Demonstration and Analysis of Tubulin Binding Sites on Centrosomes

Philippe Méda,[‡] Véronique Chevrier,[‡] Bernard Eddé,[§] and Didier Job*[,‡]

Département de Biologie Moléculaire et Structurale, Laboratoire du Cytosquelette, Institut National de la Santé et de la Recherche Médicale Unité 366, Commissariat à l'Energie Atomique, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France, and Laboratoire de Biochimie Cellulaire, Collège de France, 11 place Marcellin Berthelot, 75231 Paris Cedex 05, France

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ABSTRACT: Microtubule nucleation on centrosomes is vital to the establishment of organized microtubule arrays in cells. Despite recent advances, little is known about the sequence of molecular events which leads to microtubule assembly on centrosomes. A putative early step in the nucleation process is interaction of free tubulin dimers with centrosomes. Here, we asked if centrosomes indeed interact in a specific manner with free tubulin dimers. Using lysed cells, we show that centrosomes have a specific capacity to accumulate free tubulin molecules as compared to most other cytoplasmic cell structures. When interphasic lysed cells are incubated with rhodamine-conjugated tubulin, centrosomes emerge as conspicuous sites of tubulin accumulation while other insoluble cytoplasmic cell structures are not stained. In mitotic cells, lysed at various stages of mitosis, fluorescent tubulin stains centrosomes and other mitotic structures, such as the mitotic spindle, the midzone of the cleavage furrow, and the center part of the midbody. Fluorescent tubulin staining of centrosomes in lysed cells is not affected by addition of high concentrations of serum albumin to fluorescent tubulin solutions prior to incubation. In contrast, addition of micromolar concentrations of unlabeled tubulin, to fluorescent tubulin solutions, strongly reduces centrosomal staining. The tubulin binding capacity of centrosomes is conserved following centrosome isolation. Using quantitative methods for analysis of fluorescent tubulin binding on centrosomes, we find that centrosomes contain about 25 000 saturable tubulin binding sites. The apparent dissociation constant of tubulin−centrosome complexes is circa 5 μM. The kinetics of tubulin association with centrosomes are slow, with a half-saturation time of about 3 min and a very slow dissociation rate. Tubulin binding to centrosomes is inhibited at low temperatures, at pH above neutrality, and at NaCl concentrations above 100 mM. Our results suggest that accumulation of tubulin dimers is one intrinsic function of centrosomes. We propose that such a function is not accounted for by the presence of γ-tubulin on centrosomes and may be an important factor in the regulation of centrosome-dependent microtubule nucleation.

Many essential cellular functions, such as chromosome segregation during mitosis and the transport of vesicles and organelles in cells, rely on organized microtubule networks. Maintenance of the correct geometry and polarity of microtubule arrays is therefore essential to cell function [for review, see Cole and Lippincott-Schwartz (1995), Hyman and Karsenti (1996), and McNally (1996)].

In most animal cells, microtubules are organized by the centrosome. Centrosomes are structurally complex organelles consisting of a pair of centrioles embedded in the so-called pericentriolar material (PCM). Centrosomes have the capacity to nucleate microtubule assembly, and this property has been assigned to the PCM (Tassin *et al.*, 1985; Bré *et al.*, 1987; Maniotis & Schliwa, 1991; Buendia *et al.*, 1992; Ohta *et al.*, 1993). Centrosomes concentrate microtubule nucleation sites at a defined location in cells and ensure fidelity in tubulin assembly so as to form polymers with constant polarity and protofilament number. The slow-growing (minus) end of microtubules nucleated on centrosomes is attached to the PCM whereas the rapidly growing (plus) end is distal to the centrosomes (Heidemann & McIntosch, 1980; Heidemann, 1991). Microtubules formed through spontaneous assembly of tubulin *in vitro* have a variable number of protofilaments. Instead, microtubules nucleated from the PCM have a constant protofilament number of 13, and this ensures fidelity in the geometry of the microtubule lattice (Heidemann & McIntosh, 1980; Mitchison & Kirschner, 1984).

The protein composition and the molecular organization of the PCM are still largely unknown [for review, see Fuller *et al.* (1992), Kalt and Schliwa (1993), Rose *et al.* (1993), and Archer and Solomon (1994)], and this limits our understanding of the mechanisms which underlie the organization and activity of centrosomal microtubule nucleating complexes. Several antibodies directed against different PCM components inhibit microtubule nucleation (Moudjou *et al.*, 1991; Joshi *et al.*, 1992; Doxsey *et al.*, 1994), suggesting the existence of a multicomponent nucleation complex. Among such components, recent work has pointed to a central role for γ-tubulin.

The discovery of γ-tubulin, a minor species of tubulin, relied on genetic approaches. The γ-tubulin gene, *mipA*, was discovered as a suppressor of a conditional and lethal mutation in β-tubulin (Oakley & Oakley, 1989). Later, γ-tubulin was shown to be a conserved component of the centrosome or its equivalent in a variety of organisms and to be required for assembly of centrosomes around sperm centrioles in Xenopus egg extracts [for review, see Joshi (1994)]. Recently, γ-tubulin has been shown to be part of

* To whom correspondence should be addressed.
[‡] Institut National de la Santé et de la Recherche Médicale Unité 366.
[§] Collège de France.
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a ring complex (γ-TuRC) thought to template microtubule assembly on centrosomes (Moritz *et al.*, 1995; Zheng *et al.*, 1995). These data leave little doubt that γ-tubulin plays a central role in microtubule nucleation on centrosomes. However, we still do not know if the presence of γ-TuRC accounts for all the features of microtubule nucleation on centrosomes. For instance, available evidence suggests that γ-tubulin binds to microtubule minus ends with very high affinity. The affinity constant of the binding reaction is in the range of 10^{-10} M (Li & Joshi, 1995). On the other hand, the number of microtubules nucleated per centrosome increases at tubulin concentrations in the 10−100 μM range (Mitchison & Kirschner, 1984). Such data pose questions concerning the physicochemical factors which limit the rate of microtubule nucleation on centrosomes. Identification of these factors clearly requires knowledge of the steps and parameters of tubulin interaction with and assembly on centrosomes. Very little is presently known on the sequence of molecular events which lead to microtubule nucleation on centrosomes. It is not known, for instance, whether centrosomes first bind tubulin dimers or interact with more complex, preformed tubulin-assemblies, such as spontaneously formed microtubules or other tubulin containing complexes. As an approach to these questions, we have tested to see if centrosomes are endowed with a specific capacity to concentrate tubulin dimers as compared to other insoluble cell structures, and we have used semiquantitative binding assays to measure the parameters of tubulin interactions with isolated centrosomes.

We find that centrosomes specifically accumulate tubulin dimers. Centrosomes contain about 25 000 tubulin binding sites with a dissociation constant of circa 5 μM. We suggest that such binding sites are different from γ-tubulin itself and may play an important role in the kinetics of tubulin nucleation on centrosomes.

MATERIAL AND METHODS

Materials

All chemicals unless otherwise indicated were of the highest purity grade available from Sigma (St. Louis, MO). Phosphocellulose P11 was from Whatman (Maidstone, U.K.); ATP, GDP, and GTP were from Boehringer Mannheim (Mannheim, Germany); EIA grade Tween-20 was from Bio-Rad (Hercules, CA); 5(and 6-)-carboxytetramethylrhodamine succinimidyl ester was from Molecular Probes (Eugene, OR); anhydrous glycerol was from Fluka (Buchs, Swizterland); anhydrous ethanol and anhydrous methanol were from Carlo Erba (Val de Reuil, France).

The PCM monoclonal antibody (mAb) 6C6 was as described in Chevrier *et al.* (1992); the mAb anti-β-tubulin (clone 2.1) was from Sigma; the anti-γ-tubulin polyclonal antibody Gaëlle was kindly provided by Dr. Anne-Marie Lambert. It was generated against a peptide conserved among γ-tubulins from yeast to man (EEFATEGTDRKDVFFYC) (Joshi *et al.*, 1992). The fluorescein (FITC)-conjugated goat anti-mouse IgM F(ab)′$_2$ antibody and the FITC-conjugated goat anti-rabbit antibody were purchased from Cappel (West Chester, PA); the FITC-conjugated goat anti-mouse antibody was from Jackson (West Grove, PA).

NIH/3T3 cells were purchased from American Type Culture Collection, Rockville, MD (ATCC number CRL 1658). Culture media were from Life Technologies (Paisley, Scotland).

Methods

Cell Culture. Cells were incubated at 37 °C in a humidified incubator with 6% CO_2. Cells were grown in DMEM complemented with 10% heat-inactivated bovine fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin.

Microtubule Protein Isolation and Tubulin Purification. Microtubule protein isolation from fresh bovine brain and subsequent tubulin purification by P11 phosphocellulose chromatography were as described in Mitchison and Kirschner (1984).

Fluorochrome Labeling of Tubulin. Rhodamine-conjugated tubulin was prepared as described by Hyman *et al.* (1991). The fluorochrome to tubulin dimer ratio was between 0.8 and 1.2.

Centrosome Preparation. Centrosomes were purified from calf thymocytes as in Komesli *et al.* (1989).

Analysis of Tubulin Binding Sites on Lysed Cells. Cells grown 1−4 days on glass coverslips were lysed by immersion for 1 min at room temperature in 100 mL of lysis buffer composed of 80 mM PIPES−KOH, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100 (v/v), and 10% glycerol (v/v). Lysed cells were subsequently washed 2 × 1 min in 100 mL of lysis buffer. Previous work has shown that in such lysed cells, centrosomes retain apparently normal microtubule nucleation capacity (Lieuvin *et al.*, 1994).

Lysed cells were washed in 100 mL of PME buffer [80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid)] and further incubated 1 min at room temperature with fluorescent tubulin in PME buffer in the presence or absence of unlabeled tubulin or bovine serum albumin as indicated in the figure legends. Following incubation with tubulin, cells were briefly washed in PBS/Tween buffer, fixed for 6 min in anhydrous methanol at −20 °C, and washed again in PBS/Tween buffer. Cells were further double-stained using various primary antibodies diluted in PBS containing 1 mg/mL bovine serum albumin (BSA). Dilution factors were as indicated in parentheses. For PCM double staining, cells were sequentially incubated with mAb 6C6 (1/10 000) and FITC-conjugated goat anti-mouse IgM F(ab)′$_2$ Ab (1/250). For total tubulin double staining, incubations were with anti-β-tubulin mAb (1/250) and FITC-conjugated goat anti-mouse antibody (1/250). Double staining for γ-tubulin was with polyclonal Gaëlle antibody (1/1000) and with FITC-conjugated goat anti-rabbit antibody (1/250).

Following double staining, coverslips were dried in anhydrous ethanol, mounted in mowiol medium, and observed using a Zeiss Axioscop epifluorescence microscope (Zeiss, Lyon, France). Pictures were taken with a Nikon F-601 (Charenton-le-Pont, France) using a Kodak ASA 400 film. Negative control experiments performed with the omission of primary antibodies revealed only diffuse low-level background staining.

Assays of Tubulin Binding on Isolated Centrosomes. Centrosomes in PME buffer were incubated with fluorescent tubulin in a final volume of 30 μL, under various conditions as indicated in the figure legends. Binding reactions were

stopped by addition of 1 mL of cold PME buffer. Centrosomes were sedimented on coverslips as described in Komesli *et al.* (1989). Coverslips were briefly washed in PBS/Tween buffer, fixed for 6 min in anhydrous methanol at −20 °C, and washed again in PBS/Tween buffer. Centrosomes were further stained with mAb 6C6 and processed for microscopy analysis as described above in the case of lysed cells.

Preparation of Rhodamine-Labeled Microtubule Fragments. Rhodamine-conjugated tubulin (7.5 μM) was polymerized for 20 min at 37 °C in PEM buffer containing 5 mM $MgCl_2$, 10% glycerol, and 1 mM GTP. The microtubule suspension (100 μL) was sedimented on coverslips through a prewarmed (30 °C) 25% glycerol cushion as described in Komesli *et al.* (1989). Coverslips were briefly washed in 100 mL of warm PBS/Tween buffer and immediately fixed in anhydrous methanol prior to further processing for immunofluorescence microscopy. Polymers were not cross-linked during preparation. Cross-linking modifies rhodamine fluorescence. The majority of microtubules depolymerized during preparation. In practice, we found that the incubation conditions described above yielded a workable density of isolated polymers following centrifugation on coverslips.

Fluorescence Quantification. In assays of tubulin binding to isolated centrosomes, fluorescence was quantified using a C2400-08 Hamamatsu SIT Camera (Hamamatsu City, Japan) and a Crystal Sapphir Card from Quantel (Montigny-le-Bretonneux, France). A minimum of 10 microscopic fields per coverslip were analyzed at 1000× magnification. Each field contained 10−20 centrosomes identified by double staining with mAb 6C6. Images of tubulin−rhodamine-stained centrosomes were recorded, and the total luminance of each centrosome was measured. In all experiments, the setting of the imaging system was adjusted so as to avoid reaching saturating levels of luminance for the brightest centrosomes.

An intrinsic limitation of the imaging system is that, in such conditions, weakly labeled centrosomes yield no signal. Therefore, total luminances as measured by the system cannot be strictly proportional to actual luminances, and deviation from linearity increases when the signal decreases. Therefore, for determination of tubulin binding parameters, experiments were designed so as to include internal standards (see Results).

RESULTS

We wished to assess the existence and significance of tubulin binding sites on centrosomes. Centrosomes could lack the capacity to accumulate tubulin. Alternatively, tubulin binding could occur on centrosomes but could show strong variability as a function of the centrosome cellular environment or of the centrosome purification state. Finally, the ability to concentrate tubulin could be a stable, intrinsic property of centrosomes. To test which of these possibilities was correct, we analyzed tubulin binding on centrosomes in cells lysed at different stages of the cell cycle and on isolated centrosomes. Lysed cell experiments were also expected to yield information concerning the overall distribution of tubulin binding sites among insoluble cell structures. Such information was needed to determine whether centrosomes would prove special with regard to the capacity to accumulate tubulin molecules or if tubulin binding capacity would be a widespread property of lysed cell components.

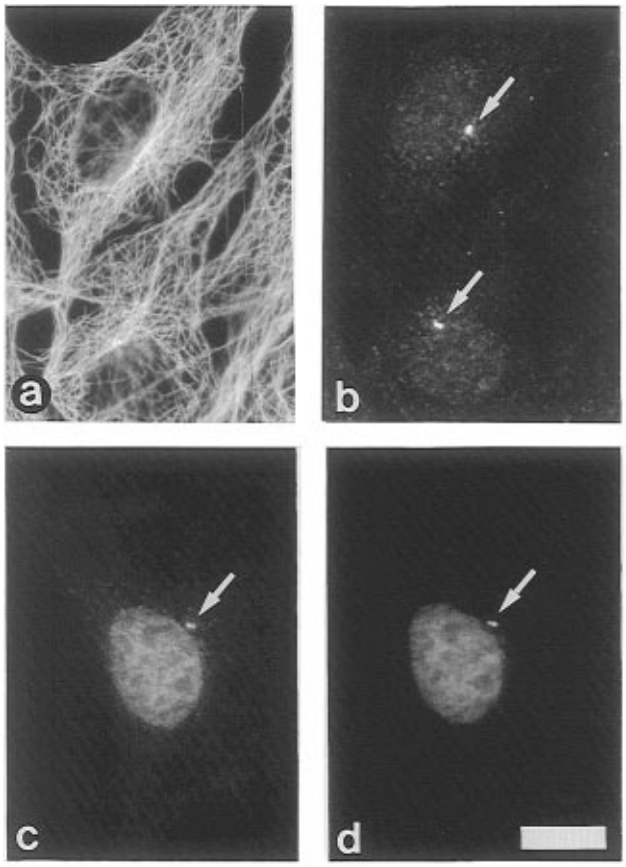

FIGURE 1: Analysis of tubulin binding sites in lysed interphase cells. Lysed NIH/3T3 cells were incubated with rhodamine-conjugated tubulin and further immunostained with either a β-tubulin antibody or a γ-tubulin antibody. (a, b) Double staining with a β-tubulin antibody (a) and tubulin−rhodamine (b). (c, d) Double staining with a γ-tubulin antibody (c) and tubulin−rhodamine (d). Except for diffuse, nonspecific, staining of nuclei, centrosomes (arrows) are the only cell structures stained by tubulin−rhodamine. Note that interphase microtubules do not bind tubulin−rhodamine. Bar, 5 μm.

Staining of Lysed Cells with Fluorescent Tubulin. All the experiments reported below were run using GDP−tubulin, in order to study tubulin binding independently of tubulin polymerization. When GTP−tubulin was used instead of GDP−tubulin, results were unchanged (data not shown), provided that temperature of the incubations was not raised above 25 °C. At 25 °C, no measurable tubulin polymerization occurred in the range of tubulin concentrations used in the present study.

Lysed NIH/3T3 cells were incubated with rhodamine-labeled tubulin and subsequently washed in PBS prior to methanol fixation. Interestingly, despite the fact that centrosomes are minor components of lysed cells, centrosomes emerged as conspicuous sites of tubulin accumulation in interphase cells (Figure 1b,d). Fluorescent tubulin actually stained centrosomes as clearly as γ-tubulin antibodies (Figure 1c,d). We also observed variable amounts of fluorescence in interphase nuclei (Figure 1b,d). Some degree of nuclear staining is a common occurrence following incubation of lysed cells with fluorescent molecules. In the present study, nuclear fluorescence occurred to a similar extent when cells were incubated with fluorescent tubulin as when cells were incubated with fluorescent IgG (Figure 1c,d). Such staining may be due either to the fact that nuclei are voluminous organelles, accumulating more background fluorescence than

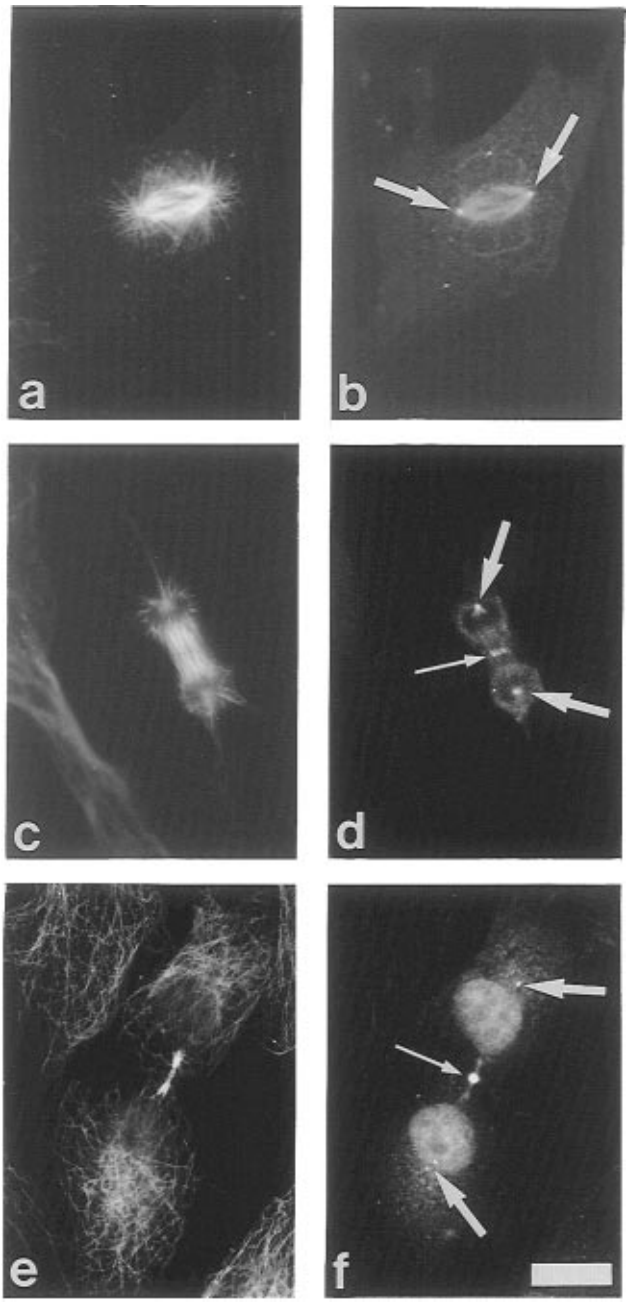

FIGURE 2: Analysis of tubulin binding sites in lysed mitotic cells. Cells were incubated with rhodamine-conjugated tubulin and immunostained with a β-tubulin antibody. Anti-β-tubulin staining (a, c, e). Tubulin−rhodamine staining (b, d, f). Centrosome (large arrows) staining with fluorescent tubulin occurs in cells lysed at various stages of mitosis. Kinetochore to pole microtubules are clearly stained by fluorescent tubulin (b). During telophase, tubulin−rhodamine accumulates in the midzone of the cleavage furrow (small arrows, d) and, subsequently, in the center part of the midbody (small arrows, f). Bar, 5 μm.

most of the other cell structures, or to the affinity of nuclear histones for IgG and tubulin (Multigner *et al.*, 1992).

The presence of bright spots was noted distinct from centrosomes, in lysed interphase cells exposed to fluorescent tubulin. Such spots have no defined location in cells, can be found outside of the cells, and most probably correspond to tubulin aggregates.

Examination of mitotic cells lysed at various stages of mitosis showed conservation of centrosomal staining at all stages of the cell cycle (Figure 2b,d,f). In metaphasic cells, fluorescent tubulin also stained the spindle (Figure 2b). Astral microtubules were not stained with fluorescent tubulin while the same polymers were brightly stained with β-tubulin antibodies. Such a staining pattern suggests that fluorescent tubulin associates preferentially with the kinetochore to pole microtubules. In telophasic cells, fluorescent tubulin stained the midzone of the cleavage furrow, in addition to centrosomes (Figure 2d). In late telophase cells, fluorescent tubulin brightly stained the center zone of the midbody (Figure 2f). Note that in Figure 2f, the centrosome signal is weak. This is because we could not find cells in which the centrosomes and the midbody were located in the exact same focal plane. Examination of many cells in late telophase actually showed persistent centrosomal staining (data not shown). We repeated tubulin staining experiments on other types of lysed cells including HeLa cells and Rat 2 cells. Results were the same in all cell types (data not shown). We conclude from these data that in lysed cells, fluorochrome-conjugated tubulin stains a limited number of well-defined cell structures including centrosomes. In interphase cells, centrosomes emerge as the only cytoplasmic cell structures clearly stained with fluorochrome-labeled tubulin.

Centrosomal staining with tubulin−rhodamine as observed in lysed cells could reflect mere absorption of tubulin on centrosomes or could result from the existence of specific, saturable tubulin binding sites on centrosomes. To test which of these possibilities was correct, we incubated lysed cells with tubulin−rhodamine in the presence of excess unlabeled tubulin. Results are shown in Figure 3c,d for interphase cells. We found that addition of 10 μM unlabeled tubulin to 5 μM rhodamine−tubulin in the incubation mixture strongly reduced the centrosomal signal (Figure 3d). In contrast, addition of high concentrations of serum albumin (up to 500 μM), to solutions containing fluorochrome-coupled tubulin, prior to the incubation with lysed cells had no effect on centrosomal staining (Figure 3f). Examination of centrosomes in mitotic cells yielded similar results (data not shown). These experiments show that centrosomes in lysed cells contain saturable tubulin sites and suggest that the tubulin concentration corresponding to half-saturation is in the micromolar range.

Analysis of Tubulin Binding Sites on Isolated Centrosomes. We tested to see if isolated centrosomes had similar properties as centrosomes in lysed cells with regard to tubulin binding. Results are shown in Figure 4. Isolated centrosomes were incubated with 5 μM rhodamine−tubulin in the absence (Figure 4a,b) or the presence of either 30 μM unlabeled tubulin (Figure 4c,d) or 500 μM albumin (Figure 4e,f). Centrosomes were subsequently stained with a PCM antibody (Figure 4a,c,e) in order to differentiate centrosomes from contaminating tubulin aggregates. Isolated centrosomes were brightly stained with rhodamine-labeled tubulin (Figure 4b). Centrosomal staining was abolished upon addition of 30 μM unlabeled tubulin to rhodamine−tubulin solutions prior to incubation (Figure 4d), but unmodified upon addition of albumin (Figure 4f). It was concluded that like centrosomes in lysed cells, isolated centrosomes contain saturable binding sites for tubulin.

We further analyzed in a quantitative way tubulin fluorescence on isolated centrosomes. To this effect, we used a SIT camera coupled to a Quantel image analysis system. A typical signal/response curve of the imaging system is shown in Figure 5. To determine such a curve, centrosomes were incubated with mixtures containing various proportions of

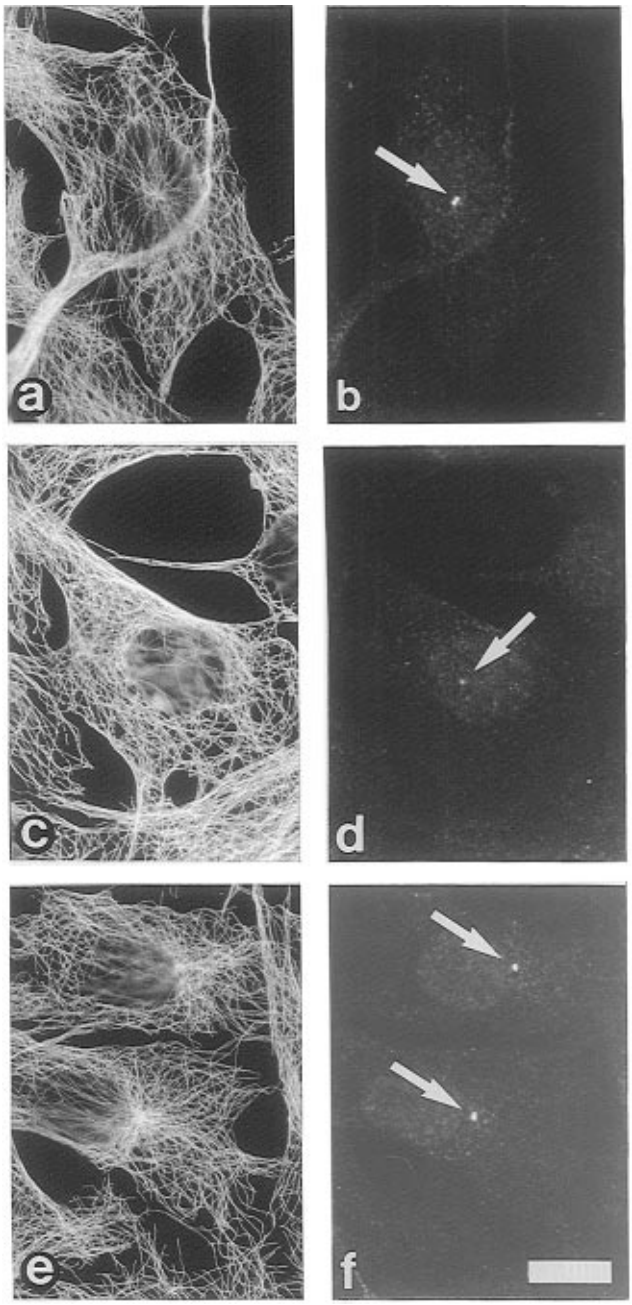

FIGURE 3: Specificity of tubulin binding sites in lysed cells. Interphase lysed cells were incubated with 5 μM fluorescent tubulin either alone (a, b) or in the presence of 10 μM unlabeled tubulin (c, d) or 500 μM bovine serum albumin (e, f) and analyzed for centrosome staining (arrows). Double staining was with an anti-β-tubulin antibody and tubulin−rhodamine. Bar, 5 μm. Anti-β-tubulin staining (a, c, e). Tubulin−rhodamine staining (b, d, f).

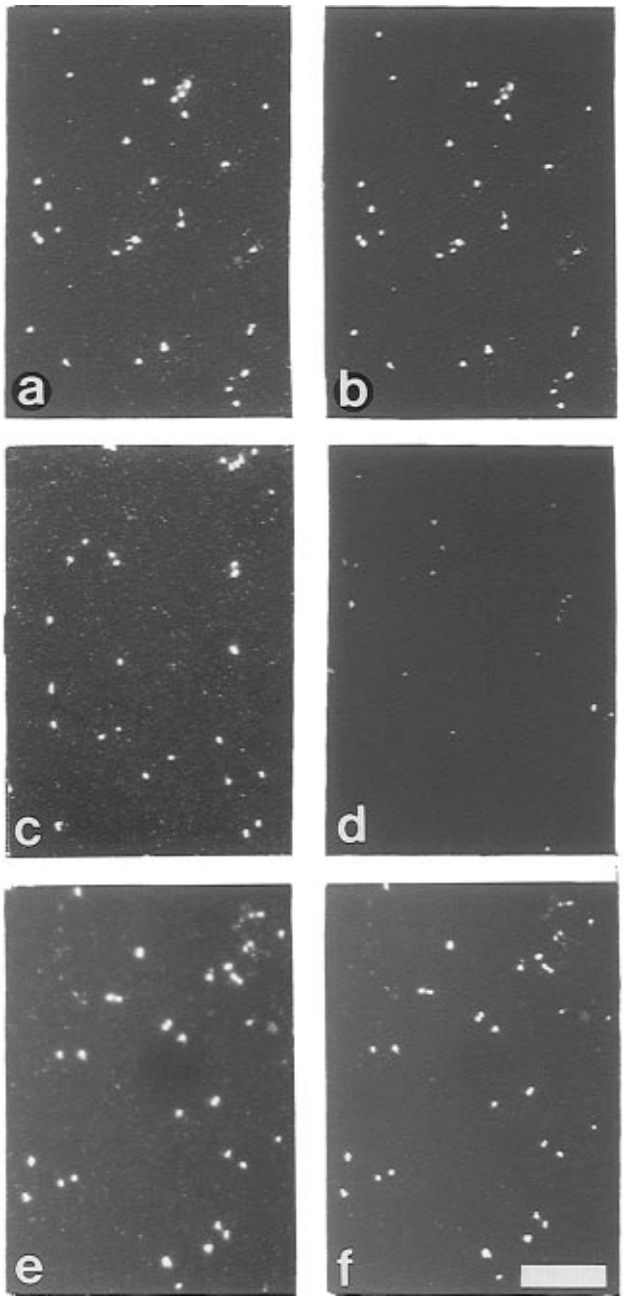

FIGURE 4: Tubulin binding to isolated centrosomes. Isolated centrosomes on coverslips were incubated with 5 μM fluorescent tubulin for 15 min at 25 °C, either alone (a, b) or in the presence of 30 μM unlabeled tubulin (c, d) or 500 μM bovine serum albumin (e, f). Double staining was with PCM mAb 6C6. Bar, 5 μm. mAb 6C6 staining (a, c, e). Tubulin−rhodamine staining (b, d, f).

tubulin−rhodamine and of unlabeled tubulin at constant 30 μM final tubulin concentration. The signal/response curve was linear for signals ≥30% of the maximum signal. At lower levels of fluorescence, the response of the imaging system dropped more rapidly than the corresponding signals.

We then repeated in a quantitative way the competition experiments shown in Figure 4. Centrosomes were incubated with 5 μM rhodamine-labeled tubulin in the presence of increasing concentrations of unlabeled tubulin (Figure 6a). Results showed a sharp decrease of fluorescence in the presence of unlabeled tubulin. The limit of detection of the imaging system was reached at unlabeled tubulin concentrations circa 10 μM.

The saturation curve of centrosomal tubulin binding sites was then determined (Figure 6b). Increasing concentrations of rhodamine-labeled tubulin were added to suspensions of isolated centrosomes. The intensity of the centrosomal signal increased sharply for tubulin concentrations in the 1−10 μM range to reach an apparent plateau at tubulin concentrations ≥30 μM. These results confirmed that tubulin binding sites on centrosomes were saturable. The dissociation constant (K_d) of the tubulin binding reaction and the number of tubulin binding sites per centrosome were then determined directly, using internal standards. The K_d value was determined as the concentration of tubulin−rhodamine yielding half-saturation of the centrosomal binding sites. The corresponding signal was determined by saturating

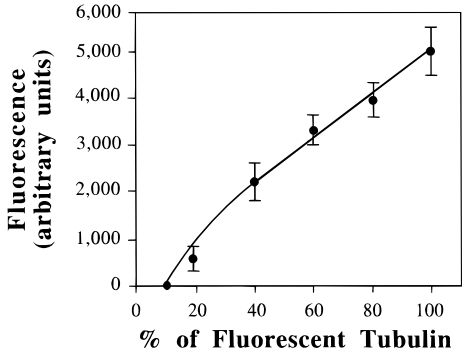

FIGURE 5: Signal/response curve of the imaging system. Isolated centrosomes were incubated for 15 min at 25 °C with 30 μM tubulin solution containing various proportions of rhodamine-labeled and unlabeled tubulin, as indicated. The signal/response curve is linear for signals ≥30% of the maximum signal.

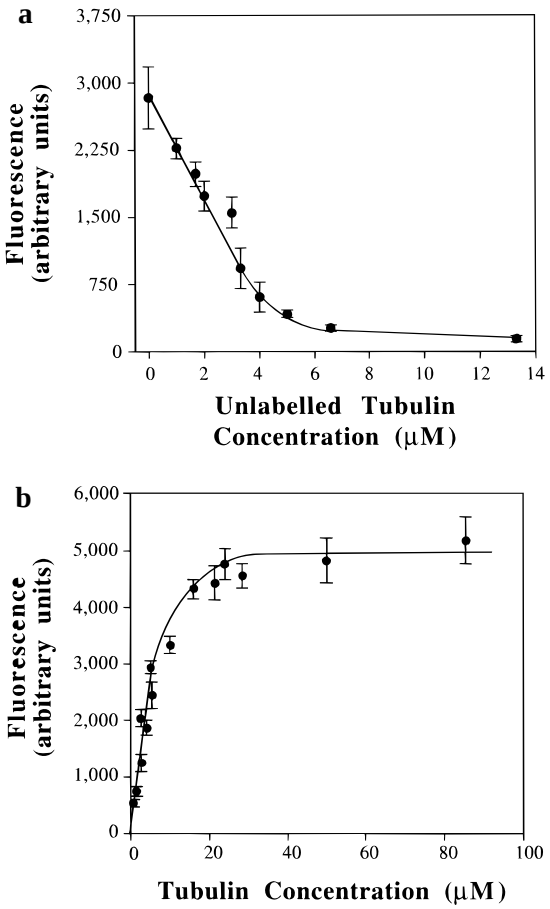

FIGURE 6: Saturability of tubulin binding sites on isolated centrosomes. (a) Competition curve. Isolated centrosomes were incubated for 15 min at 25 °C with 5 μM rhodamine-labeled tubulin, in the presence of increasing unlabeled tubulin concentrations, as indicated. The signal decreases sharply as a function of unlabeled tubulin concentration. (b) Saturation curve. Centrosomes were incubated for 15 min at 25 °C with increasing concentrations of rhodamine-labeled tubulin, as indicated. Saturation of tubulin binding sites occurs circa 30 μM tubulin as indicated by the plateau of the curve.

centrosomal tubulin binding sites with a 30 μM tubulin solution containing equal amounts of labeled and unlabeled tubulin molecules. Under the conditions used in Figure 6b, we find that dilution of fluorescent tubulin with equal amounts of unlabeled tubulin at a total tubulin concentration of 30 μM resulted in a drop of centrosome fluorescence from 4900 to 2400 units. The latter value corresponded to the signal observed when centrosomes were incubated with 0.5 μM pure fluorescent tubulin. We concluded that 0.5 μM corresponds to the tubulin concentration yielding half-saturation of centrosomal binding sites and therefore to the K_d of the tubulin binding reaction.

In order to determine the total number of tubulin binding sites on centrosomes, we needed to compare the total fluorescence of tubulin-saturated centrosomes with that of standard objects containing a known number of tubulin dimers. Another requirement was that centrosomes and standards showed roughly equal fluorescence so as to minimize problems related to nonlinearity of the signal/response curve of the imaging system. We chose to use as standards microtubules formed from pure fluorescent tubulin. Microtubules are known to contain about 1625 tubulin dimers per micrometer of length (Bayley *et al.*, 1994). Microtubules, polymerized from pure fluorescent tubulin, were sedimented onto coverslips. The average point luminance and the luminance per micrometer of length of the sedimented polymers were then determined. When centrosomes were incubated with saturating concentrations (30 μM) of the same tubulin batch, the corresponding average point luminances were higher than those of the reference microtubules. Centrosomes were therefore incubated with a series of 30 μM tubulin solutions containing increasing proportions of unlabeled tubulin. We found that the average point luminances of microtubule standards and of centrosomes were roughly equal when centrosomes were incubated with tubulin mixtures containing 1 part of fluorescent tubulin for 5 parts of unlabeled tubulin. Under such conditions, the average total fluorescence of a single centrosome was equivalent to the total fluorescence of a 2.6 μm microtubule fragment. Hence, the total number of tubulin dimers bound to saturated centrosomes was estimated to be: $1625 \times 2.6 \times 6 \approx 25\,000$.

Kinetics of Tubulin Binding to Centrosomes. The on-rate of tubulin binding to centrosomes was determined by incubating isolated centrosomes with saturating concentrations of fluorescent tubulin. At given time points, aliquots were removed, and centrosomes were centrifuged onto coverslips through a glycerol cushion, prior to quantification of centrosomal fluorescence (Figure 7a). Results showed a relatively slow binding of tubulin to centrosomes with a half-saturation time of about 3 min.

Off-rates of the tubulin binding reaction were measured by first incubating centrosomes with 5 μM tubulin−rhodamine and then adding 10 μM excess unlabeled tubulin. Figure 7b shows that over a period of 30 min at 25 °C, tubulin dissociation occurred at a very slow rate. After 30 min, centrosomes began to show structural damage.

Effectors of Tubulin Binding on Centrosomes. Tubulin binding on centrosomes was strongly influenced by temperature (Figure 8a). At 0 °C, little binding of tubulin on centrosomes occurred within 15 min, while at 37 °C saturation levels were reached within 5 min.

We investigated the possible influence of pH variations, of increasing NaCl concentrations, and of various antimicrotubular drugs on tubulin binding to centrosomes. Tubulin binding was enhanced at acidic pH and rapidly decreased at pHs higher than 6.8 (Figure 8b). Increasing NaCl concentrations to 100 or 150 mM also markedly affected the binding capacity of centrosomes (Figure 8c).

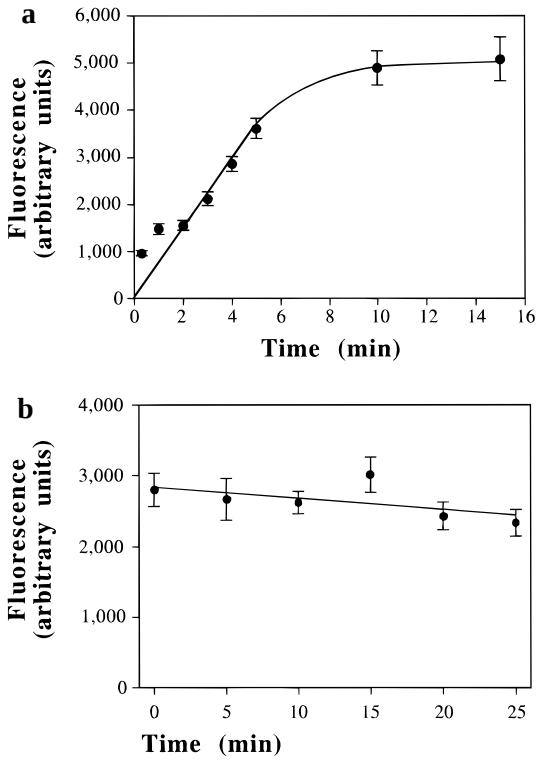

FIGURE 7: Kinetics of tubulin binding to centrosomes. (a) Centrosomes were incubated at 25 °C with 30 μM fluorescent tubulin for various amounts of time as indicated. (b) Centrosomes were incubated with 5 μM tubulin for 15 min at 25 °C to achieve half-saturation of tubulin binding sites. Unlabeled tubulin (10 μM) was subsequently added for various amounts of time as indicated. Centrosome fluorescence showed hardly detectable decrease within 30 min.

We also tested the influence of colchicine, nocodazole, and podophyllotoxin. Obviously, a specific effect of such drugs on the tubulin interaction with centrosomes would be of major interest with regard to the pharmacology of these compounds, *in vivo*. However, we only found a moderate (40%) inhibitory effect of colchicine while podophyllotoxin and nocodazole had no significant influence (data not shown).

Finally, we wondered if γ-tubulin was involved in the binding of tubulin on centrosomes. Centrosomes were preincubated with a γ-tubulin antibody prior to tubulin binding assays. The antibody was directed against a γ-tubulin peptide known to interfere with microtubule nucleation in vivo (Joshi *et al.*, 1992). This antibody had no effect on tubulin binding to centrosomes (not shown).

DISCUSSION

The aim of the present study was to characterize centrosome interaction with free tubulin dimers.

We find that when lysed interphase cells are exposed to fluorochrome-labeled tubulin dimers, centrosomes are the only obvious sites of tubulin accumulation, among insoluble cytoplasmic cell structures. Tubulin binding on centrosomes also occurs in cells lysed in mitosis. These results strongly suggest that concentrating tubulin dimers is one function of centrosomes. Our study concerned cell types in which microtubule nucleation is restricted to centrosomes. The distribution of tubulin binding sites in other cell types will clearly be of interest. For instance, microtubule nucleation sites distinct from centrosomes appear in epithelial cells during polarization, and some cells are devoid of centrosomes

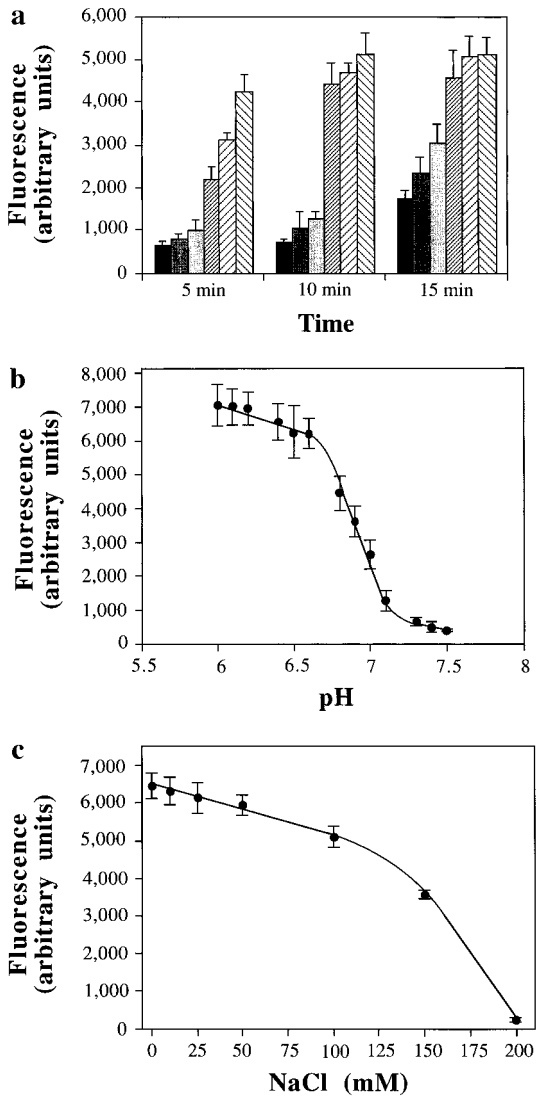

FIGURE 8: Effect of pH, temperature, and ionic strength variations on tubulin binding to isolated centrosomes. (a) Temperature effect. Isolated centrosomes were incubated at various temperatures with 30 μM rhodamine-labeled tubulin for 5, 10, or 15 min as indicated. Temperatures were the following, for each group of bars from left to right: 0 °C, 10 °C, 20 °C, 25 °C, 30 °C, and 37 °C. (b) pH effect. Isolated centrosomes were incubated for 15 min at 25 °C with 30 μM fluorescent tubulin, in PEM buffer, at various pHs as indicated. Experiments were run within the buffering range of PEM (pH 6.1−7.5). (c) NaCl effect. Isolated centrosomes were incubated for 15 min at 25 °C with 30 μM rhodamine-labeled tubulin in the presence of increasing NaCl concentrations as indicated.

(Maro *et al.*, 1985; Tassin *et al.*, 1985; Houliston *et al.*, 1987; Mogensen *et al.*, 1989; Bré *et al.*, 1990; Buendia *et al.*, 1990; Maniotis & Schliwa, 1991; Henderson *et al.*, 1995; Meads & Schroer, 1995). It would be of interest to know if in such situations tubulin nucleation sites and tubulin binding sites still colocalize.

Tubulin binding, in cells lysed in mitosis, is not restricted to centrosomes. Tubulin binding also occurs on a subset of spindle microtubules, in the central part of the cleavage furrow and in the midbody. Previous work has shown that components of centrosomes such as γ-tubulin are associated with the spindle (Lajoie-Mazenc *et al.*, 1994). Likewise, staining of the midzone of the cleavage furrow has been previously described using antibodies primarily directed against centrosomes or against components of other microtubule organizing organelles such as kinetochores (Andreas-

sen *et al.*, 1991; Compton *et al.*, 1991; Chevrier *et al.*, 1992). Such components may mediate tubulin interaction with the spindle and the midzone of the cleavage furrow. Other molecules such as microtubule-associated proteins or molecular motors may be involved.

The center region of the midbody appears as a reservoir of tubulin binding molecules, and is probably normally isolated from the intracellular environment by a membraneous organelle. This is the simplest explanation for the very bright staining of this part of the midbody following cell lysis with Triton while the same locus shows no tubulin staining in cells fixed without prior treatment with Triton (Chevrier *et al.*, 1992). However, persistent tubulin staining of the central part of the midbody in lysed cells requires that tubulin binding sites are primarily compartmentalized through association with Triton resistant structures. The high density of tubulin binding sites in the midbody may result from the concentration of proteins initially associated with the spindle and/or with other mitotic organelles which are known to subsequently accumulate in the central region of the midbody. Such proteins may include molecular motors [for review, see Vernos and Karsenti (1996)] and proteins associated with centrosomes or equivalent microtubule organizing centers (Chevrier *et al.*, 1992; Todorov *et al.*, 1992; Lajoie-Mazenc *et al.*, 1994; Shu *et al.*, 1995). These proteins may need to be inactivated through some kind of encapsulation after completion of cytokinesis.

We have found here that following isolation, centrosomes still bind tubulin in a specific, saturable, way. The K_d of the binding reaction was determined to be 5 μM. This value implies that the binding of tubulin is sensitive to variations of intracellular free tubulin concentration that might occur in the physiological range. Total tubulin concentration in cells has been measured to be in the 20 μM range (Hiller & Weber, 1978), with at least 50% of total tubulin being polymers (Olmsted, 1981). The kinetics of the binding reaction are slow with association rates in the order of minutes and very slow dissociation kinetics. As discussed below, such features are compatible with a strong "retention" effect of centrosomes with respect to tubulin accumulation. The temperature and ionic strength dependence of tubulin binding on centrosomes as observed in the present study were similar to those observed for tubulin polymerization *in vitro*. We also found a sharp decrease of tubulin binding to centrosomes when the pH of tubulin solutions was raised above the pK_a of PIPES buffer (pK_a = 6.8). Such a pH effect in PIPES-based buffers has been observed in the case of tubulin polymerization. Recent work shows a 2.5-fold increase of the critical concentration for tubulin assembly on microtubule plus ends, in PIPES buffer, when the pH of the tubulin solutions was varied from 6.8 to 7.2 (Tiwari & Suprenant, 1994). We believe that this sharp pH effect is related to a specific action of PIPES buffer on the tubulin dimer. Such specific action may vary rapidly as a function of pH around 6.8, a critical value corresponding to the pK_a of PIPES. Finally, tubulin binding was only weakly influenced by drugs such as colchicine, vinblastine, or podophyllotoxin.

The main questions which arise with regard to centrosomal tubulin binding sites concern their relationship with γ-tubulin assemblies such as γ-TuRC and the possible physiological meaning of such sites. The molecular identity of the centrosomal tubulin binding sites that we assayed could not be established in the present study. We are attempting to identify the tubulin domain involved in tubulin interaction with centrosomes using competition assays with tubulin peptides. Preliminary results show crude peptide mixtures of digested tubulin to affect tubulin binding to centrosomes (data not shown). We expect to further test active peptides for their capacity to interact with γ-tubulin or with other PCM components.

We find that centrosomes contain about 25 000 tubulin binding sites with a dissociation constant of about 5 μM. Such sites are most probably not associated with γ-tubulin. Centrosomes contain probably less than 1000 γ-tubulin molecules (Mitchison & Kirschner, 1984). Isolated centrosomes measurably nucleate about 50 microtubules. If each microtubule is templated by a γ-TuRC composed of 13 γ-tubulin molecules, centrosomes should contain roughly 650 γ-tubulin molecules. The reported K_d corresponding to the interaction of γ-tubulin with preformed microtubules end is in the 10^{-10} M range (Zheng *et al.*, 1995). In the present study, we used dimeric tubulin, not preformed microtubules, but it would be remarkable if such a difference in experimental conditions accounted for a 10^4-fold difference in the apparent K_d. Hence, there is probably at least a 10-fold difference in the number of measured tubulin binding sites to the number of γ-tubulin molecules present on centrosomes.

In view of recent evidence that microtubule growth is templated by γ-TuRCs, one may wonder about the usefulness of an additional higher capacity, lower affinity tubulin binding system, on centrosomes. Such a system may in fact be of great importance if nucleation is limited by kinetic factors, i.e., if the accessibility of γ-tubulin in γ-TuRC is a limiting factor in the nucleation process. Such kinetic limitations would provide a reasonable explanation for the apparent paradox that while γ-TuRC would be expected to be saturated with tubulin dimers at nanomolar tubulin concentrations, the number of microtubules nucleated per centrosome increases only within the 5−50 μM tubulin concentration range.

In different biological systems involving few high-affinity binding sites bound to insoluble structures, ligand targetting is greatly facilitated by a two-step binding process. In a first step, the ligand binds loosely to the insoluble structure which contains the high-affinity sites. Such loose binding has a “retention effect” (Schwartz, 1976) limiting the diffusion of the ligand molecule to a restricted part of the cell, and this greatly increases the probability of productive interaction of ligand molecules with their specific binding sites in a second step. Such a two-step binding process has long been documented in the case of DNA binding proteins (Berg *et al.*, 1982), or in the case of phages binding to cell membranes (Silhavy *et al.*, 1975). Recently, evidence has accumulated that, in the case of actin polymerization, subunit recruitment close to a nucleation site may also be central to the nucleation process (Lasa & Cossart, 1996). In *Listeria monocytogenes*, a single protein, ActA, is sufficient for actin assembly (Pistor *et al.*, 1995). ActA contains a binding domain for a focal adhesion associated protein, VASP, which itself associates with profilin. Profilin is thought to concentrate actin monomers close to a nucleation site located in a nearby domain of ActA (Chakraborty *et al.*, 1995). The apparent ability of centrosomes to concentrate tubulin dimers suggests a similar mechanism in the case of tubulin nucleation. The main parameters of the tubulin binding reaction that we

measured are compatible with this hypothesis. The K_d of the tubulin binding reaction is in reasonable agreement with the range of tubulin concentrations within which nucleation efficiency increases on isolated centrosomes. The apparent low dissociation rate of the reaction favors a strong tubulin "retention" capacity of the PCM. The relative role of γ-TuRC and of other putative centrosomal tubulin binding systems in the process of microtubule nucleation will obviously greatly benefit from further comparative studies and molecular characterization of the molecules involved in the different binding reactions.

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