

HP33: hepatocellular carcinoma-enriched 33-kDa protein with similarity to mitochondrial N-acyltransferase but localized in a microtubule-dependent manner at the centrosome

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Accepted 18 February; published on WWW 8 April 1999

SUMMARY

Using a new subtraction method and chemically induced rat hepatocellular carcinomas, we identified a hepatocellular carcinogenesis and hepatocyte proliferation-related gene designated *hp33* that encoded a 33-kDa protein. The predicted protein was similar to the bovine aralkyl N-acyltransferase and arylacetyl N-acyltransferase. HP33 was restrictively expressed in the liver and kidney, and its gene expression was stimulated in the regenerating liver as well as in hepatocellular carcinoma. Interestingly, it was demonstrated in various hepatic cells that HP33 was localized in regions surrounding the centrosome, where mitochondria were not concentrated. Moreover, its centrosomal localization was evident in the interphase but not in the mitotic phase of the cell cycle. The centrosomal localization of HP33 was dependent on microtubules, and ectopically expressed HP33 was seen at centrosomes even

in fibroblasts, which do not exhibit a typical staining pattern of HP33. The centrosomal localization of HP33 became invisible by nocodazole treatment, whereas the mitochondrial staining pattern was not affected by it. In vitro cosedimentation experiments using purified microtubules indicated that HP33 bound to MTs directly and that its MT-binding ability was dependent on the C-terminal basic domain of the protein. These results suggest that, different from early predictions based on its primary structure, HP33 has a growth- and carcinogenesis-related function that may be independent of mitochondrial function.

Key words: Hepatocellular carcinogenesis, Liver, Mitochondria, N-acyltransferase, Centrosome, Cell cycle, Microtubule, MAP

INTRODUCTION

The regulatory mechanism of ordered cell proliferation and cell cycle progression of eukaryotes is a current central subject in cell biology. The identification of factors related to this mechanism is most critical to an understanding of the above cellular behaviors, and many functional molecules including kinases, phosphatases, cyclins and check point factors have been discovered so far. However, despite the uncovering of many regulatory factors related to cell proliferation, it is still unclear what the related input (signal-generating factors) and final output (effector apparatus) molecules are. The centrosome is the most typical cellular apparatus governing cell cycle progression. The centrosome functions as a major microtubule (MT) organizing center (MTOC) of interphase and mitotic cells. The mammalian centrosome consists of a centriole and pericentriolar matrix (PCM) (Lange and Gull, 1996). The

centrosome is the nucleus of MT arrays that provide a wide variety of cellular processes, including the mechanical scaffolds of a cell, rails for vesicle transport and mitotic apparatus (Kellogg et al., 1994). Many kinds of structural proteins, MT-based motor proteins, microtubule-associated proteins (MAPs) and their regulatory factors were found to be related to centrosome/MT-systems (Paoletti and Bornens, 1997). Their ability to participate in centrosome/MT organization has been well characterized in cell-free systems and by mutation analysis. However, except for the conventional MAPs observed in nervous systems and centrosomal protein identified from the analysis of *Drosophila* mutants (Gonzalez et al., 1998; Kobayashi and Mundel, 1998), little is known about centrosomal proteins and MAPs, especially their relationship to cell proliferation and tissue differentiation.

We have been searching for molecules related to cell growth and carcinogenesis in the rat liver. The rat liver has several

advantages for this purpose, for example, hepatocytes do not usually proliferate, but they do enter into a single-round cell cycle (liver regeneration) following partial hepatectomy. Moreover, the liver is very convenient for reproducibly generating experimental hepatocellular carcinomas (HCC) (Solt and Farber, 1976). Earlier, we established a subtracted cDNA library derived from rat HCC (Kishimoto et al., 1996), in which subtraction had been conducted by use of normal and HCC-derived mRNAs. Thus, this library was assumed to include concentrated genes whose expressions were stimulated by hepatocarcinogenesis. We have isolated several carcinogenesis- and growth-related genes, including an enzyme, transcription factor and putative membrane protein (Kishimoto et al., 1996, 1998; Nakadai et al., 1998). Thus, isolation of novel genes by this method may provide new insight into mechanisms relating to proliferation, differentiation and carcinogenesis of hepatocytes.

In this work, we screened the subtracted HCC-derived cDNA library and identified a gene, named *hp33*, which encoded a protein that had a similarity to bovine N-acetyltransferase (Vessey and Lau, 1996, 1998), a mitochondrial enzyme. Contrary to their structural similarities, HP33 protein was observed at the perinuclear centrosome in hepatic cells during interphase. Moreover, localization of HP33 at the centrosome was dependent on the MT network, and the protein bound directly to MTs in vitro.

MATERIALS AND METHODS

Preparation of RNAs, cDNA library and rat hepatocellular carcinomas

The method used to prepare poly(A)⁺ RNAs and the cDNA library from normal liver and hepatocellular carcinomas (HCC) of rats was described previously (Kishimoto et al., 1996). Rat HCC was generated by the Solt-Farber method (Solt and Farber, 1976) with a few modifications (Kishimoto et al., 1996). The Solt-Farber procedure was initiated by an intraperitoneal injection of diethylnitrosamine (DEN), oral administration of 0.02% aminoacetyl fluorene (AAF), and partial hepatectomy. Liver samples were extirpated and stored in liquid nitrogen. In most cases, such rats die of HCC within 8 months after the DEN injection. All animal treatments were performed in accordance with the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education.

Northern blot hybridization

300 ng of poly(A)⁺ RNAs were used for northern hybridization. The hybridization conditions and the amount of cDNA probes were described previously (Kishimoto et al., 1996). Poly(A)⁺ RNAs from various tissues were blotted onto Multiple Tissue Northern (MTN) membrane (purchased from Clontech) and hybridization was carried out as recommended by the supplier.

Purification of full-length soluble HP33 for biochemical assay

The whole open reading frame of *hp33*, to which a histidine tag had been attached at the N terminus, was subcloned into pFastBac (Gibco) and expressed in Sf9 cells by use of the pFastBac Baculovirus Expression System (Gibco). Cells infected with histidine-tagged HP33 (His-HP33)-expressing virus were harvested, and the soluble His-HP33 was purified by Ni-NTA agarose (Qiagen). Purified His-HP33 was further purified by MonoS followed by MonoQ (Pharmacia). 0.5 mg of His-HP33 protein of >98% purity was finally obtained.

Polyclonal antibody against HP33

Histidine-tagged amino terminus (amino acids 1-185)-truncated HP33 (His-ΔHP33) was overexpressed in *E. coli* by use of the pET vector system (Studier et al., 1990). Insoluble His-ΔHP33 was purified by Ni-NTA agarose under denaturing conditions according to the user's manual, dialyzed against water, and used to generate polyclonal antibody in rabbits. The antibody was affinity-purified by the following procedure. The insoluble His-HP33 in HP33-expressing Sf9 cell lysates (see above) was purified under denaturing conditions as described above. The purified insoluble His-HP33 was subjected to a preparative SDS-PAGE, and the protein was further purified by electroelution. The purified His-HP33 was immobilized on a HiTrap column (Pharmacia), and anti-HP33 polyclonal antibody was purified by use of this His-HP33-conjugated column according to the user's manual.

Protein extraction and western blotting

Livers were homogenized with a Teflon homogenizer in a equal volume of 2× SDS sample buffer. After centrifugation at 15000 *g* for 30 minutes at room temperature, the supernatant fractions were recovered. The protein concentration of these extracts was determined by CBB-staining of protein-blotted 3MM paper with bovine serum albumin (BSA) used as a standard. HP33 in the extract was detected by the standard western blotting technique as described previously (Kishimoto et al., 1996).

Cells and transfection

The RL34 cell line (Yamada et al., 1987) was established from normal rat liver. LiNM cells (T. Sakamoto, personal communication) were established from human HCCs. 3Y1 is a rat fibroblastic cell line. LiNM and other cell lines were grown in RPMI1640 (Gibco) and Dulbecco's Modified Minimal Essential Medium (MEM) (Gibco), respectively, and both media were supplemented with 10% fetal calf serum.

An HP33-expression plasmid (pOP_{hp33}) for animal cells under the regulation of LacSwitch Inducible Mammalian Expression System (Stratagene) was constructed from the pOP13 vector. Cells were cotransfected with pOP_{hp33} and the p3'SS *lac* repressor expression plasmid by use of LIPOFECTIN (Gibco). 2 days after transfection, culture media were replaced by fresh media containing 400 µg/ml hygromycin and 800 µg/ml Geneticin (Gibco). After isolation of stable transformants, HP33 was overexpressed in the transformants by treatment with 5 mM isopropyl β-D-thiogalactopyranoside (IPTG). We obtained one stable transformant, HP33/3Y1, which was derived from 3Y1.

Indirect immunofluorescence staining

Indirect immunofluorescence staining was performed using 1:200-diluted affinity-purified HP33 antibody, 1:2000-diluted anti-α-tubulin monoclonal antibody (Sigma), 1:100-diluted anti-β-tubulin monoclonal antibody (Sigma), and 1:2000-diluted anti-γ-tubulin monoclonal antibody (Sigma). Cells on a glass coverslip were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, 5 mM EGTA, and 2 mM MgCl₂ for 8 minutes, treated with 0.5% Triton X-100 (or 1.0% Triton X-100 for anti-γ-tubulin monoclonal antibody) for 5 minutes and blocked with 1% BSA. Then, the cells were treated with the primary antibody in 1% BSA. 100-fold-diluted (in 1% BSA) FITC-conjugated donkey anti-rabbit IgG (H+L) (Jackson) or Texas Red-conjugated sheep anti-mouse IgG (H+L) (Jackson) was used for the second antibody. Finally, the cells were stained with 1 µg/ml of DAPI (Wako), mounted with 90% glycerol containing 1 µg/ml of *p*-phenylenediamine, and visualized under a fluorescence microscope (Axioskop, Zeiss). When MTs were to be depolymerized, cells were exposed to 2 µg/ml of nocodazole and subsequently incubated at 37°C for 4 hours just before fixation.

Mitochondria staining

For staining mitochondria, the medium was replaced by a fresh

medium containing 200 nM Mitotracker (Molecular Probes), and the cells were incubated at 37°C for 45 minutes just before the fixation. These Mitotracker-treated cells were subsequently immunostained as described above.

Preparation of tubulin solution

Bovine tubulin was purchased from Sigma. Tubulin solution was prepared according to the user's manual and stored at -80°C. MAP-free tubulin was freshly prepared from the whole brain of 10 rats according to the method described previously (Maekawa et al., 1991). Purified rat tubulin was stored at -80°C.

Microtubule-binding assay

For the MT-binding assay, we used the MT spin-down method described by Raff et al. (1993) with a few modifications. 20 µl (about 100 µg protein) of stocked bovine tubulin was used for one assay. Five volumes of buffer A (0.1 M MES, pH 6.5, 1 mM EGTA, 0.5 mM MgCl₂, 2.5 M glycerol, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 1 µg/ml aprotinin) was added to the tubulin solution, and the mixture was incubated on ice for 30 minutes. After centrifugation at 15000 g for 30 minutes at room temperature, the supernatant was added to 0.01 volumes of 100 mM GTP and 20 µg/ml taxol (Wako) and incubated at 37°C for 30 minutes to polymerize the tubulin. Polymerized tubulin (i.e. microtubules, MTs) were collected by centrifugation, resuspended in CX buffer (50 mM Hepes/KOH, pH 7.6, 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1 mM DTT, 0.1% NP40, 1 mM benzamidine-HCl, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 0.2 µg/ml taxol and 1 mM GTP) in the presence of the appropriate concentration of KCl, and centrifuged again. Alternatively, rat MT was prepared from 100 µg of rat tubulin by the same protocol using 2 volumes of PEM buffer (10 mM Pipes, pH 6.8, 0.1 M KCl, 0.5 mM MgCl₂ and 1 mM EGTA) instead of 5 volumes of buffer A. HP33 for the MT-binding assay was diluted with CX buffer with an appropriate concentration of KCl, incubated at 37°C for 30 minutes, and centrifuged at 15000 g for 30 minutes at room temperature to obtain 100 ng of HP33 protein/50 µl CX buffer. Finally, prepared MT and HP33 were mixed and incubated at 37°C for 30 minutes. After centrifugation at 15000 g for 30 minutes at room temperature, the supernatant was collected and added to 0.24 volumes of 5× SDS sample buffer, whereas pellets were resuspended in CX buffer. The resuspended pellets were centrifuged again and MT-pellets were obtained.

For gel filtration assays, 60 µl of bovine tubulin solution was added to 5 volumes of buffer A, centrifuged, and polymerized by essentially the same protocol used for the cosedimentation experiment. The pellets were resuspended well in 100 ml CX(-) buffer (CX buffer omitting taxol and GTP) on ice. This protein solution was mixed with 1 µg HP33 in 100 µl CX(-) buffer, and dialyzed against the same buffer. After centrifugation at 15000 g for 30 minutes at 4°C, the supernatant was subjected to gel filtration of Superdex-200 (Pharmacia). Eluates made with CX(-) buffer were fractionated and added to 0.25 volumes of 5× SDS sample buffer. In this procedure, KCl was not added to the CX buffer. The molecular mass was estimated with molecular mass markers (Pharmacia). Samples obtained in the cosedimentation assay and gel filtration were subjected to SDS-PAGE followed by immunoblotting.

In vitro translation and HP33-microtubule cosedimentation assay

Deletion mutants of HP33 were constructed by standard molecular cloning techniques and PCR. *Hp33* sequences including deletion versions lacking the histidine-tag were subcloned downstream of the T7 promoter of pBluescript(SK⁺) (Stratagene). Recombinant proteins were expressed using 1 µg template DNA and [³⁵S]methionine (Amersham) by in vitro translation using the TNT T7 Quick Coupled Transcription/Translation System (Promega). 10 µl of the reaction mixture was withdrawn and mixed with 50 µl CX buffer containing

0.1 M KCl; the mixture was then incubated at 37°C for 30 minutes. After centrifugation at 15000 g for 30 minutes at room temperature, the supernatant was used for the cosedimentation assay. Methods for preparation of the bovine MT pellets and cosedimentation experiment were basically the same as those described above. Proteins were measured with an RI imaging analyzer (BAS 1000, Fuji Film).

RESULTS

Identification of *hp33* gene

Previously, we identified one rat cDNA clone, named *no.73*, in the subtracted cDNA library. The subtraction was performed between normal liver and Solt-Farber protocol-driven hepatocellular carcinomas (HCC), and the library contained concentrated hepatocellular carcinoma-stimulated genes. Using the *no.73* clone as a probe, we screened the HCC-derived cDNA library and obtained one clone (*no.73-11*). Northern blot analysis revealed that *no.73-11* gene expression was low in normal liver but high in HCCs (Fig. 1). The size of the transcript was estimated to be about 2.0 kb. Expression of the *no.73-11* gene was stimulated over tenfold in HCC liver compared with that in the normal liver. Since the original *no.73-11* clone was only partial, we further screened the library and obtained one longer cDNA of 2279 bp, whose size was nearly consistent with that of its mRNA. Sequencing of this clone revealed one long open reading frame encoding 295 amino acids: a 33-kDa polypeptide (Fig. 2).

Homology search of the predicted amino acid sequence encoded by this gene revealed that the 33-kDa protein was similar to bovine aralkyl N-acyltransferase (ArAlk) (Vessey and Lau, 1996) and arylacetyl N-acyltransferase (AAc) (Vessey and Lau, 1998), which are known to be mitochondrial enzymes (Fig. 2). The predicted 33-kDa protein had 67% identity with AAc and 66% identity with ArAlk. However, at present, we cannot designate the 33-kDa protein as an N-acyltransferase because the sequence identity is not so high and the enzyme activity has not been detected yet. Moreover, the 33-kDa protein did not exhibit a mitochondrial localization pattern (see below). In this communication, we have named

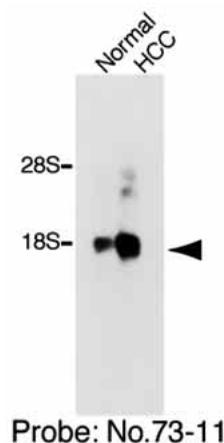


Fig. 1. Northern blot analysis of HCC-related subtracted clone *no.73-11*. 1 µg of poly(A)⁺ RNA from normal liver or HCC cells was analyzed with *no.73-11* probe. The positions of 18S and 28S rRNAs are indicated.

chemical hepatocarcinogenesis (Fig. 3B). In this assay, we used relatively small amounts of poly(A)⁺ RNA. A basal level of HP33 gene expression was observed in the normal liver (Fig. 3B, lane 1). After injection of the tumor initiator diethylnitrosamine (DEN), HP33 gene expression still remained at the initial level (Fig. 3B, lanes 2-4). However, during continuous administration of the tumor promoter aminoacetyl fluorene (AAF) after DEN injection, HP33 gene expression was remarkably stimulated (about 5-10 times), and this elevated level lasted for at least 7 months (Fig. 3B, lanes 5-8). In the course of the Solt-Farber protocol, numerous transformed hepatocytes, which represent precancerous cells, appear in the liver in a few weeks after DEN injection. These cells form hyperplastic nodules, become hepatomas, and finally occupy the whole liver in the 7th month. In the liver regeneration process, HP33 gene expression was not induced by 24 hours after partial hepatectomy but was dramatically stimulated (about 15 times) by 48 hours after surgery (Fig. 3B, lanes 9-11). These results indicate that HP33 gene expression is enhanced in accordance with hepatic growth as well as hepatocarcinogenesis.

We performed western blotting of rat liver cell extracts using the HP33 antibody, which had been confirmed to be specific for HP33 (data not shown). Western blot analysis detected one polypeptide with an apparent molecular mass of 33 kDa (Fig. 3C). This value coincided well with that predicted from the cDNA sequence. These results indicate that the HP33 protein actually exists in the liver. We found that the amount of HP33 in HCC was more than five times higher than that in the normal liver (Fig. 3C). This result strongly suggests that the HP33 protein overproduces during hepatocarcinogenesis, and the pattern fits well with those of northern blotting presented in Figs 1 and 3B.

Intracellular localization of HP33

The amino acid N-acyltransferases are mitochondrial enzymes and have significant similarity with HP33 (Fig. 2). Immunocytochemical analysis of the rat normal liver-derived cell line RL34 was performed (Fig. 4). RL34 cells in interphase were double-stained with a mitochondria-specific dye and anti-HP33 antibody. Mitochondria were observed as short, thick fibers and were distributed throughout the cytoplasm (Fig. 4A). On the contrary, anti-HP33 antibody did not stain the fibrous mitochondria but did label the perinuclear region as diffuse foci (Fig. 4B). Every cell contained a single focus beside its nucleus. Control IgG did not stain anywhere in RL34 cells (data not shown). The localization of anti-HP33 antibody-stained foci coincided with that predominantly stained by the anti- β -tubulin antibody (Fig. 4C,D). We further carried out immunostaining of RL34 by using both anti- γ -tubulin and anti-HP33 antibodies. Gamma-tubulin is one of the authentic centrosome-localizing proteins and forms an MTOC (Pereira and Schiebel, 1997). We found that HP33 sites were exactly the same as those of γ -tubulin (Fig. 4F,G). Although the region detected by the anti-HP33 antibody was slightly diffuse around the centrosome, the prominently stained regions appeared to be coincident with the foci stained by anti- γ -tubulin. We suggest that HP33 is localized at the centrosome. Accordingly, these data are not consistent with an earlier suspicion that this novel gene encodes a mitochondria-specific enzyme, as the staining pattern of HP33 did not indicate colocalization with mitochondria.

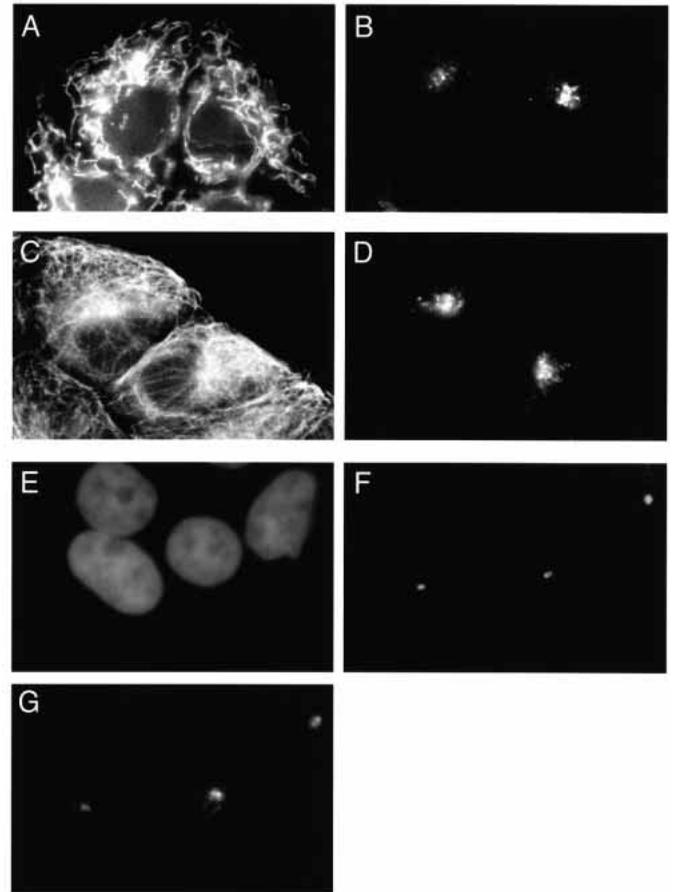


Fig. 4. Subcellular localization of HP33 in liver-derived RL34 cell. HP33 was detected by indirect immunostaining using anti-HP33 antibody (B,D,G). (A,B, C,D and E-G) Observations of the identical cell population in each of the three sets of panels. Mitochondria were stained with Mitotracker (A). Beta- (C) and gamma-tubulins (F) were detected with corresponding antibodies. DNA was stained with DAPI (E).

Cell cycle-dependent localization of HP33

Hp33 was identified as a gene whose expression varied depending on hepatocyte growth as well as on hepatocarcinogenesis (Fig. 3B). Next, we addressed the question as to whether HP33 localization is altered during cell-cycle progression. We performed immunocytochemistry for HP33 and tubulin in RL34 at different stages of the cell cycle (Fig. 5). HP33 was observed as foci localizing at the centrosome in interphase (Fig. 5A-C). At prophase, when the centrosome is segregated, HP33 was observed to spread around the duplicated centrosomes (Fig. 5D-F). The HP33 fluorescence expanded and its intensity became rather pronounced (Fig. 5F versus C). HP33 foci became invisible in metaphase and in anaphase cells (Fig. 5I,L). At telophase, however, HP33 appeared at the centrosomal position again (Fig. 5O). Localization of γ -tubulin was manifestly detected in the centrosome even in anaphase cells (Fig. 5Q), whereas the anti-HP33 antibody did not show the dot-like staining pattern in identical cells (Fig. 5R). Our results demonstrate that the centrosomal localization of HP33 depends on the phase of the cell cycle.

Centrosomal localization of HP33 in other cells

HP33 was observed tissue specifically (Fig. 3A) and was localized at the centrosome, at least in rat normal liver-derived RL34 cells (Fig. 4). We investigated how HP33 was localized in other cells, i.e. human hepatocarcinoma-derived LiNM cell lines (Fig. 6A-C) and rat fibroblast 3Y1 (Fig. 6D-F). The anti-HP33 antibody was confirmed to crossreact with the human counterpart antigen (data not shown). We found that HP33 was also localized at the centrosome in LiNM cells where MTs were nucleated (Fig. 6C). Gamma-tubulin was colocalized with HP33, and control IgG did not stain these cells (data not shown). These results indicate that the *hp33* gene is also expressed in human hepatoma cells and is concentrated at the

centrosome. The anti-HP33 antibody did not significantly stain the rat fibroblast 3Y1 (Fig. 6F). On the contrary, the anti- α -tubulin antibody stained MT arrays of these cells (Fig. 6E). We also tested other cell lines and found that the centrosome-localizing pattern was specific to hepatic cells (data not shown).

Ectopic expression of HP33 and its intracellular localization

We established 3Y1-derived HP33/3Y1 cells in which the *hp33* gene had been stably integrated and can be induced by IPTG by the *lac* switch system (see Materials and methods). IPTG treatment of HP33/3Y1 cells for 48 hours remarkably enhanced

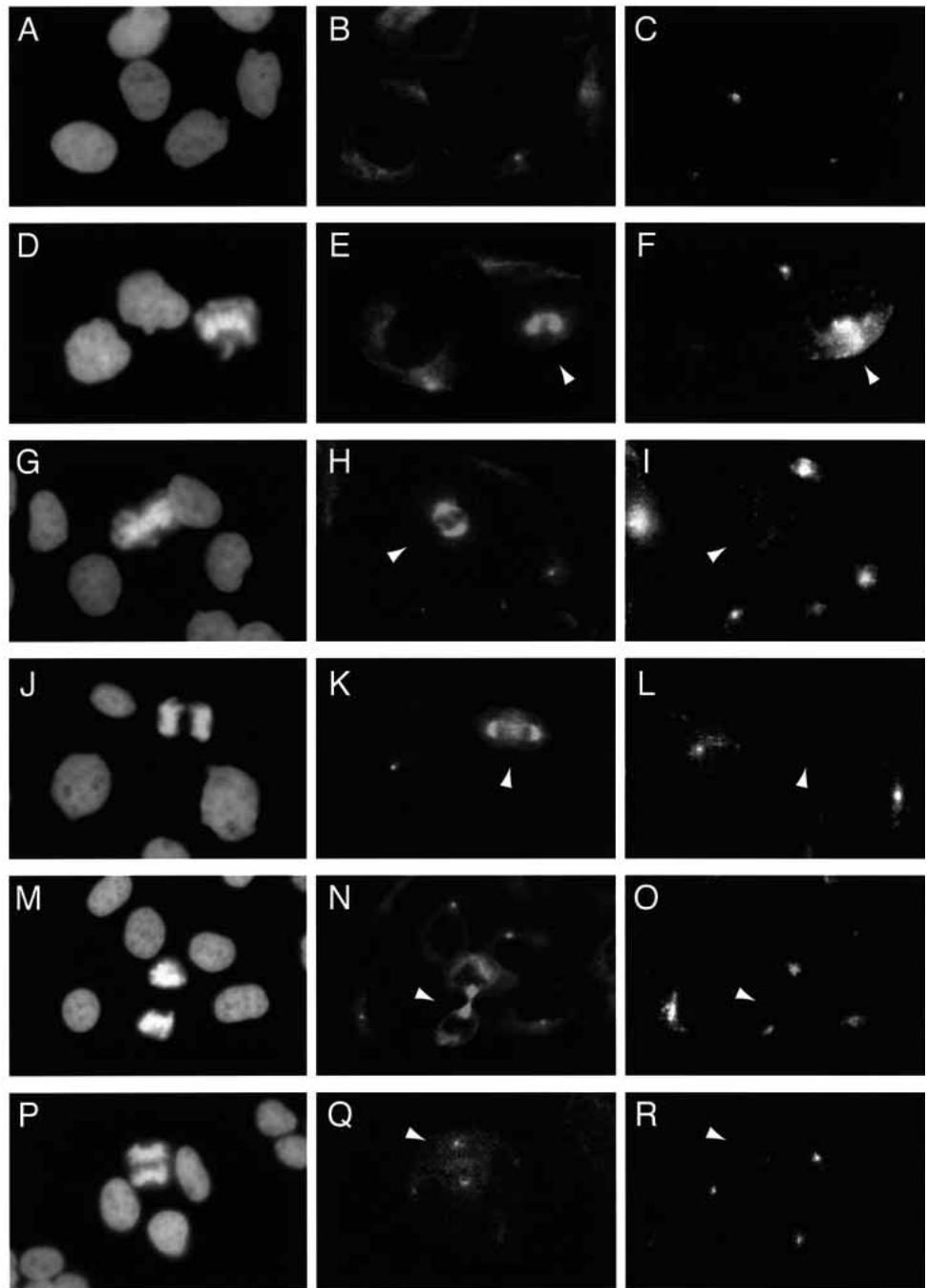


Fig. 5. Transition of HP33 localization in the cell cycle. HP33 (C,F,I,L,O,R) DNA (A,D,G,J,M,P), α -tubulin (B,E,H,K,N), and γ -tubulin (Q) in RL34 cells were visualized as described in Fig. 4. (A-C) Interphase; (D-F) prophase; (G-I) metaphase; (J-L and P-R) anaphase; (M-O) telophase. Cells that reside in an assigned cell cycle are indicated by arrowheads.

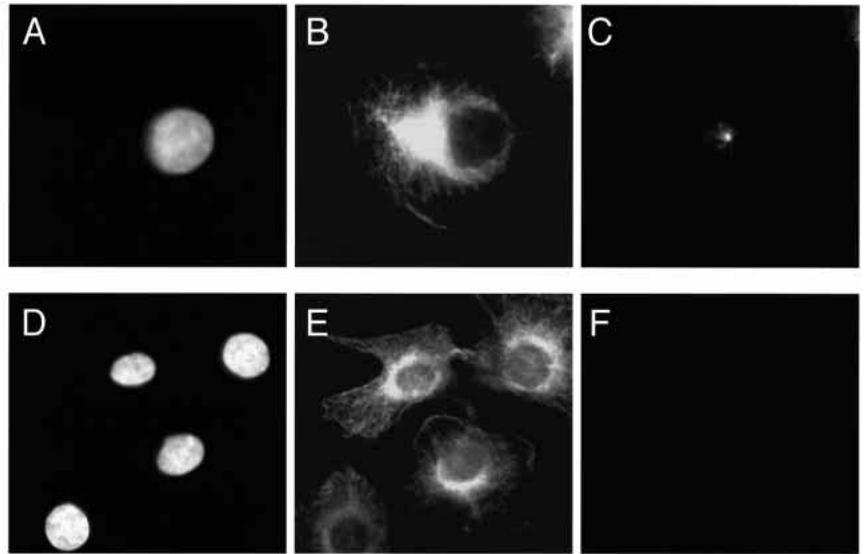


Fig. 6. Immunofluorescence assay for HP33 in other cells. HP33 in LiNM (A-C) and 3Y1 (D-F) cells was detected as described in Fig. 4. (C,F) HP33; (A,D) DNA; (B,E) α -tubulin.

expression of the HP33 protein (data not shown). Fig. 7 shows the double-staining pattern after induction. In contrast to parental 3Y1 cells (Fig. 6D-F), HP33 proteins in HP33/3Y1 were clearly recognized to be localized at a single region in the cell (Fig. 7B, open arrowhead), the position being the same as that of the MT-condensed centrosome (Fig. 7A). In these HP33-overexpressing 3Y1 cells, we observed that some MT arrays were more condensed in regions of the cytoplasm other than the centrosome (Figs 7A versus 6E) and that some HP33 proteins were present along with these disorganized MT arrays (Fig. 7B, solid arrowheads). Consequently, we suggest that HP33 is intrinsically capable of localizing in the centrosome regardless of the cell type.

Microtubule-dependent localization of HP33

To obtain more solid data about the relationship between HP33 and MTs, we used a microtubule-depolymerizing agent, nocodazole. After incubation of RL34 for 3 hours with nocodazole, most of the MTs were almost completely depolymerized (Figs 8A versus 4C). In identical cells, the centrosomal localization of HP33 disappeared (Figs 8B versus 4B,D), whereas the mitochondrial staining pattern was unaffected (Figs 8C versus 4A). In the treated cells, γ -tubulin persisted in its centrosomal localization pattern (data not shown). These results indicate that the centrosomal

localization of HP33 is dependent on a nocodazole-sensitive MT network.

Microtubule-binding ability of HP33

The above results prompted us to test a direct interaction between HP33 and MT or tubulin. We performed an *in vitro* MT-binding assay using highly purified soluble (avoiding denaturing treatments) recombinant HP33 and bovine MT (Fig. 9). First, we examined HP33-MT interaction by a cosedimentation assay under various ionic strength conditions (0-0.5 M KCl). Supernatant and pellet fractions were analyzed by SDS-PAGE followed by western blotting with anti-HP33 antibodies (Fig. 9A). HP33 coprecipitated with MTs even in 0.5 M KCl (Fig. 9A, lanes 3, 6 and 9). HP33 was not precipitated in the absence of MTs (Fig. 9A, lane 21). We further performed similar experiments using highly purified rat brain tubulin (Fig. 9A, lanes 10-18) and confirmed that HP33 coprecipitated together with rat MT at any KCl concentration used.

The above experiments did not exclude the existence of either polymerized HP33 or tubulin heterodimer-bound HP33. Moreover, it is interesting to analyze the molecular states of MTs involved in HP33 binding. We mixed MT with HP33, centrifuged it at a low speed, and then prepared supernatants that we expected to contain unpolymerized and non-

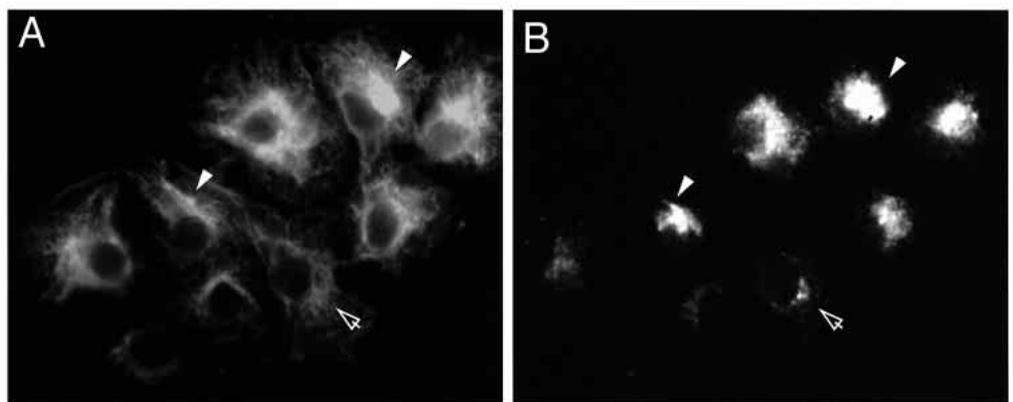


Fig. 7. Overexpression of HP33 and its cellular localization. HP33-overexpressing HP33/3Y1 cells were analyzed and observed by a fluorescence microscope. (A) α -tubulin; (B) HP33.

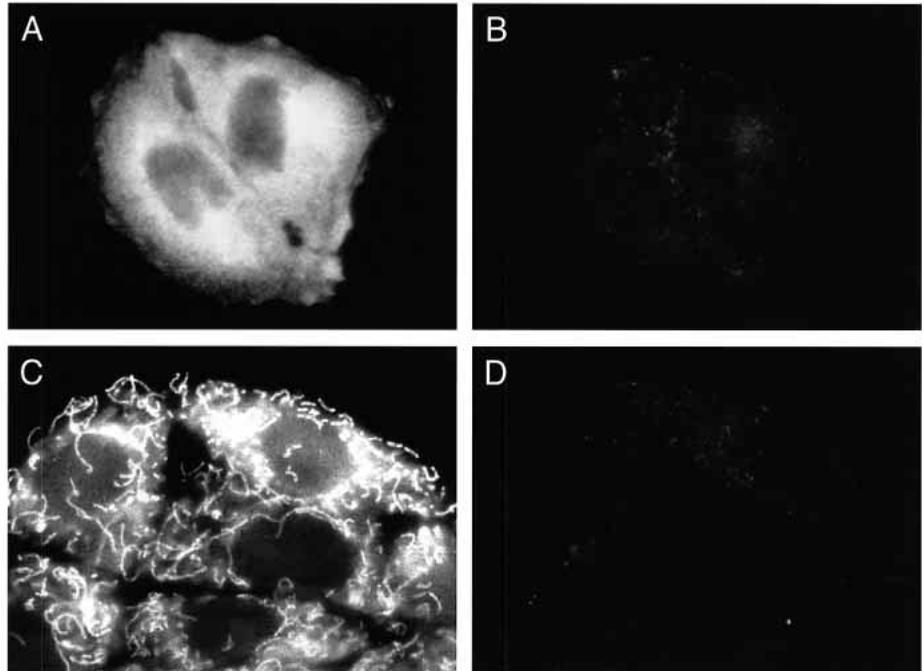


Fig. 8. Nocodazole treatment of RL34. RL34 cells were incubated with nocodazole-containing media to depolymerize the MT array (see Materials and methods). Cells were subsequently double-labeled with anti- β -tubulin (A) and anti-HP33 antibody (B), or with mitochondrial dye (C) and anti-HP33 antibody (D).

sedimenting small MTs. This supernatant was subjected to gel filtration. In a control experiment without tubulin, HP33 alone was eluted in fractions corresponding to 30 kDa, which just fits the monomer size of HP33 (Fig. 9Ba), indicating HP33 is present as a monomer. On the other hand, we found that the supernatant contained small-sized MTs (eluted in the $200 \rightarrow 2 \times 10^3$ kDa/void fractions) and dimer tubulins (eluted at around the 100-kDa fractions) (Fig. 9Bb) when the tubulin sample alone was used for the assay. Next, we analyzed an HP33-tubulin mixed sample (Fig. 9Bc). HP33 was coeluted only with slightly polymerized MTs. The fractions corresponding to tubulin dimers did not contain HP33 (Fig. 9Bc). These HP33 elution profiles were completely different from those obtained with HP33 alone. The gel-filtration assays used highly purified HP33 and tubulin, thus suggesting that HP33 binds to intact MT but not to the tubulin dimer.

Domain analysis of HP33 for microtubule-binding

We constructed six HP33 deletion mutants to delineate the MT-binding region (Fig. 10A). The constructs were subjected to a transcription/translation-coupling reaction. In vitro translation products were labeled with [35 S]methionine, and the same amount of crude translation products was mixed with purified MT and subjected to cosedimentation analysis. As stated in the figure legends, the proportion of the sample was analyzed for input or bound HP33 (Fig. 10B). All truncated versions exhibited MT-binding ability (70%) similar to that of the full-length protein except for the Δ C-Half mutant (Fig. 10C). Around 80% of the binding ability was lost in Δ C-Half, a mutant containing only the N-terminal 82 amino acids. C-Fos, as a negative control, did not bind to MT (only 20% of the binding obtained with the wild-type HP33), like the Δ C-Half (data not shown). These data suggest that the central and C-terminal domains of HP33 function independently for the MT-binding and that the MT-binding requires a particular protein module.

DISCUSSION

HP33 is a novel protein having a similarity to N-acyltransferase

We identified one gene designated *hp33* in the subtracted cDNA library in which rat HCC-related genes were supposed to be concentrated. The structure of HP33 was very similar to that of bovine amino acid N-acyltransferase (Vessey and Lau, 1996, 1998) (Fig. 2B). N-acyltransferases are present in the mitochondrial matrix space of the liver and kidney, and they generate an amino acid conjugate of carboxylic acid from CoA-thioester and amino acid (Vessey, 1997). In the present study, we demonstrated that HP33 was not localized at mitochondria but in a slightly diffused region including the centrosome (Fig. 4). Ectopically expressed HP33 was also localized at the centrosome, even in non-hepatic cell lines (Fig. 7). Additionally, the centrosomal localization of HP33 disappeared with nocodazole treatment of cells, though the intracellular distribution of mitochondria did not change (Fig. 8). Localization of mitochondria is dependent on various cytoskeletal apparatuses in addition to MTs (Langford, 1995; Kloc and Etkin, 1995; Krendel et al., 1998). The present results almost excluded the possibility that mitochondria not detectable by Mitotracker existed around the centrosome. We therefore conclude that the centrosomal HP33 is not present in the mitochondria matrix.

It remains to be clarified whether or not HP33 is a rat homologue of N-acyltransferase. However, even though HP33 has been shown to have N-acyltransferase activity, centrosomal HP33 is not likely to be responsible for detoxification, because the detoxification reaction of carbonic acids also requires closely related sequential enzymatic reactions by mitochondrial matrix proteins (Vessey, 1997). Mitochondrial enzymes have been demonstrated to have other biogenetic functions in addition to their mitochondrial function (Zorov et al., 1997; Francavilla et al., 1997; Lisowsky, 1996). For

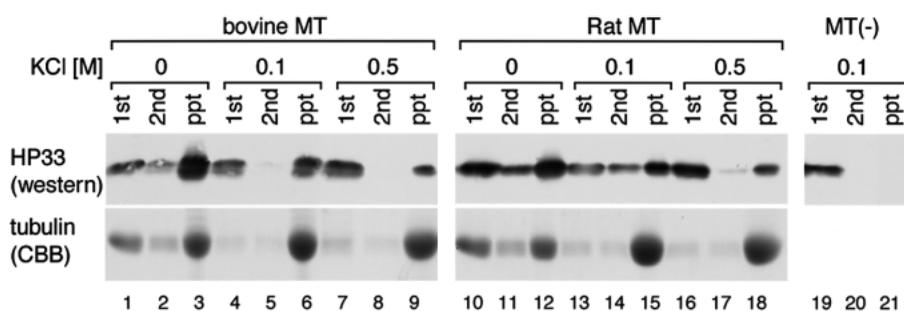
example, cytochrome *c* in the cytoplasm was suggested to activate caspase in the apoptotic signaling pathway (Li et al., 1997). Hence, HP33 might be an example of such proteins.

Microtubule-binding ability of HP33

The results of this study clearly showed that HP33 cosedimented and formed a complex with MTs in vitro (Fig. 9), because we used MAP-free MTs and pure recombinant HP33 (>98%) in the MT-binding assay. Thus, it can be concluded that HP33 binds directly to MT. The results also suggested that the MT-binding strength of HP33 is considerably stronger than that of other MAPs, because HP33 cosedimented with MT even in 0.5 M KCl (Fig. 9A, lanes 7-9

and 16-18). Fig. 10B,C shows that only Δ C-Half drastically lost the original MT-binding ability. Since Δ N had a nearly intact binding activity, we suggest that the N-terminal 82 amino acids were not critical for MT-binding. Because two domains of HP33, amino acids 83-175 and 170-295, still had significant intrinsic MT-binding activity, these two domains most likely function independently. Although these positions had no similarity to known MAPs, we suppose that MT-binding ability of HP33 may be attributed to a bulk basic atmosphere in the protein. It has been reported that a basic region was necessary for MT-binding of *Drosophila* 205-kDa MAP (Irminger-Finger et al., 1990). Indeed, the pI value of the N terminus (Δ C-Half) was specifically low, 5.8 (Fig. 10A).

A Co-sedimentation



B Gel filtration

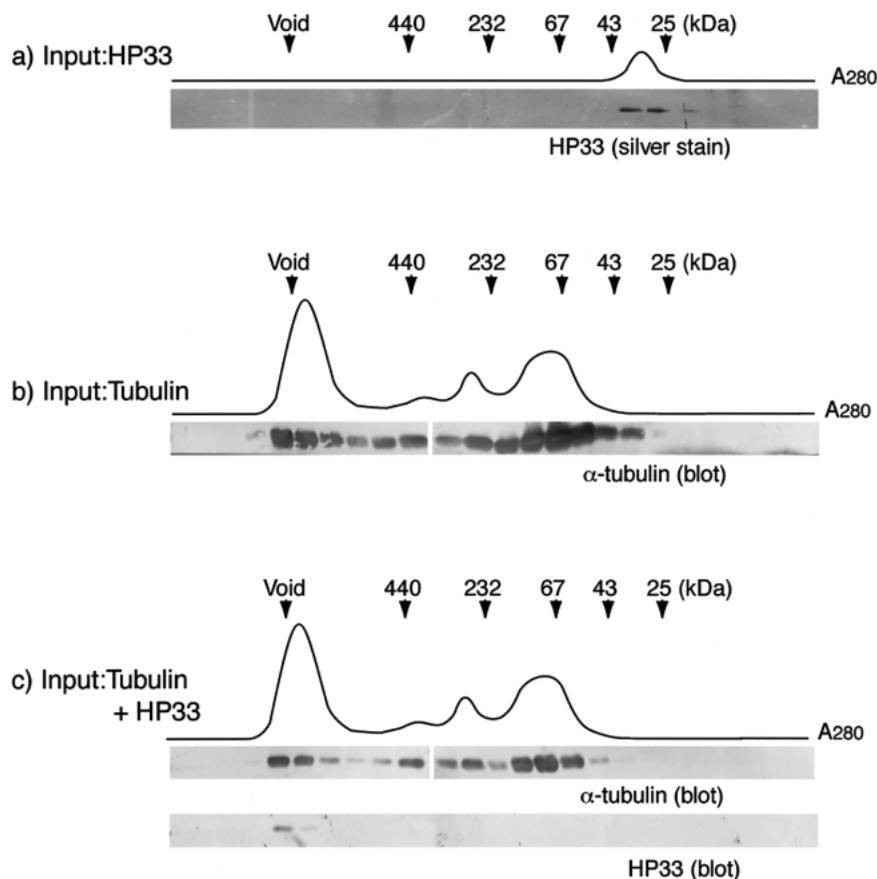
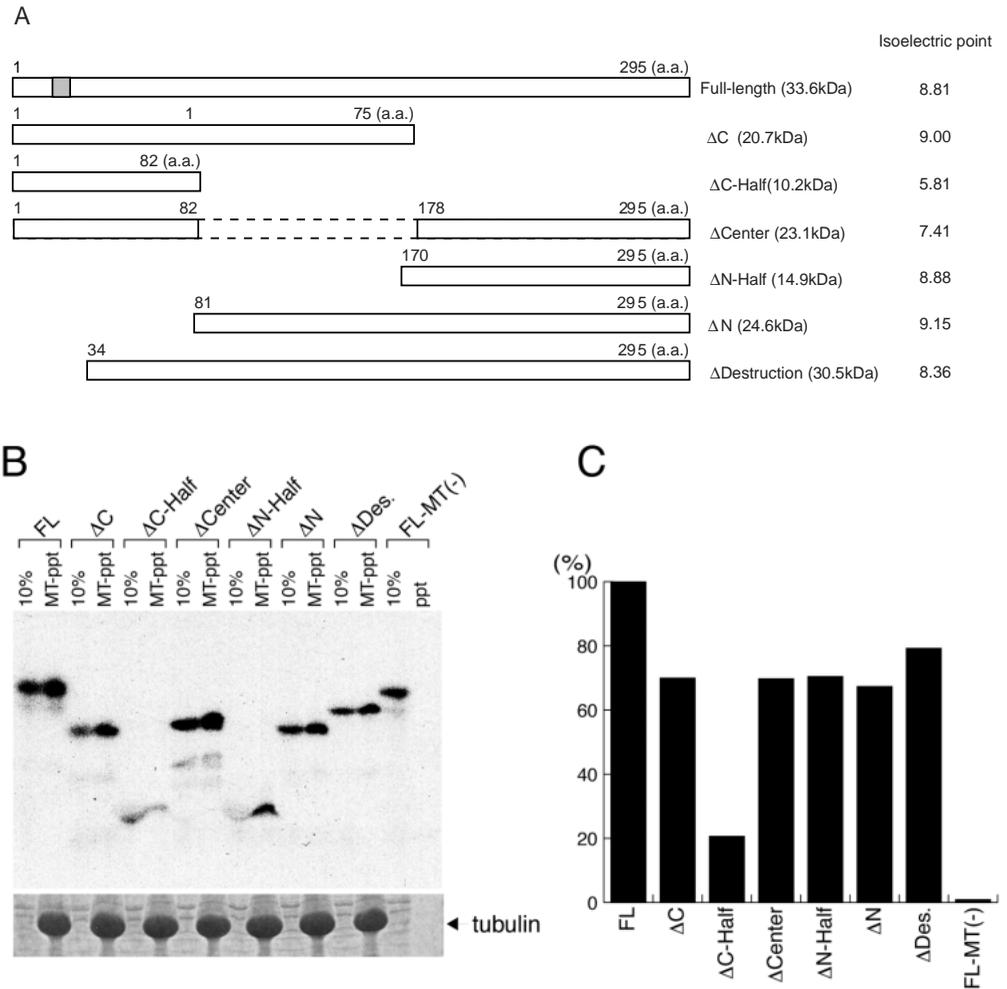


Fig. 9. Microtubule-binding assay for HP33. (A) Binding assay for HP33 and MT by cosedimentation experiment. Proteins recovered in the first and second supernatants (1st and 2nd) and precipitates (ppt) in the cosedimentation assay in the presence of various concentrations of KCl were detected by western blotting for HP33 and by CBB staining for tubulin. Bovine MT (Sigma) and freshly prepared rat MTs were examined. Lanes 19-21 are MT(-) controls for lanes 13-15. (B) Gel filtration for analyzing molecular states of HP33 and tubulin existing in the supernatant fractions in the cosedimentation experiments. Control experiments using HP33 (a) or MT (b) alone. (c) Results using both HP33 and MT. Net protein elution profiles are presented as absorbance at 280 nm (A_{280}). HP33 and tubulin in each fraction were detected by silver staining and western blotting. Positions of the void volume and marker proteins are indicated by arrowheads.

Fig. 10. Microtubule-binding analysis for HP33 deletion mutants. (A) Structure of HP33 deletion mutants used in the assay. The destruction box-like sequence is indicated by the shadow box. Δ Center has an internal deletion from amino acids 83-276. The molecular mass and isoelectric point for each construct are also presented. (B) Autoradiograph after SDS-PAGE for determination of MT-cosedimented HP33. 10% of the initial in vitro-translated HP33 proteins were used for the cosedimentation assay (10%) and precipitates with MT (MT-ppt) were subjected to SDS-PAGE and autoradiography. Results of CBB staining of the identical gel for determination of tubulin are also shown. (C) Relative MT-binding ability for each HP33 mutant. Radioactivity of specific bands in the above SDS-polyacrylamide gel were determined by an RI-imaging analyzer. Results are indicated as relative values for deletion versions (Δ version [MT-ppt/10%]) to that obtained for full-length HP33 (FL[MT-ppt/10%]).



Centrosomal localization of HP33

Since treatment of RL34 cells with nocodazole erased the centrosomal localization of HP33 (Fig. 8), the centrosomal localization is dependent on the MT array. These results are based on the assumption of a mechanistic model in which the MT-binding ability of HP33 is responsible for its centrosomal localization and thus HP33 can be localized in a region where MTs are condensed (e.g. centrosome, PCM and peripheral region of the nucleus). However, the centrosomal localization of HP33 may not be as simple as assumed because of the reasons described below. First, HP33 was not observed clearly along with MT arrays like conventional MAPs (Fig. 4). Second, HP33 was not localized at the MT-concentrated midbody in telophase (Fig. 5O). Third, ectopically overexpressed HP33 was localized at the centrosome but not at either the midbody or peripheral MT arrays (Fig. 7). Therefore, the centrosomal localization pattern of HP33 may not be directly correlated with its MT-binding ability. This situation is similar to the nuclear/mitotic apparatus protein (NuMA) that binds to MTs but is not localized along with them in mitosis (Merdes et al., 1996). Overexpressed HP33 was partially localized at the centrosome in non-hepatic cell lines (Fig. 7), suggesting that HP33 is potentially capable of binding to a certain general structure in PCM and to ubiquitous MTOC-localizing factors (e.g. minus-end directed

MT-based motor), whose localization is also dependent on the MT-network.

Cell cycle-dependent localization of HP33

Centrosomal localization of HP33 was not observed in cells during the transition from metaphase to anaphase (Fig. 6). There are several possible mechanisms underlying this phenomenon. First, an epitope in HP33 may be masked by unknown proteins in the mitotic spindle and spindle pole, which are complex structures composed of a variety of proteins (Kuriyama and Nislow, 1992). Second, HP33 metabolism can be regulated by serine/threonine kinases at prophase or during G₂/M transition, since it has many putative phosphorylation sites for PKA (Thr143), PKC (Ser16, Ser24, Thr138, Ser156 and Ser192) and CKII (Ser106, Ser145 and Thr160). Many lines of evidence show that CKII (Krek et al., 1992; Hanna et al., 1995), PKC (Wells et al., 1995; Murray et al., 1994) and PKA (Nigg et al., 1985) are related to the mitotic apparatus and progression. Third, HP33 may be degraded in mitosis, because HP33 had a destruction box-like sequence at amino acid positions 18-24 (Fig. 2A). Destruction box-containing proteins, such as Cyclin B and Cut2, undergo ubiquitin-dependent degradation during the metaphase/anaphase transition (Funabiki et al., 1996; Irniger et al., 1995). In contrast, the subcellular localization of HP33 became invisible

in metaphase, and the centrosomal localization was already observed in telophase. If the degradation of HP33 is dependent on its destruction-box sequence, HP33 may require another ubiquitin-dependent proteolysis pathway.

Tissue specificity of HP33 and its relation to hepatocarcinogenesis and liver regeneration

HP33 mRNAs were detected almost specifically in the liver and kidney, and scarcely in the lung (Fig. 3A). This tissue specificity was also observed at the protein level (data not shown). Consistent with these results, centrosomal localization of HP33 was observed tissue specifically in hepatocyte-derived cell lines (Fig. 6 and data not shown). We assume that HP33 gene expression is therefore regulated by a tissue-specific signal transduction pathway. The above arguments led us to consider the possibility that HGF (hepatocyte growth factor) is involved in this hypothetical transduction pathway. HGF is produced in mesenchyme and functions as a mitogen and morphogen for epithelial cells during development of the liver, kidney and lung (Takahashi et al., 1993; Johnson et al., 1993; Igawa et al., 1991; Montesano et al., 1991). Interestingly, these tissues are the same as those that express HP33. Additionally, HCC-stimulated HP33 was detected in the regenerating liver (Fig. 3B), and HGF is also induced by partial hepatectomy and liver damage for regeneration of a damaged liver or kidney (Matsumoto and Nakamura, 1992; Ramadori et al., 1992; Kinoshita et al., 1991). HGF stimulates multiple intracellular signaling pathways through binding to its receptor tyrosine kinase, a c-Met proto-oncogene product (Bardelli et al., 1997). HP33 may be expressed downstream of the HGF/c-Met pathway.

In general, the remaining hepatocytes enter a single round of the cell cycle after partial hepatectomy. The mitotic index reaches a peak in 30 hours after partial hepatectomy (Fischer-Szafarz and Nadal, 1977). In our study, HP33 overexpression was observed 48 hours after partial hepatectomy, a time at which DNA synthesis and cell division had almost been arrested and the cells resided in the G₁ phase (Fig. 3B). Hence, HP33 is thought to function in interphase, and this idea is consistent with the observations in the present study, where centrosomal localization of HP33 was evident when the cells were in interphase. Importantly, HGF also functions to repress cell proliferation (Tajima et al., 1991). It is possible that HP33 functions to stabilize the interphase and that hepatomas lose such an HP33-dependent regulation required for normal cell cycle progression.

We thank Drs R. Kuriyama and H. Abe for valuable discussions and critical reading of the manuscript, and Dr T. Arai for providing the anti-tubulin antibody. The cell lines used in this study were provided by Riken Cell Bank and the Japanese Cancer Research Resources Bank. This work was supported by a grant from the program Grants-in-Aid for Scientific Research on Priority Areas of The Japanese Ministry of Education, Science, Sports and Culture.

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