the *Drosophila* MT, a well known model organism, have been provided.

Two *Drosophila* MT genes have been reported, *Mtn* (at the 85E region of the third chromosome) and *Mto* (at 92E on the same chromosome); they are distant paralogues, sharing only 26% amino acid identity. *Mtn* encodes a 40 residue peptide, comprising 10 Cys, and it is present in two allelic forms: *Mtn.3* (Lys as C-terminal residue) and *Mtn1* (Glu as C-terminal residue) [13,14]. On the other hand, *Mto* encodes a 43 amino acid peptide, with 12 Cys, and was isolated from a Cd-resistant cell line [15].

Expression of these genes following metal induction appears to be significantly different. Amplification of the *Mtn* locus has been reported in unrelated natural populations and correlated to an increase of heavy metal tolerance [16]. However, *Mto* amplification is restricted to duplication and only in laboratory cell lines [15]. Transcripts of *Mtn* are mainly located in the digestive tract and Malpighian tubules of metal-fed larvae and adults. Cu and Cd are strong inducers of *Mtn* in the midgut, while Zn stimulates transcription from both genes in the Malpighian tubules. Basal expression supports a more housekeeping function for *Mto*. These expression patterns have suggested differential roles: involvement of MTO in Zn and Cu homeostasis and Zn excretion regulation, and contribution of MTN to Cu and Cd removal following oral intoxication [13,17].

Surprisingly, neither functional nor evolutionary analyses of these proteins are available. MTO has recently been purified to homogeneity, but it has not been characterized [18]. Isolation of MTN from flies, even metal-induced, has never been reported, probably due to lysosomal import and degradation after metal chelation. Recombinant synthesis was described in yeast, but only at the analytical level [19]. Here, we report heterologous expression of *Mtn* (*Mtn1*) in *Escherichia coli*, which provided highly homogenous MTN preparations. Fea-

*Corresponding author. Fax: (34)-93-4110969.
E-mail: silvia@porthos.bio.ub.es

[1] Made equal contributions to this study.

Abbreviations: CD, circular dichroism; DTNB, 5,5′-dithiobis(nitrobenzoic acid); ICP-AES, inductively coupled plasma atomic emission spectrometry; ESI-MS, electrospray ionization mass spectrometry; MT, metallothionein

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tures of in vivo-conformed Zn- and Cu-MTN complexes have been determined, as well as those of the Cd- and Cu-aggregates obtained in vitro by metal replacement to Zn-MTN. Finally, primary structure relationships with other MT were established and the physiological role and evolutionary history of the MTN protein has been re-evaluated.

2. Materials and methods

2.1. Gene cloning and recombinant protein purification

Drosophila melanogaster Mtn cDNA, cloned in pUC9, was kindly provided by Dr. G. Maroni [13]. PCR amplification of the coding region was performed using this plasmid as a template. The upstream primer MTD1 (5′-CCCAGATCTATGCCTTGCCCA-3′) introduced a *Bgl*II restriction site immediately upstream of the ATG initiation codon, and the downstream primer MTD2 (5′-CGGCGCGTCGACTCACTCGGAGCA-3′) generated a *Sal*I restriction site just after the *Mtn* stop codon. The PCR product was isolated from a 2% agarose gel, digested with *Bgl*II and *Sal*I and directionally cloned in the compatible *Bam*HI-*Sal*I sites of the pGEX4T-1 expression vector (Amersham Pharmacia), yielding pGEX-*Mtn*. DNA sequence was confirmed by automatic sequencing using an ABI 370 Automatic Sequencer and the Amersham Dye Terminator Cycle Sequencing kit. Restriction enzymes were purchased from Promega. JM105 was used as recipient strain for cloning and sequencing. To enhance the production of recombinant protein, pGEX-*Mtn* was transformed into the protease-defective BL-21 *E. coli* strain [20]. Exponentially growing 1.5 l LB cultures of the transformed strain were induced with 100 μM IPTG for 3 h, in the presence of 300 μM $ZnCl_2$ or 500 μM $CuSO_4$. The GST-MTN protein was isolated from the cell lysate by affinity chromatography, the MTN portion subsequently cleaved from the fusion protein and purified by FPLC chromatography, as described for recombinant mammalian MT [21].

2.2. Characterization of the metalated MTN species

Inductively coupled plasma atomic emission spectrometry (ICP-AES) in a Thermo Jarrell Ash, Polyscan 61E was used to measure S (182.0 nm), Zn (213.9 nm) and Cu (324.8 nm) contents and to estimate the protein concentration [21]. Thiol groups over total sulfur were determined by the 5,5′-dithiobis(nitrobenzoic acid) (DTNB) reaction [22], except in Cu-containing samples, as the presence of Cu in MT prevents SH quantification by Ellman's method [23]. Electronic absorption (UV) measurements were performed on an HP-8452A Diode array. A Jasco spectropolarimeter (Model J-715) interfaced to a computer was used for circular dichroism (CD) measurements. A Peltier PTC-351S maintained the temperature at 25°C. All spectra were recorded with 1 cm capped quartz cuvettes, corrected for the dilution effects and processed using the program GRAMS 32. Titrations of Zn-MTN with Cd(II) and Cu(I) at pH 7 were carried out following the procedures described elsewhere [21,24] and followed by UV and CD spectroscopies. Electrospray ionization mass spectrometry (ESI-MS) was also used to assess the purity and the metal binding stoichiometry of all the metalated species, as explained below.

2.3. ESI-MS

Molecular mass measurements were performed on a Fisons Platform II Instrument equipped with the Mass Lynx software, calibrated with horse heart myoglobin (0.1 mg/ml). The experimental conditions for the metalated species were as follows: 10 μl of protein solution was injected at 20 μl/min; capillary counterelectrode voltage, 4.5 kV; lens counterelectrode voltage, 1.0 kV; cone potential, 35 V; source temperature, 120°C; *m/z* range, 750–1950; scanning rate, 2 s/scan; interscan delay, 0.2 s. The carrier was a 5:95 mixture of methanol and 3 mM ammonium formate/ammonia, pH 7.5. The molecular mass of the apo-proteins was determined as described above except for the following conditions: for apo-MTN obtained from Zn-MTN, the carrier was a 1:1 mixture of acetonitrile and 0.05% trifluoroacetic acid, pH 2.5; source temperature, 60°C; for apo-MTN obtained from Cu-MTN, the carrier was a 1:1 mixture of acetonitrile and trichloroacetic acid, pH 1.5; syringe pump at 30 μl/min; source temperature, 90°C. In both cases, the capillary counterelectrode voltage was 3.5 kV, the lens counterelectrode voltage was 0.5 kV and the cone potential was 40 V. Masses for the holo-species were calculated according to [25].

2.4. Protein sequence and structure comparisons

Protein sequence alignments and tree construction were performed using Clustal W (v1.74) [26] and the PILEUP and PAUP utilities enclosed in the GCG Package (v.10) [27]. Structure visualization was carried out using the Insight II Package [28] v.97 (Byosim. Inc.). Software was supported by an Irix 64 v.6.4/Silicon Graphics Octane Workstation platform.

3. Results

3.1. Gene cloning and protein biosynthesis

DNA sequencing of the major PCR product (123 bp) confirmed that it corresponded to the *Mtn* coding region and that no nucleotide changes had been introduced. To assess GST-MTN production, total cell extracts of induced and uninduced *E. coli* BL-21 cells containing pGEX-*Mtn* were resolved on a denaturing polyacrylamide gel. After Coomassie blue staining, an extra band corresponding to the expected size of the protein fusion (30 kDa) was detected. GST-MTN was recovered from transformed *E. coli* cells grown in media supplemented with IPTG and the required metal, and it was cleaved with thrombin. The MTN portion was further purified, so that homogeneous Zn- and Cu-MTN preparations were obtained. Due to the expression system used, the recombinant protein contained two extra N-terminal amino acids (*N*-Gly-Ser), but this does not interfere with the metal binding features of MT [21]. The yield of pure MTN was 0.3 mg/l of culture and MTN concentration in the FPLC fractions was within the range of 10^{-4}–10^{-5} M.

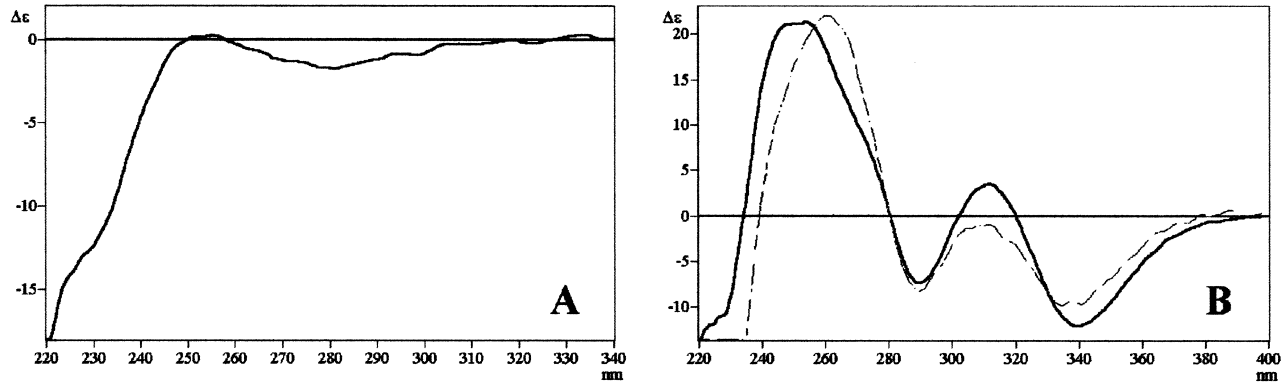

Fig. 1. (A) CD spectrum of recombinant Zn-MTN. (B) CD spectrum of recombinant Cu-MTN (solid line). The CD spectrum of the Cu-MTN species generated in vitro by total Zn/Cu replacement is also shown (dotted line).

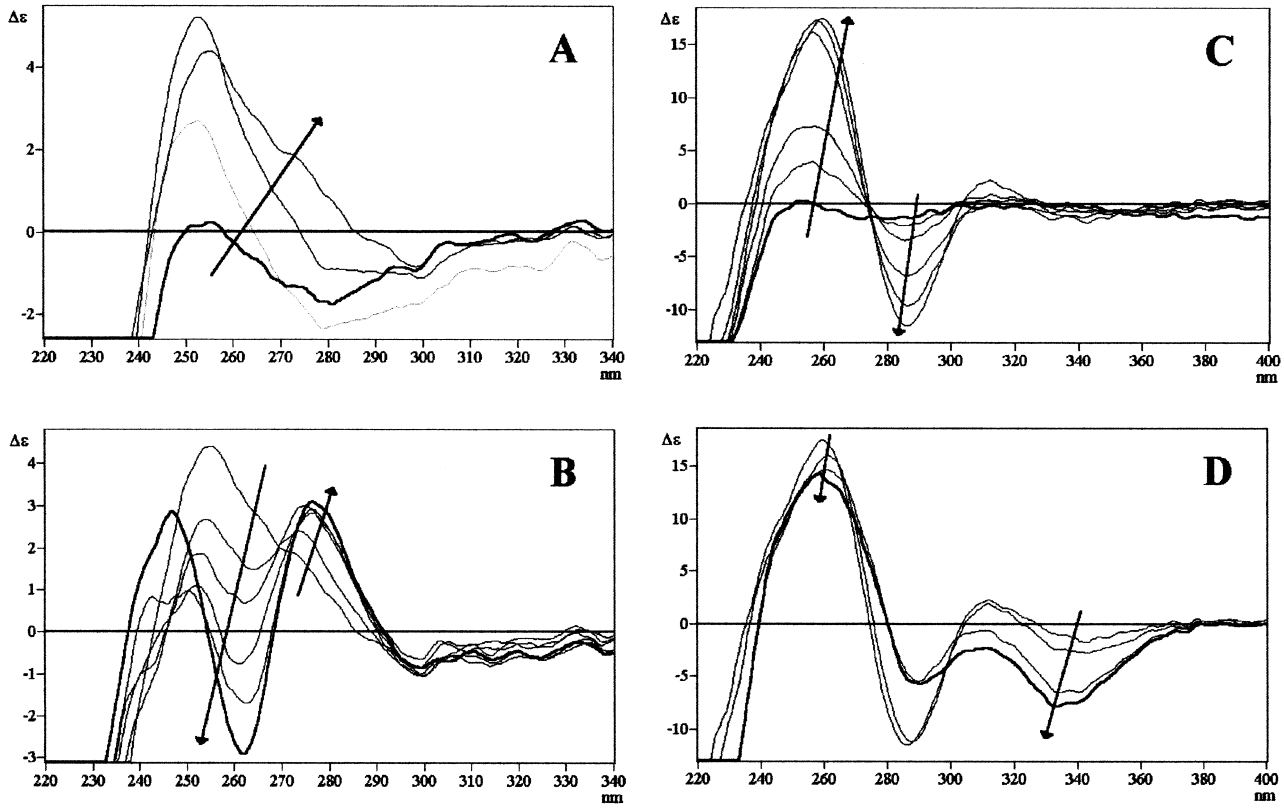

Fig. 2. CD spectra obtained during the first (A) and second (B) stage of the titration of Zn-MTN with Cd(II), 12.16 μM; and during the first (C) and second (D) stage of the titration of Zn-MTN with Cu(I), 10.98 μM at pH 7. Arrows indicate increasing Cd(II) and Cu(I) equivalents, respectively. Traces corresponding to fully loaded Zn, Cd and Cu proteins are in bold.

3.2. Characterization of the Zn-MTN and Cu-MTN synthesized in vivo

ICP-AES, Ellman's and ESI-MS results (Table 1) showed that synthesis of MTN in Zn-supplemented media leads to the formation of two metalated species: Zn_3-MTN and Zn_4-MTN. Acidification of this sample at pH 2.5 yielded a single apo-form with a molecular mass in accordance with the value calculated for the polypeptide sequence. This confirmed the integrity of the MT protein in the metal species mixture. ICP-AES and DTNB data indicated an average ratio of Zn/reduced protein of 3.5 and an oxidation degree of 35%, which could be expected from the coexistence of the two species. ESI-MS measurements carried out at pH 2.5 and 7.5 did not reveal multimers of MTN, thus pointing to the formation of intramolecular disulfides. The biosynthesized Zn-MTN species showed a low intensity and poorly resolved CD spectrum (Fig. 1A), which indicates a low degree of folding.

When *Mtn* was expressed in Cu-supplemented media, a single Cu_8-MTN species was detected by ESI-MS. The high intensity and well-defined CD spectrum (Fig. 1B) revealed an organized folding of the protein around the metal ions. However, a 7.0 Cu/protein ratio was calculated from ICP-AES (Table 1). The absence of undermetalated species detected by ESI-MS, the fact that the Cu_8-MTN species was also obtained in vitro through Zn/Cu replacement (see below) and the observation of a pure apo-protein by ESI-MS at pH 1.5 are all suggestive of the presence of a small fraction of totally oxidized MT (12.5%), while the bulk of the non-oxidized protein binds 8 Cu(I) ions.

3.3. In vitro Cd(II) and Cu(I) binding ability of Zn-MTN

The cadmium and copper binding ability of Zn-MTN was analyzed by metal replacement studies. The titration of the in vivo-synthesized Zn-MTN species with Cd(II) or Cu(I) was

Table 1
Molecular masses and zinc to protein and copper to protein ratios found for in vivo-synthesized Zn-MTN and Cu-MTN

Expression of MTN in X-supplemented media	*m* (apo-protein)[a]	*m* (X-protein)[b]	(X/protein)[c]	(X/protein)[d]
X = Zn	3995.9 ± 1.7	4253.0 ± 2.2	4	3.5
		4188.6 ± 3.1	3	
X = Cu	4003.0 ± 1.3	4496.7 ± 0.6	8	7.0

[a]Molecular mass of the apo-protein. Calculated mass based on the polypeptide sequence is 3997.49.
[b]Molecular mass of the Zn- and Cu-protein complexes. Calculated masses for neutral species with loss of two protons per zinc bound and one proton per copper bound [28] are 4251.01 (Zn_4-MTN), 4187.63 (Zn_3-MTN) and 4497.86 (Cu_8-MTN).
[c]Zinc or copper per MT molar ratio calculated from the mass difference between holo- and apo-protein.
[d]Zinc per MT molar ratio calculated from the content of zinc (ICP-AES) and cysteine thiolates (Ellman's method) and Cu per MT molar ratio calculated from the content of copper and total sulfur (ICP-AES) [21].

followed by UV (data available upon request) and CD spectroscopies (Fig. 2). ESI-MS determinations were performed at the end of each titration.

As expected, the addition of Cd(II) to the Zn-MTN solution caused replacement of the Zn(II) ions by Cd(II). Initially (Fig. 2A), a Gaussian band developed at ca. 250 nm which finally resolved in a derivative-shaped profile (Fig. 2B), indicating the presence of clustered Cd-thiolate complexes [21]. A new Gaussian-shaped band centered at ca. 275 nm developed before reaching saturation. Although there is not any bibliographic reference of absorption at this wavelength for Cd-thiolate chromophores, absorbances between 260 and 320 nm have been reported for Cd-BP1 cadystin complexes, which contain inorganic sulfur [29]. ESI-MS measurements after saturation indicated the presence of two Cd-MTN species, Cd_3-MTN and Cd_4-MTN (data not shown), whose relative abundance was similar to that observed for the Zn-MTN species. These data are consistent with a stoichiometric but non-isomorphous replacement of Zn by Cd atoms in MTN, together with the acquisition of a more organized fold in the latter.

The increase in the intensity of the CD bands during the titration of Zn-MTN with Cu(I) (Fig. 2C,D) comes from the enhancement of chirality produced by a tighter protein fold around the incoming metal ions. In the first stage of the titration (Fig. 2C), a derivative-like band with an inflexion point at 273 nm evolved. It showed a slight decrease in intensity in the second stage of the titration while a new band centered at ca. 335 nm appeared (Fig. 2D). The final CD spectrum corresponding to the fully Cu-loaded MTN species obtained in vitro practically matched that recorded for the in vivo-synthesized Cu-MTN (Fig. 1B). Molecular mass measurements of the Cu-loaded MTN species have been plagued by the common difficulties associated with the artifactual clustering and oxidation either of Cu(I) to Cu(II) ions or of thiolate to disulfide bonds [30]. However, ESI-MS results after Cu(I) saturation of MTN showed a predominant Cu_8-MTN species, together with other minor species of lower nuclearity (data not shown). These data corroborate the structural similarities observed in the CD spectra of the in vivo-synthesized and in vitro-replaced Cu-MTN species.

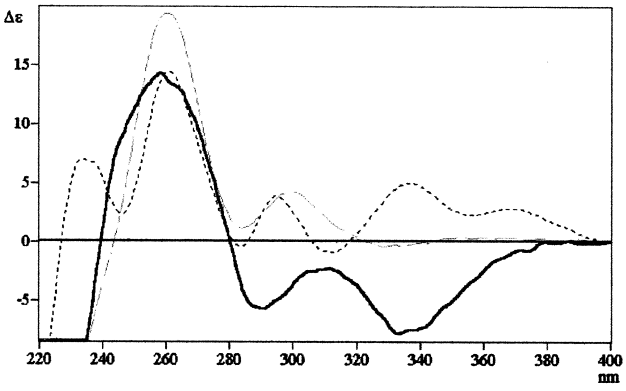

Fig. 3. Superimposition of the CD spectra of the Cu_8-MTN species generated in vitro by Zn/Cu replacement (solid line) and of recombinant mouse Cu_6-βMT (dashed line) and Cu_7-βMT (dotted line).

Table 2
(A) PILEUP output of seven MT sequences, using the Blosum 62 weight matrix for the multiple alignment construction and (B) the consensus PAUP tree constructed from the four possible maximum parsimony PAUP trees from the alignment shown in (A)

A)

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                 . . . .10 . . . .20 . . . .30 . . . .40 . . . .
MTO      ~~~~~~~~~~~~~~~~~~~~~~~~MVCKGCGTNCQCSAQKCGDNC.ACN
CUP1     ~~~~~~~~~~~~~~~~~~~~~~~~QNECHECQCQCGSCKNNE.QCQ
Mouse1   ~~~~~~~~~~~~~~~~~~~~~~~~~~MDPNCSC...STGGSC.TCT
Neuros   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~GDCGC...SGASSC.NCG
MTN1     ~~~~~~~~~~~~~~~~~~~~~~~~MPCPCGSGCKCASQATKGSC.NCG
Crab     ~~~~~~~~~~~~~~~~~~~~~~~~MPGPCCNDKCVCQEGGCKAGC.QCT
CRS5     MTVKICDCEGECCKDSCHCGSTCLPSCSGGEKCKCDHSTGSPQCKSCG

                 50 . . . .60 . . . .70 . . . .80 . . . .90 . . .
MTO      KDCQCVCKNG.PKDQC.CSNK~~~~~~~~~~~~~~~~~~~~~~~~~~~~
CUP1     KSCSCPTGCN.SDDKCPCGNKSEETKKSCCSGK~~~~~~~~~~~~~~~
Mouse1   SSCAC.KNCKC..TSCKKSCCSCCPVGCSKCAQGCVCKGAADKCTCCA
Neuros   SGCSC.SNCGS..K~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
MTN1     SDCKC.GGDKK..SAGCCSE~~~~~~~~~~~~~~~~~~~~~~~~~~~
Crab     S.CRC.SPCQKCTSGCKCATKEECSKTCTKPCSCCPK~~~~~~~~~~
CRS5     EKCKCETTCTCEKSKCNCEKC~~~~~~~~~~~~~~~~~~~~~~~~~~
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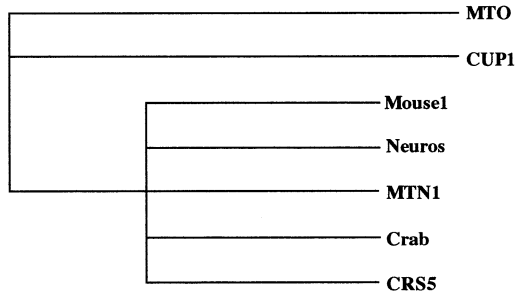
B)



Sequences are: MTO and MTN1, the two *Drosophila* MT, CUP1 and CRS5, the two *S. cerevisiae* MT; Neuros, *N. crassa* MT; Crab, *Cancer pagurus* MT and Mouse1, mouse MT 1.

3.4. Structure/function analysis

Alignments of Class II MT by conventional algorithms are questionable due to the extreme size heterogeneity of the sequences, from 25 residues for *N. crassa* up to 105 for *Tetrahymena*, and due to the variable composition of the Cys arrays: X-Cys-X-Cys-X, X-Cys-Cys-X and X-Cys-Cys-Cys-X. Thus, we focused our analysis on a more homogeneous subgroup of MT peptides containing the X-Cys-X-Cys-X motif. This included: mouse MT 1 and *N. crassa* MT as the most distant members; the two *Drosophila* forms: MTN and MTO; the two yeast forms: CUP1 and CRS5; and a representative of crustacean MT, the blue crab, all of them with a reported metal cluster structure determined by X-ray or nuclear magnetic resonance (NMR) studies.

The PILEUP alignment (Table 2), in agreement with all the Clustal W pairings obtained (data not shown), revealed the next interesting features. (1) A segment of approximately 40 residues defined a coherent core of the alignment. It comprised the complete sequence of *N. crassa* MT, in agreement with the predicted ancestral position of this fungal form among eukaryotic MT. (2) The two *Drosophila* peptides almost matched this core, but for their seven N-terminal residues, which contain two cysteines. *Drosophila* MTN corresponded to the shortest reported MT among multicellular organisms (40 residues), even shorter than those of several unicellular yeasts (*S. cerevisiae* CRS5, *Candida* MT, *Yarrowia* MT). (3) The alignment for the secreted form of *S. cerevisiae* CUP1 ended at Lys-69. The C-terminal unaligned tail, which includes 12 residues, two of which are Cys, corresponded to a

segment that does not participate in the Ag(I) clusters, according to NMR results [31,32]. (4) CRS5, the other *S. cerevisiae* MT [33], is similar in length (69 amino acids) to the vertebrate Class I MT, although it differs in amino acid composition and Cys distribution. The N-terminal segment remained unaligned and it might contribute to the second chelating domain predicted for this yeast MT [34]. (5) The two bi-dominial Class I MT, crustacean (β-β) and mammalian (β-α), were aligned by their N-terminal domains. This is also consistent with the hypothesis that the β domain is the ancestral unit in eukaryotic MT evolution. (6) In all cases, the unaligned segments comprised the X-Cys-Cys-X motifs. Therefore, this structure may have appeared later than the primeval β X-Cys-X-Cys-X motif, and may have eventually led to the formation of α-like clusters, as seen in vertebrate MT.

Unrooted trees generated from the core sequences (Table 2) by three algorithms, PILEUP, Clustal W and four PAUP pair-wise comparisons, highlight two main features. First, *Drosophila* MTO and *S. cerevisiae* CUP1 MT always cluster independently of the rest of sequences. Second, *Drosophila* MTN and *S. cerevisiae* CRS5 belong to a same tree branch, which also comprises *N. crassa* and Class I MT (crustacean and mammalian MT). From these data, an early origin of the MT duplication, which would be later responsible for the bi-domain forms, could be traced already in monocellular eukaryotes. The separate clustering of the mono-domain *N. crassa* MT and MTN1 together with the bi-dominial forms in yeast (CRS5), crab and mouse MT suggested that this was the lineage which gave rise to the β-β and β-α forms.

4. Discussion

Drosophila MTN is the shortest MT reported among metazoa. For the present, all metazoan MT have shown up to two metal binding clusters, or at least a peptide length compatible with the folding of two independent chelating domains. The fact that monocellular eukaryotes contain both mono-domain MT, the fungal (*N. crassa*, *Agaricus*) and *S. cerevisiae* CUP1 proteins, and longer peptides able to form two or more clusters, as described for yeast CRS5 and *Tetrahymena* MT, points to an early origin of the domain duplication in the eukaryotic lineage. Surprisingly, *Drosophila* MTN and MTO amino acid sequences appear closer to *Ascomyceta* than to its *Arthropoda* counterparts, i.e. crustacean MT. As for MTN the similarity can be extended to its metal binding features, it appears as an ancient form in MT evolution.

We have previously shown for mammalian βMT that preference for a given metal correlates with the degree of folding of the holo-protein recombinantly obtained from metal-supplemented cultures [35]. Data obtained in this study for recombinant Zn-MTN and Cu-MTN show the formation of a single, low oxidized Cu_8-MTN species in the presence of copper, while a mixture of oxidized, poorly folded, Zn_3- and Zn_4-MTN aggregates in the presence of zinc. Furthermore, Zn/Cu in vitro replacement led to well-defined aggregates, fully comparable to those obtained in vivo. In contrast, in vitro Zn/Cd replacement enhanced the degree of folding of MTN, but it remained still far from that achieved in the presence of Cu(I). These data indicate that MTN binds monovalent metals much more efficiently than divalent ones. The ability of these metals to fold the MTN peptide is proportional to the level of *Mtn* mRNA that they induce in metal-fed flies: Cu > Cd > Zn [17]. This is consistent with a fundamental role of MTN in copper gastric detoxification, also operative for cadmium protection, and otherwise, a poor contribution to zinc metabolism, which would be mainly contributed by MTO, as suggested before [18].

The stoichiometry of the in vivo-synthesized and in vitro-replaced Cu-, Zn- and Cd-MTN species revealed further details about the metal binding features of MTN. Spectroscopic and ESI-MS data indicated a unique, well-defined Cu_8-MTN cluster; however, a minor Cu_7-MTN species was also detected by ESI-MS in samples submitted to harsh conditions. The copper binding stoichiometry of MT is controversial. Several Cu(I) species have been reported for *N. crassa* MT [10,36], *S. cerevisiae* CUP1 [31,32,37–41] and mammalian β domain [24,35,42,43], depending on synthesis, preparation conditions and detection procedures. Differently coordinated Cu(I) ions, some in compact clusters, some in external regions, and an extreme flexibility of the thionein chain to rearrange the thiol-Cu(I) bonds would account for this heterogeneity.

The superimposition of the CD spectrum of the Cu-MTN species obtained by Zn/Cu replacement with those of the recombinant mouse Cu_6-βMT and Cu_7-βMT species [24] (Fig. 3) gives information on the stereochemistry of the Cu-MTN aggregate. On the one hand, the CD spectrum of Cu-MTN and Cu_6-βMT resembles in shape, with minor variations in the position and intensity of some bands. On the other hand, the bands at 280–400 nm corresponding to Cu-MTN are almost equal but of opposite sign to those observed for the Cu_7-βMT species. The first resemblance suggests that Cu_8-MTN and Cu_6-βMT fold similarly and allows the assumption that both contain the same $Cu^I(S_{Cys})_3$ chromophores. The coincident band of Cu_8-MTN and Cu_7-βMT at ca. 340 nm is indicative of the presence of additional digonal $Cu^I(S_{Cys})_2$ units in Cu_8-MTN. The opposite sign of the CD bands at ca. 340 nm can be explained by inverse folding of the peptide chains around these chromophores. The presence of digonal $Cu^I(S_{Cys})_2$ units is sensible taking into account that 10 S_{Cys} cannot trigonally coordinate eight Cu(I) ions.

Regarding divalent metals, a Zn_3- and Zn_4-MTN mixture was obtained. These stoichiometries match those found for the Zn-βMT and Zn-CUP1 native species, respectively [21,37,42]. In the case of MTN, the Zn/Cd replacement increased the degree of organization of the cluster, although the mixture of species (Cd_3- and Cd_4-MTN) was maintained. Analogous results were found for the Zn/Cd replacement in the β-C5H mouse MT site-directed-mutant, where substitution of Cys by His resulted in a modified βMT peptide with decreased binding capacity for Zn and Cd [44]. Thus, the coexistence of two species, even after Zn/Cd replacement, seems to be indicative of non-optimized forms for coordination of divalent ions.

Protein sequence comparisons (Table 2) define a primeval MT core, with strong similarities to the extant mono-domain fungal copper-thioneins. According to this alignment, *Drosophila* MTN should behave as a conventional copper-thionein, which is precisely what our results strongly suggest: a resemblance between *Drosophila* MTN and yeast CUP1. Both share 10 coordinating Cys and a clear preference for monovalent ions, Cu(I), with a major stoichiometry of eight Cu(I) and a less efficient capacity to bind divalent metals.

MT core sequences aligned with the N-terminal domain of Class I, namely β domain, thus identifying the ancient copper-

thioneins as β-like peptides. The reported X-Cys-X-Cys-X β domain signature is located at the core, while all Cys-Cys stretches remain outside, in agreement with their previous consideration as α peptide motifs. The position of the Cys-Cys pairs differs in each MT, consistently with changes associated with an emergent trait. Moreover, in yeast CUP1, the C-terminal Cys doublet does not contribute to the Cu(I)- and Ag(I)-aggregates [31,32], thus these Cys-Cys doublets may not yet have acquired their coordinating capacity. From our alignment, we suggest '...C-X-C-X_{5-8}-C-X_{1-2}-C-X_{2-3}-C-X-C...' as the consensus sequence for the β-like MT domain, which extends 'the central MT segment' -CxCxxxCxC- previously defined [12]. In all trees, *Drosophila* MTN clusters with yeast CRS5, whereas *Drosophila* MTO appears close to the well-characterized CUP1 yeast protein. This could be interpreted as: first, an early divergence of the MT lineages in metazoa, and second, a higher association of MTN and SRC5 to the fungal mammalian line. Finally, it highlights the need to define orthology/paralogy relationships, in order to avoid misleading comparisons. Full genome data should contribute to this end.

The identification of *Drosophila* MTN as the only metazoan MT representative of the primeval copper binding βMT domains is the first step of the knowledge of its coordination abilities. The comparative analysis of the three-dimensional structure of the Cu(I)-MTN and CUP1 metal clusters should be relevant. Moreover, the two additional Cys present in MTO when compared to MTN could be related to a putative increase in the metal binding capacity; the fact that these two terminal Cys form an α conventional array support this view. Longer MT-like proteins, as yet unknown, cannot be ruled out. If found, they would be representatives of the widely described two-domain metazoan MT. Finally, why ancient MT forms have been preserved in *Drosophila* is still an open question.

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