Chinese hamster protein homologous to human putative protein kinase KIAA0204 is associated with nuclei, microtubules and centrosomes in CHO-K1 cells

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Abstract Monoclonal antibody raised against a preparation of loach fish sperm centrosomes was used for screening of cDNA expressing library of Chinese hamster CHO-K1 cells. Two positive clones appeared to encode 628 amino acid protein fragment that was 72% identical to human KIAA0204 protein, i.e. putative protein kinase. Polyclonal antibodies raised against products of cDNA expression in *E. coli* recognized 210-kDa polypeptide in CHO-K1 cells and immunostained nuclear speckles, centrosomes and microtubules in these cells. The 210-kDa polypeptide (named MAK-L) co-sedimented with exogenous microtubules. Thus, one more protein kinase seems to be associated with the microtubule network in vertebrate cells.

Key words: Microtubule; Nucleus; Centrosome; Serine-threonine protein kinase; Expressing library of cDNA; Antibody; CHO-K1 cell

1. Introduction

Microtubules (MTs) of eukaryotic cells are dynamic polymer structures with highly variable stability [1,2]. They are key components of kinesin- and dynein-mediated cell motility, and also participate in transport of some mRNPs driven by an unknown mechanism [3]. Proper arrangement of MTs in cells is necessary for correct accomplishment of motility and transport events. MT arrays are subjected to radical cell regulation, and a number of cell factors including centrosomes, structural proteins, enzymes and ions participate in the management of MTs and constantly or temporary associate with them [1,2]. On the other hand, artificial depolymerization or extra-polymerization of MTs leads to activation of some transcription factors and cell cycle regulatory proteins [4,5]. Probably, some of cellular regulatory proteins can alternate their functions by anchoring in MTs [6,7]. The work of MT machinery is not properly understood yet, and identification of new MT proteins can shed light on MT behavior and functioning. Some MT-interacting proteins that are not abundant in the cell were revealed by immunofluorescence firstly in centrosomes and in mitotic spindles (for example see [8]), i.e. the places of a dramatically increased MT concentration. Centrosomes therefore probably should be good sources of MT-associated and MT-interacting proteins for monoclonal Abs raising [9].

We isolated a preparation of centrosomes from loach fish *Misgurnus fossilis* spermatozoa (manuscript in preparation) and raised monoclonal Abs against it. A panel of monoclonal Abs recognizing centrosomes and mitotic spindles was selected, and these monoclonal Abs were used for screening of cDNA library of Chinese hamster ovary CHO-K1 cells. We describe in this work the results obtained with SN2-3D2 Abs. The recombinant protein, discovered with these Abs, appeared to be closely related to human myeloblast putative serine-threonine protein kinase KIAA0204 recently found in the human genome sequencing work. It was shown to be localized in MTs, nuclei and centrosomes of CHO-K1 cells. Thus, probably it is a new member of the group of MT-associated and centrosome-associated protein kinases.

2. Materials and methods

All reagents were of best research grade and were purchased from Sigma, Serva and Merck if not indicated overwise.

2.1. Screening of cDNA library with monoclonal Abs, sequencing and expression of cDNAs

A mixture of monoclonal Abs raised against preparation of loach fish *Misgurnus fossilis* sperm centrosomes was used for screening of Chinese hamster ovary cells (CHO-K1 cells) cDNA expression library in the λUni-ZAP^{TM}XR vector (Stratagene). Screening procedure was made as described in [10]. The cDNAs of two positive clones (named 5-3D2 and 8-3D2) were recloned in pBluescript (Stratagene) for sequencing and to pQE31 vector (Qiagen) for expression in *E. coli*. Sequencing was made by an ABI model 373 DNA sequencer using an ABI PRISM dye terminator cycle sequencing ready reaction kit with Ampli Taq Polymerase (FS). Products of expression of cDNAs (5-3D2 and 8-3D2 polypeptides, respectively) were purified in affinity Ni-Ac agarose (Qiagen) column in the presence of 8 M urea.

2.2. Antibodies

Rabbit was immunized with 8-3D2 polypeptide by standard method described in [11], and immune serum was affinity purified using columns with 5-3D2 and 8-3D2 polypeptides conjugated with BrCN-Sepharose. Abs purified using a column with 8-3D2 polypeptide (plc3D2 Abs) were divided in a column with 5-3D2 polypeptide into plc83D2 Abs (in a flow through fraction) and plc53D2 Abs (eluted from the column). For immunostaining, these Abs were used in concentrations 50–100 μg/ml, for immunoblotting – approximately 1 μg/ml. Monoclonal Abs to alpha-tubulin DM-1A and to splicing factor SC35, secondary goat anti-rabbit IgG and goat anti-mouse IgG Abs conjugated with either tetramethylrhodamine isothiocyanate or fluoresceine isothiocyanate, or with horseradish peroxidase were purchased from Sigma. Goat anti-rabbit IgG Abs conjugated with alkaline phosphatase and donkey anti-rabbit IgG antibodies conjugated with 10 nm colloidal gold (Auroprobe-10) were purchased form Amersham.

2.3. Immunofluorescence and immunogold staining

CHO-K1 cells were grown in F10 medium (Flow Laboratories)

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supplemented with 10% of fetal bovine serum (Flow Laboratories) and 100 μg/ml of gentamicin in 5% of CO_2 and 95% air at 37°C. MT drugs were added to the culture medium before fixation: nocodazole (Calbiochem) to 5 μg/ml for 2 h, taxol (generous gift of Dr. Suffnes, National Institutes of Health) to 10 μg/ml overnight, vinblastine to 10 μg/ml overnight. For immunofluorescence staining, the cells were Triton-permeabilized as described in [12] and fixed with 0.5% glutaraldehyde. Preparations were examined in a Photomicroscope-3 (Opton).

For immunogold staining, cells were fixed as described above and then subsequently stained with plc3D2 and Auroprobe-10 Abs and processed as described in [13]. Ultrathin sections were examined in a HU-11B electron microscope (Hitachi) at 75 kV.

2.4. *Preparation of subcellular fractions, electrophoresis and immunoblotting*

All solutions used for cell fraction preparation were supplemented with protease inhibitor cocktail [14]. Cell nuclei isolation was made according to [15]. For soluble fraction preparation, cells were incubated with 1 ml of 0.5% Triton X-100 in 80 mM piperazine-N, *N'*-bis(2-ethanesulfonic acid pH 6.7, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA (PEM buffer) with 10 μg/ml of taxol during 3 min at room temperature. The supernatant was cleared by centrifugation at 140 000 × *g* during 30 min.

For co-sedimentation of CHO-K1 cell proteins with exogenous MT, CHO-K1 cells were scraped off in 1 ml of ice-cold 0.5% Triton X-100 in PEM buffer, incubated in ice during 30 min and centrifuged at 140 000 × *g* during 40 min at 4°C. Bovine brain partially purified self-polymerizing tubulin was isolated by the method described in [9]. Co-sedimentation of CHO-K1 extract with brain MT was done as described in [16]. Control sample was supplemented with 5 μg/ml of nocodazole except taxol. For salt treatment of MTs, a 4 M glycerol cushion was supplemented with 0.8 M KCl.

Electrophoresis of CHO-K1 cell homogenate or subcellular fractions was done in a Laemmli system as described in [15]; gradient 6–12% polyacrylamide gel was used. The proteins were transferred to nitrocellulose filters Hybond-C (Amersham) by a semi-dry method [15] and processed as described in [11,15]. Peroxidase was developed with diaminobenzidine/H_2O_2, alkaline phosphatase – with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium system.

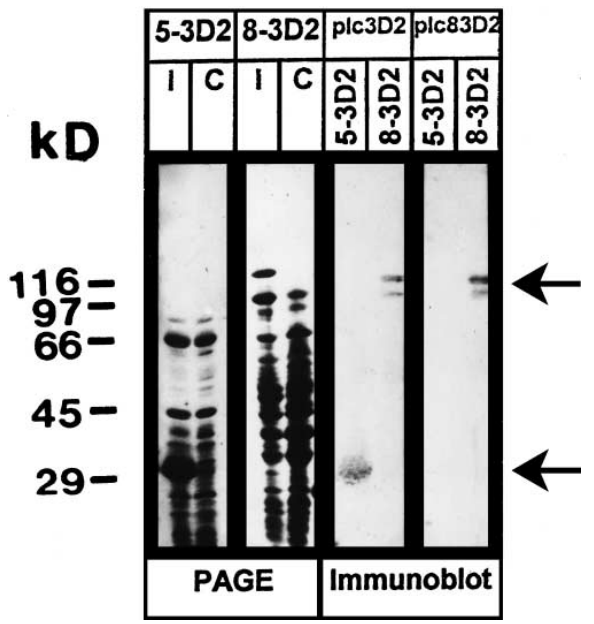

Fig. 1. Expression of 5-3D2 and 8-3D2 cDNAs in *E. coli* in pQE system and characterization of plc3D2 and plc83D2 polyclonal Abs by immunoblotting with expression products. The results show anomalous electrophoretic mobility of 8-3D2 protein fragment and specificity of Abs used. C, control (non-induced) *E. coli* cells; I, IPTG-induced *E. coli* cells. Positions of expressed fusion proteins are indicated with arrows. kD, molecular mass markers.

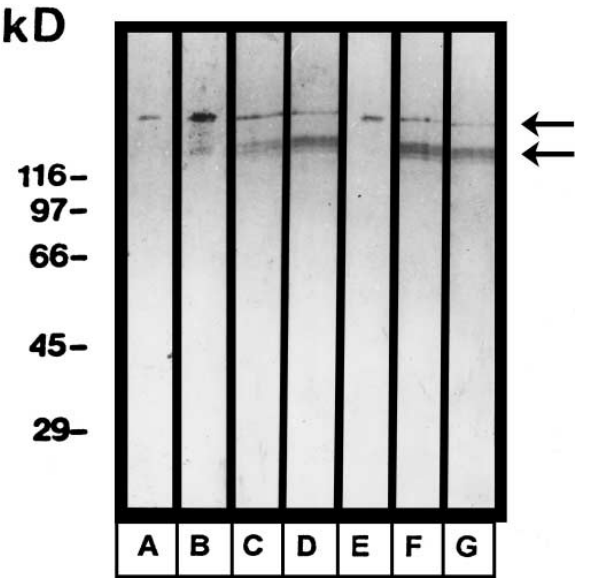

Fig. 2. Immunoblots of CHO-K1 cell homogenate and subcellular fractions. A, monoclonal SN2-3D2 Abs; B–D and F, G, plc3D2 Abs; E, plc83D2 Ab; A–C and F, cell homogenate; D, cell homogenate incubated during 2 h at room temperature; F, nuclear preparation; G, soluble fraction; A, B and E developed with peroxidase conjugate; C, D and F, G, developed with alkaline phosphatase conjugate. The 210-kDa polypeptide and subset of 150–180-kDa polypeptides (probable products of proteolytic degradation of 210-kDa polypeptide) are recognized with all Abs in all subcellular preparations and are indicated with arrows. kD, molecular mass markers.

3. Results

3.1. *Chinese hamster cell protein similar to KIAA0204 human putative protein kinase was partially cloned*

Monoclonal Abs SN2-3D2, raised against centrosomes isolated from loach fish *Misgurnus fossilis* sperms, immunostained in cultured CHO-K1 cells centrosomes and faint cytoplasmic network sensitive to nocodazole treatment (data not illustrated) and recognized 210-kDa polypeptide (Fig. 2). Two positive clones (named 5-3D2 and 8-3D2) were found by screening of Chinese hamster CHO-K1 cell cDNA expression library with these Abs. These clones contained 1256 n.p. and 1956 n.p. cDNA inserts, respectively. Sequencing of these cDNA inserts (accession number AF002245 in GenBank) revealed that most probably they were originated from one mRNA. The nucleotide sequence and deduced amino acid sequence (3D2 polypeptide) of both cDNAs were found to have 72% identity with a corresponding fragment of the recently described human myeloblast KIAA0204 protein (accession number D86959 in GenBank). Moreover, 98% identity with KIAA0204 protein was found in 200 aa reside C-terminal fragment of 3D2 protein that was identified in 8-3D2 clone and was absent from 5-3D2 clone. The KIAA204 protein was specified in GenBank as a putative serine-threonine protein kinase since it contained at least two serine-threonine protein kinase signatures in N-terminal part of molecule.

3.2. *Relative molecular mass of 3D2 protein in CHO-K1 cells was 210 kDa: immunoblotting data*

The product of expression in *E. coli* of 5-3D2 cDNA had a predicted molecular mass of 30 kDa, and it appeared in elec-

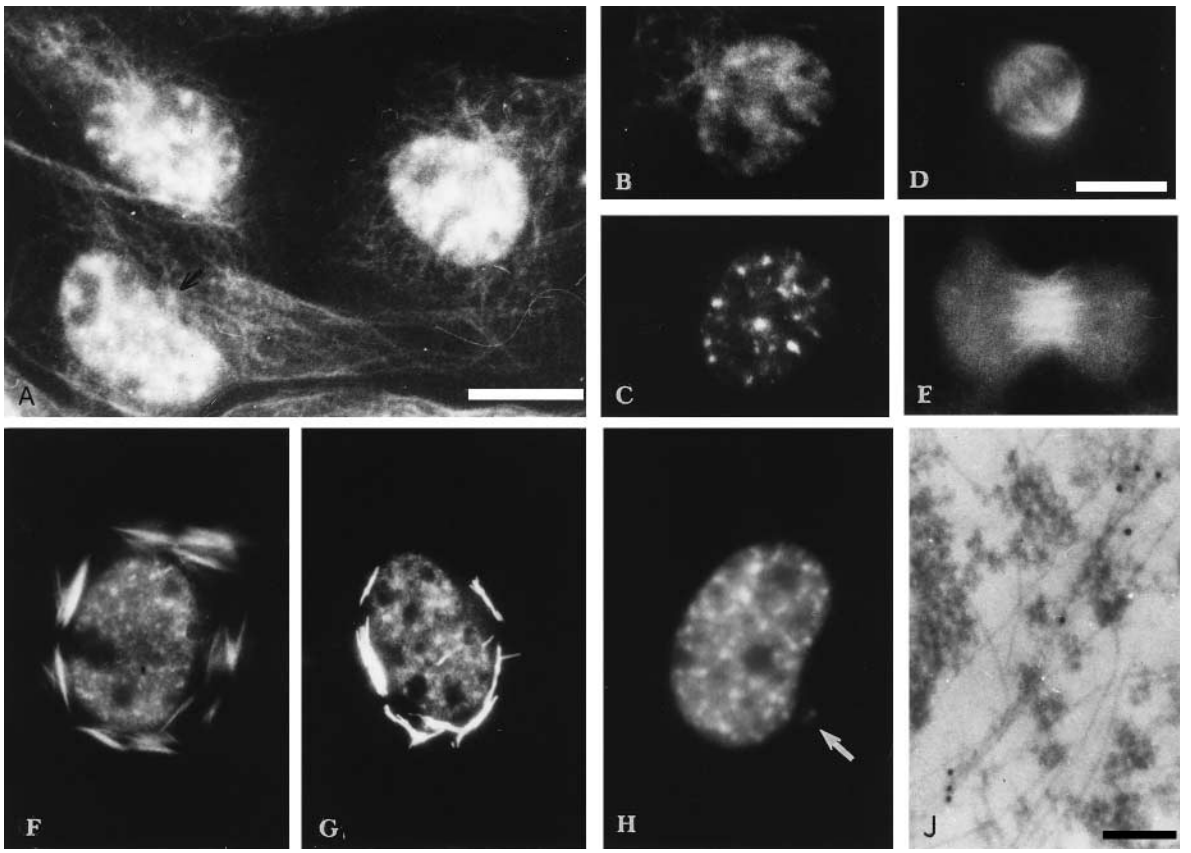

Fig. 3. Immunofluorescence (A–G) and immunogold (J) staining of CHO-K1 cells showing localization of 3D2 protein in nuclei, centrosomes and MTs. A–E and J, control cells; F, taxol-treated cells; G, vinblastine-treated cells; H, nocodazole-treated cells. D, metaphase cell; E, early telophase cell; other pictures, interphase cells. C, anti-SC35 Abs; other pictures, pcl3D2 Abs. B and C, double immunostaining. Arrows indicate centrosomes. Bars, 10 μm in A–H and 1 μm in J.

trophoregram as a 30-kDa polypeptide (Fig. 1). The product of expression in *E. coli* of 8-3D2 cDNA had a predicted molecular mass of 75 kDa, but its relative molecular mass in electrophoregram was estimated as 120 kDa (Fig. 1). Most probably, the 8-3D2 polypeptide had highly deviant mobility in the Laemmli system due to its C-terminal part highly enriched with charged amino acid resides.

The specificity of Abs plc3D2, plc83D2 and plc 53D2 was confirmed by immunoblotting with the expression products. Polyclonal Abs plc3D2 recognized both the 8-3D2 and 5-3D2 expression products (Fig. 1), the same was shown for 5-3D2 Abs (data not illustrated). Abs plc83D2 recognized the 8-3D2 product only (Fig. 1). It should be noticed that the part of the 8-3D2 polypeptide recognized specifically by plc83D2 Abs had 98% identity with the KIAA0204 protein, and plc83D2 Abs had to recognize the KIAA0204 protein if it is expressed in CHO-K1 cells.

In immunoblots of CHO-K1 cell homogenates monoclonal Abs SN2-3D2, plc3D2, plc83D2 and plc53D2 recognized one 210-kDa polypeptide, named 3D2 protein (Fig. 2). Probably, the 3D2 protein is identical or very close to the KIAA0204 protein. The KIAA0204 protein had a predicted molecular mass of 139 kDa, but as 8-3D2 protein fragment had highly deviant electrophoretic mobility, it is possible that the natural 3D2 protein also had anomalous behavior in Laemmli electrophoresis. After incubation of the cell homogenate at room temperature, a number of 180–150-kDa polypeptides recognized by plc3D2 Abs appeared in the preparation (Fig. 3), and most likely these polypeptides were products of the 210-kDa polypeptide degradation. The same 210 and 180–150 kDa-polypeptides was recognized with plc3D2 Abs in the total cell homogenate, in soluble fraction of the cells, in insoluble fraction (data not illustrated) and in nuclear preparation (Fig. 3).

3.3. 3D2 protein in CHO-K1 cells was localized in nuclei, microtubules and centrosomes: immunofluorescence and immunogold data

Plc3D2 Abs (and also plc53D2 and plc83D2 Abs; data not illustrated) immunostained in Triton-permeabilized and glutaraldehyde-fixed CHO-K1 cells nuclear speckles, the cytoplasmic fibrillar network (putative MTs) and small perinuclear dots (putative centrosomes) (Fig. 3A, B). Anti-tubulin DM-1A Abs immunostained the same network as anti-3D2 antibodies (data not illustrated). Nuclear speckles were co-localized with the nuclear speckles immunostained with anti-SC35 Abs (Fig. 3B, C); thus, the nuclear speckles were RNP-rich splicing islands. In mitotic cells, mitotic spindles and midbodies were brightly stained with plc3D2 Abs (and plc83D2 and plc 53D2 Abs, data not illustrated), and no staining of chromosomes or any cytoplasmic inclusions was seen (Fig. 3D, E). MT bundles were stained after taxol treatment (Fig. 3F), and tubulin paracrystalls were stained after vinblastine treatment (Fig. 3G). No staining of the cytoplasm except cen-

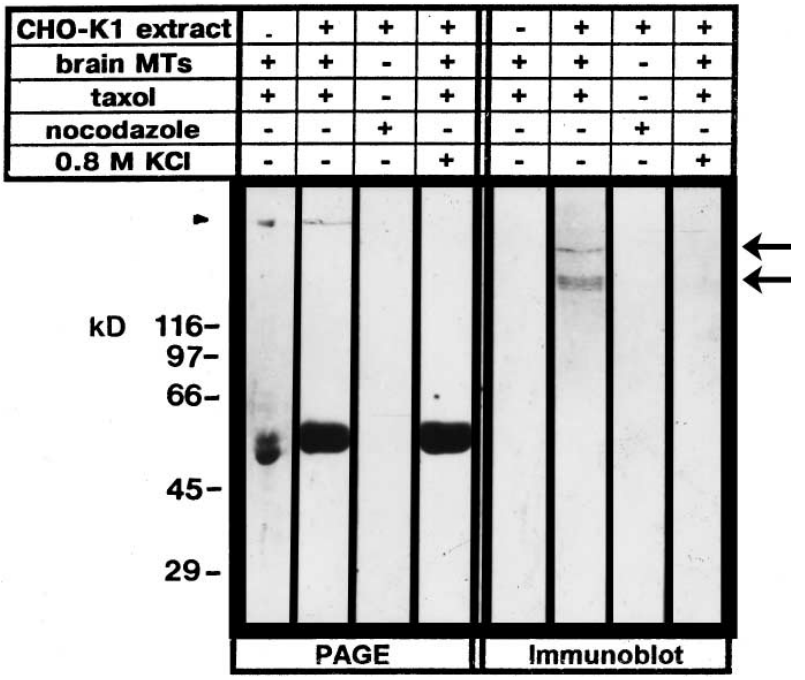

Fig. 4. Co-sedimentation of the 3D2 protein from CHO-K1 cells with brain microtubules. The pellet after sedimentation of the incubation mixture through the 4 M glycerol cushion was analyzed by immunoblotting with the plc3D2 Abs. In the table the components of incubation mixture are recorded. Arrowhead indicates MT-associated protein from bovine brain, arrows indicate 3D2 polypeptides. kD, molecular mass markers.

trosomes with plc3D2 Abs was seen after disruption of microtubules with nocodazole (Fig. 3H).

Immunofluorescence data were confirmed with immunogold ones: in CHO-K1 cells immunostained with plc3D2 Abs and immunogold, gold particles were localized along cellular MTs (Fig. 3J). It is interesting that some MTs remained unstained with plc3D2 Abs. Quite a lot of gold particles were associated with centrosomes independently from MTs (data not illustrated).

3.4. 3D2 protein of CHO-K1 cells co-sedimented with exogenous microtubules

Moreover, the 210-kDa polypeptide recognized with plc3D2 Abs co-sedimented with brain MTs through the glycerol cushion (Fig. 4). It could not sediment in the conditions of experiment in the absence of brain MTs or throughout the cushion in the presence of 0.8 M KCl (Fig. 4). The concentration of salt necessary to remove the 3D2 protein from MTs was strikingly high. This concentration was confirmed in experiments with salt treatment of permeabilized CHO-K1 cells incubated with taxol: immunofluorescence staining of 3D2 protein significantly decreased with salt concentrations above 0.6 M KCl.

4. Discussion

So, the 210-kDa 3D2 protein localized in MTs, nuclear speckles and in centrosomes was partially cloned from Chinese hamster CHO-K1 cells. This protein had strong similarity to recently sequenced human KIAA0204 putative serine-threonine protein kinase. In CHO-K1 cells 3D2 protein was recognized both with plc83D2 Abs that were directed against polypeptide nearly identical to KIAA0204 protein fragment and with plc3D2 Abs that were directed also to 3D2 polypeptide with less homology to the KIAA0204 protein. Most probably, the difference between the 3D2 and the KIAA0204 proteins was species specific, and thus they represented one and the same or very closely related proteins, putative serine-threonine protein kinases. In our further work we are going to investigate this protein or potential set of its isoforms in human cells.

The 3D2 and KIAA0204 proteins had some homology with *Drosophila* 220-kDa LK6 centrosome-associated protein kinase [8]. Interestingly, the LK6 protein kinase as a 3D2 protein had deviant electrophoretic motility and rapidly degraded in vitro. Another feature of similarity of these proteins is their interaction with centrosomes and microtubules. From data of this work we cannot distinguish between interaction of the 3D2 protein with tubulin itself or with some other MT-associated proteins since crude cell extract was used in co-sedimentation experiments. It should be noticed that 3D2 and KIAA0204 protein had some similarity with MT-associated protein MAP1B and with pericentrin in their C-terminal part.

A number of protein kinases were shown previously to interact in any way with MTs or with MT-associated proteins. They are: $p34^{cdc2}$ [17]; MAPK family [18–20]; PKC isoform PKC-ς [6]; *polo* family kinases: polo in *Drosophila* [21], cdc5 and plo1 in yeast [22,23] and Plk1 [24] and Sak [25] in mammalians; LK6 in *Drosophila* [8]; $p110^{mark}$ [26] in mammalian brain; Pk9.7 in *Xenopus gastrula* [27] and several ubiquitous protein kinases [28,29]. Experimental alternation of function of MT-interacting protein kinases can lead to dramatic disturbance of the MT network, especially in mitosis [8,27,30], and to variations of cell motility [31]. Misfunctioning of some brain MT protein kinases is expected to be one of possible roots of Alzheimer's and related diseases [32].

To our knowledge, only MAPK was shown by immunofluorescence to distribute along MTs in cultured interphase

cells. So, 3D2 protein seems to be the second described protein kinase with distinct co-localization with MTs. A part of 3D2 protein was localized in nuclei of CHO-K1 cells. Most probably, this means that the protein can translocate to the nucleus as other regulatory proteins do in response to cell signals, for example, as MAPK can [33]. The cellular function of the 3D2 protein is also under further investigation now. If the 3D2 protein is proven to be a protein kinase, we suggest to name it MAK-L, from microtubule-associated kinase, large.

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