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Characterization of a Microtubule Assembly Inhibitor From *Xenopus* Oocytes

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The dynamic properties of microtubules (MTs) are important for a wide variety of cellular processes, including cell division and morphogenesis. MT assembly and disassembly in vivo are regulated by cellular factors that influence specific parameters of MT dynamics. Here, we describe the characterization of a previously reported MT assembly inhibitor activity from *Xenopus* oocytes [Gard and Kirschner, 1987: *J. Cell Biol.* 105:2191–2201]. Video microscopy measurements reveal that the inhibitor specifically decreases the plus end growth rate of MTs and increases the critical concentration for tubulin. However, catastrophe frequency, rescue frequency, and shrinkage rates are not affected by the activity. Chromatography on Mono Q and hydroxyapatite columns has shown that the activity cofractionates with a subpopulation of tubulin. This tubulin subpopulation and the MT assembly inhibitor activity also co-migrate with a large S value (25–30S) on sucrose gradients. The high molecular weight tubulin complex and the MT assembly inhibitor activity are both developmentally regulated and disappear after oocyte maturation with progesterone. Cell Motil. Cytoskeleton 45:51–57, 2000.

Key words: microtubules; tubulin; *Xenopus;* oocyte; dynamic instability

INTRODUCTION

The dynamic properties of microtubules are critical for a wide variety of cellular functions, such as establishing and disassembling the mitotic spindle and changing cell shape during development [Brinkley et al., 1976]. The formation and disassembly of microtubule (MT) networks must be tightly regulated to ensure their correct positioning both temporally and spatially. During oogenesis in *Xenopus laevis*, MTs undergo dramatic and rapid rearrangements [Gard et al., 1995]. Interphase oogonia contain a sparse radial array of MTs, but postmitotic oocytes (stage 0) contain MTs in a cytoplasmic cap at one end of the oocyte. Stage I oocytes exhibit a disperse MT array that then changes to a complex array with MTs concentrated in the cortex. At stage III, the MTs radiate from a yolk-free region surrounding the germinal vesicle. Finally, during stage IV–VI, the MT array becomes polarized along the animal-vegetal axis, presumably to facilitate transport of material to the two poles. The oocyte volume increases by 500-fold from stage I–VI, and during this time there is also a 5,000-fold increase in the cytoplasmic pool of tubulin [Gard et al., 1995]. Thus, the oocyte faces unique challenges in regulating tubulin polymerization during this rapid period of growth.

In vivo, MTs are highly dynamic structures that are either growing by addition of tubulin dimers to MT ends or rapidly shortening [Mitchison and Kirschner, 1984; Walker et al., 1988]. MTs convert between these two phases infrequently. Abrupt and stochastic transitions from growing to shrinking (catastrophe) or shrinking to growing (rescue) have been observed for MTs both in

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vivo and in vitro. The rates of growth and shrinkage and frequencies of catastrophe and rescue are all important parameters that can be modulated to regulate patterns of MT formation and disassembly [Desai and Mitchison, 1997]. The reported rates for MT assembly/disassembly in vitro from pure tubulin are much slower than what is observed in cells, suggesting that protein factors are responsible for regulating MT dynamics in vivo [Schulze and Kirschner, 1988].

Protein modulators of MT dynamics can function in a number of ways [see review by Desai and Mitchison, 1997]. MT-associated proteins, e.g., MAP2 or tau from brain, interact with the MT polymer and stabilize it by decreasing catastrophe frequency and increasing rescue frequency [Hirokawa, 1994]. Other proteins, such as Op18/stathmin, promote catastrophes by binding to and sequestering tubulin dimers [Belmont and Mitchison, 1996; Jourdain et al., 1997; Howell et al., 1999]. These proteins are crucial during the interphase-mitosis transition when long, stable MTs become shorter and less stable. The *Xenopus* system has also been useful in the study of MT dynamics [Parsons and Salmon, 1997]. *Xenopus* egg extracts have been well characterized and used for purifying a number of MAPs that regulate MT dynamics. XMAP230, the *Xenopus* homolog of MAP4, suppresses catastrophes and decreases shortening rates [Andersen et al., 1994]. XMAP215 regulates dynamics by increasing elongation rates and decreasing rescue frequencies [Vasquez et al., 1994]. Finally, XMAP310 is the first ''rescue factor'' identified; it increases rescue frequencies and decreases shortening rates of MTs in the mitotic spindle [Andersen and Karsenti, 1997]. Thus, different MAPs fulfill distinct functions and thereby regulate MT dynamics in specific ways.

Xenopus eggs and oocytes are naturally arrested at different points in the cell cycle, and have a unique distribution of MTs [see review by Gard et al., 1995]. Although their tubulin content is equivalent, it has been shown that egg extracts support extensive MT assembly, while oocyte extracts inhibit MT assembly [Gard and Kirschner, 1987]. Even in the presence of taxol and 20 μM exogenous tubulin, oocyte extracts cannot assemble MTs in vitro. Furthermore, it was shown that oocytes, but not eggs, contain a factor that inhibits growth from MT plus ends [Gard and Kirschner, 1987]. In this study, we have further examined this factor with the goal of understanding its biochemical properties as well as how it acts upon MTs. We show that the oocyte MT assembly inhibitor increases the critical concentration for tubulin at the plus ends of MTs, but does not affect shrinkage rates or transition frequencies. We have partially purified this activity and show that it cofractionates with a large tubulin complex that is found in oocytes but not eggs.

MATERIALS AND METHODS

Extract Preparation

Xenopus laevis oocytes were harvested and prepared as described by Gard and Kirschner [1987]. Typically 25–50 ml of packed oocytes were rinsed in Ca^{+2}-free MBS (Modified Barth's Saline: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM Hepes, pH 7.5) before digestion by 10 mg/ml collagenase (Boehringer Mannheim, Inc., Indianapolis, IN). Digested oocytes were washed into lysis buffer (80 mM KPipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 5 mM NaF, 0.2 mM PMSF, and pepstatin, leupeptin, and chymostatin each at 10 μg/ml). Extracts were prepared by dounce homogenization (20 strokes, Wheaton 40 ml dounce, pestle A). Crude extracts were separated into yolk, cytoplasm, and pigment granule layers by centrifuging at 100,000*g* for 90 min in a SW55 rotor. The clear cytoplasmic layer (HSS) was removed with an 18-gauge syringe needle and cytochalasin B was added to 10 μg/ml to prevent actin polymerization. Extracts were frozen in liquid nitrogen and stored at −80°C.

Microscopy/Assays

Differential interference contrast (DIC) microscope assays were carried out on a Zeiss (Thornwood, NY) Axioplan microscope with a 63x, 1.4 N.A. objective and illumination from a 100 W Hg lamp. Flow cells were made by placing a 18 × 18 mm coverslip over two horizontal pieces of double-sided tape, 1.5 cm apart. The approximate volume of the chamber was 25–30 μl. Microtubule formation from sea urchin sperm axonemes was measured as follows: Sea urchin axonemes (10 mg/ml) were diluted 1:80 in BRB80 (80 mM K-Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$) and adsorbed onto the surface of the coverslip for 10 min at room temperature. Unbound axonemes were removed by washing the chamber with excess BRB80. A 25 μl mixture of bovine brain tubulin (20–25 μM), 1 mM GTP, and BRB80 were flowed into the chamber to begin MT polymerization from axonemes. After 10 min of growth, when plus and minus ends can be distinguished by their different MT lengths [Allen and Borisy, 1974], extract or fractions were flowed into the chamber (5 μl extract or fraction, 1 mM GTP, 25 μM bovine brain tubulin unless stated otherwise, and BRB80 to 25 μl). Measurements of dynamic instability parameters were taken over a period of 30–40 min after addition of the extract. Growth and shrinkage rates were determined by measuring lengths of MTs over time. Transition frequencies were observed for individual MTs over the same time period.

Chromatography

The Pharmacia (Gaithersburg, MD) FPLC system was used for all protein purification procedures. Typically, 8–10 ml of clarified oocyte HSS (25–30 mg/ml) was subjected to ammonium sulfate fractionation. The 0–30% ammonium sulfate cut was resuspended in 1–2 ml of column buffer, passed through a 2-μm filter, frozen in liquid nitrogen, and stored at −80°C. Typically 1 ml of ammonium sulfate cut was loaded onto a 1 ml MonoQ column in BRB80. Separation was achieved by a 0–0.5M NaCl gradient over ten 1-ml fractions. One milliliter of the 0–30% ammonium sulfate fraction was also loaded onto a 5-ml hydroxyapatite column in 10 mM K-phosphate (pH 7.2) buffer. A two-step salt elution was performed using 0.2 M and 0.4 M K-phosphate, and 22 1-ml fractions were collected, dialyzed into BRB80, and assayed for MT assembly inhibition. To test activity, fractions were dialyzed into BRB80 using a microdialyzer (Pierce Chemical Co., Rockford, IL). To rapidly test whether an extract or fraction had inhibitory activity, the following assay was performed. Axonemes were adsorbed to coverslips as described above. A mixture of extract/fraction, 1 mM GTP, 25 μM bovine brain tubulin, and BRB80 was added directly to the flow cell. After 15 min at room temperature, the number of MTs formed per axoneme was assessed. The activity was expressed as the percentage of axonemes without MTs. At least 50 axonemes were counted per assay.

SDS PAGE/Immunoblotting

Gel samples were prepared in Laemmli sample buffer (Laemmli, 1970) and loaded onto 5–15% polyacrylamide gradient gels. Gels were either Coomassie stained and destained or transferred by semi-dry blotting onto nitrocellulose. Nitrocellulose was stained with Ponceau S, rinsed in water, blocked with 5% dry milk, and incubated with primary antibody against alpha tubulin (DM1α-ICN) (1:200 dilution). HRP-conjugated mouse secondary antibody (Amersham, Arlington Heights, IL) was then incubated at 1:200 dilution for 1 h, and the signal was detected using a chemiluminescence kit (ECL-Amersham). Blots were scanned and quantitated using a UMAX scanner and NIH Image software.

Gradients

Sucrose gradients (5-ml) were prepared by layering equal volume steps of 40, 31.25, 22.5, 13.75, and 5% sucrose dissolved in BRB80. Extracts (300 μl) were layered on top and spun for 3 h at 55K rpm in a SW 55 rotor. Equal volume fractions were collected and processed either for SDS-PAGE followed by immunoblotting tubulin, or assayed for MT assembly inhibition by video microscopy. The following standards were also layered on a parallel gradient and detected by Coomassie staining of polyacrylamide gels: BSA (4.4S), catalase (11S), thyroglobulin (19.5S).

RESULTS/DISCUSSION

Gard and Kirschner [1987] previously described an MT assembly inhibitor from oocytes. In order to determine how this inhibitor functions, we measured the kinetic parameters of dynamic instability (MT growth rate, shrinkage rate, and the transition frequencies between these states) in the presence and absence of oocyte extract. This was accomplished by directly visualizing single microtubules using video-enhanced DIC microscopy. Since we observed that MTs could not grow off of axonemes in the presence of tubulin and oocyte extract, we first grew MTs off of sea urchin sperm axonemes using bovine tubulin (25 μM) at room temperature and then added 5 mg/ml of *Xenopus* oocyte high-speed supernatant (HSS) in the presence of added (25 μM) exogenous bovine brain tubulin and 1 mM GTP. The presence of exogenous bovine tubulin was required for these measurements, because the tubulin concentration in the oocyte extract was below the critical concentration and, consequently, resulted in the rapid catastrophe of all MTs. Under these conditions, video microscopy revealed that MTs continued to grow in the presence of oocyte supernatant. However, the factors in the supernatant substantially reduced the plus end growth rate from 1.5 μm/min to 0.25 μm/min ($P < 0.02$) (Fig. 1). In contrast, the minus end growth rate was unaffected by the presence of oocyte extract ($P > 0.5$) (Fig. 1).

The inhibitory factor could affect growth rate by altering the critical concentration for tubulin assembly at the plus ends of MTs. To determine whether this was the case, we measured the plus end growth rates in the presence and absence of oocyte HSS at various concentrations of added bovine tubulin (Fig. 2). In both cases, growth rate increased linearly as a function of tubulin concentration, and a linear regression fit revealed the critical concentration for assembly. The presence of oocyte HSS dramatically increased the critical concentration for tubulin assembly from 10 to 45 μM at the plus ends of MTs. The slope revealed that the second order rate constant for tubulin assembly declined from 0.04 $\mu m^{-1} min^{-1}$ to 0.009 $\mu m^{-1} min^{-1}$ in the presence of oocyte HSS.

To examine the shrinkage rate of MTs in the presence of oocyte extract, the tubulin concentration was abruptly lowered below the critical concentration by perfusing the flow cell with either buffer or oocyte extract without added tubulin and then following the decrease in MT length as a function of time. The shrinkage rates of MT plus ends in the presence and absence of oocyte extract were comparable (5.9 and 6.9 μm/min) ($P > 0.5$).

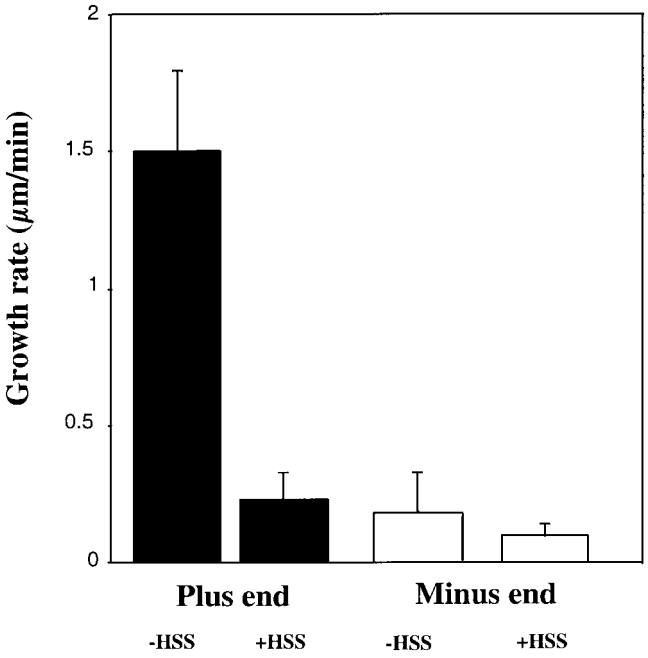

Fig. 1. MT growth rates (plus and minus end) in the presence and absence of *Xenopus* oocyte high speed supernatant (HSS) at 5 mg/ml. Measurements were carried out in the presence of bovine brain tubulin at 25 μM and 1 mM GTP at room temperature, as described in Materials and Methods. Mean growth rates were determined by measuring lengths over time for >30 microtubules. Standard deviations are shown.

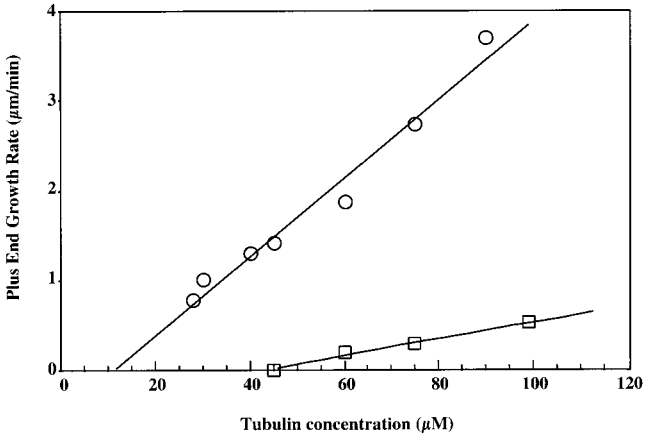

Fig. 2. Plus end MT growth rate at varying bovine tubulin concentrations in the presence or absence of *Xenopus* oocyte HSS (5 mg/ml). Growth rates were determined by measuring MT lengths over time as described in Materials and Methods. For each tubulin concentration, >25 microtubule growth rates were measured. The mean growth rates are shown. The standard error of the mean (S.E.M.) was within the symbols. *Open circles* and *squares* represent growth rates in the absence and presence of oocyte extract, respectively.

We also analyzed the transitions between MT growth and shrinkage (catastrophe and rescue) in the presence of *Xenopus* oocyte HSS and 25 μM bovine tubulin. Neither the catastrophe nor rescue frequencies differed significantly in the presence or absence of oocyte HSS (both were $\leq 0.1\ min^{-1}$).

In summary, the oocyte HSS specifically alters the plus end growth rate and the critical concentration for tubulin assembly at the plus end. From these results, we speculate that the protein factor may act by loosely capping and blocking subunit addition at the plus end. If the factor were simply sequestering tubulin and decreasing the monomer pool, one would expect to find an increase in critical concentration at the minus as well as the plus end and an increase in catastrophe frequency. We also suspect that this capping factor behaves dynamically, since we have shown that plus ends are not blocked in the presence of this activity. MT plus end-capping proteins have not been well characterized, although an unidentified minus end capping activity has been described from sea urchin eggs [Spittle and Cassimeris, 1996].

We next determined whether the oocyte MT assembly inhibitor activity was due to a protein. The activity was completely inactivated by trypsin but not RNase, indicating that it is indeed a protein and not an RNA (not shown). We then attempted chromatographic separation methods to purify the protein responsible for the activity. Since the MT assembly inhibitor greatly increases the critical concentration for tubulin assembly, we could rapidly assay for the presence of the inhibitor by scoring MTs assembled off of axonemes at a tubulin concentration of 20 μM. At this tubulin concentration, microtubules rarely assemble off of axonemes in the presence of oocyte HSS (Fig. 2). Activity in column fractions was measured by scoring the percentage of axonemes that had no MTs growing from them after 15 min at room temperature in the presence of 20 μM tubulin. Typically, addition of the oocyte HSS with 20 μM bovine tubulin resulted in 95% of axonemes without MTs, whereas <5% of axonemes had no MTs growing from them in the presence of 20 μM tubulin alone.

After ammonium sulfate fractionation of the oocyte HSS, >75% of the activity was recovered in the 0–30% fraction. This step concentrated the starting material for further column fractionation. When subjected to Mono Q or hydroxyapatite (HA) chromatography, the activity eluted in a single peak (Fig. 3A,B). The activity was purified 100-fold after the ammonium sulfate cut followed by either of these chromatographic steps. However, we were unable to retain significant activity after more than two chromatographic steps in series and hence could not purify the factor to homogeneity.

Since our earlier results suggested that the inhibitory factor could interact with monomeric tubulin (Fig. 2), we immunoblotted the column fractions for tubulin. Tubulin eluted as two peaks, suggesting a heterogeneity

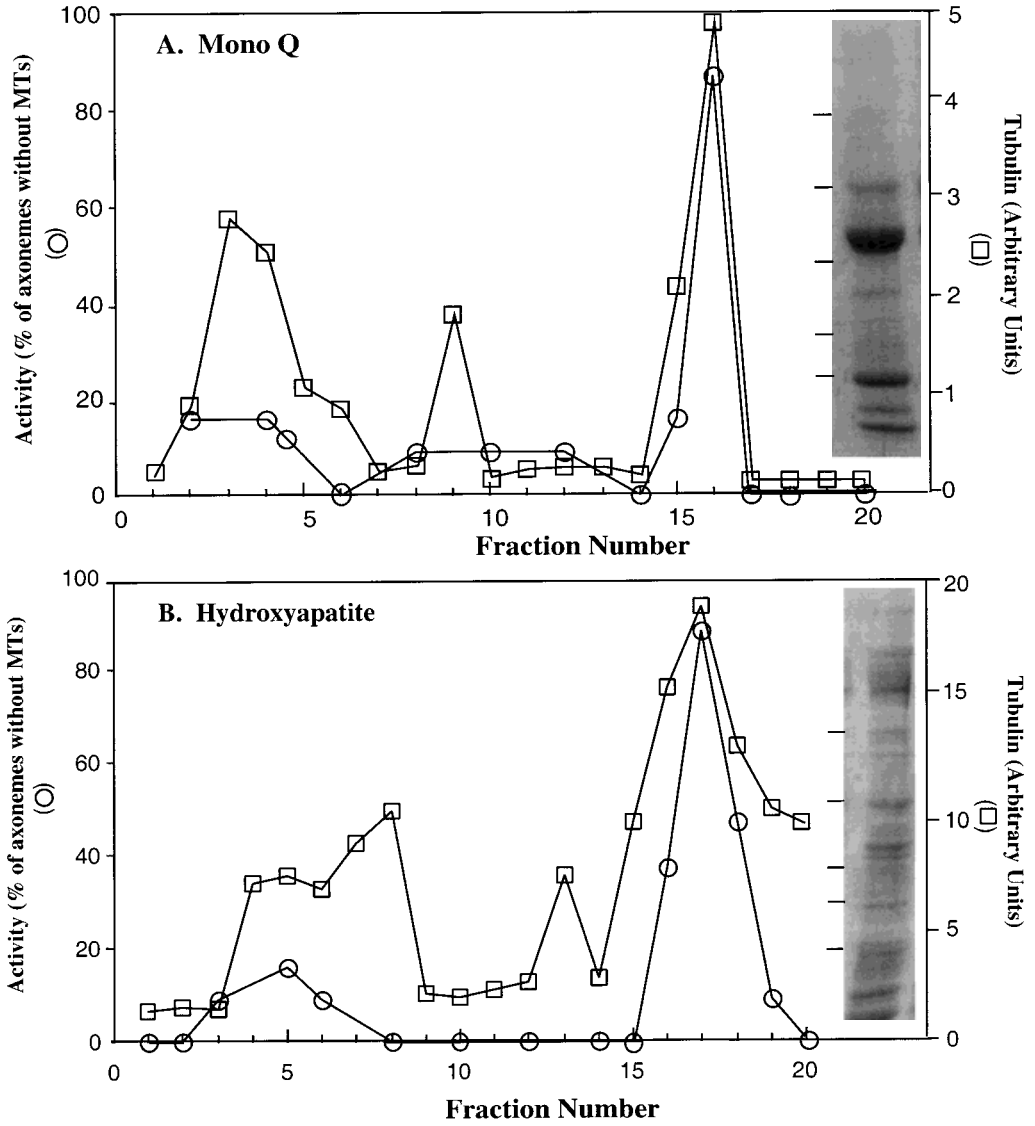

Fig. 3. Chromatography of the MT assembly inhibitor and tubulin. **A:** Mono Q Chromatography. Here, 0.5 ml of the 0–30% ammonium sulfate cut of 4 ml of oocyte HSS (25 mg/ml) was loaded onto a 1 ml Mono Q column. A 0–0.5 M NaCl gradient was run and fractions collected and assayed for MT inhibition activity (*circles*) as described in Materials and Methods. The peak activity eluted at 0.4 M NaCl. Specific activity was increased by 100-fold from the HSS to the peak Mono Q fraction. In parallel, fractions were processed for SDS-PAGE and immunoblotted with anti-alpha tubulin antibody. Blots were scanned and quantitated to show the tubulin distribution across the column (*squares*). **Inset:** 15 µl of the peak activity/tubulin fraction run on SDS-PAGE and Coomassie stained. Molecular weight markers (top to bottom) are 205, 112, 87, 69, and 57 kDa. **B:** Hydroxyapatite chromatography. Here, 0.5 ml of the 0–30% ammonium sulfate cut of 8 ml of oocyte HSS (25 mg/ml) was loaded onto a 5 ml HA column. A two-step salt elution was carried out (0.2 and 0.4M KPi) as described in Materials and Methods and fractions were collected and assayed for MT inhibitor activity (*open circles*). The activity eluted at 0.4M KPi. In parallel, fractions were also processed for SDS-PAGE and immunoblotted as in Figure 3a. Quantitation of the immunoblot shows the distribution of tubulin across the column (*open squares*). The peak fraction (**inset**) is shown after Coomassie staining. Molecular weight markers are (top to bottom) 205, 112, 87, 69, and 57 kDa.

of tubulin or an association of tubulin with other proteins. In both the Mono Q and HA columns, the MT inhibitory activity cofractionated with one of the two tubulin populations. We also immunoblotted fractions for Op18, gamma tubulin, and the chaperonin Hsp90, and did not find any of these proteins cofractionating with the inhibitory activity. The tubulin sequestering/MT destabilizing protein CKAP1 [Tokito et al., 1997] also did not cofractionate with the inhibitory factor.

We also determined the sedimentation properties of the inhibitory factor by sucrose gradient fractionation. We found that the activity sedimented at >20S on a 5–40%

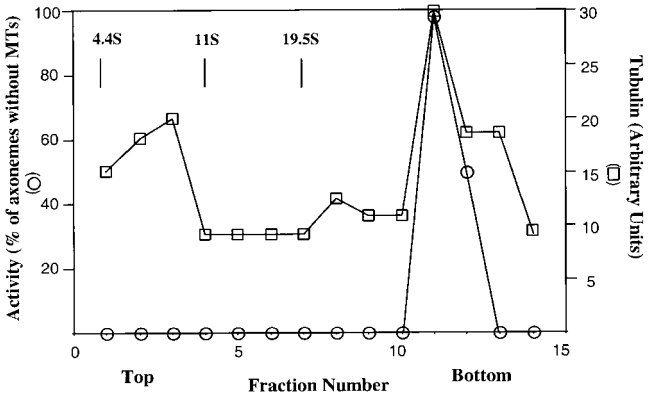

Fig. 4. Sucrose density gradient fractionation of tubulin and MT inhibitory activity in oocyte HSS. Extracts (300 µl) were layered on top of a 5–40% sucrose gradient and centrifuged as described in Materials and Methods. Equal volume fractions were collected and analyzed for tubulin content by immunoblotting (*squares*) or microtubule plus end growth inhibition by video microscopy (*circles*).

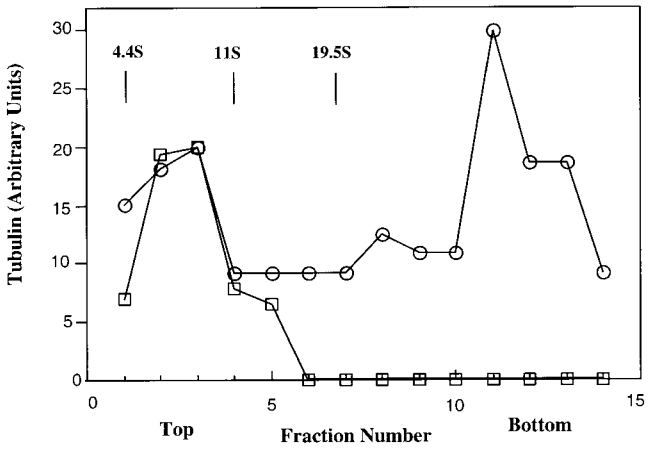

Fig. 5. Sucrose density gradient fractionation of tubulin in egg and oocyte extracts. Equivalent amounts of *Xenopus* egg extract or oocyte extract (200 µl) were layered on a 5–40% sucrose gradient and centrifuged as described in Materials and Methods. Equal volume fractions were collected and processed for SDS-PAGE, then immunoblotted with anti-alpha tubulin antibody. Blots were scanned and quantitated to indicate the distribution of tubulin throughout the gradient. *Open squares:* Tubulin distribution of the egg extract. *Open circles:* Tubulin distribution of the oocyte extract.

sucrose gradient (Fig. 4), suggesting that the activity may reside in a large protein complex. Since the activity copurified with a subpopulation of tubulin by Mono Q and HA chromatography, we immunoblotted the gradient fractions for tubulin as well. Surprisingly, tubulin in oocyte extracts migrated both at 6S, the size expected for the alpha-beta heterodimer, but also as a large complex (Fig. 4). In several gradients, the position of this larger tubulin complex was not well-defined, although it consistently sedimented at >20S and generally comigrated with the inhibitory activity. We also compared *Xenopus* egg and oocyte extracts on parallel sucrose density gradients. As mentioned earlier, egg extracts do not contain the MT assembly inhibitor activity. As shown in Figure 5, egg extracts only contained the expected 6S form of tubulin and no higher molecular weight form. Oocytes matured in vitro with progesterone also contained only 6S tubulin, and did not exhibit any MT assembly inhibitor activity (not shown). These results suggest that the higher molecular weight form of tubulin is not an artefact of homogenization or buffer conditions, but represents a form of tubulin unique to oocytes.

To ascertain the nature of the large tubulin complex, oocyte HSS was treated with 10 µg/ml nocodazole prior to sucrose gradient centrifugation. Nocodazole treatment did not alter the S value or amount of the high molecular weight tubulin (data not shown). Thus, the high molecular weight tubulin is unlikely to be polymerized MTs. In addition, we found that exogenously added rhodamine-labeled bovine brain tubulin could be incorporated into this large tubulin complex (not shown). This result suggests that the >20S tubulin complex is capable of incorporating added tubulin dimers. It is interesting that the MT assembly inhibitor cofractionated with a subfraction of tubulin in three separate fractionation procedures. This tubulin subspecies exists as a large complex in oocytes. Interestingly, the MT assembly inhibitor activity and the high molecular weight tubulin complex are both oocyte specific and disappear after maturation. These correlations raise the possibility that the high molecular weight tubulin complex may be responsible for the inhibitory activity. However, purification of the activity is required in order to substantiate this speculation.

The physiological role of the inhibitory activity described in these and other [Gard and Kirschner, 1987] studies is not clear. However, the regulation of this activity suggests that it serves an important role for the oocyte. Given the fact that oocyte MTs are rapidly reassembled after cold-induced depolymerization [Gard et al., 1995] it does not appear that this factor completely prevents MT assembly in vivo. Rather, it may function as a loose capping protein that modulates MT dynamics during oocyte development. Further biochemical studies of this protein complex are required to determine the mechanism and physiological role of this activity.

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