J. Microbiol. Biotechnol. (2003), **13**(6), 912–918

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Detection of Mitotic Centromere-Associated Kinesin (MCAK) During Cell-Cycle Progression of Human Jurkat T Cells Using Polyclonal Antibody Raised Against Its N-Terminal Region Overexpressed in *E. coli*

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Received: May 27, 2003
Accepted: September 27, 2003

Abstract Mitotic centromere-associated kinesin (MCAK), which is a novel kinesin with a central motor domain, is believed to play a role in mitotic segregation of chromosome during the M phase of the cell cycle. In the present study, it is shown that a rabbit polyclonal antibody has been produced using the N-terminal region (187 aa) of human MCAK expressed in *E. coli* as the antigen. To express the N-terminal region in *E. coli*, the MCAK cDNA fragment encoding N-terminal 187 aa was obtained by PCR and was then inserted into the pET 3d expression vector. Molecular mass of the N-terminal region overexpressed in the presence of IPTG was 23.2 kDa on SDS-PAGE, and the protein was insoluble and mainly localized in the inclusion body that could be easily purified from the other cellular proteins. The N-terminal region was purified by electro-elution from the gel after the inclusion body was resolved on the SDS-PAGE. The antiserum obtained after tertiary immunization with the purified protein specifically recognized HsMCAK when subjected to Western blot analysis, and showed a fluctuation of the protein level during the cell cycle of human Jurkat T cells. Synchronization of the cell-cycle progression required for recovery of cells at a specific stage of the cell cycle was performed by either hydroxyurea or nocadazole, and subsequent release from each blocking at 2, 4, and 7 h. Northern and Western analyses revealed that both mRNA and protein of HsMCAK reached a maximum level in the S phase and declined to a basal level in the G1 phase. These results indicate that a polyclonal antibody raised against the N-terminal region (187 aa) of HsMCAK, overexpressed in *E. coli*, specifically detects HsMCAK (81 kDa), and it can analyze the differential expression of HsMCAK protein during the cell cycle.

Key words: Human MCAK, motor protein, pET 3d vector, polyclonal anti-MCAK, differential expression, cell-cycle progression, Jurkat T cells

Kinesin-related proteins (KRPs) represent a fundamental component of the machinery for organelle movement along microtubules in eukaryotic cells. The structure of KRPs is similar to that of myosin II in that they possess two heavy chains as well as two light chains per active motor, two globular head motor domains, and an elongated coiled-coil responsible for heavy chain dimerization. There are at least ten different subfamilies of KRPs based on the position of a motor domain that plays a central role in moving cargo along microtubules by utilizing ATP. However, mechanisms underlying the function of individual KRPs remain not clearly understood [20].

Chromosomal segregation during mitosis is a critical intracellular transport event required for progression through the M phase. This segregation is believed to be accomplished through the dynamics of the mitotic spindle that is composed of an array of microtubules as well as associated KRPs. A family of KRPs localized to the mitotic spindle appear to contain a centrally located motor domain, while most kinesins described previously possess either a NH2- or COOH-terminal motor domain. The first identification of KRP that contained a central motor domain was the murine KIF-2 gene that was cloned from brain tissue [2], whose function has been determined as a transporter of membranous organelles in neuron [12]. Another novel kinesin with a central motor domain was among the kinesins of CHO cells, which were identified using antipeptide antibodies to the conserved regions of the kinesin motor domain [24]. Since this kinesin was associated with the centromeric region of mitotic chromosomes during mitosis, it was designated mitotic centromere-associated kinesin (MCAK). Further investigations of the hamster MCAK revealed that it is located in the kinetochore, suggesting that it may coordinate the dynamics of kinetochore microtubules [19, 20]. Sequentially, *Xenopus* kinesin central motor 1 (XKCM1) that is localized in mitotic centromeres as well as spindle poles and regulates microtubule dynamics

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during mitotic spindle assembly [21], and diatom spindle kinesin 1 (DSK1) that is localized in the central spindle and is involved in anaphase spindle elongation [23], have been identified as members of the central motor family of KRPs.

Recently, human MCAK (HsMCAK) cDNA was first cloned from human Jurkat T cells. As determined by DNA sequence analysis, the clone appeared to contain an open reading frame of 2,169 bp, which encodes a 723-amino acid protein with a molecular mass of 81 kDa [9]. Northern blot analysis employing the cDNA as probe revealed that human MCAK-specific mRNA is expressed in tissues containing dividing cells, such as thymus, testis, small intestine, colon (mucosal lining), and placenta, and that the expression of HsMCAK-specific mRNA may be induced in the S phase to support cellular proliferation. The functional role of human MCAK as a motor protein in cellular proliferation remains largely unknown, possibly due to unavailability of its specific antibody. Analysis of the amino acid sequence deduced from the nucleotide sequence of HsMCAK revealed that the kinesin motor domain signature sequence (GS)-(KRHSTQ)-(LIVMF)-x-(LIVMF)-(IVC)-D-L-(AH)-G-(SAN)-E [3-5, 7, 18] was in position 484-495, and that the ATP/GTP-binding site motif A consisting of a (AG)-x(4)-G-K-(ST) [15, 22] was located in position 346-353. While the overall sequence homology of human MCAK is 79.2%, 50.4%, 63.2%, and 33.7% to hamster MCAK, murine KIF-2, *Xenopus* XKCM1, and diatom DSK1, respectively, the sequence homology within the motor domain shows 89.4%, 78.5%, 83.6%, and 45.0% identity to individual counterparts, respectively.

In the present study, rabbit polyclonal antibody was raised using the N-terminal region of human MCAK expressed in *Escherichia coli* system as the antigen, and differential expression of MCAK during cell-cycle progression of human Jurkat T cells was subsequently determined by Western blot analysis. The results showed that the polyclonal antibody obtained after tertiary immunization was able to specifically recognize human MCAK protein with a molecular mass of 81 kDa at 2,000-fold dilution, when subjected to Western blot analysis. The level of MCAK protein reached a maximum in the S phase of the cell cycle to support the cellular proliferation and was tightly regulated at the transcriptional level.

MATERIALS AND METHODS

Bacterial Strain and Vector Plasmid

E. coli BL21(DE3)pLysS [*hsd*S gal (λcI*ts*857 *ind*1 *Sam*7 *nin*5 lacUV5-T7 *gene*1) *pLys*S] and the protein expression vector pET 3d were purchased from Promega (Madison, WI, U.S.A.). The recombinant pET 3d plasmid harbouring a human MCAK cDNA fragment encoding the N-terminal 187 aa in the *Nco*I/*Bam*HI site was designated pET 3d-N-HsMCAK.

Expression of the N-Terminal Region (187 aa) of Human MCAK in *E. coli* and Purification

To insert the cDNA fragment encoding the N-terminal of HsMCAK into the *Nco*I/*Bam*HI site of pET 3d vector, polymerase chain reaction was performed in the presence of HsMCAK cDNA as well as both *Nco*I-forward primer 5'-AATGGCCATGGACTCGTCGCTT-3' and *Bam*HI-reverse primer 5'-AGGATCCTCACCGAACTGAGTTC-3' (*Nco*I/*Bam*HI sites are underlined). PCR conditions were as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1.5 min, and extension at 72°C for 2 min. The amplified cDNA fragment was cloned in the *Nco*I/*Bam*HI site of the pET 3d vector, resulting in pET 3d-N-HsMCAK, in which the cDNA was placed under a strong T7 promoter in sense orientation. The plasmids were then introduced into *E. coli* BL21(DE3)pLysS [*hsd*S gal (λcI*ts*857 *ind*1 *Sam*7 *nin*5 lacUV5-T7 *gene*1) *pLys*S, and transformants were selected on LB plates containing ampicillin (50 μg/ml). The synthesis of the N-terminal of HsMCK protein in *E. coli* was induced by isopropyl β-D-thiogalactopyranoside (IPTG) as previously described [17]. To identify and localize the N-terminal HsMCAK protein produced by the *E. coli* transformant, the bacterial culture was fractionated into three portions, including total cell lysate, soluble protein fraction, and inclusion bodies (pellet) as described [11], and an equivalent amount of each fraction was electrophoresed on 11% SDS polyacrylamide gel. The presence of HsMCAK protein was determined by staining with Coomassie brilliant blue. The protein accumulated in the insoluble inclusion body fraction was purified from the gel by electroelution after SDS-polyacrylamide gel electrophoresis.

Immunization of Rabbit With the N-Terminal Region of HsMCAK

Five-hundred microliters of the N-terminal region of HsMCAK (200 μg) purified by electroelution was mixed with an equal volume of Freund's complete adjuvant, and were injected intramuscularly at the thigh muscle of the two rear legs of a rabbit. For secondary and tertiary immunization, the protein mixed with Freund's incomplete adjuvant was injected into the rabbit in the same manner every four weeks. The bleeding was done 10 days after each immunization to test antibody titer.

Arrest of Cell-Cycle Progression of Jurkat T Cells by Hydroxy Urea or Nocadazole

Continuously proliferating Jurkat T cells (4×10^5/ml) were cultured in RPMI 1640 medium with 1 mM hydroxy urea for 20 h to block the cell-cycle progression at the G1/S boundary [1], whereas Jurkat T cells were cultured in the

presence of 0.1 μg/ml of nocadazole for 20 h to arrest at the G2/M boundary [6]. To release the cells from the arrest points and to continue the cell-cycle progression, the cells treated with hydroxyurea or nocadazole were layered over FBS, centrifuged, washed three times with 1× HBSS containing 2% FBS, and then incubated in RPMI 1640 medium up to 7 h. The culture medium used for human Jurkat T cells was RPMI 1640 (Bethesda Research Laboratories) containing 10% FBS (Upstate Biotechnology Inc., Lake Placid, NY, U.S.A.), 20 mM HEPES (pH 7.0), 5×10^{-5} M β-MeOH, and 100 μg/ml gentamycin.

Flow Cytometric Analysis

Cell-cycle progression of resting T cells following polyclonal activation was analyzed by flow cytometry as described elsewhere [14]. Approximately 1×10^6 T cells were suspended in 100 μl of PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended with 12.5 μg of RNase in 250 ml of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37°C for 30 min before staining of the cellular DNA with 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content, based on increased red fluorescence.

Northern Blot Analysis

Total RNA was extracted and isolated by solubilization in guanidine thiocyanate as described elsewhere [10]. Equivalent amounts of total RNA were electrophoresed on 1% formaldehyde-agarose gels and transferred to GeneScreen Plus membranes. The nylon membrane as well as human Multiple Tissue Northern Blot were hybridized in ExpressHyb solution at 68°C for 2 h with cDNA probe radiolabeled with [^{32}P]dCTP by the random primer labeling method [8], and washed according to the manufacturer's instruction.

Cell Lysate, Protein Quantitation, and Western Blot Analysis

The cells were suspended in lysis buffer (137 mM NaCl, 25 mM MOPS, 15 mM EGTA, 15 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 2.5 μg/ml E-64, 0.1% Triton X-100, pH 7.2), disrupted by sonication, and extracted at 4°C for 30 min, as described elsewhere [13]. After centrifugation at 14,000 rpm for 20 min, the supernatant was obtained as cell lysate. Protein quantitation was performed using the Micro BCA kit (PIERCE, Rockford, IL, U.S.A.). Total 15 μg of cell lysates were subjected to electrophoresis on 4-12% NuPAGE gradient gel with MOPS buffer and electrotransferred to Immobilon-P membrane (Millipore Corporation, Bredford, MA, U.S.A.). The membrane was allowed to react with individual primary antibodies and then with horseradish peroxidase conjugated with Donkey anti-rabbit IgG antibody.

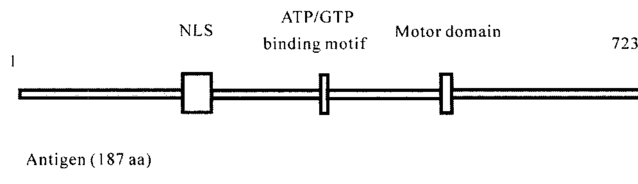

Fig. 1. Location of the three conserved domains, nuclear localization (NLS) domain, kinesin motor domain, and ATP/GTP-binding site motif, in the amino acid sequence of human MCAK.

The antigen molecule used to raise a rabbit polyclonal antibody was the N-terminal region (187 amino acid residues) of the MCAK protein.

Detection of each protein was visualized by ECL Western blotting detection system according to the manufacturer's instructions (Amersham, Arlington Heights, IL, U.S.A.).

RESULTS AND DISCUSSION

Construction of Recombinant Plasmid pET 3d-N-HsMCAK

To produce human MCAK in *E. coli*, the cDNA fragment corresponding to N-terminal 187 amino acid residues was inserted into the *NcoI*/*Bam*HI site of an expression vector pET 3d and designated pET 3d-N-HsMCAK. Since the amino acid sequence deduced from the nucleotide sequence of HsMCAK showed three conserved domains, including the nuclear localization (NLS) domain [25], kinesin motor domain [3-5, 7, 18], and ATP/GTP-binding site motif A in the middle region of the protein [15, 22], it was decided to

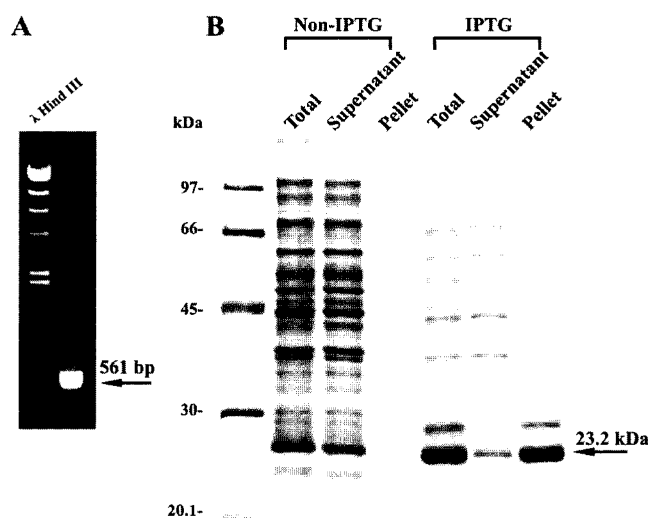

Fig. 2. Electrophoresis of HsMCAK cDNA fragment encoding the N-terminal 187 amino acid after amplification by PCR (A), and identification of the N-terminal HsMCAK protein after expression in *E. coli* (B).

employ the N-terminal 187 amino acid residues, whose sequence is more unique than other regions for HsMCAK (Fig. 1). The MCAK cDNA fragment required to construct pET 3d-N-HsMCAK was obtained by PCR. When PCR was done with HsMCAK cDNA in the presence of both *Nco*I-forward primer and *Bam*HI-reverse primer, a 561-bp PCR product was amplified (Fig. 2A). The amplified PCR product was digested with *Nco*I/*Bam*HI and purified by extraction with buffer-saturated phenol buffer and precipitation with 2.5 volume of cold ethanol. Subsequently, the purified PCR product was ligated with pET 3d vector that was linearized by the same restriction enzymes. After the ligation mixture was used for transformation of *E. coli* BL21(DE3)pLysS, the transformant containing the pET 3d-N-HsMCAK was obtained. From the transformant, the pET 3d-N-HsMCAK was isolated, and the nucleotide sequence of inserted coding region for N-terminal region (187 aa) of HsMCAK was confirmed by DNA sequence analysis (data not shown). The *E. coli* transformant containing pET 3d-N-HsMCAK was designated *E. coli* pET-N-HsMCAK.

Identification and Localization of the N-Terminal Region of HsMCAK in *E. coli*

For identification and localization of the N-terminal HsMCAK protein produced in *E. coli* pET-N-HsMCAK, the strain was cultured with shaking in LB media, containing ampicillin (50 μg/ml) and chloramphenicol (20 μg/ml), at 37°C and, when O.D at 600 nm reached 0.4, 0.3 mM IPTG was added and cultivation was continued for an additional 4 h. The *E. coli* expression system employing pET vectors was developed not only to overexpress target DNAs under the control of a T7 promotor and T7 RNA polymerase, but also to regulate the expression of target DNAs by placing a T7 RNA polymerase gene under control of *lac*UV5 promotor, which is inducible by IPTG [17]. In addition, the expression system was constructed to express target DNAs from its own translation start and not as a fusion protein. By using the above conditions for *E. coli* system with pET 3d vector, the N-terminal HsMCAK was expressed at a high level and its molecular mass appeared to be 23.2 kDa, as expected from the amino acid sequence (Fig. 2B). In addition, the bacterial culture was fractionated into three fractions to elucidate the localization of the N-terminal MCAK protein in the bacterial cells. As shown in Fig. 2B, the HsMCAK protein induced in the presence of 0.3 mM IPTG was mainly localized in the inclusion body. However, the N-terminal HsMCAK was barely detectable in the soluble fraction. Under these conditions, *E. coli* pET-N-HsMCAK was able to express a basal level of the HsMCAK protein in the absence of IPTG. These results demonstrate that the expression of the N-terminal region of HsMCAK protein is successfully induced by IPTG in *E. coli* pET-N-HsMCAK, and the protein is mainly localized in the inclusion body.

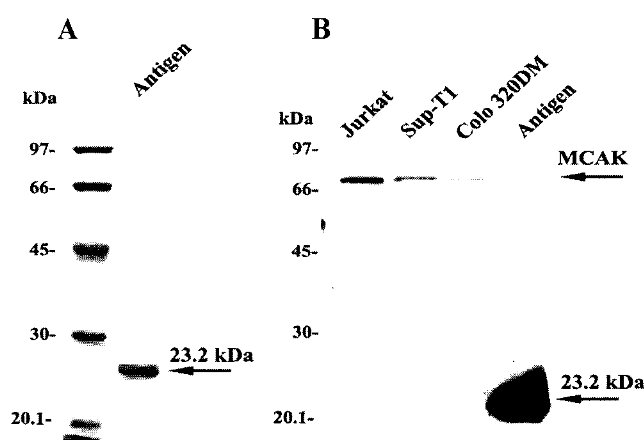

Fig. 3. Confirmation of the purified N-terminal HsMCAK protein on SDS-PAGE (A), and Western blot analysis of the intact human MCAK (81 kDa) as well as the N-terminal HsMCAK (23.2 kDa) using a rabbit polyclonal antibody raised against the N-terminal HsMCAK protein (B).

Production of Rabbit Polyclonal Antibody Using the N-Terminal Region of HsMCAK as the Antigen

For purification of the N-terminal HsMCAK protein, the inclusion body was harvested from the culture of *E. coli* pET-N-HsMCAK. Since the incusion body fraction appeared to contain an additional contaminated 28 kDa protein upon SDS-PAGE, which was distinct in molecular mass from 23.2 kDa of the N-terminal HsMCAK, the protein was further purified by electroelution from the gel. As shown in Fig. 3A, the purified HsMCAK protein was confirmed on 11% SDS-PAGE. In order to produce a rabbit polyclonal antibody against the N-terminal HsMCAK protein, 200 μg of the purified protein mixed with adjuvant was injected intramuscularly for each primary, secondary, and tertiary immunization. To evaluate the antibody titer by Western blot analysis, bleeding from the rabbit was done 10 days after each immunization. Since the antiserum obtained 10 days after tertiary immunization in 2,000-fold dilution was able to detect specifically the N-terminal HsMCAK antigen as well as the whole molecule of HsMCAK protein with molecular mass of 81 kDa in the cell lysates of human leukemia Jurkat T cells, lymphoma Sup-T1, and colon adenocarcinoma COLO 320DM, the animal was sacrificed and the antiserum was recovered (Fig. 3B). It is noteworthy that the antibody appeared to immunoprecipitate the intact form of 81 kDa HsMCAK protein in the cell lysate (data not shown). Although it is generally accepted that immunization with a soluble form of antigen would be better than using insoluble form in the inclusion bodies for producing an antibody which can recognize a natural intact form of the protein, these results indicate that a rabbit polyclonal antibody raised against a denatured form of the N-terminal HsMCAK was able to recognize the intact form of HsMCAK protein.

Dectection of HsMCAK mRNA and Protein During Cell-Cycle Progression of Jurkat T Cells

Although the functional role of MCAK is believed to be associated with mitotic segregation of chromosome during the M phase of the cell cycle based on the result from hamster MCAK, it still remains unclear whether the cellular role of the human MCAK is similar to that of hamster MCAK involved in chromosome segregation, or whether human MCAK has an additional role during cell proliferation.

If the human MCAK is the homologue of hamster MCAK and its physiological role resembles that of hamster MCAK, it is expected that the expression of human MCAK reaches a maximum in the M phase, because the chromosome segregation occurs in the M phase of the cell cycle. To test this prediction, it was decided to investigate the expression level of HsMCAK during the cell-cycle progression of continuously growing Jurkat T cells. In order to synchronize Jurkat T cells in the specific stage during the cell-cycle progression, two cell-cycle blocking agents, such as hydroxyurea for blocking at the G1/S transition point [1] and nocadazole for blocking at G2/M transition point [6] were employed. While the continuously growing Jurkat T cells were composed of 56% of the G1 cells, 36% of the S phase cells, and 8% of G2/M phase cells, treatment of Jurkat T cells with hydroxyurea or nocadazole for 20 h efficiently blocked the cells in late G1 or at the G2/M boundary, and thus approximately 65% of the cells remained at the G1 and 83% of the cells at the G2/M phase. Since both hydroxyurea and nocadazole are known as reversible blocking agents of the cell-cycle progression, reversal of the blocking was easily accomplished by washing the cells and resuspending in the reagent-free medium at 37°C. The blocked cells could synchronously continue the cell-cycle progression at least for 7 h after the release from the blocking. To obtain Jurkat cells at the specific stages of

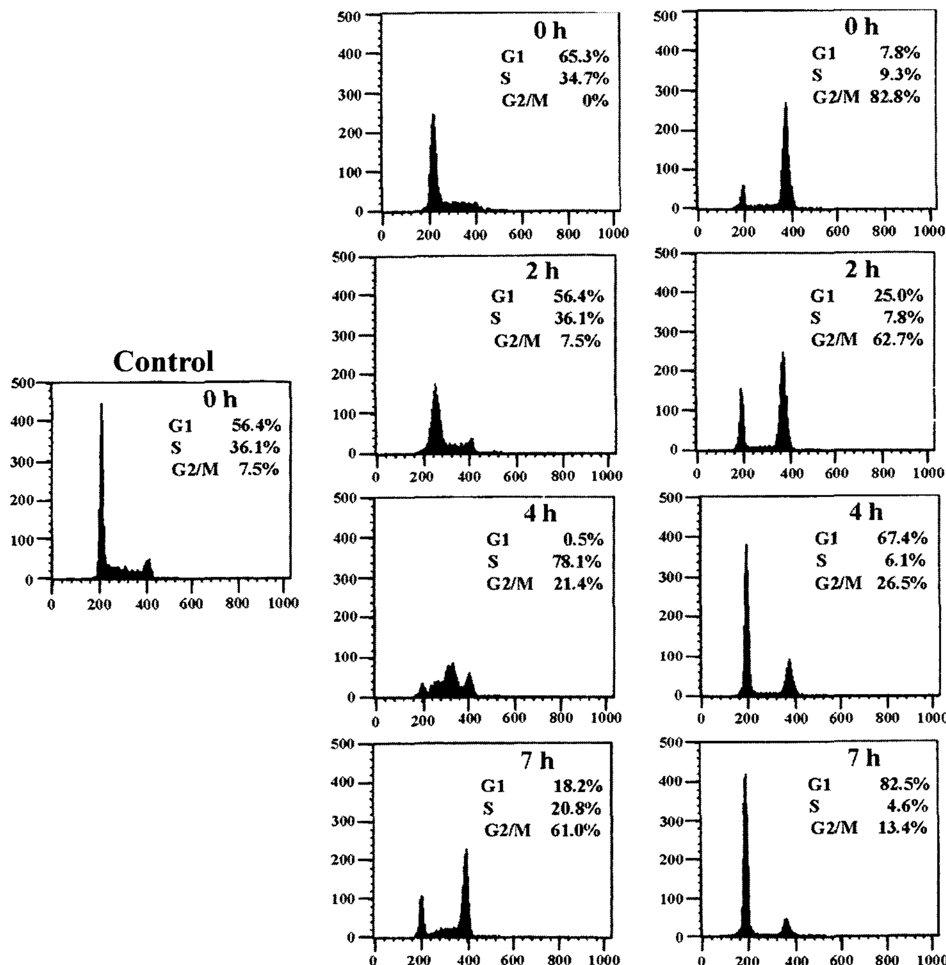

Fig. 4. Flow cytometric analysis of the cell-cycle distribution of continuously growing Jurkat T cells.
Jurkat T cells were cultured in RPMI 1640 medium with 1 mM of hydroxyurea for 20 h to arrest at the G1/S boundary or with 0.1 μg/ml of nocadazole to arrest at the G2/M boundary, and the cells were released from the arrest points for the indicated time periods. Jurkat T cells under various conditions were harvested and stained with propidium iodide, and an equal number of cells (10^4) were analyzed by flow cytometry.

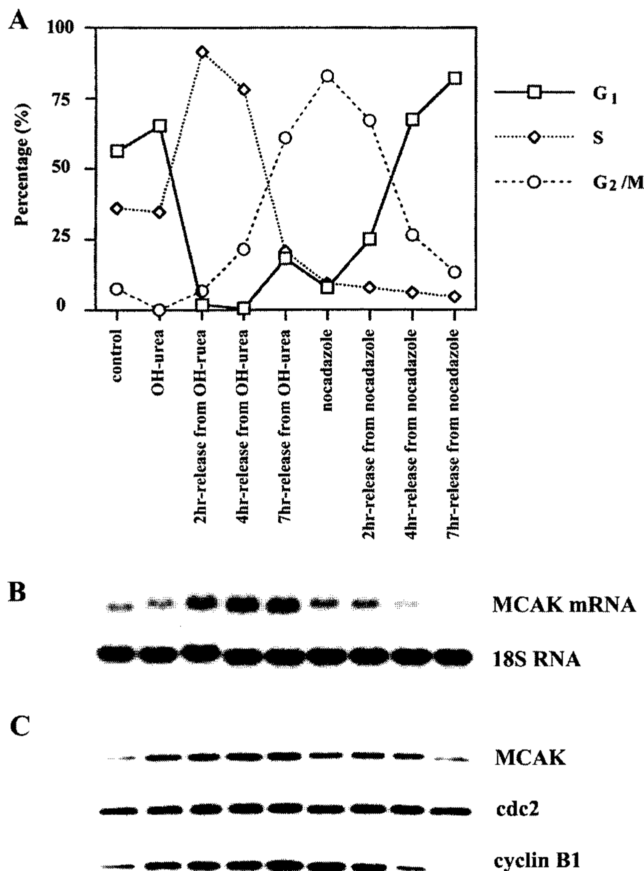

Fig. 5. Cell-cycle distribution (A), and Northern (B) and Western (C) analyses of the human MCAK, cdc2, and cyclin B1 in Jurkat T cells blocked in the G1/S boundary by treatment of hydroxyurea or blocked at the G2/M boundary by nocadazole, and released from each blocking point at various time periods.

Fifteen micrograms of total RNA or 20 µg of cell lysate extracted from Jurkat T cells untreated (lane 1), cells blocked with hydroxyurea (lane 2) and released from the blocking at 2 h (lane 3), 4 h (lane 4), and 7 h (lane 5) or cells blocked with nocadazole (lane 6) and released from the blocking at 2 h (lane 7), 4 h (lane 8), and 7 h (lane 9) were analyzed as described in Materials and Methods.

the cell cycle, the cells were released from the blocking point for 2, 4, or 7 h. Subsequently, the cells were processed for RNA extraction. Northern blot analysis revealed that the expression of MCAK-specific mRNA reached a maximum level in late G1 and S phase, and declined to the basal level as the cells exit from the S phase (Figs. 5A and 5B). The basal level of the MCAK mRNA expression was sustained until the cells traversed through G2/M and entered to G1 phase. The expression pattern of MCAK protein during the cell-cycle progression appeared to be essentially the same as that of MCAK mRNA. Under these conditions, the protein level of two cell-cycle regulatory proteins, cdc2 and cyclin B, which are known to form a complex and play an important role as the M phase promoting factor in the cell cycle [16], was also determined. The level of cdc2 protein was relatively constant throughout the cell-cycle progression, whereas cyclin B1 level fluctuated and reached a maximum in the S-G2/M phase and declined to an undectectable level as the cells exited from M phase and moved into G1 phase. These results demonstrate that the expression of human MCAK may be regulated at the transcription level during the cell-cycle progression and reaches a maximum level in the S phase.

In summary, the above results demonstrate that the N-terminal 187 amino acid residues of HsMCAK are specific enough to raise a polyclonal antibody that can specifically detect the intact HsMCAK with a molecular mass of 81 kDa. The results also indicate that the level of HsMCAK protein reaches a maximum in the S phase of the cell cycle to support the cellular proliferation and is regulated at the transcriptional level.

Acknowledgement

This work was supported by Korea Research Foundation Grant (KRF-2000-015-DP0313).

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