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A cytoskeleton-associated protein, TMAP/CKAP2, is involved in the proliferation of human foreskin fibroblasts ☆

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Abstract

Previously, we reported the cloning of a cytoskeleton-associated protein, TMAP/CKAP2, which was up-regulated in primary human gastric cancers. Although TMAP/CKAP2 has been found to be expressed in most cancer cell lines examined, the function of CKAP2 is not known. In this study, we found that TMAP/CKAP2 was not expressed in G0/G1 arrested HFFs, but that it was expressed in actively dividing cells. After initiating the cell cycle, TMAP/CKAP2 levels remained low throughout most of the G1 phase, but gradually increased between late G1 and G2/M. Knockdown of TMAP/CKAP2 reduced pRB phosphorylation and increased p27 expression, and consequently reduced HFF proliferation, whereas constitutive TMAP/CKAP2 expression increased pRB phosphorylation and enhanced proliferation. Our results show that this novel cytoskeleton-associated protein is expressed cell cycle dependently and that it is involved in cell proliferation.

Keywords: TMAP; CKAP2; G1; Cell cycle; Cytoskeleton; Proliferation

In a previous study, we reported the cloning of a microtubule-associated protein, TMAP/CKAP2, and of its alternatively spliced variant, both of which were up-regulated in primary human gastric adenocarcinomas [1]. The frequency of TMAP/CKAP2 mRNA over-expression in primary human gastric adenocarcinomas was about 60%, but its protein expression was detected in all benign and malignant tumors examined. The average number of TMAP/CKAP2-immunoreactive cells in adenocarcinomas was about 50%, whereas no protein was detected in normal mucosal cells. Furthermore, the expression of this gene was detected in all gastric cancer cell lines examined [1]. Others also cloned the same gene as an up-regulated sequence in two of six diffuse B-cell lymphomas and as an antigen presented in cutaneous T-cell lymphoma, and subsequently named it LB1 and Se20-10, respectively [2,3].

Although TMAP/CKAP2 was reported to be up-regulated in malignancies, its role in carcinogenesis is entirely unknown. Its amino acid sequence does not suggest any function, since it contains no known protein motifs and shows no sequence homology with any other known proteins. The only property of TMAP/CKAP2 suggested is an association with the cytoskeleton. Maouche-Chretien et al., suggested that exogenously-introduced LB1 associated with cytoskeletal structure [3]. When we transfected TMAP/CKAP2 into HeLa cells, we observed that it localized to the microtubule-organizing center (MTOC), and that it formed thread-like structures, which overlapped considerably with α-tubulin, thus suggesting that TMAP/CKAP2 is associated with microtubules [1]. Given this

☆ *Abbreviations:* CKAP2, cytoskeleton-associated protein 2; TMAP, tumor-associated microtubule-associated protein; HFF, human foreskin fibroblast.

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observation, we named the gene TMAP (tumor-associated microtubule-associated protein). We recently confirmed that endogenous TMAP/CKAP2 co-precipitates with microtubules (unpublished observations). In addition, it has been reported that mouse TMAP/CKAP2 has a microtubule-stabilizing effect [4]. Based on its suggested association with the cytoskeletal structure, this gene was named cytoskeleton-associated protein 2 (CKAP2) by the human genome project. However, the sequence of TMAP shares no homology with cytoskeleton-associated protein 1 (CKAP1) [5], and no characteristics of the gene product are known, except for its association with microtubules. Therefore, we will refer to this gene as TMAP/CKAP2 throughout this paper.

Here, we report that the expression of TMAP/CKAP2 is dependent on the progression of the G1 cell cycle, and that the knockdown or over-expression of TMAP/CKAP2 reduces or enhances the proliferation of HFFs.

Materials and methods

Cell culture, transfection, transduction and cell cycle analysis. HeLa and NIH-3T3 cells were purchased from ATCC (Manassas, VA, USA) and HFF (primary human foreskin fibroblast) cells were kindly provided by Dr. J. Yang (Samsung Medical Center, Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37 °C under 5% CO_2. NIH-3T3 transfection was performed using Lipofectamine Plus™ Reagent (Invitrogen) following the manufacturer's instructions. For adenoviral infection, subconfluent HFFs were incubated for 2 h in serum-free DMEM, which was then replaced with 10% FBS-containing DMEM. To synchronize HFF cells in the G1 phase, subconfluent cells were cultured in serum-free DMEM for 3 days, or cultured to complete confluency in 10% FBS-containing DMEM. Cells were collected at indicated times after serum addition to serum-starved HFFs or after re-plating confluent HFFs at low density, and Western blot analysis was performed as described previously [1]. For cell cycle analysis, cells grown on 60-mm dishes were collected by trypsinization and centrifuged at 1000 rpm for 10 min. The cells were then resuspended and washed twice in PBS, and finally fixed in 50% ethanol overnight in suspension. Cells were incubated with 200 μg/ml RNAse (Roche Diagnostic) for 20 min at room temperature, and 100 μg/ml propidium iodide was then added. Finally, cells were analyzed using a FACScan (Becton–Dickinson).

Adenovirus vector and plasmid construction. Adenoviruses encoding TMAP/CKAP2 and BFP were made using a pAdEasy™ kit (QBIOgene; Carlsbad, CA, USA) as previously described [6]. The TMAP/CKAP2 open reading frame was produced by PCR, and mutated at the *Pac*I restriction site by site-directed mutagenesis. Recombinant pShuttle-CMV TMAP was produced by inserting a blunt-ended 2046 bp PCR product into the *Eco*RV site of the pShuttle-CMV vector. Adenovirus vector was constructed by homologous recombination of pShuttle-CMV plasmid containing the TMAP/CKAP2 insert and pAdEasy vector, using the *Escherichia coli* strain BJ5183. pAdenovator-CMV5-IRES-BFP plasmid (QBIOgene) was used to produce control adenovirus. The cloned recombinant pAdEasy plasmids were then transfected into HEK293 cells to produce recombinant viruses. The titers of recombinant virus stocks were determined by using the cytopathic effect test in HEK293 cells. TMAP/CKAP2 and BFP proteins encoded by the viruses are referred to as AV-TMAP and AV-BFP, respectively. pcDNA-HisA-TMAP plasmid was constructed by inserting PCR-amplified TMAP ORF into the *Eco*RI and *Bam*HI sites of pcDNA-HisA vector (Clontech).

Knockdown of TMAP/CKAP2 with morpholino antisense oligonucleotide or siRNA. The expression of endogenous TMAP/CKP2 was suppressed by transfecting HeLa and HFF cells with 9 nmol (per well in six-well plates) of morpholino-based antisense oligonucleotide 5′-cggtg tgctcatcgtagcctcgggt-3′, according to the manufacturer's instructions (GeneTools) in 1.5 ml of serum-free DMEM. After incubating for 3 h, the medium was replaced with 10% FBS-containing DMEM. Twenty-four hours after transfection with anti-TMAP morpholino, immunoblotting and FACS analysis were performed. A siRNA oligonucleotide targeting TMAP/CKAP2 was generated by annealing the following sequences: sense, 5′-GUUCUAUCUUGGCGCUAAAdTdT-3; antisense, 5′-UUU AGCGCCAAGAUAGAACdTdT-3′. For a negative control, a siRNA oligonucleotide targeting luciferase (sense, 5′-CUUACGCUGAGUA CUUCGAdTdT-3′; antisense, 5′-UCGAAGUACUCAGCGUAAGdT dT-3′) was used. Transfection of the siRNA duplex (final concentration, 200 nM) was carried out using DharmaFect (Dharmacon) according to the manufacturer's instructions. Immunoblotting for TMAP/CKAP2 and α-tubulin was performed 24 h after transfection. For the cell proliferation assay, cell numbers were counted at indicated time points after replating the siRNA-transfected cells on a six-well plate at a density of 1×10^4 cells per well.

Immunocytochemistry. Cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 10 min. After washing in PBS, cells were incubated for 1 h in a mixture of rabbit anti-TMAP polyclonal sera [1] at 1:200 and anti-α-tubulin monoclonal antibody (Sigma) at 1:4000. Primary antibodies were detected by incubating the cells with Cy2-conjugated anti-rabbit IgG (Molecular Probe) or Cy3-conjugated anti-mouse IgG (Rockland) for 45 min at room temperature. Representative images of stained cells were obtained using a LSM 510 confocal microscope (Carl Zeiss).

Western blot analysis. Equal amounts of cell lysates were fractionated on 8% or 12% SDS–PAGE gels and transferred to membranes, which were immunoblotted for 2 h either with rabbit anti-TMAP polyclonal sera [1] at 1:1000, with rabbit anti-Phospho-pRB polyclonal antibody (Cell Signaling) at 1:1000, with mouse anti-pRB monoclonal antibody (BD Biosciences) at 1:1000, with rabbit anti-p27 polyclonal antibody (Santa Cruz Biotechnology) at 1:500, or with mouse anti-α-tubulin monoclonal antibody (Sigma) at 1:10,000. After incubation with anti-rabbit or anti-mouse IgG-HRP conjugate (Amersham), membranes were incubated for 5 min with WestPico reagent (Pierce, Rockford, IL, USA). Western blot images were obtained using a LAS-1000 chemiluminescence imaging system (Fuji Film).

Quantitative reverse transcriptase-polymerase chain reaction. Total RNA was extracted from HFFs at the indicated times using Trizol (Invitrogen), and 2 μg of each RNA sample was converted into cDNA using thermostable reverse transcriptase (Invitrogen). A 570-bp sequence from TMAP cDNA was amplified using *Taq* polymerase (Perkin-Elmer) and 5′-ATTGAAGAGATGCGACACAC-3′ and 5′-TTATGTTGTATC AGCCTCATA-3′ as upstream and downstream primer, respectively, for 27 cycles {95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s}. A 302-bp sequence from human G3PDH cDNA was amplified using *Taq* polymerase and 5′-CTTCAACAGCGACACCCACTCCTC-3′ and 5′-GGC CCCTCCCCTCTTCAA-3′ as upstream and downstream primers, respectively, for 20 cycles {95 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s}. PCR products were electrophoresed on 1.2% agarose gels containing 5 ng/ml ethidium bromide. The fluorescence intensities of the corresponding DNA bands were quantified using a BAS imaging system (Fuji Film).

Results

Expression of TMAP/CKAP2 in dividing primary human fibroblasts

In our previous study, we were unable to detect TMAP/CKAP2 in normal gastric mucosal cells [1], which suggested that TMAP/CKAP2 expression might be low in normal cells. Consequently we decided to examine the expression of TMAP/CKAP2 in cultured normal human cells. When

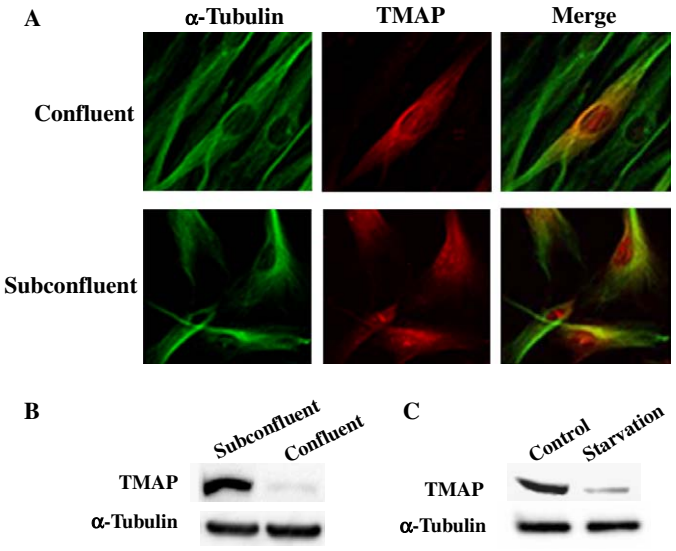

Fig. 1. TMAP/CKAP2 is expressed in dividing HFFs. (A) Confluent or subconfluent HFFs were stained with anti-TMAP or anti-α-tubulin antibodies. (B) Confluent and subconfluent HFFs and (C) serum-starved HFF cell lysates were analyzed by Western blotting using polyclonal anti-TMAP antibody or monoclonal anti-α-tubulin antibody as a loading control.

we stained primary human foreskin fibroblasts (HFFs) at the confluent stage with an antibody against TMAP/CKAP2 [1], we observed that only a few cells were positively stained, whereas α-tubulin was expressed in all cells (Fig. 1A, top). Since most cancer cell lines that divided continuously expressed TMAP/CKAP2 [1] and confluent HFF cells arrested at G1 [7–9] did not express TMAP/CKAP2, we presumed that TMAP/CKAP2 is expressed in dividing cells, but not in non-dividing cells. To test this presumption, we re-plated confluent HFF cells, and found that TMAP/CKAP2 was expressed in most of the HFFs one day after plating (Fig. 1A, bottom), and this result was confirmed by Western blotting. The amount of TMAP/CKAP2 in HFFs was almost undetectable at confluence, whereas it was well expressed in subconfluent cells (Fig. 1B). Even at a subconfluent stage, HFF cells expressed low levels of TMAP/CKAP2 if they were deprived of serum for three days (Fig. 1C).

Expression of TMAP/CKAP2 is dependent on G1 progression

The expression of TMAP/CKAP2 in dividing cells, but not in G0/G1 arrested cells, suggested that TMAP/CKAP2 is expressed sometime after the initiation of the cell cycle. Therefore, we examined the expression of TMAP/CKAP2 in HFFs, which had been released from G0/G1 arrest. First, we cultured HFFs without serum for three days to synchronize them at G0/G1 [10]. After adding serum to culture medium, we examined the time-dependent expression of TMAP/CKAP2 by Western blotting (Fig. 2A). The presence of TMAP/CKAP2 was barely detectable in serum-deprived HFFs. After adding serum, TMAP/CKAP2 remained low for 12 h, but then clearly increased at 16 h. We also examined the phosphorylation of pRB-1 and the level of cdk inhibitor p27 as indicators of G1 progression [8]. As expected, pRB was not phosphorylated in serum deprived HFFs, but its phosphorylation was observed 9 h after adding serum and then gradually increased up to 24 h, whereas the amount of p27 was drastically reduced 6 h after adding serum. HFF cells entered the S phase about 18 h after serum addition (data not shown).

These results indicate that TMAP/CKAP2 expression is dependent on the progression of the G1 cell cycle. Since it was possible that TMAP/CKAP2 was induced by the direct activation of transcription factors by serum, we examined the expression of TMAP/CKAP2 in HFF cells that had been arrested at G0/G1 by confluent culture (Fig. 2B). TMAP/CKAP2 up-regulation was observed 16 h after plating, and the amount rapidly increased until 24 h (Fig. 2B). The amount of TMAP/CKAP2 continuously increased until the G2/M phase, after which it rapidly decreased (data not shown). On the other hand, the phosphorylation of pRB was elevated 12 h after plating and peaked at 20 h, whereas p27 expression was clearly decreased 12 h after plating. In accordance with this p27 reduction, the amount of cyclin D1, an important mediator of the G1/S transition [11], started to increase 6 h after plating and then gradually increased until 24 h. HFFs entered the S phase approximately 20 h after plating (Fig. 2D), indicating that TMAP/CKAP2 up-regulation occurred in the late G1 phase and preceded G1/S transition.

We then examined TMAP/CKAP2 mRNA expression levels by RT-PCR (Fig. 2C). Its mRNA was barely detectable in confluent cells. But the mRNA level was apparently increased at 12 h after plating and peaked at 16 h, and lasted until 24 h. This indicates that the observed up-regulation of TMAP/CKAP2 was the result of an increase in its mRNA level, probably due to transcriptional activation, and that the expression of TMAP/CKAP2 is regulated in a cell cycle-dependent manner.

The constitutive expression of TMAP/CKAP2 enhanced cell proliferation

The G1 cell cycle-dependent expression of TMAP/CKAP2 suggested that TMAP/CKAP2 may play a role in G1 progression. To study the function of TMAP/CKAP2 in G1 progression, we examined the effect of the constitutive expression of TMAP/CKAP2 in HFFs. Because HFFs have low transfection efficiency, we used adenoviral vector to maximize the number of cells expressing TMAP/CKAP2. We then compared the effects of adenovirus vector-encoding TMAP/CKAP2 (AV-TMAP) with control adenovirus-encoding BFP (AV-BFP). Since it has been well reported that adenovirus vectors suppress the cell cycle [12], we used an amount of vector (5 MOI),

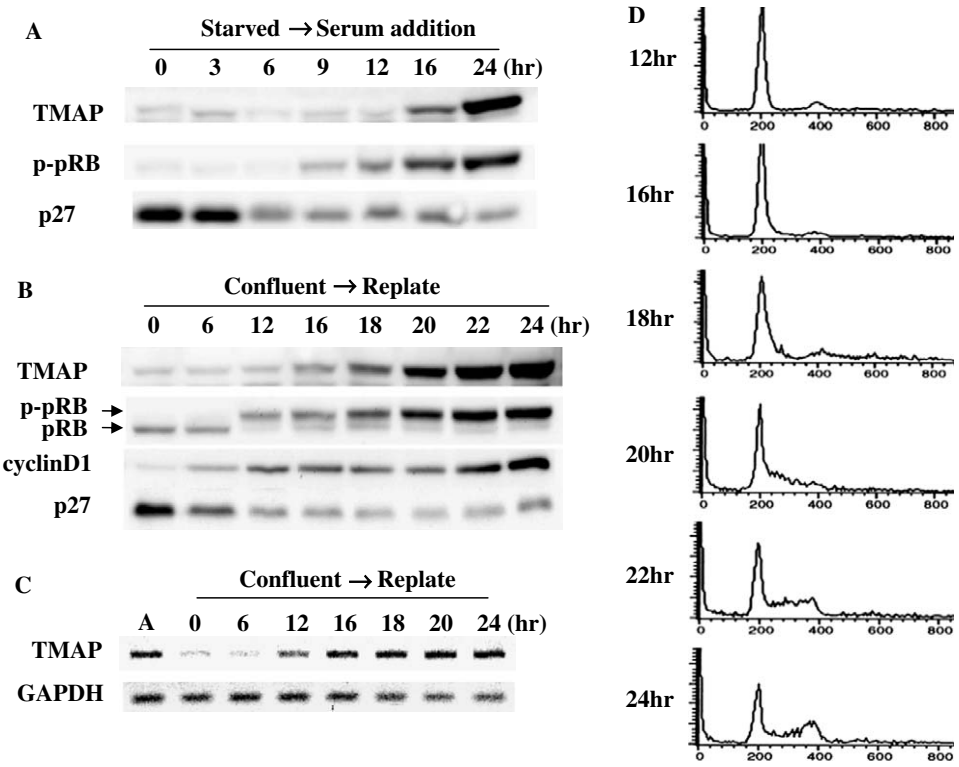

Fig. 2. The cell cycle-dependent expression of TMAP/CKAP2 in HFFs. HFFs were synchronized at G0/G1 by serum starvation for 3 days or by confluent culture. (A) After initiating cell cycle by adding serum, cell lysates (30 μg of proteins/lane) at each time point were analyzed by Western blotting with anti-TMAP, anti-phospho-pRB and anti-p27 antibodies. (B) After initiating cell cycle by replating confluent cells at low density, cell lysates were analyzed at indicated times by Western blotting with anti-TMAP, anti-phospho-pRB, anti-cyclin D1 and anti-p27 antibodies. (C) The expression of TMAP at various time points after replating confluent HFFs were analyzed by RT-PCR. (D) The ploidy of cells at the various sampling times were analyzed by FACS using PI staining after plating confluent HFFs at low density.

which minimally affected the cell cycle, but which expressed enough TMAP/CKAP2.

We first examined pRB phosphorylation and the amounts of p27 in AV-TMAP and AV-BFP infected cells 16 h after re-plating, because phosphorylated pRB upregulation and p27 downregulation were apparent 16 h after re-plating confluent HFFs. Little increase in the amount of phosphorylated pRB was observed in AV-BMP infected cells, although the amount of p27 had substantially decreased. However, in AV-TMAP infected cells, phosphorylated pRB levels clearly increased, and p27 down-regulation was more prominent than that in AV-BMP infected cells at 16 h after re-plating (Fig. 3A). These results indicated that the constitutive expression of TMAP/CKAP2 might promote G1 progression.

To confirm that the constitutive expression of TMAP/CKAP2 is capable of enhancing cell proliferation, we examined the growth rates of HFF cells after infecting them with AV-BFP or AV-TMAP. Both AV-BFP and AV-TMAP infected cells reached confluence at day 8, but AV-TMAP infected cells proliferated more rapidly than AV-BFP infected cells (Fig. 3B). At days 3 and 5, the numbers of AV-TMAP infected cells were $2.43(\pm0.16)\times10^5$ and $8.96(\pm0.59)\times10^5$, respectively, while those of AV-BFP infected cells were $7.8(\pm0.4)\times10^4$ and $3.84(\pm0.24)\times10^5$, respectively. The proliferation of AV-TMAP infected cells slowed down after day 6, when they were about 75% confluent, and stopped on reaching confluency. We also compared the sizes of pcDNA-TMAP and pcDNA-vector transfected NIH-3T3 cell colonies (Fig. 3C), and found that pcDNA-TMAP colonies were more than twice as large as pcDNA colonies (2.2 ± 0.6). These results indicate that the constitutive expression of TMAP/CKAP2 enhances cell proliferation, due to a promotion of G1 progression.

Knockdown of TMAP/CKAP2 reduced cell proliferation

After we observed that the forced expression of TMAP/CKAP2 enhanced G1 progression and cell proliferation, we attempted to knockdown TMAP/CKAP2 in HFFs. Treatment with TMAP/CKAP2 antisense oligonucleotide significantly reduced TMAP/CKAP2 protein in HFFs in a dose-dependent manner (Fig. 4A). In this experiment, we used 9 nmol/well of antisense oligonucleotide, which reduced the amount of TMAP/CKAP2 by 90%. Knockdown of TMAP/CKAP2 resulted in the down-regulation of phosphorylated pRB and the up-regulation of p27 protein, indicating that the G1/S transition was retarded in antisense-transfected cells (Fig. 4B). Since TMAP/CKAP2 increased during late G1, the knockdown of TMAP/CKAP2 probably prevented a further increase in phosphorylated pRB and the down-regulation of p27 during

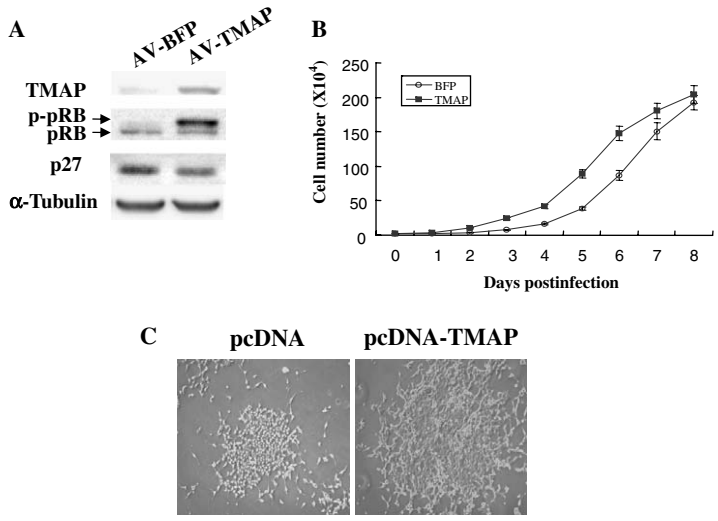

Fig. 3. Constitutive TMAP/CKAP2 expression enhanced HFF and NIH-3T3 cell growths. (A) HFFs were infected with adenovirus-encoding BFP (AV-BFP) or TMAP (AV-TMAP) at an M.O.I of 5, after replating at a density of 2×10^4 per well in six-well plates. Sixteen hours after replating, cells were analyzed by Western blotting for TMAP/CKAP2, mobility retarded pRB (phosphorylated pRB), and p27 using specific antibodies. α-Tubulin was included as a loading control. (B) HFFs were infected with AV-BFP or AV-TMAP as in (A). Cell numbers were counted daily until day 8. Results are expressed as the means ± SDs of triplicate cultures. (C) NIH3T3 cells were transfected with pcDNA vector or pcDNA-TMAP and selected with G418 (500 μg/ml) for 8 days. Phase-contrast photographs were then taken.

the late G1 and G1/S transition. These results suggest that TMAP/CKAP2 is involved in the progression of the G1 cell cycle in HFF cells.

We next studied the effect of TMAP/CKAP2 knock down on cell proliferation. We treated HFFs with antisense or sense oligonucleotides after plating 1×10^4 cells per well in six-well plates (Fig. 4C). Sense-treated cell numbers increased rapidly, whereas antisense-treated cell numbers did not increase substantially for 5 days. At day 8, the number of HFFs treated with antisense had increased to $4.7(\pm 0.7) \times 10^5$, which was only 31.7% of that of the sense-treated cells ($14.8(\pm 0.9) \times 10^5$). We also confirmed the limited proliferation of antisense-treated HFF cells by microscopy. At day 8, sense-treated HFF cells were almost confluent, but antisense-treated cells were far from confluency (Fig. 4D).

In order to validate the result of the antisense experiment described above, we also attempted to suppress the expression of TMAP/CKAP2 using a siRNA targeting a different site within TMAP/CKAP2 mRNA. As shown in Fig. 4E, the TMAP/CKAP2 siRNA efficiently suppressed the level of TMAP/CKAP2 in HFFs. Following transfection with siRNAs, we replated the cells on six-well plates at a density of 1×10^4 cells per well and counted the number of cells at days 1, 4, and 5, during which the suppression of cell proliferation was evident following the antisense treatment. TMAP/CKAP2 siRNA-transfected cells showed a significant retardation in the rate of cell growth, compared to mock-transfected or luciferase siRNA-transfected cells. At day 5, the number of HFFs transfected with TMAP/CKAP2 siRNA only reached $1.8 \pm 0.5 \times 10^5$, while that of the control cells was $3.5 \pm 0.7 \times 10^5$ (Fig. 4F), clearly indicating that the siRNA-mediated suppression of TMAP/CKAP2 reduced the rate of proliferation of HFFs. Taken together, these results demonstrate that silencing TMAP/CKAP2 in HFFs results in a significant reduction in the rate of cell proliferation, presumably due to a delay in G1 progression.

Discussion

The TMAP/CKAP2 gene has been reported to be up-regulated in B-cell lymphoma [3] and in stomach cancer [1], and is also highly expressed in many cancer cell lines. These reports suggest that TMAP/CKAP2 may be involved in the highly proliferative trait of cancers. However, our results indicate that TMAP/CKAP2 may participate in cell cycle progression, and not in the abnormal proliferation of cancer cells. In primary HFF cells, the expression of TMAP/CKAP2 depended on the state of cell division. Confluent or serum-starved HFFs, which were arrested at the G0/G1 phase, did not express TMAP/CKAP2, whereas subconfluent or dividing HFFs did (Fig. 1). This means that TMAP/CKAP2 is expressed in normal primary cells if they divide, and that the expression of TMAP/CKAP2 is specific to dividing cells, rather than to cancer cells. These findings also explain why normal gastric mucosa does not express TMAP/CKAP2, whereas tumors and many cancer cell lines express considerable amounts [1]. As cancer cells lose the ability to stop at G1 and divide continuously, they always express TMAP/CKAP2.

Different expression patterns of TMAP/CKAP2 according to the state of cell division led us to investigate the cell cycle-dependent expression of TMAP/CKAP2. Our results show that the expression of TMAP/CKAP2 is cell cycle-dependent (Fig. 2), i.e., its expression was barely detectable at

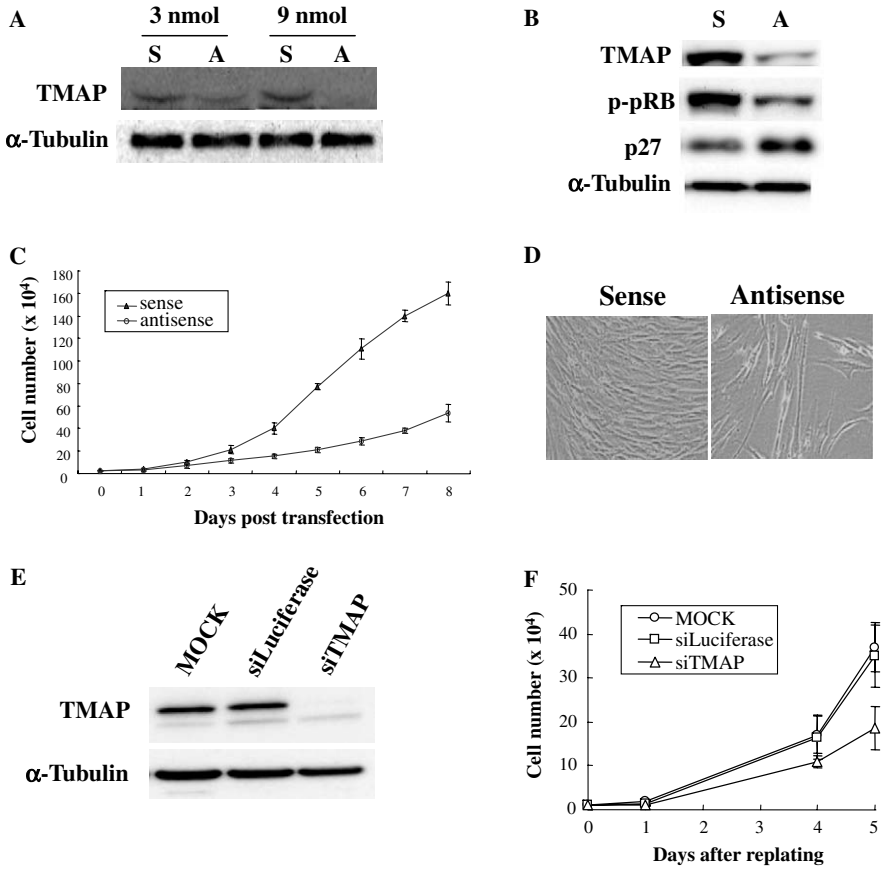

Fig. 4. Knockdown of TMAP/CKAP2 reduced cell growth in HFFs. (A) Twenty four hours after transfecting morpholino antisense TMAP oligonucleotide (3 and 9 nmol per well in a six-well plate) into HFFs, TMAP/CKAP2 was quantitated by Western blotting. (B) HFFs were transfected with 9 nmol of antisense oligonucleotide, and 24 h later, TMAP/CKAP2, phosphorylated pRB, and p27 were quantitated by Western blotting with specific antibodies. (C) After transfecting HFFs with 9 nmol of sense and antisense oligonucleotides, cells were plated at a density of 1×10^4 per well in six-well plates. Cell numbers were counted daily until day 8, and (D) phase-contrast photographs were taken on day 8. (E) Twenty four hours after transfecting siRNA targeting TMAP/CKAP2 (siTMAP) or luciferase (siLuciferase) into HFFs, the protein level of TMAP/CKAP2 was assayed by Western blot analysis. For the mock treatment control (MOCK), cells were transfected without siRNA. (F) After transfection of siRNAs, the HFFs were replated on six-well plates at a density of 1×10^4 cells per well. Cell numbers were counted at days 1, 4, and 5. The results shown are representative of at least three independent experiments (A, B, D, and E), and are expressed as the means ± SD of triplicate cultures (C,F). α-Tubulin was included as a loading control (A, B, and E).

G0/G1, began to increase significantly during the late G1 phase, and reached its peak at G2/M, which implies that the functions of TMAP/CKAP2 may be related to cell cycle progression. In an analysis of genes expressed in human cell cycle, TMAP/CKAP2 was observed as a member of G2/M cluster in HeLa cells [13]. Therefore, it is quite possible that TMAP/CKAP2 has roles in both the G1 and G2/M phases of the cell cycle. We are currently investigating the role of TMAP/CKAP2 in the G2 to M transition.

The constitutive expression of TMAP/CKAP2 enhanced, whereas its knockdown reduced, HFF proliferation (Figs. 3 and 4), indicating that TMAP/CKAP2 is involved in the proliferation of these cells. At present, we do not know the mechanism underlying cell proliferation regulation by TMAP/CKAP2, but one possibility is that TMAP/CKAP2 facilitates G1 progression. In the present study, the constitutive expression of TMAP/CKAP2 enhanced the phosphorylation of pRB and reduced the level of p27 in HFFs, suggesting that it may affect the levels of these two key molecules in G1-S progression. However, TMAP/CKAP2 does not appear to directly cause pRb phosphorylation or p27 reduction. Moreover, the constitutive expression of TMAP/CKAP2 did not induce pRb phosphorylation and had no effect on p27 in G0/G1-arrested HFF cells (unpublished observations), and hence did not induce the further proliferation of HFFs after they had reached confluence (Fig. 3). Therefore, there is a possibility that the observed changes in the levels of pRB and p27 are the consequences of G1 progression promotion by TMAP/CKAP2.

Since TMAP/CKAP2 co-localizes with microtubule and has a microtubule stabilizing effect [1,4], the effect of TMAP/CKAP2 on cell proliferation could be due to its effect on the cytoskeleton. The activation of the Ras-ERK pathway by growth factor receptors and integrin is a key for G1 progression into the S phase [reviewed in 14]. However, cell shape-dependent growth studies [15,16] and cytoskeleton disruption studies suggest that the

cytoskeleton should be intact for cells to pass through the restriction point and enter the S phase [17–19]. Therefore, the microtubule-stabilizing effect of TMAP/CKAP2 could have some role in cell cycle progression, but this possibility remains to be elucidated.

Recently, Tsuchihara et al., reported that TMAP/CKAP2 expression is p53 dependent, and that TMAP/CKAP2 over-expression activates p53-mediated cell cycle arrest [20]. These results contradict our results and previous reports, which have demonstrated TMAP/CKAP2 up-regulation in human cancers [1–3]. We also observed that TMAP/CKAP2 was highly expressed in UV-irradiated cells. However, this phenomenon was observed even in p53-deficient HeLa cells; moreover, the introduction of p53 did not result in a further TMAP/CKAP2 increase in HeLa cells (unpublished observations). Since the expression of TMAP/CKAP2 peaks in the G2/M phase, we presume that TMAP/CKAP2 accumulates in UV-irradiated cells, because most of the cells are arrested at the G2/M phase. p53 overexpression could induce the accumulation of TMAP/CKAP2 by blocking the cell cycle at G2/M, and there is a possibility that the excessive expression of TMAP/CKAP2 could disrupt the cell cycle by stabilizing cytoskeletal structures.

Acknowledgments

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