Identification of a 100 kDa Microtubule-Associated Protein from Xenopus Eggs

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ABSTRACT—A 100 kDa protein was identified by a combination of microtubule affinity chromatography and microtubule co-sedimentation from *Xenopus* egg extracts. The 100 kDa protein was expressed in Stage VI oocytes and early embryos, and then decreased at tailbud stage. The 100 kDa protein was found in adult organs such as brain and liver. Immunofluorescent microscopy revealed that the 100 kDa protein was mainly detected in spinal cord, notochordal sheath, optic cup, lens and cement gland in tailbud embryos. In *Xenopus* A6 cells, the 100 kDa protein showed filamentous networks in the peripheral cytoplasm and uniform distribution around the nucleus. During cell division, the 100 kDa protein was localized to the mitotic apparatus. The 100 kDa protein may have some roles in microtubule dynamics, organization of the mitotic apparatus and maintenance of cell shape.

INTRODUCTION

A microtubule is a polar and dynamic structure (Kirschner and Mitchison,1986; Wade and Hyman, 1997). Microtubule-based structures mediate the wide range of cellular processes. Microtubules serve as rails for the transport of organelles (Hirokawa, 1998). At the onset of mitosis, microtubule network is reorganized into the mitotic apparatus (Hyman and Karsenti, 1996; Walczak and Mitchison, 1996). Microtubule-based processes also have important roles during early development. In *Xenopus laevis*, such processes include transport of certain RNAs to the vegetal cortex during oogenesis (Yisraeli *et al.*, 1990; Zhou and King, 1996), movement of the germ plasm (Ressom and Dixon, 1988; Robb *et al.*, 1996), dorsal-ventral axis specification (Elinson and Rowning, 1988) and new membrane formation during early cleavages (Danilchik *et al.*, 1998). Microtubule-associated proteins (MAPs) and microtubule motor proteins have been shown to regulate the organization of microtubule structures and to function through these structures (Hyman and Karsenti, 1996; Walczak and Mitchison, 1996; Hirokawa, 1998).

To understand the regulation of microtubule function during cell cycle or development, it is important to identify proteins that interact with microtubules and analyze their biochemical activity on microtubule dynamics and structures. *Xenopus* eggs and egg extracts are widely used for studying microtubule dynamics *in vivo* (Houliston and Elinson, 1991; Schroeder and Gard, 1992) and *in vitro* (Belmont *et al.*, 1990;

Verde *et al.*, 1990; Sawin and Mitchison, 1991). In this study, several proteins interacted with microtubules were identified by a combination of microtubule affinity chromatography and microtubule co-sedimentation from *Xenopus* egg extracts. Among them, a 100 kDa protein was found to associate with microtubules *in vitro* and *in vivo*. During development, 100 kDa protein was enriched to the spinal cord, notochordal sheath, optic cups, lens and cement gland of tailbud stage embryos. During cell division of *Xenopus* cultured cells, 100 kDa protein associated with interphase microtubule arrays and with mitotic apparatus. These results suggest the roles of the 100 kDa protein in organization and stabilization of the cell shape and the mitotic apparatus.

MATERIALS AND METHODS

Preparation of oocytes, eggs, embryos and organs

Xenopus female frogs were anesthetized on ice. Ovaries were isolated and treated with 2% collagenase (Wako Pure chemicals, Osaka) in modified Barth's solution (MBS; 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM Ca (NO_3)$_2$, 2.4 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) to remove follicle cells. Oocytes were staged according to Dumont (1972). Eggs were obtained by injecting human chorionic gonadotropin (Teikoku Zoki Ltd., Tokyo). For artificial fertilization, a sperm suspension from freshly minced testes in modified Steinberg's solution (MSS; 60 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO_3)$_2$, 0.83 mM $MgSO_4$, 5 mM Hepes, pH 7.4) was added to the eggs. Embryos were cultured in MSS at room temperature and staged according to Nieuwkoop and Faber (1967). Organs were removed from anesthetized frogs and were extensively washed with MSS and then two times with BRB80 (1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 80 mM Pipes, pH 6.8). They were homogenized with Potter-Elvehjem homogenizer in ten volumes of BRB80 and spun at 12,000g for 10 min at 4°C. The protein concentration was determined by the protein assay (Bio-Rad, Hercules, MA) using bovine serum albumin (BSA) as a standard.

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Preparation of egg extracts, microtubules and microtubule-associated proteins

Dejellied eggs were activated by a pulse of 12 V of alternating current in MSS and then washed three times with XB (0.1 M KCl, 1 mM MgCl$_2$, 0.1 mM CaCl$_2$, 50 mM sucrose, 10 mM Hepes, pH 7.7). They were packed by centrifugation at 600g at 4°C for 30 sec. Packed eggs were crushed in a minimal volume of XB by centrifugation at 10,000g for 15 min at 4°C. Extracts were supplemented with protease inhibitors (1 μg/ml leupeptin, pepstatin, chymostatin and 1 mM PMSF), ATP regenerating system (Murray, 1991) and DTT at 0.5 mM and stored at –80°C. Thawed low-speed extracts were supplemented with 5 μg/ml cytochalasin B and then centrifuged at 105,000g for 1 hr at 4°C. High-speed supernatants were supplemented with 1 μM taxol and incubated at 20°C for 5 min. Two additional aliquots of taxol were then added over a 10 min period to bring the final taxol concentration to 5 and 20 μM. The taxol-stabilized microtubules and associated proteins were pelleted by centrifugation at 80,000g for 30 min at 20°C through 40% sucrose cushions prepared with BRB80 plus 5 μM taxol and 1 mM GTP. The pellets were suspended in BRB80 containing 1 mM ATP, 1 M KCl and 20 μM taxol to 1/5 of the original volume of the extracts. After 30 min incubation at 20°C, eluted proteins were separated from microtubules by centrifugation at 80,000g for 30 min at 20°C through 40% sucrose cushions. The pellets were used as microtubules.

In the experiments shown in Fig. 4A, the microtubule pellets with associated proteins were suspended in BRB80 plus 20 μM taxol and following additions; 1 mM ATP; 0.1 M KCl; 0.5 M KCl. The supernatants were concentrated in Ultrafree CL with molecular weight cutoff of 10,000 (Millipore, Bedford, MA) and dialyzed against BRB10 (BRB80 made with 10 mM Pipes). Proteins were precipitated with 10% trichloroacetic acid (TCA), neutralized with 1 M Tris and dissolved in SDS sample buffer, and analyzed by SDS-PAGE.

Preparation of microtubule affinity columns

Microtubule affinity columns were prepared according to the method of Kellogg *et al.* (1989) with slight modification. Microtubules prepared as above were suspended in C buffer (1 mM MgCl$_2$, 1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 50 mM Hepes, pH 7.6) containing 20 μM taxol and 1 mM GTP. Taxol-stabilized microtubules (3–4 mg) were mixed with a 1:1 mixture of Affigel 10 (Bio-Rad, Hercules, USA) and Toyopearl 650-M (Tosoh, Tokyo) previously equilibrated with C buffer. BSA columns were prepared in the same manner and used as the control. In each case, about 70% of the input protein were bound to the column. The bed volume of the columns used were 5 ml and their protein contents were about 0.5 mg/ml for both microtubule and BSA columns.

Microtubule affinity chromatography

Low-speed extracts (4 ml) were diluted six times with C buffer and centrifuged at 105,000g for 1 hr at 4°C. The supernatant was applied first to the BSA column and washed with C buffer plus 25 mM KCl at a flow rate of 10 ml/hr. The flow through fraction was then applied to the microtubule column. After washing the column with C buffer plus 25 mM KCl, proteins bound to each columns were eluted in three steps with C buffer containing 1 mM ATP, 0.1 M KCl and 0.5 M KCl. Proteins in each fraction were precipitated with 10% TCA, neutralized with 1 M Tris as described above and analyzed by SDS-PAGE. For microtubule co-sedimentation experiments, each fraction was concentrated with Ultrafree CL, dialyzed against C buffer plus 25 mM KCl and adjusted to 1 ml.

Microtubule co-sedimentation experiment

Each fraction eluted from a microtubule affinity column was concentrated and dialyzed as above. They were separated into half and mixed with or without 50 μg of taxol-stabilized microtubules in C buffer containing 1 mM GTP and 20 μM taxol at final volume of 1 ml, and then incubated for 30 min at 20°C. They were centrifuged at 80,000g

for 30 min at 20°C. Proteins in the supernatants were precipitated with 10% TCA, neutralized with 1 M Tris and dissolved in SDS sample buffer. Pellets were directly dissolved in SDS sample buffer. They were analyzed by SDS-PAGE.

Preparation of antisera

The proteins eluted from microtubule affinity columns were separated by SDS-PAGE and the gel was stained with Coomassie Brilliant Blue (CBB). After destaining, gel was treated with 2% glutaradehyde in water for 1 hr at room temperature and washed extensively with water. Each protein band of interest (about 30 μg of protein) was excised with razor blade and homogenized in 0.4 ml of sterile phosphate-buffered saline (PBS; 110 mM NaCl, 1.9 mM KCl, 8 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, pH 7.4) by passing through the connected syringes. For the first immunization, gel slurry was emulsified with an equal volume of Freund's complete adjuvant while the remaining immunizations were with Freund's incomplete adjuvant. Mice were intraperitoneally injected at one-week intervals. The presence of specific antibody was tested by immunoblotting and immunofluorescent staining.

Immunofluorescent staining

Xenopus A6 cells were cultured in ASF (Ajinomoto, Tokyo)/H$_2$O/ fetal calf serum at a ratio of 50:40:10 at 23°C with 5% CO$_2$. They were fixed with 3.7% formaldehyde in PBS for 5 min, and treated with 0.1% Triton X-100 in PBS for 5 min. They were incubated with antisera (1:50 dilution) for 1 hr. After washing with PBS plus 0.05% Tween 20, protein was detected by rhodamine-labeled rabbit anti-mouse IgG or FITC-labeled goat anti-mouse IgG. DNA was stained by 0.1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Nacalai Tesque, Kyoto).

Xenopus embryos were fixed and embedded in polyester wax according to Houliston and Elinson (1991), and 8 μm thick sections were cut, expanded using 0.1% amylopectin, dewaxed with 100% ethanol, rehydrated through 70% and 30% ethanol and processed for immunofluorescent staining using the antisera or monoclonal anti-tubulin antibody (DM1α, Seikagaku Co., Tokyo).

Immunoblotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in Tris-buffered saline (TBS; 140 mM NaCl, 0.1% Tween 20 and 25 mM Tris, pH 7.4) containing 20% skim milk. After washing with TBS, membrane was incubated with antisera (1:100 dilution) for 1 hr at room temperature. Alkaline phosphatase-conjugated anti-mouse antibody was used as the secondary antibody.

## RESULTS AND DISCUSSION

Identification of the 100 kDa protein

To identify proteins that interacted with microtubules, first attempt was made by adding taxol to *Xenopus* egg extracts and both microtubules and associated proteins were collected by centrifugation (Vallee, 1982; Vallee and Bloom, 1983). However, they might contain proteins that bound to microtubules non-specifically in *in vitro* conditions. To distinguish these proteins from genuine microtubule-binding proteins as possible, combination of two methods, affinity chromatography followed by microtubule co-sedimentation was adopted. Taxol-stabilized microtubules were crosslinked to the column resin. High-speed supernatants of egg extracts were first applied to a control BSA column to remove non-specific binding and flow through fraction was then chromatographed on the microtubule affinity column. The bound proteins were eluted sequen-

tially with 1 mM ATP, 0.1 M KCl or 0.5 M KCl, and analyzed by SDS-PAGE (Fig. 1). Many proteins were specifically eluted from microtubule columns. Little protein was bound to the control BSA columns. Then proteins in each fraction were tested for their ability to precipitate with microtubules. Taxol-stabilized microtubules were added to each fraction eluted from the columns. After incubation, microtubule-bound and unbound proteins were separated by centrifugation and analyzed by SDS-PAGE (Fig. 2). In these experimental conditions, recovery of the proteins with molecular weight higher than 200 kDa was very low, perhaps due to the proteolytic degradation during preparation in relatively high temperature or in acidic condition (see Materials and Methods). Two proteins of 40 kDa and 35 kDa in ATP fraction and a protein of 100 kDa in 0.1 M KCl fraction were specifically precipitated with microtubules (indicated with dots in Fig. 2). These proteins could bind to microtubules by themselves or together with other protein(s) in the same fraction. When fractions eluted with ATP and salts from a microtubule column were combined together and the mixture was processed for co-sedimentation experiment (lanes indicated with ATP/KCl in Fig. 2), no additional proteins could be found to bind to microtubules in this study.
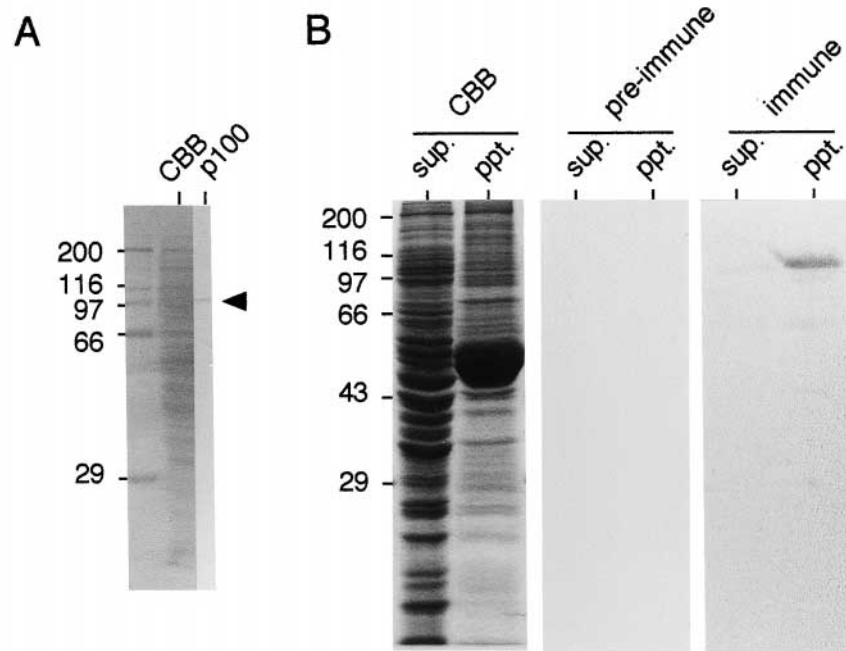
The 100 kDa protein (p100) in 0.1 M KCl fraction from microtubule affinity column could be easily recognized after CBB staining (indicated with a dot in Fig. 1). To characterize p100 further, antisera were generated by immunizing mice with gel purified protein. Figure 3 shows the specificity of an antiserum raised against p100. The antiserum recognized
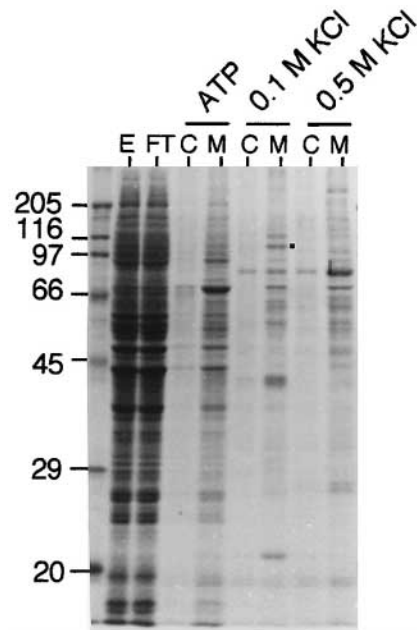

Fig. 1. Analysis of the proteins eluted from a microtubule affinity column and a BSA control column. High-speed supernatant was loaded onto the columns and bound proteins were eluted sequentially with 1 mM ATP, 0.1 M KCl and 0.5 M KCl. E; high-speed extpract, FT; flow through from a microtubule affinity column, C; control BSA column, M; microtubule affinity column. The proteins in each fraction were resolved by SDS-PAGE and stained with Coomassie Brilliant Blue. Numbers on the left indicate the molecular weights $\times 10^{-3}$ of marker proteins. A dot in 0.1 M KCl fraction from the microtubule affinity column indicates the 100 kDa protein analyzed in this study.
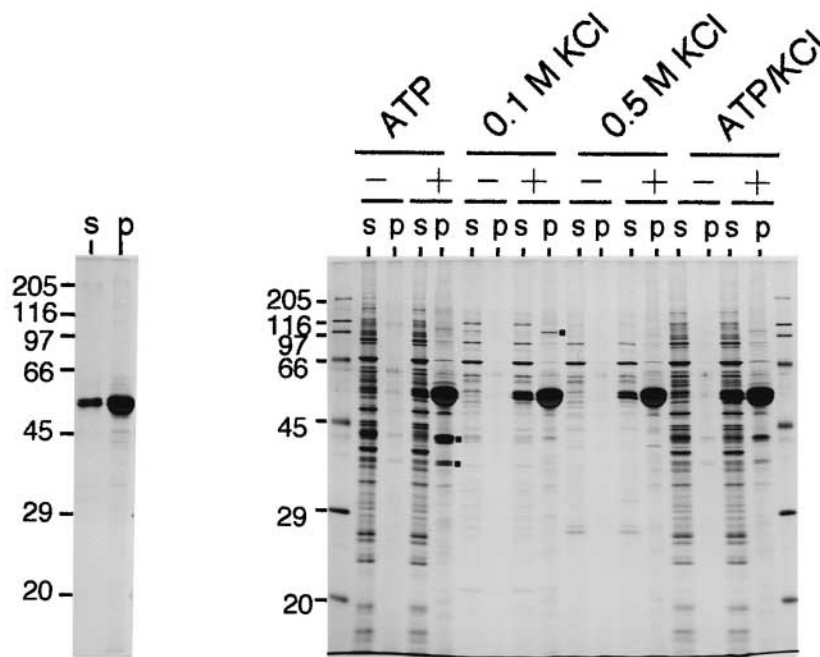
Fig. 2. Microtubule co-sedimentation experiments. (right) Proteins eluted with 1 mM ATP, 0.1 M KCl or 0.5 M KCl from a microtubule affinity column were incubated with (+) or without (–) taxol-stabilized microtubules (final concentration, 50 μg/ml). They were separated into supernatants (s) and precipitates (p) by centrifugation, analyzed by SDS-PAGE and silver staining. ATP/KCl indicates the experiment in which three fractions (ATP, 0.1 M and 0.5 M KCl) were mixed together and used for microtubule co-sedimentation. Dots indicate the proteins of 40 kDa and 35 kDa in ATP fraction and a protein of 100 kDa in 0.1 M KCl fraction which were specifically precipitated with microtubules. (left) The experiment with only taxol-stabilized microtubules used in this study.

Fig. 3. Specificity of a mouse antiserum raised against 100 kDa protein in 0.1 M KCl fraction from microtubule affinity column. (A) High-speed supernatant was electrophoresed, transferred onto a PVDF membrane and proved with the antiserum. CBB; proteins visualized by CBB staining, p100; immunoblotting with the anti-p100 antiserum. Arrowhead indicates the position of p100. (B) High-speed supernatant was incubated in the presence of taxol and separated by centrifugation. The supernatant (20 μg) and pellet (taxol-stabilized microtubules with MAPs; 5 μg) were electrophoresed, stained with CBB (left) or they were transferred onto the PVDF membrane and probed with pre-immune serum (middle) or antiserum (right). Only p100 was recognized by anti-p100 antiserum. There was no signal when the membrane was reacted with pre-immune serum.
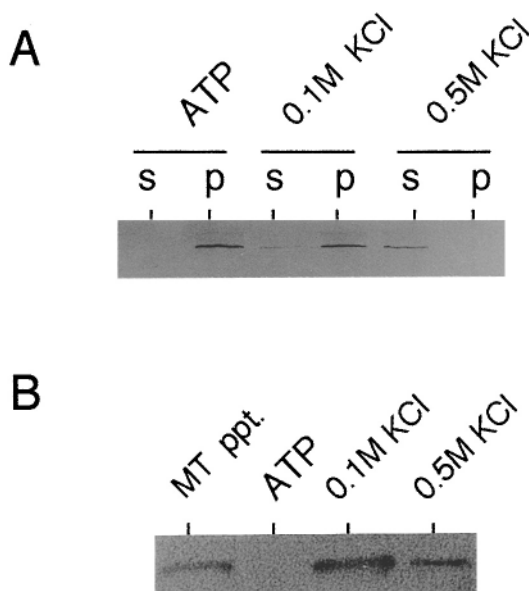

Fig. 4. Microtubule binding of p100 *in vitro*. (A) Taxol-stabilized microtubules with associated proteins were separated from high-speed supernatant, extracted by 1 mM ATP, 0.1 M KCl or 0.5 M KCl and analyzed by immunoblotting with anti-p100 antiserum. p100 was extracted from taxol-stabilized microtubules by 0.5 M KCl. (B) Fractions from a microtubule affinity column were probed with anti-p100 antiserum. Most of p100 was eluted by 0.1 M KCl and a portion of p100 was eluted by 0.5 M KCl. MT ppt.; taxol-stabilized microtubules with associated proteins.

p100 in *Xenopus* egg extracts and taxol-stabilized microtubule pellets (Fig. 3A, B). The binding property of p100 to taxol-stabilized microtubules was tested as shown in Fig. 4. Microtubule pellets were extracted with ATP or salts, separated by centrifugation and the presence of p100 was analyzed by immunoblotting after SDS-PAGE. Treatment of taxol-stabilized microtubule pellets with ATP or 0.1 M KCl could not release p100 from microtubules. All of p100 was released with 0.5 M KCl (Fig. 4A). When eluates from microtubule column were analyzed with the anti-p100 antiserum by immunoblotting, most of p100 was present in 0.1 M KCl fraction and residual amount of p100 was found in 0.5 M KCl fraction (Fig. 4B). The discrepancy of KCl concentration that could release p100 from taxol-stabilized microtubules between these experiments may be due to the conformation of microtubules whether they attached to the column resin or not.

Expression of the 100 kDa protein

Immunoblot analysis of adult *Xenopus* organs showed that p100 was expressed in brain and weakly in liver (Fig. 5A). There were no reactive species in heart and muscle. Expression of p100 during development was also examined (Fig. 5B). The 100 kDa protein was present in stage VI oocytes. Almost equal amount of p100 was present from stage VI oocytes to neurulae (stage 20). In tailbud embryos (stage 28), total amount of p100 was reduced. At this stage, several tissues expressed p100 (see below). Expression of p100
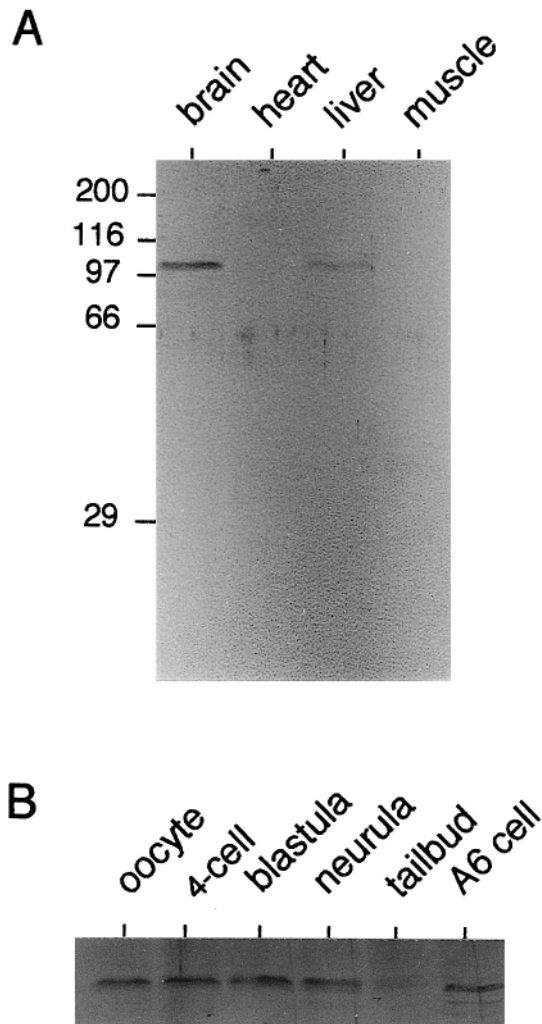
Fig. 5. Expression of 100 kDa protein in the adult *Xenopus* organs, early embryos and A6 cultured cells. (A) p100 was expressed in brain and weakly in liver, but not in heart and muscle. Equal amount of proteins (50 µg) were electrophoresed and proved with anti-p100 antiserum. (B) Expression of p100 during development and in *Xenopus* A6 cells (50 µg protein was loaded onto each lane). Stage VI oocytes were collected from ovary and embryos were harvested at 4 cell stage (4-cell), stage 9 (blastula), stage 20 (neurula) and stage 28 (tailbud). Almost equal amount of p100 was expressed until neurula stage and then decreased. A6 cells also expressed the 100 kDa protein.

seemed to be switched from maternal stock to tissue specific control between neurula and tailbud. The *Xenopus* A6 cultured cells also expressed p100 (Fig. 5B).

**Intracellular localization of the 100 kDa protein**

Immunoblot analysis (Fig 5B) showed that p100 was abundant between oocytes and neurula stage embryos. Subcellular distribution of p100 during these stages was examined by indirect immunofluorescent microscopy. Despite of its presence in eggs and early embryos, p100 was not localized to any recognizable structures including sperm astral microtubules shortly after fertilization, cytoplasmic microtubule ar-

rays and mitotic apparatus (data not shown). Clear localization of p100 was not seen until tailbud stage from which the amount of p100 decreased (Fig. 5B). In tailbud (stage 28) embryos, p100 was abundant in spinal cord and was especially enriched in ventro-lateral portion where axons would grow outward (Fig. 6B). Also, p100 was enriched in the region around notochord corresponding to the notochordal sheath (Fig. 6B). In developing eye (Fig. 6D) and cement gland (Fig. 6F) of later stage embryos (stage 34), p100 staining was observed to outline the cells forming each tissues. The reason why p100 did not associate with microtubule structure *in vivo* during early development is not known. Phosphorylation of MAPs causes reduction of their affinity for microtubules (Shiina *et al.*, 1992; Andersen *et al.*, 1994; Masson and Kreis, 1995). The association of p100 with microtubules may be suppressed by post-translational modifications such as phosphorylation during early developmental stages. From these observations, p100 appears to maintain the cell shape and confer mechanical strength on these tissues during later development.

Amphibian embryos have large yolk granules and the presence of them makes it difficult to recognize subcellular structures. Figure 5B showed that p100 was expressed in *Xenopus* cultured A6 cells, so the localization of p100 in A6 cells was examined (Fig. 7). In the cultured cells, p100 was found as the filamentous networks in the peripheral region of the cell and was evenly distributed around the nucleus (Fig. 7A').

Many MAPs and microtubule motor proteins have been shown to associate with the mitotic apparatus during mitosis (Andersen *et al.*, 1994; Houliston *et al.*, 1994; Boleti *et al.*, 1996; Walczak *et al.*, 1996; Walczak *et al.*, 1997; Andersen and Karsenti, 1997). The distribution of p100 during mitosis was also examined by immunofluorescence staining (Fig. 7B'–E'). In prometaphase cells, p100 was localized to the centrosomal regions and microtubules extended from them (Fig. 7B'). During metaphase and anaphase, p100 was localized to the mitotic spindle but astral microtubules were less or not stained (Fig. 7C', D'). At telophase, centrosomal region and the remnant of the spindle were stained (Fig. 7E').

Intracellular distribution of p100 resembles known proteins in *Xenopus* such as Eg5, XMAP215 and XMAP230 (Gard and Kirschner, 1987; Andersen *et al.*, 1994; Houliston *et al.*, 1994). However, they are able to associate with the microtubule structures during early development (Houliston *et al.*, 1994; Gard *et al.*, 1995) and their molecular weights are higher than p100. Tau is one of the well characterized microtubule-associated protein and also binds to interphase microtubule arrays and mitotic spindle (Connolly *et al.*, 1977). Tau has several variants, lower molecular weight (50,000–70,000) and higher molecular weight (100,000–125,000) (Drubin *et al.*, 1985; Viereck *et al.*, 1988). Antibodies to tau reacted with protein bands of 100–130 kDa and 50–70 kDa in *Xenopus* brain (Gard and Kirschner, 1987; Viereck *et al.*, 1988), however there was no reactive polypeptides in *Xenopus* egg and liver (Gard and Kirschner, 1987). The antisera raised against p100
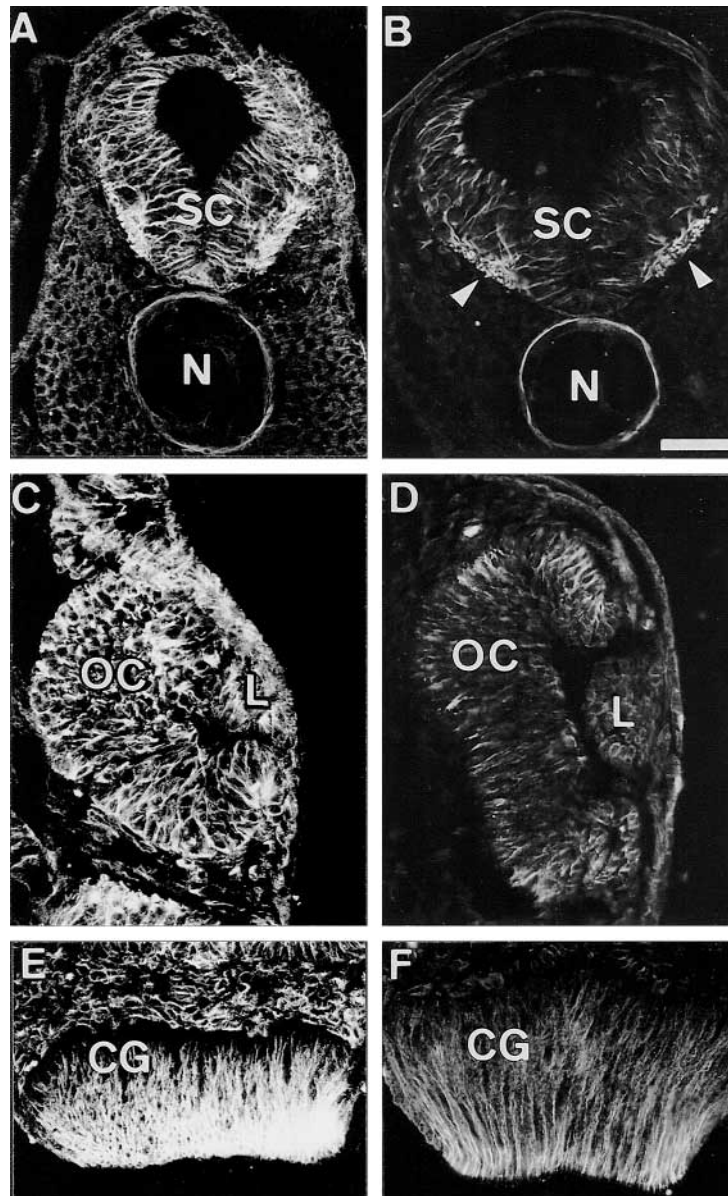
Fig. 6. Immunofluorescent staining of tailbud stage (stage 28 and stage 34) embryos. Sections were stained with anti-tubulin antibody (A, C, E) or with anti-p100 antiserum (B, D, F). (A, B) Dorsal side of the transverse sections of stage 28 embryos. (A) Almost all cells were labeled by anti-tubulin antibody. (B) p100 was enriched in spinal cord cells and notochordal sheath. In spinal cord, p100 appeared to be localized to the structure underlying the cell membrane and was especially enriched in the ventro-lateral portion of the spinal cord (arrowheads). Perhaps axons of growing neurons were stained. Anti-p100 antiserum staining was also seen in the region around notochord corresponding to the notochordal sheath. (C-F) Sections of stage 34 embryos through optic cap and lens (C, D) and cement gland (E, F). Both antibodies revealed the similar filamentous distribution in these tissues. SC; spinal cord, N; notochord, OC; optic cup, L; lens, CG; cement gland. Bar, 50 μm.

recognized only p100 and did not react with polypeptides corresponding to the low molecular weight form of tau in the eggs and brain (Figs 3A and 5). So p100 appears to be different from *Xenopus* high molecular weight form of tau.

Recently, a 100 kDa microtubule-binding protein, TPX2, was identified and purified from *Xenopus* egg extracts (Wittmann *et al.*, 1998). TPX2 mediates the binding of a *Xenopus* kinesin-like protein, Xklp2, to microtubules (Wittmann *et al.*, 1998). Most of TPX2 was eluted from taxol-stabilized microtubules by 200-300 mM NaCl (Wittmann *et al.*, 1998).

Molecular weight and microtubule-binding property of TPX2 resembles p100, while localization of TPX2 during the cell cycle has not been determined. At present, whether TPX2 and p100 described here are the same protein is not known.

MAPs and microtubule motor proteins have been shown to affect microtubule dynamics and spindle morphology (Shiina *et al.*, 1992; Andersen *et al.*, 1994; Vasquez *et al.*, 1994; Walczak and Mitchison, 1996; Andersen and Karsenti, 1997). It should be tested how the binding of p100 to microtubules affects microtubule dynamics and morphology of the mitotic
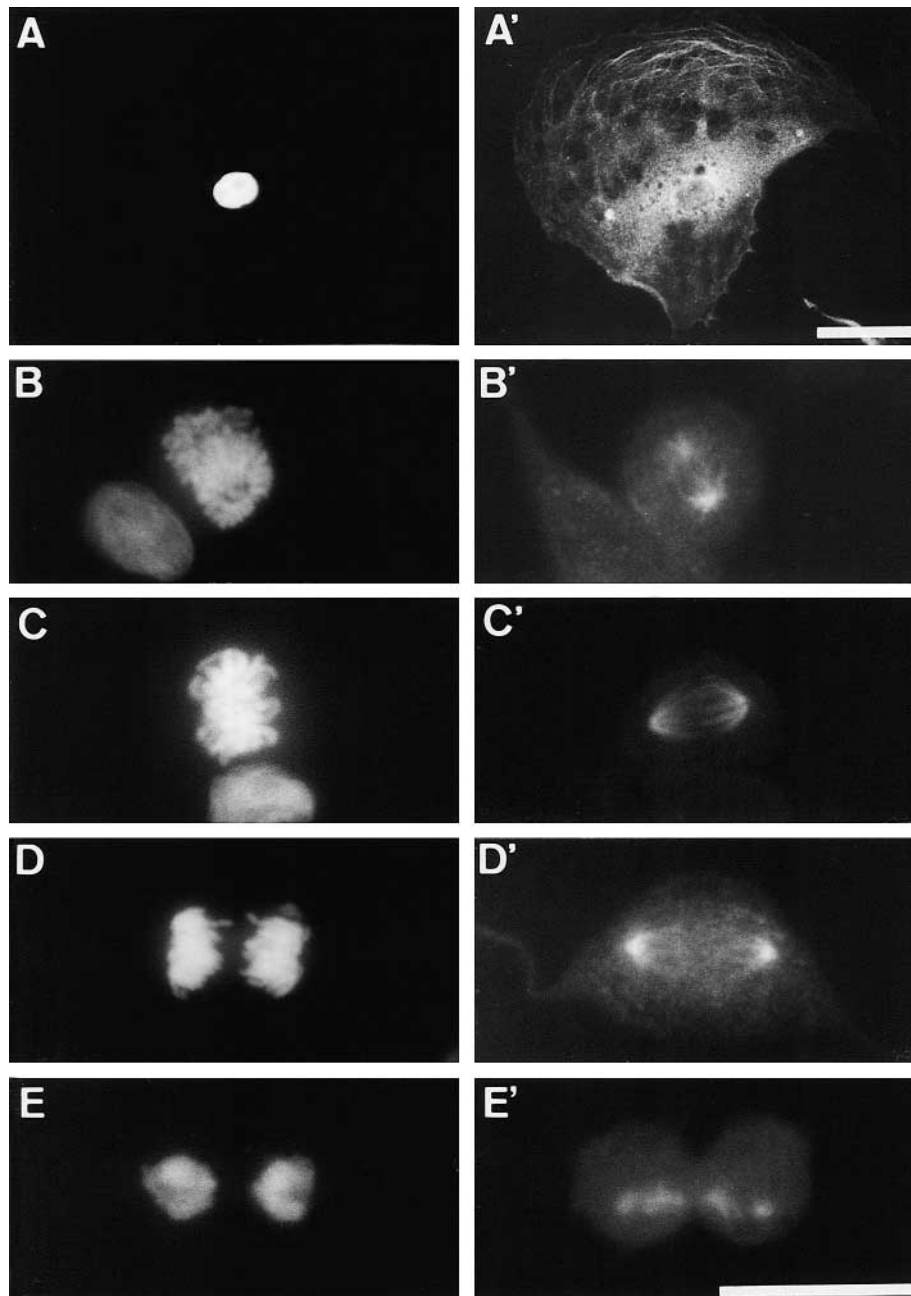
Fig. 7. Distribution of p100 in *Xenopus* cultured A6 cells during the cell cycle. A6 cells were stained with DAPI to visualize DNA (A, B, C, D and E) and with anti-p100 antiserum (A', B', C', D' and E'). In interphase cell (A, A'), p100 was stained as filamentous networks in the peripheral region of the cell. A uniform distribution was observed around the nucleus. Bar, 10 μm. At prometaphase (B, B'), centrosomes and microtubules extending from them were strongly stained. During metaphase (C, C') and anaphase (D, D'), p100 was localized to the spindle microtubules. During telophase (E, E'), remnant of the spindle and centrosomes were stained by anti-p100 antiserum. Bar, 10 μm.

apparatus.

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