# Human 76p: A New Member of the γ-Tubulin–associated Protein Family

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***Abstract.*** The role of the centrosomes in microtubule nucleation remains largely unknown at the molecular level. γ-Tubulin and the two associated proteins h103p (hGCP2) and h104p (hGCP3) are essential. These proteins are also present in soluble complexes containing additional polypeptides. Partial sequencing of a 76-kD polypeptide band from these complexes allowed the isolation of a cDNA encoding for a new protein (h76p = hGCP4) expressed ubiquitously in mammalian tissues. Orthologues of h76p have been characterized in *Drosophila* and in the higher plant *Medicago*. Several pieces of evidence indicate that h76p is involved in microtubule nucleation. (1) h76p is localized at the centrosome as demonstrated by immunofluorescence. (2) h76p and γ-tubulin are associated in the γ-tubulin complexes. (3) γ-tubulin complexes containing h76p bind to microtubules. (4) h76p is recruited to the spindle poles and to *Xenopus* sperm basal bodies. (5) h76p is necessary for aster nucleation by sperm basal bodies and recombinant h76p partially replaces endogenous 76p in oocyte extracts. Surprisingly, h76p shares partial sequence identity with human centrosomal proteins h103p and h104p, suggesting a common protein core. Hence, human γ-tubulin appears associated with at least three evolutionary related centrosomal proteins, raising new questions about their functions at the molecular level.

Key words: γ-tubulin • centrosome • microtubule • cytoskeleton • nucleation

Most of the microtubules of the cytoskeleton are nucleated on specialized organelles called microtubule organizing centers (MTOCs)[1]. These centers show distinct morphological appearances in evolutionary distant organisms, but it is not yet clear whether these various aspects reflect intrinsic biochemical differences or result from the different organization of evolutionary conserved proteins.

The spindle pole body (SPB) of *Saccharomyces* has been analyzed extensively at the functional and structural levels. Included in the nuclear envelope (Byers and Goetsch, 1975), it contains several layers composed of a small number of distinct proteins (Bullitt et al., 1997). As for all MTOCs, the SPB contains a γ-tubulin–related protein (Tub4p) (Marschall et al., 1996; Spang et al., 1996). γ-Tubulin is present at the minus extremities of the microtubules, but does not participate in the overall structure of the microtubule walls (Stearns et al., 1991; Melki et al., 1993; Li and Joshi, 1995; Zheng et al., 1995). Several experimental results demonstrate that γ-tubulin is required for the nucleation process (Oakley and Oakley, 1989; Oakley et al., 1990; Horio et al., 1991; Stearns et al., 1991; Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994; Shu and Joshi, 1995; Zheng et al., 1995), but the mechanism of nucleation and of determination of the number of microtubule protofilaments are still unknown (Erickson and Stoffler, 1996; Zheng et al., 1997). In the SPB, Tub4p interacts with two other proteins called Spc97p and Spc98p (Geissler et al., 1996; Knop et al., 1997). These three proteins are also present in a 6S soluble complex composed of at least two molecules of Tub4p, one molecule of Spc97p, and one molecule of Spc98p (Knop et al., 1997). The nucleation of the intranuclear and cytoplasmic microtubules involves the interaction of this complex with the proteins Spc110p and Spc72p, which are strictly localized to the inner and the outer plaques of the SPB, respectively (Knop and Schiebel, 1997, 1998). Hence the 6S γ-tubulin complex appears to be a precursor of the material involved in the nucleation of both intranuclear and cytoplasmic microtubules (Pereira et al., 1998).

The knowledge of the overall composition and structure of the centrosome is less advanced than in the case of the yeast SPB (Zheng et al., 1995). Interestingly, a protein cross-reacting with anti–Spc110p antibodies (Tassin et al., 1997) and two proteins that exhibit a significant amino acid similarity with Spc97p and Spc98p are present in the

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1. *Abbreviations used in this paper:* DAPI, 4′,6-diamidino-2-phenylindole; EST, expressed sequence tag; MTOC, microtubule organizing center; SPB, spindle pole body; TuRC, γ-tubulin ring complex.

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centrosomes and in γ-tubulin cytoplasmic complexes of vertebrates and *Drosophila* (Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998; Oegema et al., 1999). In contrast to the yeast 6S γ-tubulin complex, the largest complexes obtained from vertebrate and *Drosophila* involve more than three proteins (Zheng et al., 1995; Détraves et al., 1997; Oegema et al., 1999). They present a sedimentation coefficient of 32S and a ring and/or spiral appearance under the electron microscope (γ-TuRC or γ-tubulin ring complex) (Zheng et al., 1995; Oegema et al., 1999). These complexes bind to the microtubule minus ends and promote the assembly of the α/β-tubulin heterodimers (Zheng et al., 1995; Oegema et al., 1999). Tomographic analysis of the images obtained by electron immunolocalization of γ-tubulin in *Drosophila* centrosomes suggests that the γ-tubulin ring complexes constitute the microtubule nucleation sites of the pericentriolar material (Moritz et al., 1995). In somatic mammalian cells, the γ-tubulin complexes exhibit a large size heterogeneity (Debec et al., 1995; Moudjou et al., 1996; Détraves et al., 1997), but show the same overall composition as *Xenopus* γ-TuRC (Détraves et al., 1997). Besides γ-tubulin, these complexes contain other polypeptide chains with apparent molecular masses of 50, 76, 105, 135, and 195 kD in denaturing electrophoretic conditions (Détraves et al., 1997). The α/β-tubulin heterodimer (50 kD) is present in the *Xenopus* γ-TuRC and in the complexes isolated from mammalian brain (Zheng et al., 1995; Détraves et al., 1997), but is absent in the complexes isolated from cultured cells (Murphy et al., 1998). The 100-kD polypeptide band has been recently shown to contain the two Spc97p (h103p = hGCP2) and Spc98p (h104p = hGCP3) vertebrate homologues (Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998). At least a doublet was observed in the 76-kD band from *Xenopus* γ-TuRCs (Zheng et al., 1995), but the proteins have not been characterized. The partial microsequencing of the 76-kD band from pig brain γ-tubulin complexes allowed us to characterize a ubiquitous centrosomal protein in human (h76p), *Drosophila* (d75p), and *Medicago* (m85p), which is a new member of the yeast and vertebrate Spc97p and Spc98p[2] family.

## Materials and Methods

### Purification of the γ-Tubulin Complexes

Preparations of sheep and pig brain microtubule proteins obtained by two cycles of assembly and disassembly were used to isolate γ-tubulin complexes by immunoaffinity chromatography (Détraves et al., 1997). γ-Tubulin complexes obtained from pig brain were used in all experiments involving mammalian complexes.

2. Protein nomenclature. Three names have been already given to the animal orthologues of *Saccharomyces* Spc97p and Spc98p (Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998; Oegema et al., 1999). (a) The Spc97p orthologues, named hGCP2 in human (human γ-tubulin complex protein 2) and Dgrip84 in *Drosophila* (*Drosophila* γ ring protein 84), are named here h103p and d84p, respectively. (b) The Spc98p orthologues, named hGCP3 and HsSpc98 (human Spc98) in human, Xgrip109 in *Xenopus* (*Xenopus* γ ring protein 109), and Dgrip91 in *Drosophila* (*Drosophila* γ ring complex protein 91), are named here h104p, x109p, and d91p, respectively. In the absence of the 76p orthologue in *Saccharomyces*, the different orthologues of human 76p (hGCP4 or Hgrip76 according to previous nomenclature) were referred to as h76p, x76p, d75p, and m85p for human, *Xenopus*, *Drosophila*, and *Medicago*, respectively.

### Microsequencing of the 76-kD Protein

Proteins from pig brain γ-tubulin complexes were separated by one dimensional gel electrophoresis under denaturing conditions, blotted onto PVDF membrane (Problott, Perkin-Elmer), and visualized by Ponceau S (Sigma Chemical Co.). The sequence analysis of the 76-kD band, carried out using a gas–liquid sequencer (model 467A; Applied Biosystems), allowed the determination of the first 20 amino acids of a single protein. In addition, the 76-kD band was excised from Coomassie blue–stained gels, digested by porcine trypsin, and the resulting peptides were purified and sequenced as described (Sagliocco et al., 1996).

### Cloning and Sequencing of the cDNA of Human 76p and of its Drosophila and Medicago Orthologues

The first 20–amino-terminal amino acids of the 75-kD band from pig γ-tubulin complexes were encoded by a human expressed sequence tag (EST) (AA115 396). The 5′ and 3′ sequences of this clone were used to obtain a full-length cDNA from a human neuroblastoma bank using a PCR approach. This cDNA was sequenced (big dye terminator on 373 DNA sequencer with internal nucleotides). The deduced 2-kb open reading frame (ORF) showed perfect matches with the peptide sequences obtained from purified 75- kD band. The *Drosophila* EST (accession number AA694820) that showed a significant similarity to h76p, allowed by genome walking the successive identification of one genomic (nucleotides 73101–75475; ACC005113) and three additional EST (accession numbers AA536481, AA540317, and AA536269) clones coding for the *Drosophila* orthologue (d75p) of h76p. Sequences obtained from the previously identified EST and genomic clones, together with additional sequences experimentally obtained from the AA540317 (full-length) and AA694820 cDNA clones, allowed us to generate a full-length coding sequence for d75p. The gene coding for the d75p (AC005113) contains seven introns and eight exons (73101–73169, 73227–73371, 73430–73715, 73777–73936, 73997–74503, 74568–74940, 74997–75270, and 75337–75475). The full-length cDNA clone from *Medicago* was isolated by library screening using a radioactive probe corresponding to the 850-bp EcoRI insert fragment of the EST 660560 that encoded a polypeptide showing high homology with the h76p and d75p proteins. The probe was hybridized against $8 \times 10^5$ plaque-forming units of a *Medicago truncatula* root tip cDNA library (Dr. A. Niebl and Dr. P. Gamas, LBRPM, UMR CNRS 215, Castanet-Tolosan, France). Phage plaques were plated onto nitrocellulose membranes (Optitran BA-S-85; Schleicher & Schuell, Inc.) according to the manufacturer's instructions. Prehybridization, hybridization, and washings were performed according to Sambrook et al. (1989). 30 positive clones were detected after initial screening. Four clones containing a presumably full-length 2.8-kb cDNA insert were retained for further characterization and one of them was sequenced.

### Recombinant h76p

The 2-kb ORF of the h76p (AA115396) was amplified by PCR using the 5′ primer, 5′ GCGCGCGAGCTCATCCACGAACTGCTCTTGGCT 3′, and the 3′ primer, 5′ GCGCGCAAGCTTTCACATCCCGAAACTGCCCAG 3′. The PCR fragment was digested with the restriction enzymes SacI and HindIII and inserted into the plasmid pQE30 (Qiagen) resulting in a plasmid (pQE30-p76) coding for a h76p tagged with 6-His at the amino terminus. The bacterial vector pQE30-p76 was introduced in the M15(pREP4) *Escherichia coli* host strain and protein expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The insoluble fusion protein was purified either as inclusion bodies (Sambrook et al., 1989) and used for the purification of antibodies, or solubilized in 8 M urea, purified on a nickel agarose column (QIAexpressionist; Qiagen) and used for immunizations and quantifications. Alternatively, after induction by 0.1 mM IPTG for 3 h at 25°C, and centrifugation of the bacterial extract at 100,000 *g* for 45 min, soluble h76p was recovered in the supernatant where it represented ≈10% of the proteins. Buffer exchange was performed through a prepacked Sephadex G-25M column (Pharmacia).

### Transient Expression of GFP-h76p Fusion Protein in Mammalian Cells

The 2-kb h76p-ORF was amplified by PCR using the 5′ primer, 5′ GCGCGCAAGCTTATCCACGAACTGCTCTTGGCT 3′, and the 3′ primer, 5′ GCGCGCAAGCTTTCACATCCCGAAACTGCCCAG 3′.

The PCR product was digested with the restriction enzyme HindIII and inserted into the plasmid pEGFP-C3 (CLONTECH Laboratories). The resulting plasmid (pEGFP-C3-p76) expressed the h76p with the enhanced green fluorescent protein coding sequence fused at the amino terminus (GFP-h76p). Monkey kidney COS cells, cultured in DME (GIBCO BRL) with 7% FBS (Sigma Chemical Co.) in plastic flaskets (9 $cm^2$) were transfected (50% efficiency) with 1 μg of plasmid pEGFP-C3-p76 using the DEAE dextran/chloroquine procedure (Corbani et al., 1995). COS cells overexpressing the GFP-h76p (103 kD) exhibited a single polypeptide of apparent molecular mass of ≈100 kD, recognized by anti–h76p and anti–GFP antibodies (Fig. 4, top). Comparison of the immunolabeling with a standard curve raised with recombinant h76p showed that the GFP-h76p represented ≈0.4% of total proteins (Fig. 4, top), whereas the endogenous 76p remained below the detection limit, i.e., <0.003% of total proteins in control COS cells. Since the percentage of transfection was ≈50%, transfection resulted in an average expression ≈200-fold above the basal level. Alternatively, HeLa cells were transfected using the calcium phosphate procedure (Sambrook et al., 1989) and observed 65 h after transfection.

### *Antibodies*

Rabbit antibodies recognizing h76p were raised against h76p overexpressed in *E. coli* (R7629/30) and against three synthetic peptides linked to thyroglobulin (Sigma Chemical Co.) as described (Julian et al., 1993). The antibodies R72 were obtained against the peptide KQLRELQSLRLIEEEN corresponding to the region 233–248 of h76p, the antibodies R801 against the peptide RQEDTFAAELHRGGC that contains 10 amino acids corresponding to the region 304–313 of h76p, and the antibodies R190 against the peptide YNKYYTQAGGTLGSFG corresponding to the carboxy-terminal region 651–666 of h76p. Antibodies specific to h76p were purified on recombinant h76p as described (Lajoie-Mazenc et al., 1994). Specificity was checked by preincubation with the immunizing peptide (50 μg per ml). Antibodies against γ-tubulin were raised, either in rabbits (R74, R75) or in guinea pigs (C3), against a 19–amino acid peptide corresponding to the carboxy-terminal region of human γ-tubulin (Julian et al., 1993; Lajoie-Mazenc et al., 1994) and purified on recombinant γ-tubulin (Lajoie-Mazenc et al., 1994). The antibodies R82, raised in rabbits against the carboxy-terminal region of *Arabidopsis* γ-tubulin (CVDEYKASESPDYIKWG), do not cross-react with vertebrate γ-tubulin. A rat mAb (YL1/2) (Kilmartin et al., 1982) directed against α-tubulin was used to reveal the α/β-tubulin heterodimer. mAbs against neurofilament proteins were obtained from Dr. D. Paulin (Institut Pasteur, France). Anti–GFP antibodies were purchased from CLONTECH Laboratories. Affinity-purified antibodies were used in all experiments involving immunoblotting or immunofluorescence labeling.

### *Immunofluorescence*

The immunolocalization of h76p in PtK2 cells (Julian et al., 1993) was performed after a 6-min fixation in cold (−20°C) methanol. Double immunofluorescence staining of h76p and γ-tubulin was performed with rabbit antibodies (R801) and guinea pig antibodies (C3), respectively. Staining of γ-tubulin (R75) or microtubules (YL1/2) in COS cells overexpressing fluorescent GFP-h76p was performed after a 1-min permeabilization in a microtubule stabilizing medium and fixation in 3.7% formaldehyde (Lajoie-Mazenc et al., 1994). The staining was revealed with FITC and TRITC goat antibodies (Immunotech), whereas nuclei and chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI: 0.2 μg per ml). Preparations were dried, mounted in Mowiol (Rodriguez and Deinhardt, 1960), sealed, and observed by epifluorescence with a Zeiss Axiophot microscope equipped with 40× (NA 1.30) plan-neofluar objectives, a 2× Optovar, a 4× TV camera, and a stabilized excitation beam. Images, recorded in the linear dynamic range by a Nocticon camera (LH 4015; Lhesa), were digitized (100 frames averaging) with an image processing system (Sapphire from Quantel) and recalculated using a linear function (Stretch program). The specific maximal fluorescence raised by h76p antibodies over the centrosome was determined (Luminance program) as the difference between the maximal fluorescence of the centrosome and the background fluorescence measured at the immediate proximity of the centrosome.

### *In Vitro Assembly of Xenopus Sperm Asters*

Extracts were prepared using *Xenopus* oocytes arrested in the metaphase of the second meiotic division (Murray and Kirschner, 1989; Morin et al., 1994). Beads of protein A–Sepharose 4B (Pharmacia Biotech Sverige) carrying h76p (R801) and γ-tubulin (R74) rabbit antibodies were used for depletion experiments. Schematically, 50 μl of protein A–Sepharose beads were saturated for 2 h at 4°C with 2 ml of a solution of casein at the concentration of 5 mg/ml. They were incubated overnight at 4°C with 150 μl of rabbit serum (R801, R74) diluted fourfold in PBS, and washed successively in PBS, PBS with 0.1% Triton X-100, PBS, and acetate buffer (100 mM potassium acetate, 2.5 mM magnesium acetate, pH 7.2). The oocyte extract (50 μl, 60–80 mg/ml), diluted with 1 vol of acetate buffer containing an ATP-regenerating system and protease inhibitors, was centrifuged for 5 min at 10,000 *g*, mixed with the protein A–Sepharose beads, and incubated for 1 h at 4°C. After bead removal by centrifugation, the supernatant was used for microtubule assembly in the presence of permeabilized *Xenopus* spermatozoa (Blow and Laskey, 1986; Félix et al., 1994). Probing the supernatant of oocyte extracts treated with beads carrying 76p (R801) antibodies, showed that these beads effectively removed x76p from extracts diluted to 12 mg/ml, but only partially depleted extracts diluted to 25 mg/ml. The nucleation experiment was performed at 22°C by incubating 15,000 sperm nuclei (1 μl) in 20 μl of oocyte extract (25 mg/ml) and 0.5 μl of rhodamine-labeled tubulin (Hyman et al., 1991). At 5-min intervals, 2-μl samples mixed with 6 μl of Hoechst 33258 (1 μg/ml) were observed by fluorescence microscopy, and the percentage of sperm heads with an aster was determined. A sperm aster was defined by the nucleation of 15–60 microtubules at the extremity of the sperm nucleus (Félix et al., 1994). Alternatively, 75,000 permeabilized spermatozoa (5 μl) were incubated with 5 μM nocodazole for 20 min in 50 μl of oocyte extract, and then fixed with 200 μl of 3.7% paraformaldehyde in 80 mM Pipes pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$. Immediately, 50-μl samples were centrifuged at 200 *g* for 5 min onto a coverslip (12-mm-diam) pretreated with a solution of 0.01% poly-L-lysine (Sigma Chemical Co.), postfixed for 6 min with cold methanol (−20°C), and processed for immunolabeling. The recruitment of 76p on permeabilized spermatozoa was quantified in the same conditions as before in the presence of 5 μM nocodazole. The spermatozoa were centrifuged through a cushion of 0.6 ml 25% glycerol in Pipes buffer for 5 min at 5,700 *g* before processing for SDS-PAGE.

## *Results*

### *The 76-kD Polypeptide Band of the γ-Tubulin Complexes Isolated from Mammalian Brain Contains a New Evolutionary Conserved Protein*

γ-Tubulin complexes were purified from mammalian brain by affinity chromatography with γ-tubulin antibodies (Détraves et al., 1997). Besides γ-tubulin and the α/β-tubulin heterodimer, the γ-tubulin protein complexes obtained from pig and sheep brain contain four polypeptide bands (see Fig. 2 A). The band migrating at 76 kD in pig brain γ-tubulin complexes was isolated by SDS-PAGE, blotted and submitted to microsequencing. The terminal amino acid sequence xIHELLLALSGYPGSIFTxN was identified in a putative ORF at the 5′ end of a human EST (accession number AA115395). The corresponding cDNA was obtained by PCR from a human neuroblastoma cDNA library and entirely sequenced. It contains a single ORF (h76p = hGCP4) coding for a polypeptide of 667 amino acids with a calculated mass of 76,090 and a pI of 6.14 (Fig. 1). This ORF also contains the two sequences, xxEDTFAAExHR and xEILPTY, previously obtained from the tryptic digests of the 76-kD polypeptides from pig (sequence 1) and sheep (sequences 1 and 2).

Searches in databases also identified ESTs coding for apparented proteins in evolutionary distant organisms such as *Drosophila* (accession number AA694820), the moss *Physcomytrella* (accession number EJ225509), and the higher plant *Medicago* (accession number AA660560). A full-length cDNA sequence was first obtained for the *Drosophila* orthologue. The corresponding *Drosophila* gene con-

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h76p MIHELLLAL SGYPGSIFTWNKRSG LQVS                QDFPFLHPSETSVLNRLCRLGT   50
d75p MIHDLLLACRSHNPEQL GI K AFN ETTVI             DQF  IHPCEREIFMDIIKIIK   48
m85p MLHELLLAL LGYTGDLI IDRRDNNL  SANTPISDECTFKLAPDISF  IDPSDRELIERIITLG   61

h76p DYIR    FTEFIEQYTGHVQQQDHHPSQQG  QGGLHGIYLR  AFCTGL D  SVLQPY        100
d75p VY QEVEQFT HS    SGR  KSDTH GELP  D SLHGYYLLNLA  KGI EM ALEEYY        94
m85p FYYRELERFSAK     S RNLNWIRSENANPLENKEKPSVYRRALA  NGIVEILAV   Y       112

h76p RQALLDLEQEF LGDPHLSISHVNYFLDQFQLLFPSVMVVV E    QIKS QKIHG            150
d75p AE IGRLEK YCLGNERNSLSYV YNA LY AKFP LLVFMRNL ITEIHVLN LRG            143
m85p SSSILHIEQLL LSETMPILATVTQGLNKFFSLLPPLYELILKIERGDIRGGE LLN          167

h76p CQILETVYKHSC G GLPPVRSALEKILAV  CHGVMYKQLSAWMLHGLLLDQH             200
d75p CVLLHNL HQQCEH GDIQLEKAI KII MKPVKNAFFSSLAHWLLFGVIDDVH             193
m85p   LLH   KK C HCGVPELQTCIQRLL WHG HQVMYNQLASWMVYGILEDRH             212

h76p EEFFI KQGPSSGNVSAQPEEDEEDLGIG GLTGKQLREL QDLRLIEEENML              250
d75p SEFFI KFTPTDA V          DGS SFSKSATCS LLSAE K NPEDYIW              231
m85p GEFFISRQEGRD  V          ENS S SHQEI SEKLSRLSTADASLSDW              251

h76p APSLKQFSLRVEILPSYIPVRVAEKILFVGESVQMFE       NQNVNL  TR KGSIL        300
d75p      QYEVNMSQLPGFFSIVLAEKVLFVGQTVLVFK     MGR NVKV   KNKTDPL        277
m85p    HMGFHISLDMLPEYIPMRVAESILFAGKAVRVLRNPSPSFLSQDDVYPQEPK RFPKIHGFEG  313

h76p K N QEDTFAAELHRLKQQPLFSLV     DFEQ VVDRIR STVAEHLWKLMVEES  D L     350
d75p        AAKLAELDS  DD IYQLWSGRESEFFKMVVD L  SNE DTINVFRLEKVIID IKNYVSA  331
m85p RFNFQREPIINTGMRV EDLLPQ  SEAD KIENMLLD LKESSEF H  KRSFECAV DSIQAIAAS 372

h76p               L GQLKIIKDFYLLGRGELFQAFID TAQHMLKTPPT AVTEHDVNVAFQ QS A    400
d75p RLSEIAVNEVDLERQMGLIKDFFLLGRGEFYLEFC  S Q MVGTMETYRE ERFKNVIRSFE LA    391
m85p HLWQLVVVRADLNGHLKALKDYFLLAKGDFFQCFLEESRQ LMRLPPRQSTAEADIMVPFQLASL   436

h76p HKVL LDDDNLLP L LHLTIEYHGKEHK             ADATQAREGPSRETSPREAPASGW   450
d75p ATVTGITDD LD KFSL ICQRS T                 AEPDDTSD             FNFL   424
m85p KTI G EEDKYFSKVSLRMP SY GITVKPSLLNVPKATSAAAD GISGASISNASSEMSVDGW     495

h76p AALGLS  YKVQWPLHILFTPAVLEKYNVVFKYLLSVRR VQAELQHCWALQ M              500
d75p   QGLSLKYEYEWPLNLLFSPTTIERYNNIFRFLLIIR TYQYEIQRVWAKQTW              475
m85p   DGIALEYSIEWPLHLFFTQEVLSRYLKVFQYLLRLKRT QMELEKLWAS VMHQYHSIFA      553

h76p  QRK  HLKSNQTDA    IK  WRLRNHMAFLVDNLQYYLQVDVLESQFSQLLHQ  I NS      550
d75p RA KS  AKDVPPNN     K IITLRNYLMFFLNNMQYYIQVDVLESQFG ILMNV IK SR      526
m85p KNKKSDQDKSPITQQRDQRFRSMWRVREHMAFLIRNLQFYIQVDVIESQW NIL QSHIQD       612

h76p  TRDF ESI RLAHDHFLSNLLAQSFILLKPVFHCLNEILDLCHSFCS LVSQ N              600
d75p  S DF EVIQR AHTVFLANVLSHCFLLNESETQ LN V       TG   SQ N              564
m85p  SHDFTELVG  FHQEYLSALISQTFLDIGSVSRILDGIMKLCLQFCWNIENQDN             664

h76p LGPLDERGAAQ L SILVKGFSR  QSSLLFKILSS VRNHQIN S                   DLAQLLLRLD 650
d75p RNPIY  GTLLKLFGICEK FAHMTQTKDPSDDLEDEV D QLNESFGVQIASLIQLLVDVKSASCLGP LSQLLLRLD 637
m85p                    FS   NTSELEHIAEE F NKKSN S    LYTILR  SSRLAGSQRTPFLRRFLLRLN 711

h76p YNKYY TQAG GTLGSFGM             Stop                                 667
d75p FNCWF S ASHNT SA                Stop                                 650
m85p LNSFFESTA KEVMNVVRPRPTFPGLNQR   stop                                 739
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*Figure 1.* Comparison of the amino acid sequence of human 76p (h76p) with its *Drosophila* (d75p) and *Medicago* (m85p) orthologues. The alignments have been performed using both amino acid similarities (L/I/V/W/M, K/R/H, A/T/S/P/G, D/E/Q/N, and F/Y) and the conservation of the expected structural motifs (Callebaut et al., 1997). Similarities are shown by shaded areas, while bold letters indicate identical amino acids. Consensus amino acids between the three orthologue proteins are underlined. The deduced nucleotide sequences of h76p, d75p, and m85p have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers AJ249677, AJ249678, and AJ249679, respectively.

tains eight exons coding for a protein of 650 amino acids (d75p). This protein exhibits ≈33% sequence identity with h76p, and shows comparable calculated mass (74,900) and pI (5.12). The presence of an orthologue of h76p in *Medicago* (m85p) was confirmed by the isolation and sequencing of the corresponding full length cDNA. It codes for a protein of 739 amino acids, with a calculated mass of 85,300 (m85p) and a pI of 6.23. m85p exhibits ≈34 and 31% sequence identity with its human and *Drosophila* counterparts, respectively. Several common amino acid motifs were identified between the three protein orthologues, particularly the conserved amino-terminal sequence (Fig. 1).

The identity of the h76p with the major protein of the 76-kD band of pig brain γ-tubulin complexes was further assessed by antibodies raised against recombinant h76p (R7629/30) and several peptide motifs of h76p: 233–248 (R72), 303–313 (R801), and the carboxy-terminal amino acids 651–666 (R190) (Fig. 2 A). The labeling specificity was checked by competition with the immunizing peptides. Moreover, h76p antibodies (R801) specifically immunoprecipitated γ-tubulin from pig brain γ-tubulin complexes in agreement with the presence of both proteins in the same complexes (Fig. 2 B). The 76p was specifically recognized by R190 antibodies in mammalian cell extracts (PtK2 cells) in which it represents, similarly to γ-tubulin, ≈0.02% wt/wt of the cellular proteins (Fig. 2 C). Antibodies R801 and R190 (Fig. 2 D) specifically labeled a single band at 76 kD in oocyte extracts (x76p), suggesting that the sequence of this protein was conserved in vertebrates. Moreover, x76p and γ-tubulin were present in similar amounts and represented ≈0.02% wt/wt of the proteins of the oocyte extract (Fig. 2 D).

### *The 76p Is a Minor Protein of the Purified γ-TuRCs*

Since h76p was associated to γ-tubulin in mammalian protein complexes, it could be assumed that it was present in the fairly homogeneous γ-TuRCs from *Xenopus* oocytes (Stearns et al., 1991; Zheng et al., 1995). An oocyte extract was sedimented on a sucrose gradient and both 76p and γ-tubulin were revealed by immunoblotting. The distribution of x76p (R801 antibodies) was coincidental with the

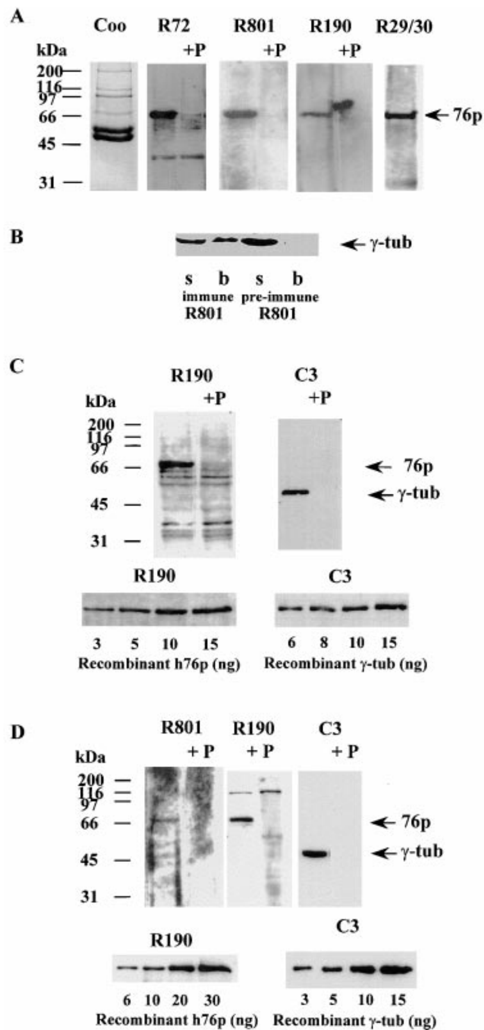



*Figure 2.* Characterization of 76p in the γ-tubulin complexes and in cell extracts. (A) Antibodies against h76p labeled the 76-kD polypeptide band. Pig brain γ-tubulin complexes submitted to SDS-PAGE were either stained with Coomassie blue (15 μg of γ-tubulin) or immunoblotted (50 ng of γ-tubulin) with antibodies R72, R801, R190, and R29/30. All antibodies labeled the polypeptide at ≈76 kD. The specificity of the labeling was controlled by preincubation with the immunizing peptide (+P). (B) Presence of 76p and γ-tubulin in the same protein complexes. Pig brain γ-tubulin complexes (250 ng of γ-tubulin) were immunoprecipitated with immune or preimmune h76p antibodies (R801). The immunoprecipitates (protein A–Sepharose beads, lanes b) and the corresponding supernatants (lanes s, one fifth of the initial volume) were analyzed by SDS-PAGE and immunoblotted with γ-tubulin antibodies (C3). A fraction of γ-tubulin specifically coimmunoprecipitated with 76p. (C and D) Detection and determination of the amount of 76p and γ-tubulin in PtK2 cell extracts (C) and *Xenopus* oocyte extracts (D). Total extracts (50 μg of proteins) were submitted to SDS-PAGE and immunoblotted with antibodies against 76p (R190 and R801) or against γ-tubulin (C3). The specificity was controlled in the presence of the immunizing peptide (+P) and the amounts of 76p (R190) and γ-tubulin (C3) were assessed by comparison with a range of purified recombinant h76p and *Xenopus* γ-tubulin. Both extracts contained equivalent amounts of 76p and γ-tubulin.

peak of γ-tubulin (C3 antibodies). Both proteins were exclusively recovered in complexes larger than 19 S (Fig. 3 A) corresponding to the γ-TuRCs as defined by Zheng et al. (1995). Immunoprecipitation of the fractions of the sucrose gradient corresponding to the γ-TuRCs by h76p antibodies (R190) and immunoblotting with γ-tubulin antibodies (C3) further demonstrated that both proteins were present in the same complexes (Fig. 3 B). However, this experiment did not exclude the possibility that a fraction of x76p assembled in complexes devoid of γ-tubulin and cosedimented with the γ-TuRCs. To check this possibility, γ-tubulin from an oocyte extract (7 mg/ml) was immunoprecipitated with rabbit antibodies (R74). Despite the inability to analyze the immunoprecipitate with rabbit 76p antibodies, the experiment showed the disappearance of both γ-tubulin (C3 antibodies) and x76p (R190 antibodies) from the supernatant (not shown). Hence, in oocyte extracts, most γ-tubulin and x76p are expected to be present in γ-TuRCs in an apparent ratio of ≈1 (Fig. 2 D). In contrast, stoichiometry determinations based on Coomassie staining suggested that the 76-kD polypeptide band corresponded to a minor protein both in *Xenopus* (Zheng et al., 1995) and mammalian brain purified γ-tubulin complexes (Détraves et al., 1997). Using defined amounts of γ-tubulin complexes and recombinant h76p as an internal standard, we confirmed that 76p was approximately fivefold less abundant than γ-tubulin in preparations of pig brain γ-tubulin complexes (Fig. 3 C). Loss of 76p in some γ-tubulin complexes and/or selective loss of some γ-tubulin complexes during purification is a likely possibility. To assess whether all purified γ-tubulin complexes contained h76p, mammalian γ-tubulin complexes were sedimented and the resuspended pellet was repeatedly subjected to immunoprecipitation either with R801 or with R190 antibodies. In each case, the first cycle of 76p depletion removed some γ-tubulin from the preparation, but further cycles failed to strongly deplete the supernatant from γ-tubulin (Fig. 3 D, M + R801 and M + R190). However, a similar result was obtained with the native γ-TuRCs directly immunoprecipitated from a crude *Xenopus* oocyte extract (Fig. 3 D, X + R190). Hence, both purified and native γ-tubulin complexes are likely to be heterogeneous. The inaccessibility of the 76p in some γ-tubulin complexes could account for these observations although two antibodies raised against distinct regions of 76p were used. Alternatively, the average stoichiometry of 76p in γ-TuRCs could be different from the actual stoichiometry in individual γ-TuRC.

Most of the purified γ-tubulin complexes bind to microtubule minus extremities (Zheng et al., 1995; Détraves et al., 1997). Since 76p is present in a fraction of γ-tubulin complexes, we investigated whether 76p could bind to microtubules. After incubation of pig brain γ-tubulin complexes (≈100 ng of γ-tubulin) with taxol-stabilized microtubules (450 μg), free and bound γ-tubulin complexes were readily separated by differential sedimentation (at 62,000 *g* for 5 min) and analyzed by SDS-PAGE and immunoblotting with γ-tubulin (C3) and 76p (R801) antibodies (Détraves et al., 1997). At 0 and 37°C in the absence of tubulin, the γ-tubulin complexes failed to sediment and both 76p and γ-tubulin remained in the supernatant. The same result was observed when γ-tubulin complexes were mixed with

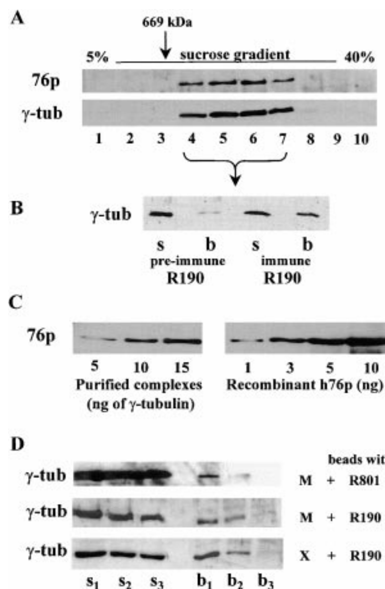



*Figure 3.* Biochemical properties of 76p. (A) 76p cosediment with γ-tubulin in *Xenopus* γ-TuRCs. An oocyte extract (1 mg of protein) was submitted to a sedimentation in a 5-ml linear 5–40% sucrose gradient (160,000 *g* for 4 h and 15 min at 0°C). The fractions (0.5 ml) were analyzed by SDS-PAGE and immunoblotting with γ-tubulin (C3) and 76p (R801) antibodies. Both proteins cosediment with an apparent mass corresponding to the γ-TuRCs (the arrow corresponds to the sedimentation of thyroglobulin at 669 kD or 19 S). (B) Presence of 76p in *Xenopus* γ-TuRCs. The presence of 76p in γ-TuRCs was assessed by the immunoprecipitation of γ-tubulin with x76p. Fractions 4–7 of the sucrose gradient (A) corresponding to γ-TuRCs were immunoprecipitated by immune or preimmune 76p antibodies (R190). The protein A–Sepharose beads (b) and a fraction of the supernatants were analyzed by SDS-PAGE and immunoblotted with γ-tubulin antibodies (C3). Protein A–Sepharose beads carrying preimmune antibodies failed to immunoprecipitate γ-tubulin, while a small fraction of γ-tubulin was recovered linked to the beads carrying the immune antibodies. (C) Determination of the amount of 76p in purified mammalian γ-tubulin complexes. Pig brain γ-tubulin complexes, submitted to SDS-PAGE, were immunoblotted with h76p antibodies (R801), and the amount of 76p was determined by comparison with a range of recombinant h76p. (D) Immunodepletion of a small fraction of γ-tubulin complexes by h76p antibodies. High molecular masses purified pig brain γ-tubulin complexes (M), obtained by centrifugation through a 40% sucrose cushion (≈500 ng of γ-tubulin) and a *Xenopus* oocyte extract (X, ≈2 mg of protein), were submitted to three successive cycles of immunodepletion with h76p antibodies (beads with R801 and R190 for purified mammalian γ-tubulin complexes and beads with R190 for *Xenopus* extract). Then the successive protein A–Sepharose beads (lanes $b_1$–$b_3$) and supernatant fractions (lanes $s_1$–$s_3$, 1/10 and 1/25 of the initial volume for mammalian γ-tubulin complexes and *Xenopus* extracts, respectively) were analyzed by SDS-PAGE and immunoblotting with γ-tubulin antibodies (C3).

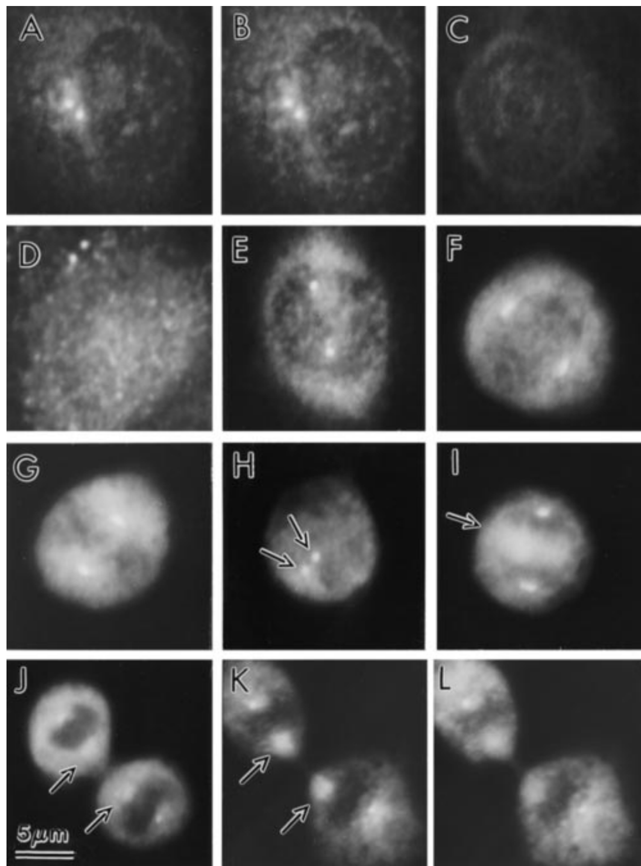
pure tubulin unable to assemble both at 0 and 37°C. In contrast, a definite amount of 76p and γ-tubulin sedimented in the presence of microtubules assembled in the presence of taxol (not shown). These observations suggested the following: (1) 76p could bind directly or indirectly to microtubules; and (2) 76p neither acts as an inhibitor of the fixation of γ-tubulin to microtubules nor is released from the complexes during their binding to microtubule.

### *The 76-kD Protein Is Localized to the Centrosome and Spindle Poles*

The centrosomal localization of 76p was assessed by immunofluorescence staining with three polyclonal antibodies (R801, R190, and R629/30) using cold methanol-fixed PtK2 cells. In all cases, 76p colocalized with γ-tubulin (Fig. 4 A) to the centrosome, which appeared as a diplosome during interphase (Fig. 4 B), and to the spindle poles at the different stages of mitosis (Fig. 4, E–J and L). Both in interphase and mitosis, the labeling raised by h76p antibodies was specific as shown by the absence of staining when the antibodies were preincubated with the immunizing peptide (Fig. 4 C). Observation of the immunofluorescent figures showed that the amount of 76p to the centrosome transiently increased during mitosis. The difficulty to exactly determine centrosome limits prevented the accurate determination of the overall fluorescence (average fluorescence × area), and led us to choose the maximal centrosomal fluorescence as a quantitative parameter (Fig. 4, bottom). The maximal fluorescence, which did not vary significantly in interphase, increased ≈6-fold from prophase to prometaphase/metaphase and decreased thereafter to its interphase level in late telophase. A similar variation has been previously reported for γ-tubulin (Lajoie-Mazenc et al., 1994). As was the case for γ-tubulin (Marschall et al., 1996), h103p and h104p (Murphy et al., 1998), treatment with colcemid (2 μM for 2 h) did not modify the presence of 76p in the interphase centrosome (Fig. 4 D) and its recruitment during mitosis (Fig. 4 H).

As for γ-tubulin (Lajoie-Mazenc et al., 1994), 76p was not only a centrosomal protein, but also relocalized during mitosis. Antibodies R190 directed against the carboxy-terminal region of h76p showed that some 76p was present in the metaphase spindle (not shown), although this localization was not observed with antibodies R801 (Fig. 4 G), in the midzone between the two separating chromosomal masses in anaphase (Fig. 4 I, arrow) and in telophase (Fig. 4 J, arrows) as previously observed with γ-tubulin (Lajoie-Mazenc et al., 1994). In contrast to γ-tubulin (Julian et al., 1993) and h104p (Tassin et al., 1998), which are transiently present in the two regions corresponding to the minus ends of the microtubules constituting the midbody (Fig. 4 K, arrows), in no case the various antibodies against h76p revealed the presence of this protein (Fig. 4 L).

The subcellular localization of 76p was observed in methanol-fixed cells, but no specific immunostaining was obtained using other fixation procedures. Neither formaldehyde- and glutaraldehyde-fixed PtK2 cells nor permeabilized cells with and without formaldehyde fixation showed a centrosomal staining when probed with the different h76p antibodies, whereas in all cases γ-tubulin antibodies decorated the centrosome. To confirm the centrosomal localization of h76p, a fusion protein between GFP and h76p (GFP-h76p) was transiently overexpressed in COS cells. The 103-kD fusion protein was specifically detected in protein extracts from transfected cells by immunoblotting with both h76p (R801) and GFP antibodies



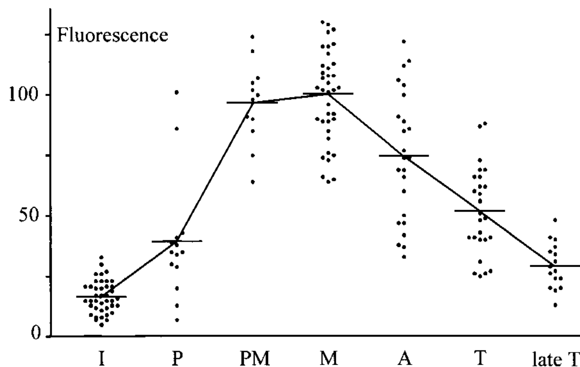




*Figure 4.* Centrosomal localization of 76p during the cell cycle. (Top) Localization of 76p. PtK2 cells were labeled with antibodies against 76p (R801) and γ-tubulin (C3). (A and B) Double immunofluorescent labeling showing the colocalization of γ-tubulin (A) and 76p (B). (C) Specificity of the interphase labeling checked by preincubating h76p antibodies with the immunizing peptide. (D) Localization of 76p to the interphasic centrosome after a 2-h treatment in the presence of 2 μM colcemid that disassembled all the microtubule cytoskeleton. (E and F) Localization of 76p to the two separating centrosomes in prophase before and after the disappearance of the nuclear envelope, respectively. (G) Localization of 76p to the poles of the metaphase spindle. (H) Localization of 76p to the two centrosomes (arrows) in colcemid-treated mitosis. (I and J) Localization of 76p to the spindle poles in anaphase (I) and telophase (J). Note the relocalization of 76p between the two chromosomal masses (I and J, arrows). (K and L) Double immunofluorescent labeling of a cell undergoing cytokinesis; γ-tubulin (K) and 76p (L) are present at the centrosomes, but in contrast to γ-tubulin (K, arrows), 76p is absent at the two minus extremities of the midbody. (Bottom) Transient mitotic recruitment of 76p to the spindle poles. The maximal intensity of fluorescence (arbitrary units) raised by R801 antibodies over the centrosome in a single preparation of PtK2 cells was determined in interphase (I) for each of the two dots constituting the diplosome and in mitosis for each of the two mitotic poles (P, prophase; PM, prometaphase; M, metaphase; A, anaphase; T, telophase; and late T, late telophase). Average values are indicated by horizontal bars and experimental values are indicated by circles.

(Fig. 5 A). Since 50% of the cells were transfected, the average overexpression was ≈200-fold as judged with a range of recombinant h76p. In interphase cells, the GFP-h76p was present at the centrosome where it colocalized with γ-tubulin (Fig. 5 D), whereas in mitotic cells the GFP-h76p localized at the spindle poles and was absent from the mitotic spindle (Fig. 5, B and C). Hence, the presence of the GFP moiety at the amino terminus of h76p did not modify its localization to the interphase and mitotic centrosomes. Since the transfection method used in PtK2 cells induced multipolar mitoses (Fig. 5), we repeated these experiments applying another method to HeLa cells. Overexpression of h76p in HeLa cells (not shown) confirmed its immunolocalization and also failed to induce evident modifications of the microtubule cytoskeleton morphology. However, 72 h after transfection, 80% of HeLa cells expressing GFP-h76p exhibited a nuclear fragmentation characteristic of apoptosis (Fig. 5, E and F), whereas only 12% were observed in cells expressing GFP. This suggests that h76p overexpression could be deleterious to cells by analogy with toxic effects due to Spc97p and Spc98p overexpression in yeast (Geissler et al., 1996; Knop et al., 1997).

### *The γ-TuRCs Containing 76p Are Necessary for In Vitro Nucleation of Microtubule Asters*

It has been previously demonstrated that the γ-TuRCs present in *Xenopus* oocyte extracts (Stearns et al., 1991; Zheng et al., 1995) are recruited by sperm basal bodies, where they are necessary for the nucleation of microtubule asters (Félix et al., 1994; Stearns and Kirschner, 1994). In agreement with its centrosomal localization, immunofluorescence staining demonstrated that 76p was present in the basal bodies of permeabilized *Xenopus* spermatozoa incubated in an oocyte extract (Fig. 6 A). The 76p was present at the extremities of the elongated sperm nuclei where it colocalized with γ-tubulin. The function of 76p (Fig. 6 B, left) was compared with γ-tubulin (Fig. 6 B, right) using permeabilized spermatozoa incubated at 22°C in an oocyte extract and challenged for their ability to nucleate microtubule asters. About 85–90% of spermatozoa nucleated a microtubule aster when incubated in a competent oocyte extract (Fig. 6 B, diamond). When the extract was partially depleted with antibodies against either γ-tubulin (R74) or h76p (R801), only 17–19% and 0–10% of the basal bodies assembled a microtubule aster, respectively (Fig. 6 B, open circle). These inhibitions were specific since they did not occur when the immunodepletion was conducted in the presence of the respective immunizing peptides (≈76 and 83%, respectively; Fig. 6 B, open triangle), preimmune antibodies (≈84 and 72%, respectively; Fig. 6 B, closed circle) or antibodies that are

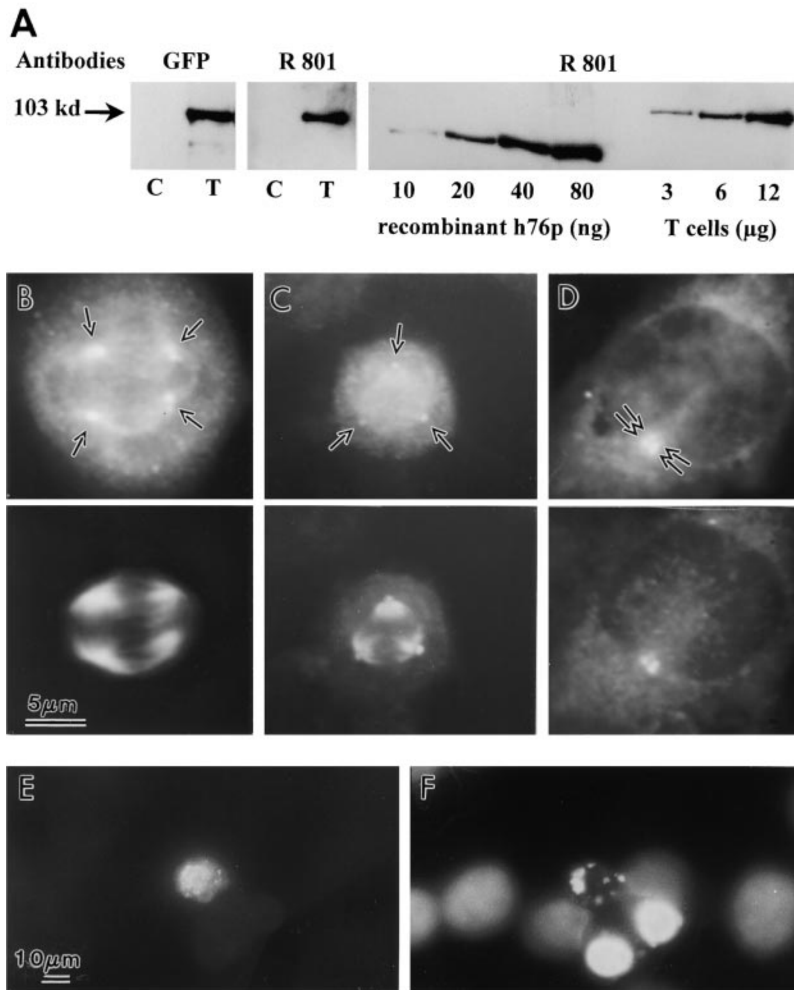



*Figure 5.* Centrosomal localization of GFP-h76p in mammalian cells. Cells were observed 65 h after transfection with a plasmid expressing the fusion protein GFP-h76p. (A) Presence of GFP-h76p in COS-transfected cells. In control (C) cell extracts (20 μg), no polypeptide bands were detected both by anti–GFP (GFP) and anti–76p antibodies (R801). In transfected cells (T) both antibodies labeled the 103-kD fusion protein. The average expression of GFP-h76p in transfected cell extract (T cells) was determined with R801 antibodies by comparison with a range of recombinant h76p. (B–D) Centrosomal localization of GFP-h76p in COS-transfected cells. In mitotic cells, the GFP-h76p (B and C, upper row) localized at the spindle poles as shown by a coimmunofluorescent staining with α-tubulin antibodies (B, lower row), and γ-tubulin antibodies (C, lower row). In interphase cells, the GFP-h76p (D, upper row) colocalized with γ-tubulin (D, lower row) although the fluorescence raised by GFP-h76p appeared broader than the γ-tubulin staining. The transformed cells exhibited numerous multipolar spindles and showed frequently two diplosomes localized in close proximity in interphase, but controls performed with the vector expressing GFP alone suggested that the increase in centrosome number was a consequence of the transfection treatment rather than because of h76p overexpression. (E–F) Appearance of apoptotic figures in transfected HeLa cells. The transfected cells expressing GFP-h76p (E) exhibited a nuclear fragmentation shown by DAPI staining (F).

unrelated to *Xenopus* centrosomal proteins (≈79%; Fig. 6 B, closed inverted triangle). Moreover, addition of mammalian γ-tubulin complexes to γ-tubulin– or 76p-depleted oocyte extracts restored their capacity to induce asters on sperm basal bodies (≈77 and 78%; Fig. 6 B, square).

It was likely that 76p and γ-tubulin were recruited by the sperm basal bodies. The recruitment kinetics of these two proteins, followed in the presence of 5 μM nocodazole during a 20-min incubation period (Fig. 6 C), paralleled the assembly of microtubule asters (Fig. 6 B). The specificity of the recruitment of γ-tubulin and 76p was further studied after a 20-min incubation period (Fig. 6 D). Barely detectable in permeabilized spermatozoa (Sp) (Fig. 6 D, lane 1), both γ-tubulin and 76p were revealed when the spermatozoa were incubated in a crude oocyte extract (Fig. 6 D, lane 2, Sp + E). The same amounts of γ-tubulin and 76p were observed in spermatozoa incubated in an oocyte extract treated either with h76p antibodies (R801) incubated in the presence of the immunizing peptide or with R801 preimmune antibodies (Fig. 6 D, lanes 4 and 5). Partial immunodepletion of the oocyte extract with h76p antibodies (R801) appeared highly efficient, and resulted in a severe drop in the accumulation of 76p and to a less extent of γ-tubulin in incubated spermatozoa (Fig. 6 D, lane 3). These observations demonstrated that 76p, like γ-tubulin, was recruited to the sperm basal bodies and that the recruitment was independent of the presence of microtubules as observed during mitosis. The quantity of 76p in permeabilized spermatozoa and spermatozoa incubated in an oocyte extract was quantified by immunoblotting by comparison with a range of recombinant h76p (Fig. 6 E) ≈0.45 ng (± 0.01, $n = 4$) and ≈4 ng (±0.5, $n = 4$) of 76p were observed per $10^6$ spermatozoa before and after incubation in an oocyte extract, respectively. This ≈9-fold increase of 76p in nucleation-competent spermatozoa strongly suggests that this protein participates to the maturation of the basal bodies as previously suggested for γ-tubulin and the *Xenopus* orthologue (Xgrip109) of h104p (Félix et al., 1994; Stearns and Kirschner, 1994; Martin et al., 1998).

Since the stoichiometry of 76p in native γ-TuRCs could be variable, 76p could act as a limiting factor in the nucleation of asters especially after its immunodepletion. This hypothesis was strengthened by addition of recombinant h76p to a partially x76p-depleted extract (Fig. 6 B, closed triangle). Although the 76p-depleted extract promoted the assembly of ≈0–10% asters, addition of recombinant h76p partially restored their formation to ≈50%. In contrast, addition of recombinant h76p to a partially γ-tubulin–depleted extract failed to restore the capacity of the basal

bodies to nucleate asters: ≈19 and 21% without and with recombinant h76p, respectively. These observations suggested that recombinant h76p could complement a 76p-depleted extract, but not a γ-tubulin–depleted extract. Therefore, it is likely that 76p does not act directly in aster nucleation, but participates in the assembly of active centrosomes. It is possible that recombinant h76p could bind to the γ-TuRCs still present in the partially x76p-depleted *Xenopus* extract and, thus, restore the nucleating activity. But the capacity of recombinant h76p to restore aster nucleation in a partially x76p-depleted extract (≈50%) was lower than the capacity of mammalian γ-tubulin complexes (≈78%) although the amount of recombinant h76p was ≈4,000-fold higher than the amount of 76p added when mammalian γ-tubulin complexes were used. The presence of inactive recombinant h76p in the preparation could account for this difference. Alternatively, some other limiting factors from the 76p-depleted extract could be necessary to recover complete nucleation.

## Discussion

Soluble γ-tubulin complexes are expected to constitute the functional unit of the MTOCs (Zheng et al., 1995). The characterization of their constituents is an essential step towards the elucidation of the molecular mechanisms of microtubule nucleation. We report the identification of the h76p protein, which is a new evolutionary conserved centrosomal constituent associated with γ-tubulin. The h76p was initially characterized by microsequencing from mammalian brain γ-tubulin complexes. The amino-terminal and internal amino acid sequences as well as the mass distribution of the peptides resulting from tryptic digestion (not shown) revealed only the 76p in the electrophoretic band of the pig brain γ-tubulin complexes migrating at 76 kD. The multiple polypeptide bands observed in the

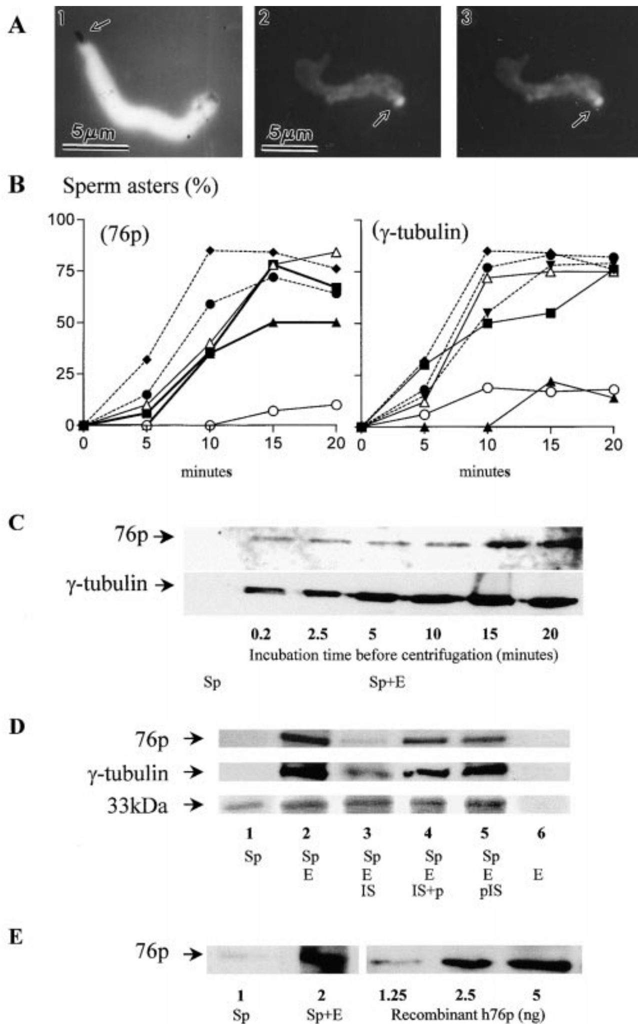



*Figure 6.* The 76p complexes are necessary for aster formation by permeabilized *Xenopus* spermatozoa. (A) Presence of 76p in sperm basal bodies after incubation in an oocyte extract. (1) Localization of 76p, labeled by antibodies R801 (black spot shown by the arrow), at the extremity of the sperm nuclei stained with DAPI (white). (2) Colocalization of 76p and (3) γ-tubulin at the extremity of the sperm basal body (arrows) (R801 and R75 antibodies, respectively). (B) Kinetics of aster nucleation by the sperm basal bodies. Depletion of 76p (left) and γ-tubulin (right) were conducted in the same experiment. (diamond) Control, spermatozoa incubated in an untreated *Xenopus* oocyte extract; (open circle) spermatozoa incubated in extract depleted with h76p (R801) or γ-tubulin (R74) antibodies; (open triangle) spermatozoa incubated in extract depleted with h76p or γ-tubulin antibodies in the presence of the corresponding immunizing peptide (500 μg/ml); (closed circle) spermatozoa incubated in extract depleted with h76p and γ-tubulin preimmune antibodies; (inverted triangle) spermatozoa incubated with antibodies unrelated to *Xenopus* centrosomal proteins (R82 directed against the carboxy-terminal region of *Arabidopsis* γ-tubulin); (square) spermatozoa incubated in h76p- or γ-tubulin–depleted extract further complemented with purified pig brain γ-tubulin complexes (corresponding to 2 ng of γ-tubulin and 0.4 ng of 76p), and (closed triangle) spermatozoa incubated in a 76p- or γ-tubulin–depleted extract complemented with an extract containing recombinant h76p (1.5 μg of recombinant h76p). No effects were observed when an extract devoid of recombinant protein was used (not shown). (C) Recruitment kinetics of 76p and γ-tubulin to the sperm basal bodies. Spermatozoa (Sp, 40 μg) were incubated in the oocyte extract in the presence of 5 μM nocodazole. Samples (Sp + E) taken at various times were centrifuged at 4°C for 5 min. 76p and γ-tubulin, recruited to spermatozoa, were revealed after SDS-PAGE by immunoblotting (R801 and R74 antibodies, respectively) and compared with untreated spermatozoa (Sp). (D) Specificity of the recruitment of the 76p and γ-tubulin to the sperm basal bodies. γ-tubulin and 76p were detected by immunoblotting with R74 and R801 antibodies, whereas the polypeptide band at 33 kD, observed after Coomassie blue staining, was used as a standard to check the recovery of sperm heads. (control, lane 1, Sp) Nonincubated spermatozoa (Sp, 40 μg), (lane 2, Sp + E) spermatozoa incubated for 20 min in the oocyte extract (E), (lane 3, Sp + E + IS) spermatozoa incubated in oocyte extract depleted with R801 immune serum (IS), (lane 4, Sp + E + IS + p) spermatozoa incubated in oocyte extract depleted with the R801 immune serum in the presence of the immunizing peptide (p), (lane 5, Sp + E + pIS) spermatozoa incubated in the oocyte extract depleted with R801 preimmune serum (pIS), and (lane 6, E) oocyte extract incubated alone showing that the recruitment is linked to the presence of spermatozoa. (E) Quantification of the amount of 76p in immature and mature sperm basal bodies. Spermatozoa (Sp, 160 μg) and spermatozoa incubated for 20 min in the oocyte extract in the presence of 5 μM nocodazole (Sp + E, 40 μg) were immunoblotted with R801 antibodies and the amount of 76p was assessed by comparison with a range of purified recombinant h76p.

76-kD range in *Xenopus* γ-tubulin complexes (Zheng et al., 1995) could result from posttranslational modifications of a highly predominant constituent. Alternatively, it is not possible to exclude the presence of other proteins since we detected the light neurofilament protein in the 76-kD band of sheep brain γ-tubulin complexes both by microsequencing and immunoblot (not shown).

Identification of numerous human and murine ESTs homologous to 76p (accession numbers AA790714, T33250, AA190050, AA352362, W27984, AA115395, A1025270, and AA893682) reveals that 76p mRNA is present in various tissues (skin, mammary glands, liver, heart, retina, colon, testis, and placenta) and could be ubiquitously expressed in animal cells, as are γ-tubulin and the two other γ-tubulin–associated proteins, h103p (hGCP2) and h104p (hGCP3). In somatic cells, 76p, like γ-tubulin (Stearns et al., 1991), represents ≈0.02% of the total cellular proteins. It is not only present in soluble *Xenopus* γ-TuRCs and heterogeneous mammalian γ-tubulin complexes, but like γ-tubulin, h103p and h104p, is a bona fide centrosomal protein. This is demonstrated by immunofluorescence staining of 76p in PtK2 cells and mature *Xenopus* sperm basal bodies, and the localization of GFP-h76p fusion protein in COS cells. Moreover, like γ-tubulin (Stearns et al., 1991), h103p and h104p (Murphy et al., 1998), 76p is associated with the centrosome independently of the presence of microtubules. An average of $\approx 4 \times 10^4$ molecules of 76p per centrosome was present in sperm basal bodies incubated in an oocyte extract, a value similar to the number of molecules of γ-tubulin per *Xenopus* centrosome (Stearns et al., 1991). Moreover, 76p is not only an integral centrosomal protein, but relocalized in the mitotic apparatus like γ-tubulin (Julian et al., 1993; Lajoie-Mazenc et al., 1994) and h104p (Tassin et al., 1998), although the localization of these proteins can differ as observed in the midbody.

Besides its centrosomal location, 76p associates in vitro with a microtubule similar to γ-tubulin, h103p, and h104p (Zheng et al., 1995; Murphy et al., 1998). Hence, 76p does not act as an inhibitor of the interaction between γ-tubulin and the α/β-tubulin heterodimers and neither dissociates before the binding of the γ-tubulin complexes to the centrosome nor inhibits their binding. The amounts of 76p (this report), γ-tubulin (Stearns et al., 1991), h103p, h104p (Murphy et al., 1998), and pericentrin (Doxsey et al., 1994) on the centrosome increase concomitantly in early mitosis. The involvement of 76p in microtubule nucleation processes is further demonstrated by the failure of *Xenopus* sperm basal bodies to assemble asters when incubated in 76p-depleted oocyte extracts and the recovery of the capacity to assemble asters after addition of mammalian γ-tubulin complexes or recombinant h76p. This observation confirmed that, in addition to γ-tubulin (Félix et al., 1994; Stearns and Kirschner, 1994), other proteins of the γ-TuRC, 76p (this report) and the *Xenopus* orthologue (Xgrip109) of h104p (Martin et al., 1998) are necessary for the nucleation process.

In purified γ-tubulin complexes, 76p is at least fivefold less abundant than γ-tubulin, in agreement with previous quantifications of the 76-kD band by Coomassie staining (Zheng et al., 1995; Détraves et al., 1997). The loss of 76p and/or the loss of some γ-tubulin complexes during purification could account for this observation since equivalent quantities of γ-tubulin and 76p are observed in the native γ-TuRCs present in *Xenopus* oocyte extracts. But the number of 76p in γ-tubulin complexes could be also heterogeneous as suggested by several observations. First, using two different antibodies, we failed to immunoprecipitate all γ-tubulin in preparations of mammalian γ-tubulin complexes and in *Xenopus* oocyte extracts. Second, addition of recombinant h76p to an x76p-depleted *Xenopus* oocyte extract partially restored the capacity of basal bodies to nucleate asters.

Orthologues to human 76p were cloned from insects (d75p) and angiosperms (m85p) (Fig. 1), and are likely present in mosses (EST, AJ225509). The identity between the three sequenced orthologue proteins varies from ≈31 to 34%. Therefore, the 76p proteins seem to generally occur in eukaryotic cells. The identification of a 76p orthologue in higher plants is particularly of note. The exact nature of the MTOCs remains poorly understood in plants. However, γ-tubulin has been identified in a variety of plants and found to localize at numerous microtubule nucleation sites (Liu et al., 1994; Joshi and Pavelitz, 1996). The isolation of the m85p gives additional support to an evolutionary conservation of the constituents of the MTOCs. Sequence alignments based on the entire sequence of h76p, d75p, and m85p, or on highly conserved sequences between these three proteins failed to identify a 76p orthologue in *Caenorhabditis* and *Saccharomyces* genomes. Although it could be argued that the yeast spindle pole body could differ from the centrosome and that the 76p has been lost during the course of evolution, this view would not apply to the typical centrosome of nematodes. Rather, the inability to detect a orthologue in *Caenorhabditis* could result from a rapid divergence of 76p as previously observed for γ-tubulin (Burns, 1995).

Alignments based on sequence homologies and on hydrophobic cluster analysis (Callebaut et al., 1997) showed that the protein 76p exhibits significant sequence similarities with the two related centrosomal human h103p and h104p (Fig. 7) and their *Drosophila* (Dgrip84 and Dgrip91) and yeast orthologues (Spc97p and Spc98p) that are associated with γ-tubulin in common protein complexes (Geissler et al., 1996; Knop et al., 1997; Martin et al., 1998; Murphy et al., 1998; Tassin et al., 1998; Oegema et al., 1999). The amino-terminal regions of h103p (hGCP2) and h104p (hGCP3) are absent from 76p orthologues (Fig. 7 A), and the homologies are distributed throughout the complete sequence of 76p (Fig. 7 B). In the common region between these proteins, the identities between h76p and h103p (≈23%) or h104p (≈27%) were comparable with the identity observed between h103p and h104p (≈33%), suggesting that this region corresponds to the core of these three γ-tubulin–associated proteins (Fig. 7). Hence, it is likely that these proteins originate from a unique gene family that diverged early in the evolution of eukaryotic cells. Identities between h76p, h103p, and h104p could possibly imply some common functional properties such as the positioning of γ-tubulin at the MTOCs (Geissler et al., 1996; Knop and Schiebel, 1997, 1998; Knop et al., 1997). It is tempting to speculate that h76p, h97p, and h98p could orient the binding of γ-tubulin through specific interactions with different docking pro-

**A**

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                          Regions of homology(see B)
h76p                1===================================667
h97p  1===========216======  ====  ======================827====902
h98p 1============249===========  ==========  ===========855===907
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**B**

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h76p     *MIHELLLALS GYPGS IFTWNK   R SGLQVSQDFPFLHPS  ETSVLNRLCRLGT   50
h97p -----AVVEDLLYVLVGVDGRYVSA QPLAGRQSRTFLV DPNLDL SIRE  LVHRILPVA   267
h98p -----ALVRDILYVFQGIDGKNIKMNN  T ENC YKVEGKANLSR SLRDTAV  RLSELGW   299

h76p  DYIRFTEFIEQYTGHVQQQDHHPSQQGQGGLHGIY  L RAFCTGLDSVLQPY          100
h97p ASYSAVTRFIEEKSS                   FEYGQVNHALAAAMRTLVK EH         303
h98p LH NKIRRYTDQRS  LDRSFGLVGQSFCAALHQELREYYRLL SVLHSQLQLED         350

h76p RQALL D  LEQEF LGDPHLSISHV NYFLDQFQLLFPSVMVVVEQ I KSQKIHG        150
h97p  LILVSQ  LEQLHRQGL  LSLQKLW FYI QPAMRTMDILASLATSVDKGECL G        352
h98p DQG V NLGLESS  LTLRRL L  VWTY   DPKIR   LK TLAALVDHCQGRKG        393

h76p  CQILETVYKH SCGGLPPV RSAL EKILAVCHGVMYKQ LSAWMLHGLLLDQH        200
h97p GSTLSLLHDRSFSYTGDSQAQE  LCL YLTKAASAPYFEVLEKWIYRGIIHDPY        404
h98P G ELASAV HAYTKTGD PYMRS LVQHILSLV SHPVLSFLYRWIYDGELEDTY        443

h76p EEFFIKQGPSSGNVSAQPEEDEEDLGIGGLTGKQLRELQDLRL IE EENML          250
h97p SEFMV                        EEHELR  KE RIQEDYNDKYW            428
h98p HEFFV                               ASDPTV   KTDRLW            460

h76p APSLKQ FSLRVEILPSYIPVRVAEKILFVGESVQMFENQNV   NLTRKGS IL        300
h97p     DQRYTIVQQQIPSFLQ KMADKILSTGKYLNVVRE   CGHDVTCPVAKEI        475
h98p     HDKYTLRKSMIPSFMTMDQSRKVLLIGKSIN FLHQ VC HDQT PTTK M        506

h76p KNQEDTFAAELHRLKQQPLFSLV D  F EQVVDRIRSTVAEHLWKLMVE    ESD L   350
h97p                    IYT LKERAYVEQI EK  A FNYAS KVLLDFLMEEKE L   509
h98p   IAVTKSAESPQDAAD LFT DLENAF  Q  GKIDAAYFETS KYLLDVL NKKYSL   555

h76p LGQLKIIKDFYLLGRGELF QAFID TAQHMLKTPPTAVTE HDVNVAFQQSA        400
h97p VAHLRSIKRYFLMDQGDFFVH FMDLAEEELRK PVEDITPPR LE ALLELA        558
h98p LDHMQAMRRYLLLGQGD FIRHLMDLLKPELVR PATTLYQ HNL TGILETA        604

h76p HKVLLDDDNLLPL LHLTIEYHGKEHKADATQ  ARE GPSRET S       PREAPASGW 450
h97p LRMSTANTDPFKDDLKIDLMPH  DLI   TQLLRV LA  IETKQ EKAMAHADPTELALSGL 613
h98p V                          RATNAQFDSPEILRRLDVRLLE  VS   PGD   TGW 635

h76p AALGLS  YKVQWPLHILFT PAVLEKYNVVF K YLLSVRRVQAELQHC  WAL Q  M    500
h97p EA   FSFDYIVKWPLSLIINRKAL TRYQMLFRHMFYC  KHV  ERQLCSVW ISN K    663
h98p DV   FSLDYHVDGPIATVFTRECM SHYLRVFN  FLWRAKRM  EY ILTD  IRKGHM  685

h76p  QRKH LKSNQT DAIKWRLRNHM  A     FLVDNLQYYLQVDVLE S QFSQLLHQINS  550
h97p TAKQHSLHSAQWF AGAFTLRQRML      NF VQNIQYYMMFEVMEPTWH  ILEKNLKS  715
h98p CNAKL LR  NMPEFSGV LHQCHILASEMVHFIHQ MQYYITFEVLECSWD ELWNK VQQ  740

h76p TRDFESIRLAHDHFLSN LLAQSFILL KPVFHCLNEILDLCHS FCSLVS QN        600
h97p ASNIDDVLGHHTGFL DTCLKD  CMLTNPELLKVFSKLMSVCVMFTNCM  QK        764
h98p AQDLDHIIAAHEVFL DTIISR  CLL DSDSRAL  LNQLRAV FDQIIELQNA       788

h76p LGPLDERGAAQLSILVKGFSR QSSLLFKILSSV RNHQI   NSDLAQLLLRLD        650
h97p  FTQSMKLDGELGG Q TLEH  STVLGLPAGAEERARK    ELARKHLAEHAD        810
h98p QDAI YR AA LEELQR   RLQFEEKKKQRE IEGQWGVTAAEEEEENKRI GE        835

h76p YNK YYTQA GGTL  GSFGM   Stop                                  667
h97p TV QLVS GFEATI  NKFDK----                                     827
h98p F KESIPKMCSQLRILTHFYQ----                                     855
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*Figure 7.* Comparison of the amino acid sequences of human 76p, 103p (h97p) and 104p (h98p). (A) Regions of homology of the three proteins (shaded area). (B) Sequence comparison in the regions of homology. Identical and homologue amino acids are shown by bold letters and by shaded areas, respectively. Consensus amino acids between h76p, h103p[1] and h104p[1] are underlined. Comparison between Figs. 1 and 7 shows that the consensus amino acids between h76p, d75p, and m85p and the consensus amino acids between h76p, h103p, and h104p are 18% identical. The alignments have been performed using both the similarities of sequences and the conservation of the expected structural motifs (Callebaut et al., 1997).

teins and possibly specify the nucleation of different microtubule arrays as observed for Spc98p at the inner plaque of the yeast spindle pole body (Nguyen et al., 1998). The mode of interaction between h76p and the other components of the human γ-tubulin complexes together with the analysis of *Drosophila* d75p mutants could shed some light on the function of h76p, and are currently under investigation.

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