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Biochemical and Biophysical Research Communications 315 (2004) 196–203
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Identification and molecular cloning of *Tetrahymena* 138-kDa protein, a transcription elongation factor homologue that interacts with microtubules in vitro

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Received 18 December 2003

Abstract

Macronucleus of *Tetrahymena* divides amitotically, although in a microtubule-dependent fashion. Besides the localization study and pharmacological study of macronuclear microtubules, mechanism of the macronuclear division is poorly understood. A biochemical search for microtubule-associated protein was attempted from the isolated macronucleus. Improvement on macronucleus isolation method and microtubule coprecipitation assay led to the cloning of p138, a new homologue of transcription elongation factor FACT (facilitates chromatin transcription) 140 kDa subunit. DNase treatment test of macronuclear extract and the sequence of p138 suggested that p138 is associated with chromosome in the macronucleus. The release tests of p138 from microtubules indicated that p138 is released from microtubules in the presence of ATP but not in the presence of AMP-PNP. Together, the results suggest a novel function of FACT homologue, that p138 interacts with both microtubules and chromosome.

Keywords: *Tetrahymena*; p138; Macronucleus; Division; Microtubules; Transcription elongation factor; FACT; Cdc68; DUF140

Most ciliates have two functionally distinct nuclei: the micronucleus and the macronucleus. The micronucleus is a germinal, transcriptionally silent nucleus that contains large metacentric chromosomes. The macronucleus is a somatic, transcriptionally active, polygenomic nucleus composed of several hundreds of short chromosomes which are derived by fragmentation of the micronuclear chromosomes during conjugation [1–3]. During cell division, the micronucleus divides by a typical mitosis, while the macronucleus divides by an alternative mechanism known as amitosis, which involves constriction of the parental nucleus and division into two roughly equal daughter nuclei [4,5]. This division takes place without dissolution of the nuclear envelope, in the apparent absence of chromosome condensation and without the formation of a mitotic spindle. The mechanism of chromosome segregation during amitosis is unknown.

Although the dividing macronucleus appears to lack a microtubular spindle, microtubules have been implicated in amitosis [6–12]. The major difficulty in understanding the role of microtubules in amitosis has been lack of data on the organization of microtubules at the whole cell level. In the previous study, we were able to dramatically improve cell fixation and permeabilization conditions for *Tetrahymena* cells which allowed systematic analysis of macronucleus-associated microtubules [13,14]. It appeared that although the macronucleus divides amitotically, even this simplified mode of nuclear division involves an assembly of elaborate array of intra- and extranuclear microtubules. We suggested that these microtubules play multiple roles, including chromatin partitioning, macronuclear positioning, and constriction.

To understand the mechanism of microtubule-driven mode of macronuclear division, we made an attempt of isolating microtubule-associated proteins from the macronucleus. Since microtubules are assembled in macronucleus only while the macronucleus divides, microtubule-associated protein from macronucleus is

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.043

expected to be involved in macronuclear division. This search led to the cloning of p138 protein that is homologous to the human transcription elongation factor FACT 140 kDa subunit. Characterization tests of p138 suggested the interaction of p138 and microtubules is affected by ATP.

Materials and methods

Cell culture. Cultivation of *Tetrahymena thermophila* strain B2086 and the amicronucleate strain of *Tetrahymena pyriformis* strain W were performed as already described [15].

Isolation of macronucleus. Tetrahymena pyriformis was grown to mid- to late-log-phase in 4 liters of culture medium. Cells were collected by centrifugation at 2000*g* and washed with medium A (4% Gum Arabic, 30 mM Pipes, pH 6.9, 2 mM EGTA, 1.5 mM $MgCl_2$, and 0.1 M sucrose). Cells were resuspended in 1800 ml medium A containing 25 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 mM dithiothreitol. 1.5 pellet volume of 10% NP-40 was added to cell suspension and blended with Sorvall blender (Omni-mixer, Newton, PA, USA) for 1 min until cells were completely disrupted. Lysate was dispensed in three 1 liter centrifuge bottles and layered on 200 ml of medium B (4% Gum Arabic, 30 mM Pipes, pH 6.9, 2 mM EGTA, 1.5 mM $MgCl_2$, and 1.0 M sucrose) per each bottle. Macronuclei were collected in the layer of medium B by centrifugation at 1100*g* for 30 min. Lower half of medium B layer was collected and centrifuged at 3000*g* for 10 min to precipitate macronuclei. Macronuclei were resuspended and washed twice with medium A by centrifugation at 800*g* for 10 min. Isolated macronuclei were washed with MTs buffer (40 mM Pipes, pH 6.9, 1 mM EGTA, and 1 mM $MgCl_2$) by centrifugation at 800*g* for 10 min. All procedures were done at 4 °C. Macronuclei were then immediately subjected to the SDS–PAGE and Western blot or immunofluorescence microscopic analysis. Macronuclei for coprecipitation assay were stored for no more than 1 month at −80 °C.

Electrophoresis and immunoblotting analysis. SDS–PAGE was carried out according to the method of Laemmli [16]. Isolated macronuclei were solubilized with 8 M guanidine–HCl containing 10% of 2-mercaptoethanol and 0.1 M Tris–HCl (pH 7.5), and dialyzed against 7 M urea, as described by Hirabayashi et al. [17]. Protein sample was subjected to SDS–PAGE. Proteins were stained with Coomassie Brilliant Blue R-250. For immunoblotting analysis, proteins resolved by SDS–PAGE were transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bellerica, MA, USA). The membranes were incubated with monoclonal anti-chick α-tubulin antibody DM1a (Amersham International plc, Little Chalfont, Buckinghamshire, UK, 1:500 dilution). The immunoblots were visualized with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Tago, Burlingame, CA, USA, 1:2000 dilution) using a BCIP/NTB phosphatase substrate system (KPL, Gaithersburg, MD, USA).

Indirect immunofluorescence microscopy. Indirect immunofluorescence microscopic analysis of α-tubulin from isolated macronucleus was performed as previously described with modifications [14]. DNA was stained with 0.5 μg/ml DAPI instead of propidium iodide. Sample images were acquired using an AQUACOSMOS imaging system (Hamamatsu Photonics, Shizuoka, Japan) and Axioskop (Zeiss, Oberkochen, Germany).

Microtubule coprecipitation assay. Isolated macronuclei were lysed by sonication (Cell Distributer, Tomy Seiko, Tokyo, Japan) in MTs medium containing 25 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 mM dithiothreitol, 1% Triton X-100, and 10 mM EDTA. To disrupt chromosome, 15 mM $MgCl_2$ and 1 mg/ml DNase I were added and incubated for 15 min at 37 °C. Solubilized proteins were collected in the supernatant by centrifugation at 100,000*g* for 30 min. Porcine brain tubulin was purified using phosphocellulose column [18], polymerized at 37 °C for 30 min, and stabilized by 20 μM paclitaxel (Wako Pure Chemical Industries, Osaka, Japan). Microtubules were added to macronuclear extract and incubated for 45 min at 5 °C in the presence of 3 mM AMP-PNP. Sample was layered on MTs buffer containing 10% sucrose, 0.5 μM GTP, and 1 mM AMP-PNP, and then centrifuged at 35,000*g* for 30 min. Precipitate was resuspended in MTs buffer containing 0.1 M NaCl, 0.5 μM GTP, and 1 mM AMP-PNP, then incubated for 15 min and then centrifuged at 35,000*g* for 30 min. Precipitate was resuspended in MTs buffer containing 0.3 M NaCl, 0.5 μM GTP, and 5 mM ATP, then incubated for 15 min and centrifuged at 35,000*g* for 30 min. Precipitate was resuspended in MTs buffer containing 0.5 M NaCl, 0.5 μM GTP, and 5 mM ATP, then incubated for 15 min, and centrifuged at 35,000*g* for 30 min. The supernatants from each centrifugation were subjected to SDS–PAGE analysis.

Cloning of 138 kDa protein. Macronuclei collected from 150 liters of culture were subjected to microtubule coprecipitation assay. The fraction of 0.3 M NaCl + ATP eluate was resolved by SDS–PAGE. Proteins were visualized by Coomassie staining. The 138 kDa protein band was cut from a gel, digested by lysyl endopeptidase, and fractionated by HPLC using reverse-phase column, and micro sequencing of the derived peptides was performed (APRO Life Science Institute, Naruto, Tokushima, Japan). Sequences of three peptides, Fractions 59, 60, and 87 were obtained and used to search the *T. thermophila* genomic DNA database at TIGR (The Institute for Genomic Research, Rockville, MD, USA) using BLAST (Basic Local Alignment Search Tool). DNA encoding three peptides was found from scaffold DNA (ID 1173226) within 1.8 kb region. Open reading frame of this region was estimated by BLAST search at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA). RNA was isolated from mid-log phase *T. thermophila* using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First strand cDNA synthesis was performed with SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). The polymerase chain reaction (PCR) and the rapid amplification of cDNA ends (RACE) were performed using first strand cDNA as template DNA and primers 5′-GATGGACGCAGAACAAGAAATGCC-3′ and 5′-AATCCTCTGGGGGAAGACCAGCTTC-3′ were used. For RACE amplification, primers 5′-GGCATTTCTTGTTCTGCGTCCATC-3′ (5′-RACE) and 5′-GAAGCTGGTCTTCCCCCAGAGGATT-3′ (3′-RACE) were used with manufacturer's primers. PCR products were cloned into pDrive vector (Qiagen, San Diego, CA, USA) and sequences were analyzed. Homologous proteins were searched at Genome Net (Kyoto University Bioinformatics Center, Kyoto, Japan) using BLAST search. Multiple alignment of homologous proteins was calculated by CLUSTAL W Multiple Sequence Alignment Program (version 1.8) at Genome Net.

Results

Isolated macronucleus contained α-tubulin

To search intra-macronuclear microtubule-associated proteins, we performed an isolation of macronuclei from *T. pyriformis*. Isolation of macronucleus was performed as described in the Materials and methods. Isolated macronuclei were evaluated by Western blot and immunostaining of α-tubulin (Fig. 1). In contrast to macronucleus in dividing phase, microtubules were absent in the macronucleus in interphase, suggesting the influx of tubulin took place in macronucleus during the macronuclear division [14]. Although, every isolated macronucleus from the log phase cells contained tubulin, while some of the macronucleus was significantly stained

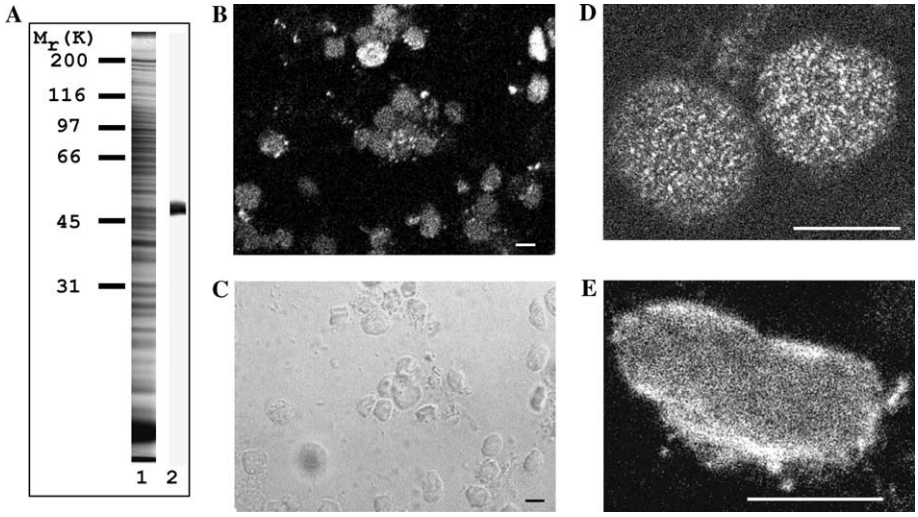

Fig. 1. Alpha-tubulin in the isolated macronucleus. Proteins in isolated macronucleus were analyzed by 10% SDS–PAGE and visualized with Coomassie staining (A, lane 1). α-Tubulin immunoblot indicates the presence of α-tubulin within the macronucleus (A, lane 2). Isolated macronuclei were immunostained with anti-α-tubulin antibody. Brighter staining was observed from some of the macronucleus, particularly from enlarged and oval shaped macronucleus (B). (C) Differential interference image of isolated macronucleus shown in (B). Closeup image of α-tubulin immunofluorescence from isolated macronucleus (D,E). Tubulin was present in every macronucleus, including the round macronucleus which is assumed to be in the interphase (D). Deformed macronucleus showed bright staining of tubulin near the periphery of macronucleus, which is assumed to be in the dividing stage (E). Bars 10 μm

(Fig. 1B). Significantly stained macronuclei were oval, indicating that they are macronuclei from dividing cells. This observation suggests the presence of tubulin in macronucleus throughout the cell cycle, including the interphase, and becoming enriched at macronuclear division. The presence of tubulin highly ensured that the isolated macronuclei might also contain proteins associated to microtubules.

One hundred and thirty eight kDa protein was identified from the macronuclear extract by microtubule coprecipitation assay

In the previous study we showed that microtubules are formed in macronucleus only during the macronuclear division [14]. This led to the idea that microtubule-associated proteins in macronucleus are most likely to be associated with macronuclear division. Isolated macronuclei were disrupted and proteins interacting with chromosomes were released by DNase treatment subsequently collected in the supernatant of ultracentrifugation. Sample was performed to microtubule cosedimentation assay in the presence of AMP-PNP, non-hydrolyzing analogue of ATP. Proteins released from microtubule pellet were analyzed on SDS–PAGE (Fig. 2). Proteins including 138 kDa were released from microtubules by a MTs buffer containing 0.3 M NaCl and 5 mM ATP. We termed 138 kDa protein as p138 from its relative molecular weight.

Next, we tested whether these proteins were specific to DNase treatment from the macronuclear extract preparation. Microtubule coprecipitation assay with macronuclear extract prepared without the DNase treatment showed that p138 did not interact with microtubules (Fig. 2B, lane: -DNase). Mock experiment, without microtubules, also did not show p138, indicating that precipitation of p138 depended on the presence of microtubules (Fig. 2B, lane: -MTs). These results suggested that p138 is a component of macronucleus and interacts with both microtubules and chromosome.

The release of p138 from microtubules was depending on ATP (Fig. 2C). In the presence of AMP-PNP, p138 was not released from microtubules at 0.3 M NaCl releasing buffer (Fig. 2C, lane: 2), while p138 is released from microtubule at 0.3 M NaCl releasing buffer in the presence of ATP (Fig. 2C, lane: 6). On the other hand, p138 was released from microtubules when ATP was used for 0.1 M NaCl washing buffer (Fig. 2C, lane: 3). Thus, interaction of p138 and microtubules was likely to depend on ATP hydrolysis.

Molecular cloning and sequence analysis revealed that p138 is a homologue of human FACTp140

Macronuclei were collected from 150 liters of *Tetrahymena* culture. Macronuclear extract was subjected to microtubule cosedimentation assay and p138 was isolated from SDS–PAGE gel. Since N-terminus of this protein was protected, inner amino acid sequences were determined from peptides isolated from lysyl-endopeptidase digests of p138 (Table 1). None of these amino acid sequences had significant homology to known proteins. We then searched from genome DNA sequence database of *T. thermophila* for genomic DNA

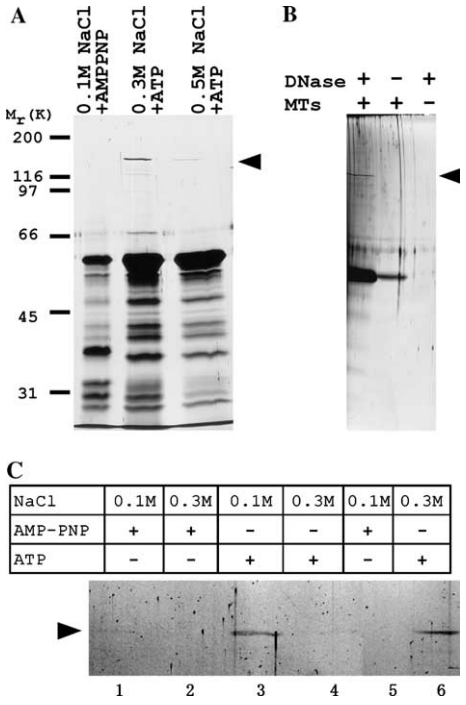

Fig. 2. Microtubule coprecipitation assay of macronuclear extract. (A) p138 was released from microtubule precipitate in the presence of 0.3 M NaCl and 5 mM ATP. Proteins coprecipitated with microtubule were released by stepwise elution performed with the exchange of buffers 0.1 M NaCl + 1 mM AMP-PNP, 0.3 M NaCl + 5 mM ATP, and 0.5 M NaCl + 5 mM ATP. Released proteins were analyzed by SDS–PAGE. (B) SDS–PAGE pattern of 0.3 M NaCl + ATP fraction from coprecipitation assay performed with macronuclear extract prepared with (+) or without (−) DNase treatment and with (+) or without (−) microtubules. One hundred and thirty eight kDa protein was only detected in the presence of both DNase treatment and microtubules. (C) p138 was released from microtubules in ATP-dependent manner. Stepwise elution of proteins from microtubules coprecipitation assay was performed with 0.1 M NaCl and 0.3 M NaCl, in the various AMP-PNP/ATP conditions (three different sets of elution: 1–2, 3–4, and 5–6). First, eluate of 0.1 M NaCl + AMP-PNP (1), followed with 0.3 M NaCl + AMP-PNP (2). Second, eluate of 0.1 M NaCl + ATP (3), followed with 0.3 M NaCl + ATP (4). Third, eluate of 0.1 M NaCl + AMP-PNP (5), followed with 0.3 M NaCl + ATP (6). Release of p138 from microtubules did not depend on salt concentration. Arrowheads indicate p138. Proteins were visualized with silver staining.

Table 1
Amino acid sequences determined from p138

Fraction	Amino acid sequences	Sequence ID
59	EYNIVFVE	1173226
60	QLLSVWNK	1173226
87	(E/N)(V/F)(E/D)EGMVFNVVVGFDNLV	1173226

Lysyl endopeptidase digested p138 was fractionated and amino acid sequence was determined from polypeptides in Fractions 59, 60, and 87. First three amino acids from Fraction 87 were undetermined due to the detection of multiple amino acids. The gene encoding these polypeptides was identified from *T. thermophila* genome data base at The Institute for Genome Research using BLAST search program.

sequences encoding the peptide sequences. One of the genomic DNAs had DNA sequence encoding all three of peptides within the 1.8 kb region. Its open reading frame was predicted from the BLAST search result. The cDNA of p138 was cloned by RT-PCR and RACE and the cDNA sequence was determined (Fig. 3A). The GenBank Accession No. for p138 is AY504817. Deduced amino acid sequence of this gene product encoded all three of polypeptides analyzed from p138. The identity of polypeptides was 78%, 100%, and 93% for Fractions 59, 60, and 87, respectively. Although the polypeptide sequences obtained from *T. pyriformis* p138 did not perfectly match the deduced amino acid sequence, we conclude that this gene encodes *T. thermophila* p138.

BLAST homology search of p138 revealed a similarity to proteins responsible for chromatin specific transcription elongation (Fig. 3C). The identity of p138 and these proteins was between 36% and 39%. They are known to interact with chromatin and unfolds nucleosome to enable the transcription of specific genes or enable the DNA replication [19–25]. Whether *Tetrahymena* p138 has the similar function is unknown, the sequence similarity suggests that p138 may interact with chromatin as well as the other homologues. Together, our results suggest that *Tetrahymena* p138 is a homologue of FACTp140 and may interact with both microtubules and chromatin structure.

Discussion

We describe here the cloning of a 138 kDa protein from *Tetrahymena*, p138, which interacts with microtubules in vitro. The results from microtubule coprecipitation assay indicated two important features of p138. The coprecipitation of p138 required a macronuclear extract prepared with DNase treatment. Interaction of p138 and microtubules was ATP dependent, possibly by a hydrolysis of ATP. These results suggest that *Tetrahymena* p138 interacts with both chromosomal structure and microtubules.

Although our first aim was to obtain kinesin from macronucleus, p138 turned out to be a homologue of human transcription elongation factor FACT 140 kDa subunit, yeast Spt16/Cdc68, *Xenopus* DUF140, etc. (Fig. 3). They are known to form heterodimers with SSRP1, Pob3, and DUF87, respectively, to access the chromatin structure [20,26,27]. Preliminarily, we have made a search for the candidates of the p138 partner. Gene search using Pob3 amino acid sequence by BLAST from TIGR *Tetrahymena* genome database showed a candidate of Pob3 homologous gene (~30% identity, ~50% similarity, data not shown). This highly indicates that *Tetrahymena* p138 has a similar function to FACT and its homologues as a chromosome binding protein.

In spite of the sequence similarity, FACT140, Cdc68, and DUF140 have a variety of functions. FACT complex is required for transcript elongation through

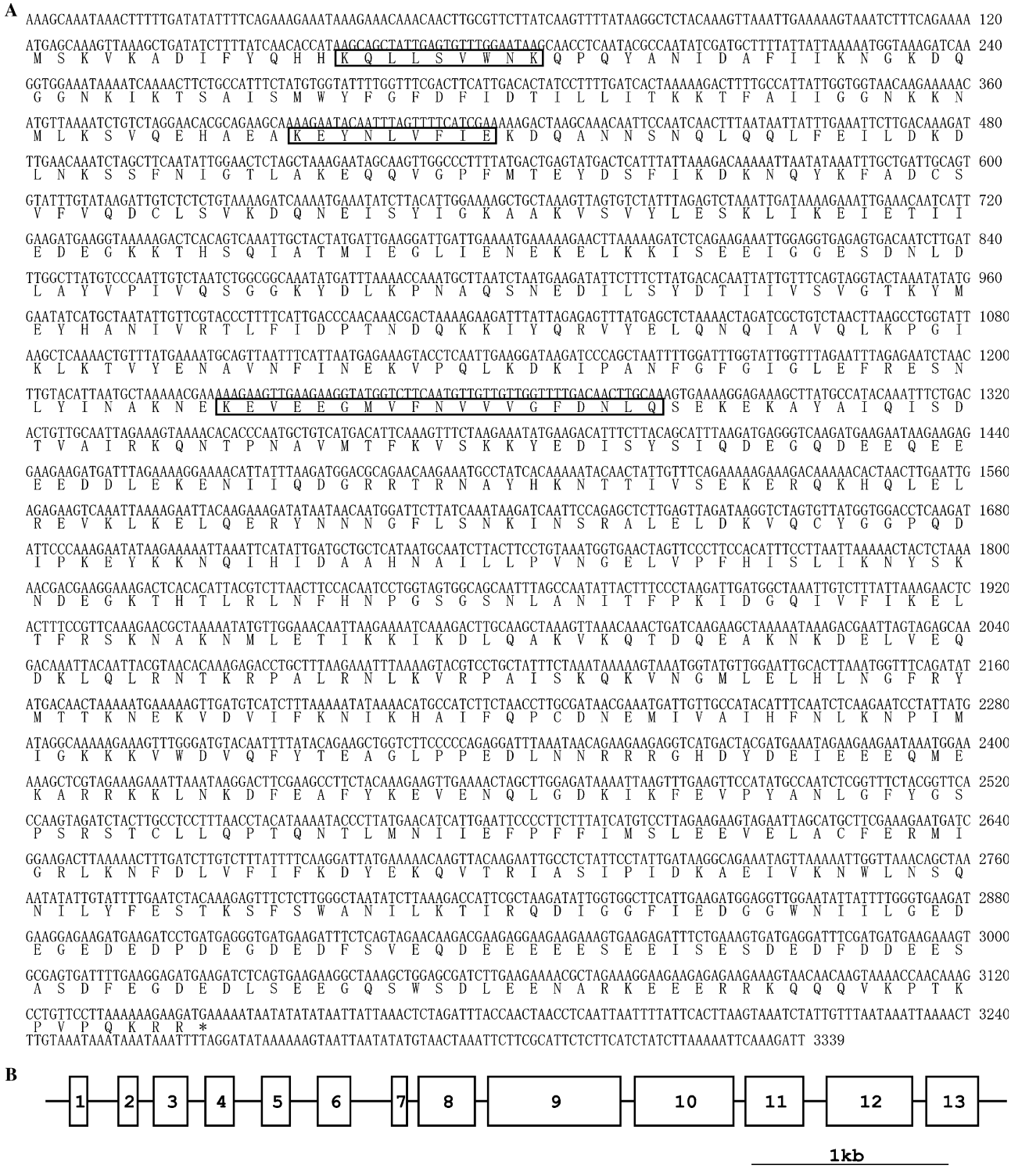

Fig. 3. Deduced amino acid sequence of p138 protein. A cDNA sequence (upper column) and deduced amino acid sequence of *T. thermophila* p138 (A). Boxed areas denote the regions of determined amino acid sequences shown in Table 1. First 'K' in each box is potential lysine expected from the lysyl endopeptidase digestion. Open reading frame consists 1007 amino acids. Exon–intron map (B). Exons are shown in boxes. Multiple sequence alignment of p138 and its homologous proteins. '*' indicates positions with identical amino acids (C). ':' indicates that 'strong' group is fully conserved. '.' indicates that 'weak' group is fully conserved. The strong and weak groups are defined as strong score >0.5 and weak score $\leqslant$0.5 that occur in the Gonnet Pam250. Conserved regions are denoted with boxes. The GenBank accession numbers for the proteins are Tt p138 (AY504817) FACT140 Hs (AAD43978), FACT140 Mm (AAL04452), DUF140 Xl (BAA76334), DRE4 Dm (AAQ22585), Spt16 Dm (BAC54898), Cdc68 Sp (T40813), T12H20 Mm (T01906), GTC102 Zm (AAN41252). Tt, *T. thermophila*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe*; Zm, *Zea mays*.

C

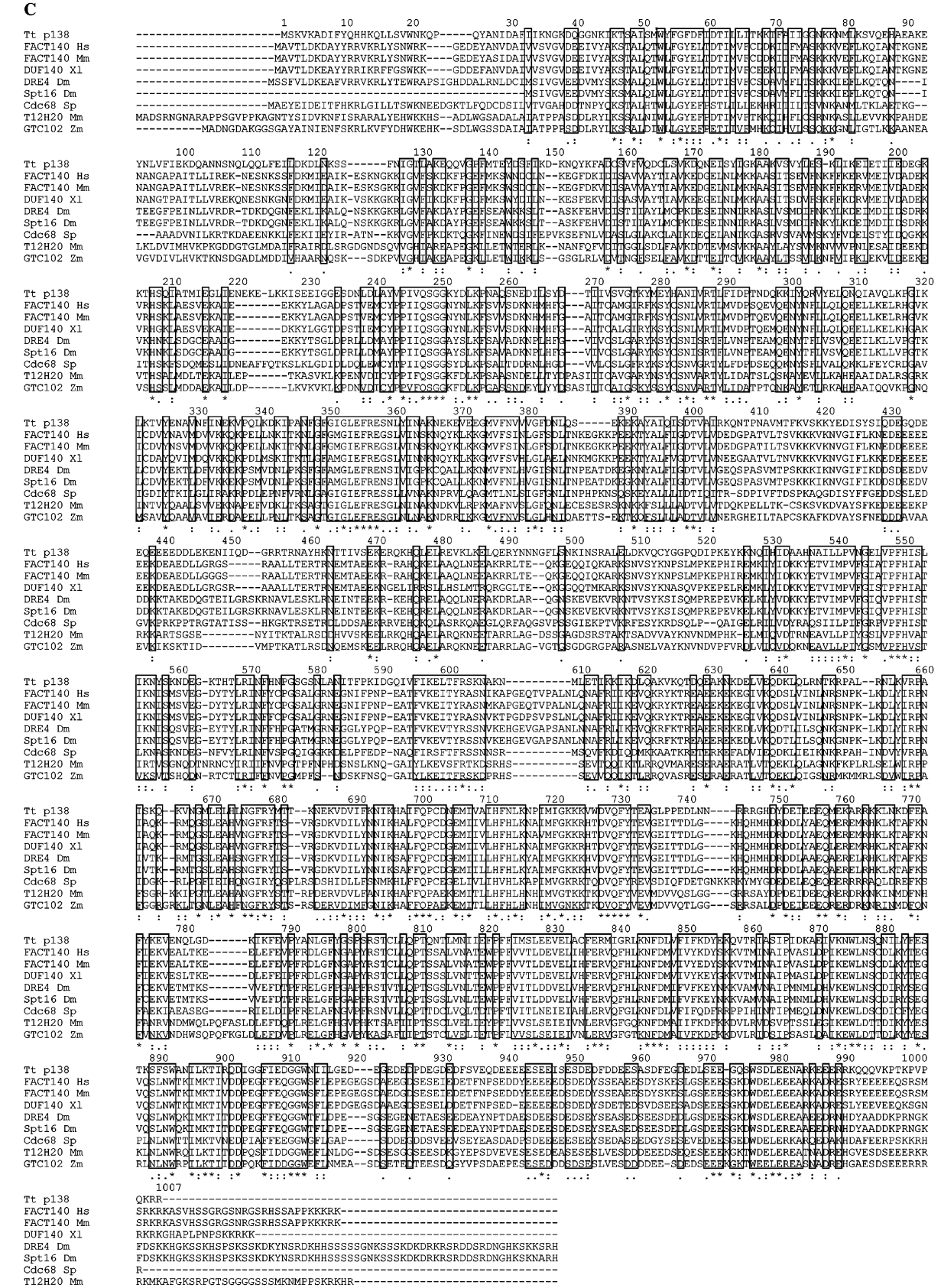

Fig. 3. (*continued*)

nucleosomes by RNA polymerase II in vitro, helping the removal of histone H2A/H2B dimers through a nucleosome core particle [23]. FACT complex is also required for DNA repair [28]. Yeast Cdc68 complex has been reported to affect the cell-cycle regulation at the checkpoint of G1 phase and is also required for DNA replication [19,26,27,29]. DUF complex has been proposed to play a role in DNA replication in *Xenopus* egg extracts [20]. In contrast to the reported function of FACT, Cdc68, and DUF complexes, our study showed the interaction of p138 and microtubules, which implies a new role of FACT homologue. Amino acid sequence of p138 did not have any significant region for microtubule binding or ATPase domain. It is likely that microtubule binding of p138 is indirect interaction. The chromatin binding activity of FACT is not affected by ATP [21]. This suggests that, if p138 behaves like FACT, the ATP-dependent binding target of p138 is other than chromatin. Hence, it is likely that there is an ATP regulated protein(s) between p138 and microtubules. This has to be examined for further understanding.

Kinesin is a well-known microtubule binding protein and some of them plays crucial role in mitosis [30]. In the presence of AMP-PNP, kinesin is strongly bound to microtubules, hydrolyzes ATP to move its self on microtubules, and becomes released from microtubules. ATP-dependent release of p138 from microtubules was similar to the behavior of kinesins. On the other hand, the substrate binding of chaperones show a similar behavior to that of kinesins. For example, polypeptide is released from Hsp90 in the presence of ATP but not in the presence of AMP-PNP [31]. Sequence analysis of p138 did not show any significant similarity with kinesins or any chaperones. We hypothesize that kinesin or chaperones mediate the interaction of p138 and microtubules, thus showing a similar behavior to ATP and AMP-PNP.

We have speculated in the previous study that macronuclear chromosome interacts with microtubules in spite of the lack of kinetochore in the macronuclear division [14]. This was speculated from the correlation of DNA pattern and microtubule localization during the macronuclear division. Microtubules are assembled in macronucleus in limited time, while the macronucleus divides. Hence, function of intra-macronuclear microtubule-associated protein is most likely to play a role macronuclear division. We suggest p138 is a candidate of protein that intermediates between chromosome and the intra-macronuclear microtubule while the intra-macronuclear microtubules separate the chromosomes during the macronuclear division.

Although recent studies revealed the function of FACT, the relationship of transcription elongation and histone modification remains unclear [32]. Acetylation and deacetylation of histone are another key factor of nucleosome regulation. Cell mating process of *Tetrahymena* undergoes differentiation of the micronucleus to the macronucleus through genomic rearrangements [1–3]. Several lines of evidences argue that histone modification is important for nuclear differentiation and genomic rearrangements [33,34]. Hence, study on *Tetrahymena* p138 may contribute not only to the understanding of macronuclear dividing mechanisms, but to a further understanding of nucleosome displacement and gene rearrangements.

Acknowledgments

We thank all members of Numata's laboratory for helpful discussions and advice. This work was supported in part by the Sasakawa Scientific Research Grant from the Japan Science Society (14-188) to K.F.

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