Human tastin, a proline-rich cytoplasmic protein, associates with the microtubular cytoskeleton

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Tastin was originally identified as an accessory protein for trophinin, a cell adhesion molecule that potentially mediates the initial attachment of the human embryo to the uterine epithelium. However, no information regarding tastin's function is available to date. The present study is aimed at understanding the role of tastin in mammalian cells. Hence, we examined the intracellular localization of tastin in human cell lines transfected with an expression vector encoding influenza virus haemagglutinin (HA)-tagged tastin. Ectopically expressed HA-tastin was seen as a pattern resembling the fibres that overlap the microtubular cytoskeleton. When HA-tastin-expressing cells were cultured with nocodazole to disrupt microtubule (MT) polymerization, tastin was dispersed to the entire cytoplasm and an MT sedimentation assay showed tastin in the supernatant; however, tastin was sedimented with polymeric MTs in cell lysates not treated with nocodazole. Sedimentation assays using HA-tastin

mutants deleted at the N- or C-terminus revealed MT-binding activity associated with the N-terminal basic region of tastin. A yeast two-hybrid screen for tastin-interacting proteins identified Tctex-1, one of the light chains of cytoplasmic dynein, as a tastin-binding protein. Immunoprecipitation and Western-blot analysis confirmed binding of HA-tagged tastin and FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys epitope)-tagged Tctex-1 in human cells. Furthermore, *in vitro* assays have demonstrated the binding between a fusion protein, glutathione S-transferase-Tctex-1, and *in vitro* translated ³⁵S-labelled tastin. As Tctex-1 is a component of a MT-based molecular motor, these results suggest that tastin plays an important role in mammalian cells by associating with the microtubular cytoskeleton.

Key words: dynein, embryo implantation, microtubule-associated protein, trophinin.

INTRODUCTION

Human tastin was identified as a trophinin-assisting protein by expression cDNA cloning in which COS-1 cells transfected with a cDNA library from trophoblast-like cells were selected for calcium-independent cell-adhesion activity [1]. When trophinin and tastin are co-expressed in COS-1 cells, the transfected cells exhibit homophilic cell-adhesion activity, whereas expression of either trophinin or tastin alone does not induce adhesion activity. Trophinin is an intrinsic membrane protein that mediates homophilic self-binding. Tastin is a cytoplasmic protein associated with the trophinin-cell-adhesion molecule complex [1,2]. These molecules are strongly expressed in cells involved in embryo implantation and early placental development in humans [1,3]. These results suggest the involvement of the trophinin-cell adhesion molecule complex in human embryo implantation.

Morphological observations of the human embryo implantation site suggest that the initial attachment of trophectoderm cells of the blastocyst induces a subsequent stronger adhesion between embryonic cells and maternal epithelial cells [4,5]. Thus the embryo-uterus adhesion not only supports the physical foeto-maternal junction but also generates intracellular signalling cascades, necessary for morphological and functional changes in embryonic cells, and uterine epithelial cells required for the

developing placenta [5,6]. The cellular processes of embryo implantation resemble those of malignant tumour cell metastasis and invasion. Thus tumour cell invasion may share some molecular mechanisms with the trophoblast invasion of maternal uterine tissue [7]. Differentiation of embryonic cells subsequent to an implantation period has been a model for epithelial polarization and differentiation [8,9]. We hypothesize that defining the nature of trophinin-mediated cell adhesion and molecular dissection of this complex will provide us with useful information to understand embryo implantation as well as tumour metastasis in humans. As trophinin is a unique cell-adhesion molecule, studies of the trophinin complex could shed light on the biochemistry of cell-adhesion molecules.

Although several histochemical and genetic studies of the trophinin complex have been undertaken [3,10–12], the molecular dissection of the trophinin complex is not complete, and no information on the structure of tastin relative to its function is available at present. Tastin (Figure 1A) is an 83 kDa protein, rich in proline residues (16% of the total amino acids). As tastin has no similarity to other known proteins, the peptide sequence of tastin does not suggest its function. In the present study, we focused on intracellular localization of tastin and identification of the associated molecules, and we report that human tastin associates with microtubules (MTs) in mammalian cells and may

Abbreviations used: cdc(2), cell division control; CPP32, procaspase 3; FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys epitope; GST, glutathione S-transferase; HA, influenza virus haemagglutinin; MAP, microtubule-associated protein; MT, microtubule; NP40, Nonidet P40; SH3, Src homology 3.

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Figure 1 Tastin constructs

(A) Structure of human tastin. (Upper panel) Schematic representation of tastin, which is composed of 778 amino acids. Proline-rich sequences (PXXP) that potentially bind to an SH3 domain are indicated by crosses [32]. The location of the consensus sequences for cdc(2) and mitogen-activated protein kinases (S/TPXK/R and L/PXS/TP respectively) is also marked [33]. D, cdc(2) kinase; M, mitogen-activated protein kinase. Other potential phosphorylation sites are as indicated previously [1]. (Lower panel) Electrostatic property of tastin. Arginine, histidine and lysine residues of tastin are scored +1, aspartic acid and glutamic acid residues are scored -1 and the others are scored 0. The sum of the scores of 33 continuous residues was divided by 33. The density of positively and negatively charged amino acids in a sliding window of 33 residues was plotted. (B) Constructs of tastin and its deletion mutants are used in the present study. An HA tag is represented by a striped box. Deletions in ΔC and ΔN mutants are indicated with connecting lines. The calculated pI values of the constructs are also listed.

directly interact with Tctex-1, one of the light chains of a MT-based motor, i.e. cytoplasmic dynein.

EXPERIMENTAL

Tastin and control constructs

Full-length cDNA encoding human tastin was isolated as described previously [1]. Deletions of tastin cDNA (Figure 1B), which included appropriate restriction sites for cloning, were generated by PCR. These cDNAs were inserted together with an influenza virus haemagglutinin (HA)-tagged nucleotide sequence into pBluescript II KS (Stratagene, La Jolla, CA, U.S.A.) with the tag at the C-terminus of each peptide (Figure 1B). After verification by DNA sequencing, the resulting cDNA constructs were cloned into pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.) by using *HindIII*–*NotI* sites. The cDNA construct for HA-tagged full-length tastin was also inserted into the pPOI3CAT vector of the LacSwitch mammalian inducible expression system (Stratagene).

The open reading frame of human ribosomal protein L4 cDNA (GenBank® Nucleotide Sequence Database accession no.

L20868) was amplified from a human foetal brain cDNA library (Clontech Laboratories, Palo Alto, CA, U.S.A.) by PCR and subcloned into pCMV-Tag 2B (Stratagene) as described previously [13].

Antibodies

Antibodies were obtained as follows: mouse monoclonal antibodies against α-tubulin (clone B-5-1-2), β-actin (clone AC-15) and a FLAG (FLAG, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys epitope) tag (M5) were from Sigma; polyclonal rabbit anti-HA antibody was from BabCO (Richmond, CA, U.S.A.); mouse monoclonal anti-cytokeratin-8 antibody was from Dako (Carpinteria, CA, U.S.A.); and mouse monoclonal anti-CPP32 antibody (CPP32, procaspase 3) was from MBL (Nagoya, Japan).

Cells and culture

Human embryonic kidney 293T cells, human cervix HeLa cells, human colon adenocarcinoma SW480 cells, African green monkey kidney COS-1 cells and Chinese hamster ovary K1 cells

were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained according to the manufacturer's instruction.

Transfection was performed by using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. To achieve stable expression of tastin, SW480 cells were co-transfected with the pOPI3CAT-based tastin expression vector and the lac-repressor expression vector containing the cytomegalovirus immediate-early promoter, which was a derivative of p3'SS (Stratagene). The cells were selected in 0.4 mg/ml of hygromycin B and 0.4 mg/ml of G418. Surviving clones were screened by immunostaining with antibodies against the lac repressor (Stratagene) and then against the HA tag after treatment with 2 mM isopropyl β -D-thiogalactopyranoside for 20 h at 37 °C. Since three independent clones thus obtained showed the same results in immunocytochemistry, one clone was utilized for further analysis after induction of tastin by isopropyl β -D-thiogalactopyranoside as described above.

Specific perturbation of cytoskeletal components in cells

For MT depolymerization [14], cells in the medium were first kept for 30 min at 4 °C and then nocodazole (Sigma) was added at a concentration of 33 μ M. After a further 30 min incubation at 4 °C, the cells were incubated for 60 min at 37 °C. For MT stabilization, cells were incubated for 2 h at 37 °C in the presence of 20 μ M taxol (Molecular Probes, Eugene, OR, U.S.A.). For microfilament disruption, cells were treated with 20 μ M cytochalasin B (Sigma) for 15 h at 37 °C.

Immunocytochemistry

The cells grown on coverslips were rinsed with PBS and fixed with 0.5 % glutaraldehyde in 10 mM Mes (pH 6.0) containing 0.138 M KCl, 3 mM MgCl₂ and 2 mM EGTA [15]. The cells were then permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 10 min. The free aldehyde groups were reduced by using 0.1 % sodium borohydride in PBS for 10 min at room temperature. After blocking with 1 % BSA, the cells were incubated with primary antibodies for 1 h at room temperature in PBS containing 10 % normal goat serum (Sigma) and 1 % BSA. The cells were then incubated with appropriate secondary antibodies, including goat anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate (Accurate, Westbury, NY, U.S.A.) and goat anti-mouse IgG conjugated with fluorescein (Cappel, Durham, NC, U.S.A.) for 30 min at room temperature in PBS, 0.5 M NaCl, 1 % normal goat serum and 1 % BSA. For the detection of F-actin, rhodamine-phalloidin (Molecular Probes) was added to the secondary-antibody solution. The cells were examined under a Zeiss Axioplan fluorescence microscope.

Subcellular fractionation

The cells were pre-treated according to either of the following two procedures, and soluble and insoluble proteins were separated after cell lysis. (a) MT-depolymerizing condition: MTs in the cells were depolymerized with nocodazole as described above. The cells were then washed twice with PBS, lysed with TNE buffer (50 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, 1 % NP40, 1 mM EDTA, 1 mM PMSF, 10 μ g/ml aprotinin) for 3 min at 4 °C. The cells were then centrifuged at 16000 *g* for 5 min at 4 °C. (b) MT-stabilizing condition: cells were incubated with taxol as described above, washed twice with 100 PEM [0.1 M Pipes (pH 6.6) containing 1 mM EGTA/1 mM MgSO₄] and were lysed for 1 min at room temperature with 100 PEM containing 1 % NP40, 20 μ M taxol, 1 mM GTP, 1 mM PMSF

and 10 μ g/ml aprotinin. The lysate was then centrifuged at 16000 *g* for 5 min at room temperature. The supernatant thus obtained was clarified by centrifugation at 100000 *g* for 60 min at 4 °C. The precipitates were washed twice with lysis buffer. These fractions were then subjected to immunoblotting by SDS/PAGE and electroblotting [13].

MT co-sedimentation assay using cell lysates

Purified bovine brain tubulin dimer (> 99 % purity; ICN Bio-medicals, Aurora, OH, U.S.A.) was polymerized in 100 PEM containing 20 μ M taxol and 1 mM GTP for 15 min at room temperature and used for the following assay. Tastin-stable transfectants were treated under MT-depolymerizing conditions, washed twice with PBS and lysed with TNE buffer. After a brief centrifugation at 16000 *g* for 5 min at 4 °C, the lysate was further clarified by ultracentrifugation. The supernatant was dialysed at 4 °C against 0.1 M Pipes (pH 6.6) containing 1 mM EDTA, 0.25 mM PMSF and 2.5 μ g/ml aprotinin and centrifuged again at 100000 *g* for 60 min at 4 °C. After the addition of EGTA, MgSO₄ and poly(ethylene glycol) at the final concentrations of 1 mM, 10 mM and 1 % respectively, the lysate was incubated for 20 min at 30 °C and centrifuged at 100000 *g* for 20 min at 30 °C to remove insoluble materials. Taxol, GTP and bovine brain MTs were added to the supernatant at the final concentrations of 20 μ M, 1 mM and 1 mg/ml respectively. The mixture was incubated for 10 min at 30 °C and centrifuged at 100000 *g* for 10 min at 30 °C. After collection of the supernatant, the pellet was washed twice with 100 PEM (pre-warmed at 30 °C) containing 1 % NP40, 20 μ M taxol and 1 mM GTP. The supernatant and pellets were dissolved in the same volume of 1 $\times$ SDS/PAGE sample buffer and analysed by immunoblotting.

In vitro transcription–translation and MT-binding assay

In vitro transcription–translation from the tastin and ribosomal L4 expression vectors was performed by using the TNT-coupled reticulocyte lysate system and RNasin (Promega, Madison, WI, U.S.A.), and L-[³⁵S]methionine (10 μ Ci/ml; NEN, Wilmington, DE, U.S.A.) according to the TNT kit manufacturer's instructions.

The *in vitro* transcription–translation product was dissolved in 100 PEM containing 1 % Triton X-100 and 1 % poly(ethylene glycol), kept on ice for 30 min and centrifuged at 100000 *g* for 60 min at 4 °C. Taxol, GTP and bovine brain MTs were added to the supernatant and the mixture was incubated and centrifuged to obtain MT-unbound and MT-bound fractions as described above. These fractions were dissolved in the same volume of SDS/PAGE sample buffer and analysed by SDS/PAGE. The gels were dried at 60 °C on Whatman 3 MM chromatography paper and subjected to autoradiography. The band intensity of the autoradiograms was measured by laser densitometry using NIH Image software.

RNA dot-blot analysis

The 3'-terminal 0.35 kb fragment of the open reading frame of tastin cDNA was isolated from the tastin expression vector and random-primed by using the Prime-It labelling kit (Stratagene) and [α -³²P]dCTP (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) for hybridization. A human RNA master blot (catalogue no. 7770-1, lot no. 8020237; ClonTech Laboratories) was hybridized, washed and subjected to autoradiography according to the manufacturer's instructions.

Yeast two-hybrid assay

By using pGilda to encode the full length of tastin as the bait, a human foetal brain cDNA library (Invitrogen) was screened by the method of Matsuzawa et al. [16]. Briefly, cells of the EGY48 strain of *Saccharomyces cerevisiae* (MAT α , trp1, ura3, his, leu2::plexApo6-leu2) were transformed by the LiCl method using 0.12 mg of the pJG4-5 cDNA library. Clones were formed on leucine-free Burkholder's minimal medium plates containing 2% galactose and transferred to Burkholder's minimal medium plates containing leucine and 2% glucose. A filter assay was performed for β -galactosidase measurements to select sample transformants. These sample cDNAs were sequenced and subjected to a BLAST search (National Center for Biotechnology Information, Bethesda, MD, U.S.A.).

Immunoprecipitation

cDNA encoding full-length Tctex-1 was subcloned from the pJG4-5 clone into pCMV-Tag2B in order to express N-terminally FLAG-tagged Tctex-1. After transfection, the cells were extracted for 30 min on ice with TNE buffer and were centrifuged at 16000 g for 5 min at 4 °C. The lysates were pre-treated with Protein G-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C and then centrifuged. The supernatant was incubated with appropriate antibodies for 2 h on ice. After the addition of Protein G gel, samples were incubated for 1 h at 4 °C and centrifuged. Protein G-bound immune complexes were washed five times with the same buffer. Immune complexes were eluted by boiling in SDS/PAGE sample buffer and immunoblotted.

In vitro binding assay using Tctex-1 protein produced in bacteria

To prepare Tctex-1 fused with glutathione S-transferase (GST), Tctex-1 cDNA was subcloned into the pGEX-KG vector [17]. *Escherichia coli* DH5 α was transformed with the expression vector, and a GST fusion protein was produced and purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The beads containing immobilized fusion proteins were equilibrated for 3 h at 4 °C with Tris/HCl buffer (pH 7.5) containing 20 mg/ml BSA, 0.5% Triton X-100, 0.5 M NaCl and 1 mM EDTA. ³⁵S-Labelled proteins produced by *in vitro* transcription-translation were then added to the gel suspension and incubated for 3 h at 4 °C with gentle agitation. The resin was washed four times with Tris/HCl buffer (pH 7.5) containing 0.5% Triton X-100, 0.5 M NaCl and 1 mM EDTA. The proteins that were bound to the resin were then eluted by boiling in SDS/PAGE sample buffer. An equivalent amount of each fraction was analysed by SDS/PAGE and autoradiography.

RESULTS

Intracellular localization of ectopically expressed tastin in mammalian cells

In a previous study [1], we showed the subcellular localization of tastin associated with cytokeratin. In trophinin-expressing cells, tastin may be associated with cytokeratin through bystin, a cytoplasmic protein that directly binds to trophinin, tastin and cytokeratin [2]. To understand the function of tastin, we examined the subcellular localization of tastin by ectopic expression of tastin in human cell lines. A mammalian expression vector encoding tastin, which was HA-tagged at the C-terminus, was transiently transfected into COS-1 cells (Figure 1). In this way, tastin expression could be visualized by immunocytochemistry using an anti-HA antibody. In a preliminary experiment, HA-

tastin expressed in COS cells was seen as a pattern resembling fibres (results not shown), suggesting that tastin co-localizes with the cytoskeleton. Tastin-expressing COS-1 cells were then doubly stained with an anti-HA antibody and with reagents for specific detection of either MTs, cytokeratin or F-actin. This experiment indicated that tastin co-localizes with MTs (Figure 2A, panels 1 and 2) but not with cytokeratin or F-actin (results not shown). This co-localization was also observed in mitotic cells (Figure 2B). Immunostaining for tastin overlapped with spindle MTs, especially strongly with their centrosome region. Co-localization of tastin with MTs was not limited to COS-1 cells. Tastin expressed transiently in 293T, HeLa and Chinese-hamster ovary K1 cells but stably in SW480 cells overlapped with MTs (results not shown). Furthermore, co-localization of tastin with MTs was not affected by the disturbance of actin fibres with cytochalasin B (results not shown), whereas tastin was dispersed within the cytoplasm after depolymerization of MTs by nocodazole (Figure 2A, panels 3 and 4). These results suggest that tastin associates specifically with MTs in mammalian cells.

Co-sedimentation of tastin with MTs in lysates of cells stably expressing HA-tastin

Co-localization of tastin with MTs suggests that tastin is a new MT-associated protein. To investigate further the association of tastin with MTs, we first examined the subcellular localization of tastin under MT-depolymerizing and MT-polymerizing conditions, using SW480 colon carcinoma cells stably transfected with HA-tastin cDNA. To solubilize MTs and microtubule-associated proteins (MAPs), tastin-stable transfectants were treated with nocodazole at low-temperature conditions, which induce MT-depolymerization. The cells were then lysed with a buffer containing 1% NP40 and separated into soluble and insoluble fractions by centrifugation. Under these conditions, α -tubulin was completely fractionated into the supernatant, indicating that the MT components became soluble. Tastin was also found in the supernatant (Figure 3A, left column).

Under MT-stabilized conditions, cells stably expressing HA-tastin were treated with taxol. The cells were then lysed in 1% NP40 and centrifuged as described above. The results show that tastin co-sedimented with MTs (Figure 3A, right column). After the taxol treatment, the localization of cytokeratin 8 and β -actin was not affected, since these proteins showed little difference in supernatant-precipitate distribution under MT-depolymerized and MT-polymerized conditions. Actin detected in the supernatants is probably of the monomeric G-form, as it is known that a large amount of monomeric actin (30–80% of the total actin) is present in cells [18]. It is unlikely that MTs polymerized by taxol artificially increased pelleted tastin under the MT-stabilized condition, since a cytoplasmic protein CPP32 was completely recovered from the supernatant under this condition (Figure 3A, bottom blot of the right column) [13]. Thus results obtained by these fractionation experiments support the hypothesis that tastin specifically associates with MTs in stably transfected SW480 cells.

Binding activity of tastin to MTs in vitro

To test whether detergent-solubilized tastin bound to MTs *in vitro*, cell lysates containing HA-tastin were mixed with MTs prepared from purified bovine brain tubulin (see the Experimental section for details). SW480 cells stably expressing HA-tastin were treated under MT-depolymerizing conditions to release MAPs from MTs. The cells were then lysed with a buffer containing 1% NP40 and centrifuged. The supernatant was then incubated with exogenously added MTs. The mixture was

Figure 2 Tastin co-localizes with MTs

COS-1 cells were transfected with expression vectors for full-length tastin (Figure 1B, FL) and subjected to double-immunofluorescence microscopy (panels 1 and 2). Other transfected cells were incubated under MT-depolymerizing conditions before immunofluorescence microscopy (panels 3 and 4). The cells were fixed with glutaraldehyde, permeabilized with Triton X-100 and were double-stained with polyclonal anti-HA antibody (panels 1 and 3) and monoclonal anti- α -tubulin antibody (panels 2 and 4). (A) Interphase, (B) mitotic phase. Bars, 20 μ m.

subjected to ultracentrifugation to precipitate MTs. This experiment showed that tastin co-sedimented with exogenous MTs (Figure 3B, lane 2), whereas tastin remained in the supernatant when exogenous MTs were not provided. This result excludes the possibility of self-polymerization of tastin or co-sedimentation of tastin with any molecule other than MTs (Figure 3B, lane 4). Therefore tastin protein in solution binds to MTs in the presence of 1 % NP40.

Association of tastin with MTs was also assayed by using tastin protein translated *in vitro*. Full-length 35 S-labelled tastin (Figure 1B, FL) was produced by *in vitro* transcription-translation and incubated with pure MTs in the presence of 1 % Triton X-100. The mixture was ultracentrifuged to pellet the MTs. As shown in Figure 4 (bar 1 from left), almost all labelled tastin co-sedimented with MTs. However, labelled tastin did not precipitate at all when MTs were not included (Figure 4, bar 3). These results suggest that tastin polypeptide can directly bind to MTs. However, since the tastin preparations used in the above experiments were derived from either SW480 cell lysates or

rabbit reticulocyte lysates, they could include proteins that bridge the interaction between tastin and MTs.

Identification of the MT-binding region in the tastin polypeptide

Binding of full-length HA-tastin to MTs was inhibited by 0.5 M NaCl (Figure 4, bar 2 from left). Hence, the electrostatic interaction must be partially responsible for binding between tastin and MTs. Although the calculated pI of tastin is neutral, the N-terminal half of the molecule is basic and the C-terminal half is acidic (Figure 1A). To determine which part of the protein binds to MTs, two HA-tagged deletion mutants (Δ N and Δ C; see Figure 1B) were subjected to a MT-binding assay *in vitro*. Δ C strongly bound to MTs and this binding was inhibited by 0.5 M NaCl (Figure 4, bars 4 and 5 from left). In contrast, only 10 % of Δ N bound to MTs (Figure 4, bar 2 from right). The binding of tastin to MTs appears to be more specific than merely an electrostatic interaction, because ribosomal protein L4, a strongly basic protein with a calculated pI of 11.7, hardly associated with

Figure 3 Association of tastin with MTs

(A) Subcellular fractionation. SW480 cells stably expressing tastin were treated under MT-depolymerizing conditions, lysed in TNE buffer and centrifuged to separate the supernatant (S) and the pellet (P). Other stable transfectants were treated under MT-stabilized conditions, lysed in 100 PEM buffer containing 1% NP40 and centrifuged. An equivalent amount of each fraction was subjected to immunoblotting with antibodies against the HA tag, α -tubulin, cytokeratin 8, β -actin and CPP32. The detected proteins are indicated on the left of the panels. (B) Co-sedimentation of tastin with MTs. A lysate of SW480 cells stably expressing tastin was incubated with bovine brain MTs, taxol and GTP, and subjected to ultracentrifugation to separate the supernatant (S, lane 1) and pellet (P, lane 2). As a control, the lysate was incubated with taxol and GTP, but MTs were not treated in the same manner (lane 3, supernatant; lane 4, pellet). An equivalent amount of each fraction was analysed by immunoblotting with the anti-HA antibody.

Figure 4 *In vitro* MT-binding assay of tastin and its mutants

In vitro translated tastin, its deletion mutants and ribosomal protein L4 were incubated either with bovine brain MTs (MTs+) or without MTs (MTs-) in the presence of the indicated concentration of NaCl and were ultracentrifuged to separate the supernatant (S) and pellet (P). An equivalent amount of each fraction was subjected to SDS/PAGE and autoradiography. *In vitro* translated products in the supernatant and pellets were measured by densitometry of the autoradiograms. Binding (%) = P/(S+P). Each bar represents the means $\pm$ S.D. for three independent experiments. The structures of FL, Δ C and Δ N are indicated in Figure 1(B).

MTs at low NaCl concentrations (bar 1 from right). Taken together, this result indicates that strong binding of tastin to MTs is achieved by a specific structure conformed by the basic N-terminal half of tastin.

Table 1 Expression of tastin in human normal tissues

The human RNA master blot was probed with 32 P-labelled tastin cDNA. Samples at the same expression level are listed in alphabetical order.

Relative expression level	Tissues
High	Bone marrow, colon, foetal liver, foetal thymus, small intestine, testis and thymus
Low	Appendix, foetal brain, foetal heart, foetal kidney, foetal lung, foetal spleen, lymph node, ovary, placenta and stomach
Undetected	Adrenal gland, aorta, bladder, brain, heart, kidney, liver, lung, mammary gland, pancreas, peripheral leucocyte, pituitary gland, prostate, salivary gland, skeletal muscle, spinal cord, spleen, thyroid gland, trachea and uterus

Tctex-1, a component of the MT-based molecular motor, directly associates with tastin

Although the results described above suggest that tastin associates with MTs directly, it is also possible that tastin associates with MTs indirectly through a third component present in the binding assay described above. The fact that tastin has no similarity with other MAPs also suggests the possibility that the binding between tastin and MTs is indirect. Tastin has no SH2 (Src homology 2)/or SH3 domains, but has many potential SH3 domain-binding sites (Figure 1A), further suggesting that tastin associates with cytoplasmic proteins other than MTs. To find such potential interactors, we screened a yeast two-hybrid cDNA library for tastin-binding proteins.

Previous studies of the expression of human tastin have been limited to human placenta tissues [1,3]. To choose interactor libraries suitable for screening, we examined the detailed tissue distribution of tastin using multiple-tissue hybridization (Table 1). Expression of tastin was not detected in most adult tissues, such as brain and heart, consistent with the previous study [1]. Tastin was not detected in the uterus, which may be related to the fact that tastin in the human uterus is expressed for a limited period associated with embryo implantation [1]. Our results showed that tastin is highly expressed in foetal liver and thymus, but moderately expressed in several foetal tissues including brain. Therefore we chose a yeast two-hybrid interactor library from foetal brain to screen for tastin-binding proteins.

A yeast expression vector pGilda, encoding HA-tagged tastin fused with LexA, was used as a bait. Expression of the fusion protein in yeast cells transformed with the bait vector was confirmed by immunoblotting of their lysates using anti-HA and anti-LexA antibodies (results not shown). From a screen of 1.2×10^7 transformants, 48 sample transformants were identified. The sequence of the samples revealed that seven clones encoded a partial- or full-length form of human Tctex-1, originally identified as a gene for the mouse *t* complex sterility locus [19]. The overlapping region of the clones was from Glu-8 to the C-terminal Ile, including almost all of the Tctex-1 peptide sequence. Tctex-1 did not bind to control proteins, including Bcl-2, Bax and Fas (Table 2), excluding the possibility that the interaction was non-specific.

Remarkably, tubulins were not selected in this library screening, despite the fact that tubulin cDNAs are abundant in the human foetal brain library. This result indicates that tastin bound either specifically to the polymeric form of MTs or indirectly to MTs. Bysin, another trophinin-assisting protein bound

Table 2 Interaction between tastin and Tctex-1 in yeast

Yeast two-hybrid assay experiments were performed by using EGY48 cells transformed with the prey and bait vectors indicated below. After growth for 2 days at 30 °C on histidine- and tryptophan-deficient medium containing glucose or galactose as a carbon source, a β -galactosidase filter assay was performed. The vectors used for control experiments are described in [16]. +, galactosidase positive; —, galactosidase negative; n.t., not tested.

Prey (pJG4-5)	Carbon source	Bait (pGilda)				
		Tastin	Bcl-2	Bax	Fas	Vector only
Tctex-1	Glucose	—	—	—	—	—
	Galactose	+	—	—	—	—
Bax	Glucose	—	—	n.t.	n.t.	n.t.
	Galactose	—	+	n.t.	n.t.	n.t.

Figure 5 Association of tastin with Tctex-1

(A) Human 293T cells were transiently transfected with vectors expressing HA-tagged tastin and Flag-tagged Tctex-1 and subjected to immunoprecipitation using a mouse monoclonal anti-FLAG antibody. Immunoprecipitation using unrelated mouse IgG was also performed as a control. (Panel IB) Immunoblotting of the transfectant lysates. The antibody used for detection is indicated on the top of each blot. Lane 1, 293T cells transfected with vectors expressing tastin and Tctex-1; lane 2, mock-transfected 293T cells. The migration positions of protein molecular mass markers (Daichi Pure Chemicals, Tokyo, Japan) are indicated. (Panel IP) Immunoblotting of immune complexes. Detected proteins are indicated on the left. Lane 1, immune complex obtained with anti-FLAG antibody; lane 2, immune complex obtained with control antibody. (B) *In vitro* translated tastin and ribosomal protein L4 (Input) were incubated with immobilized Tctex-1 fused with GST (GST–Tctex-1) and GST. After washing, proteins bound to the resin were eluted by boiling in SDS/PAGE sample buffer. An equivalent amount of each fraction was subjected to electrophoresis and autoradiography. (Left) Autoradiograms of the binding assay. The *in vitro* translated products used for the assay are indicated on the left. Lane 1, input; lane 2, fraction bound to GST–Tctex-1; lane 3, fraction bound to GST. (Right) Electrophoretic patterns of GST–Tctex-1 (lane 1) and GST (lane 2). The same volume of each resin used for the binding assay was subjected to SDS/PAGE (10% gel) and Coomassie Brilliant Blue staining. The migration positions of protein molecular mass markers are indicated on the left of the panel.

to tastin [2], was not identified in this two-hybrid screening. Since the expression level of bystin was very low in various tissues except the female reproductive organs [2,3], few clones of bystin cDNA, if present, might have been included in our library.

We then tried to determine whether the tastin–Tctex-1 interaction took place in mammalian cells. Hence, two mammalian expression vectors for HA-tagged tastin and FLAG-tagged Tctex-1 were transiently co-transfected into 293T cells. After immunoprecipitation of the transfectant lysates, a complex including tastin and Tctex-1 was detected by using anti-FLAG antibodies but not by using the control antibody; this indicated that tastin associated with Tctex-1 in human cells (Figure 5A).

To confirm the association between tastin and Tctex-1 *in vitro*, Tctex-1 was produced in bacteria and subjected to a binding assay with 35 S-labelled tastin produced *in vitro* by transcription–translation (Figure 5B). Tastin bound to GST–Tctex-1 but not to GST itself, and Tctex-1 did not bind to an unrelated protein, ribosomal L4. These results strongly suggest that the binding of tastin to Tctex-1 is direct and specific. The binding between tastin and Tctex-1 appears to be strong because binding was detected in the presence of 0.5 % Triton X-100 and 0.5 M NaCl.

Tctex-1 is one of the light chains of a dynein motor in many tissues [20,21]. Since dynein associates with MTs, these results suggest that tastin associates with MTs through dynein. Thus our results imply that the function of tastin is closely linked to that of MTs.

DISCUSSION

The present study was undertaken to characterize further the function of tastin. We have described previously [1] the trophoblast-assisting cell-adhesion activity of tastin in human trophoblastic cells and endometrial epithelial cells. Considering tastin's expression in several tissues unrelated to embryo implantation in humans (Table 1), it is possible that tastin plays an additional role. The present study demonstrated the MT-binding activity of tastin in several cell types that do not express endogenous trophinin, bystin and tastin. The biological significance of this activity of tastin is at present unknown. However, the MT-binding activity of tastin suggests that tastin may play a role in cell proliferation, as one of the major functions of MTs is to form spindle fibres during mitosis. The co-localization of tastin with mitotic spindles (Figure 2B) supports this hypothesis.

MTs are essential for all eukaryotic cells to proliferate and survive. On the other hand, expression of tastin is limited to certain cell types of human tissues, i.e. adult testis, thymus, small intestine, colon and bone marrow (Table 1). Genomic and EST database searches show that tastin is only found in mammals, and the number of EST clones in mice and rats is limited. We have recently demonstrated [12] significant differences in the expression pattern of trophinin in humans and mice. Although gene products of trophinin are expressed in both humans and mice, trophinin is not expressed in the mouse trophoblast, in sharp contrast with human trophinin. Future studies need to address whether tastin has functions unique to humans, as well as the functional consequence of its binding with MTs, whose expression and function are widely conserved.

The present study shows that tastin's N-terminal region, which is rich in basic amino acids, is responsible for binding tastin to MTs. Although tastin has no similarity to known MAPs, the use of a basic region for MT binding has been demonstrated for classic MAPs, including MAP 2 and τ [22]. Similar to these proteins, the basic region of tastin is probably bound to the acidic tails of α - and β -tubulins in MTs by an electrostatic interaction. A role of classic MAPs is stabilization of MTs [22].

In the case of tasin, however, no MT stabilization activity was observed. Therefore overexpression of tasin did not render MTs resistant to low temperature (Figure 2A) or nocodazole treatment (results not shown). Interestingly, only the basic region of the N-terminus has potential mitogen-activated protein kinase and cell division control [cdc(2)] kinase serine/threonine phosphorylation sites (Figure 1A). MT-binding sites of some MAPs are phosphorylated by these kinases *in vitro* and *in vivo*, and this decreases the affinity of MAPs to MTs [23]. Association of tasin with MTs might also be modulated by phosphorylation.

Since tasin may play a role in trophoblastic cells in creating efficient cell adhesion foci of trophinin [1], tasin's ability to associate with MTs and cytoplasmic dynein may be viewed as a role of the cell-adhesion molecule complex. Cell adhesion molecules such as integrins and cadherins transmit cell surface signals through protein kinases and the actin cytoskeleton [24–26]. For example, engagement of integrin-mediated cell-matrix adhesion tethers bundles of actin filaments to achieve stronger avidity and form a focal adhesion complex. It is possible that tasin associated with trophinin utilizes MTs as an anchor to stabilize trophinin–trophinin adhesion. Moreover, another trophinin-assisting protein, bystin, activates trophinin by interaction with cytokeratin fibres [2]. A previous study [2] shows that the interaction between trophinin, bystin and tasin becomes stronger when cytokeratin is included. It is therefore possible that in embryo implantation between 2 cytokeratin-expressing cells, i.e. trophoctoderm cells and endometrial epithelial cells, tasin associates with cytokeratin through bystin, to promote efficient adhesion of trophinin. Since tasin exhibits MT-binding activity and has potential protein-kinase-phosphorylation sites (Figure 1A), it is expected to play an active role after initial trophoblast adhesion, specifically in trophoblast proliferation and invasion.

The present study also shows that tasin interacts directly with Tctex-1, a light chain of cytoplasmic dynein. Dyneins were constructed from approx. 520 kDa heavy chains that consisted of multiple AAA domains (ATPases associated with cellular activities) [20]. The dynein heavy chains exhibited both ATPase and MT motor activities. The N-terminal 160 kDa of each heavy chain forms a stem that interacts both with additional heavy chains and an intermediate-chain–light-chain complex to form the basal cargo-binding domain of the enzyme. MT-associated dynein is a minus-end directed transporter, and its light chains, including Tctex-1, determine the cargo specificity of the motor [20,27]. Minus-ends of MTs contact the apical cell surface of polarized epithelia [27,28]. This raises the possibility that tasin connects trophinin with dynein to transport trophinin to the apical surface of epithelial cells by using the MT-based transport system. Indeed, in human placental chorionic villous trophoblast, tasin is seen exclusively at the apical side of plasma membranes [3]. Trophinin is also detected at the apical side of trophoblasts in human placenta [3]. On the other hand, dynein has been reported to be a physical force generator in cellular activities such as mitosis [29]. Co-expression of trophinin and tasin in COS-1 cells caused clustering of trophinin on the cell surface [1]. These results suggest another possibility that tasin in a trophinin complex interacts with a dynein motor to receive the forces that cluster trophinin on the cell surface. Obviously, further molecular dissection is required to test these hypotheses and to show the physiological relevance of the binding activity of tasin to MTs and Tctex-1.

Recently [30], tasin has been identified as a prostate cancer marker. Since the processes of embryo implantation differ significantly among different mammalian species and tasin may be unique to humans, expression of tasin in prostate cancer [30]

suggests the possibility that some human cancers undergo processes unique to humans. The absence of trophoblastic tumours in mice and the observation that choriocarcinoma is the most aggressive cancer in humans [31] suggest unique roles for trophinin and tasin in the human trophoblast and in malignant cancer.

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