Taxol-induced Microtubule Aster Assembly in Mitotic Extracts of Xenopus Eggs: Requirement for Phosphorylated Factors and Cytoplasmic Dynein

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Abstract. Taxol, a microtubule stabilizing drug, induces the formation of numerous microtubule asters in the cytoplasm of mitotic cells (De Brabander, M., G. Geuens, R. Nuydens, R. Willebrords, J. DeMey. 1981. Proc. Natl. Acad. Sci. USA. 78:5608-5612). The center of these asters share with spindle poles some characteristics such as the presence of centrosomal material and calmodulin. We have recently reproduced the assembly of taxol asters in a cell-free system (Buen-dia, B., C. Antony, F. Verde, M. Bornens, and E. Karsenti. 1990. J. Cell Sci. 97:259-271) using extracts of Xenopus eggs. In this paper, we show that taxol aster assembly requires phosphorylation, and that they do not grow from preformed centers, but rather by a reorganization of microtubules first crosslinked into bundles. This process seems to involve sliding of microtubules along each other and we show that cytoplasmic dynein is required for taxol aster assembly. This result provides a possible functional basis to the recent findings, that dynein is present in the spindle and enriched near spindle poles (Pfarr, C. M., M. Cove, P. M. Grissom, T. S. Hays, M. E. Porter, and J. R. McIntosh. 1990. Nature (Lond.). 345:263-265; Steuer, E. R., L. Wordeman, T. A. Schroer, and M. P. Sheetz. 1990. Nature (Lond.). 345:266-268).

Taxol, first identified as an experimental antitumor agent (Wani et al., 1971) was subsequently reported to arrest HeLa cells and mouse fibroblasts in the G2 and M-phase of the cell cycle (Schiff and Horwitz, 1980). Because this drug lowers the critical tubulin concentration for microtubule assembly (Schiff et al., 1979), it was inferred that the mitotic block was due to microtubule stabilization. Further investigations by De Brabander et al. (1981) have shown that taxol has a complex effect on microtubule organization in living cells. In interphasic PtK2 cells, the drug induced the spontaneous assembly of microtubules leading to the formation of long microtubule bundles and to the loss of centrosome-nucleated microtubules. When these cells entered mitosis, the mitotic spindle did not form and the long microtubule bundles were replaced by numerous small asters. The duplicated centrioles were present in the center of two of these asters away from the chromosomes. The other asters did not contain centrioles. Similar observations have been made in fertilized Xenopus eggs (Heidemann and Gallas, 1980), in mouse oocytes (Maro et al., 1985) as well as in epithelial canine kidney (MDCK) cells (Buendia et al., 1990). This suggested that in vivo, microtubules were not irreversibly stabilized by taxol. Instead, taxol seemed to promote the nucleation of microtubules that remained partially dynamic since at the onset of mitosis the bundles were disrupted and replaced by small asters. Hexyleneglycol, another compound known to reduce the critical tubulin concentration for microtubule assembly (Rebhun and Sawada, 1969) also induced the formation of microtubule asters specifically in the mitotic cytoplasm of sea urchin eggs and disrupted the spindle (Endo et al., 1990). Therefore, lowering the critical tubulin concentration for microtubule assembly has a differential effect in interphase and mitosis. In interphase, this merely results in extra-microtubule assembly and longitudinal bundling, whereas in mitosis, the spindle does not assemble and microtubules polymerize in aster-like structures.

Several lines of evidence suggest that the center of mitotic taxol asters is similar to spindle poles. In PtK2 cells, the center of the asters contains an accumulation of calmodulin (De Brabander et al., 1981) as normal spindle poles do (De Mey et al., 1980). In mouse oocytes microtubule asters assemble from foci containing material recognized by an anticentrosome antibody and these foci participate in the assembly of spindle poles later on in development (Maro et al., 1985). In HeLa and PtK2 cells, an antigen that originates from the cytoplasm (POPA) and relocates onto the spindle poles during mitosis is found in the center of taxol-induced microtubule asters (Sager et al., 1986). In MDCK cells, another antigen normally present at spindle poles (CTR2611) is also relocated in the center of taxol-induced asters (Buendia et al., 1990). Finally, Endo et al. (1990) had shown that the center of microtubule asters assembled in mitotic sea urchin eggs treated with hexyleneglycol contained microtubule-nucleating granules found in spindle poles. Therefore, it seems of interest to understand how these
Materials and Methods

Frog Eggs

Xenopus laevis females were injected with 100 U PMSG (Chronogest 500, Intervet, Holland) at least 3 d before use. Frogs were induced to lay eggs by injection of human chorionic gonadotropin (HCG, Sigma Chemical Co., St. Louis, MO) the night before. After injection with HCG, the frogs were kept in 1 liter of 0.1 M NaCl to avoid egg activation. The eggs were collected and rinsed several times in MMR/4 (1 × MMR is 0.1 M NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM Heps, 1 mM EDTA, pH 7.2) and the jelly coat removed in 2 % cystein (pH 7.8). Metaphase extracts were prepared from nonactivated eggs and interphase extracts from eggs activated by an electric shock (Karsenti et al., 1984a) and incubated for 90 min in 1 × MMR in the presence of cycloheximide (100 μg/ml).

Preparation of the Extracts

100,000 g supernatants were prepared from interphase and metaphase II-arrested eggs and stored in liquid nitrogen as described by Félix et al. (1989). The buffer used to prepare the extracts contained 100 mM K-Acetate, 2.5 mM Mg-Acetate, 60 mM EGTA, 10 μg/ml cytochalasin D, 1 mM DTT (pH 7.2). Packed eggs were crushed by centrifugation at 10,000 g for 15 min in a minimum volume of buffer (the buffer was diluted about threefold by the cytoplasm). An ATP regenerating system (1 mM ATP, 10 mM creatin phosphate, 80 μg/ml creatin phosphokinase, final concentration) was required for UV photocleavage of dynein and the MgATP concentration was 8-12 μM.

Microtubule Regrowth and Visualization by Immunofluorescence Microscopy

Microtubule polymerization was studied in 15-μl aliquots of extracts incubated at room temperature without or with taxol (0.1 μM). 2-μl aliquots were diluted in 50 μl of histone kinase assay buffer during the incubation at room temperature to monitor the histone kinase activity (Félix et al., 1989). Microtubules were fixed by dilution of the extract (15 μl) in 1 ml of 0.25 % glutaraldehyde (2 % M. grade) in RGI (80 mM Pipes, 1 mM EGTA, 1 mM MgCl2, 1 mM GTP, pH 6.8, with KOH). After 6 min at room temperature, the suspension was layered on a 5-ml cushion of 25 % glycerol (vol/vol) in RGI (RGI without GTP) in 15-ml corex tubes and centrifuged at 22°C for 20 min at 10,000 g. The pellet was washed once and resuspended in the same buffer containing 5 μM taxol and 10 mM MgATP. After a 30-min incubation at room temperature, the ATP-eluted material was separated from the microtubules by a 15-min centrifugation at 30,000 g at 22°C. 200 μl of the ATP-eluted fraction were loaded on a 4-ml, 5-25 % sucrose gradient and centrifuged at 100,000 g for 16 h in a SW 60 rotor. 200-μl fractions were collected from the bottom of the tube and analyzed by SDS-PAGE.

Preparation of Calf Brain Cytoplasmic Dynein

Cytoplasmic dynein was prepared from calf brain according to the procedure of Amos (1989), using 2 mM AMP-PNP instead of triplyphosphate. For final purification, sucrose gradient centrifugation (described above) was used instead of gel filtration.

EM

Microtubules fixed and centrifuged on coverslips as described above were postfixed in 1% EM grade glutaraldehyde in cacodylate buffer (100 mM) for 30 min, rinsed several times in cacodylate buffer, and treated with 0.25 % tannic acid in cacodylate buffer for 10 min. Coverslips were then immersed in 5 % OsO4 in cacodylate buffer for 1 h, rinsed several times in water, and stained en bloc with 0.5 % uranyl acetate. The samples were then dehydrated in ethanol, flat embedded in epon, sectioned, and observed in a Philips 400 electron microscope.

Preparation of Cytoplasmic Dynein from Xenopus Eggs

10,000-g extracts from nonactivated or activated eggs were incubated in the presence of 1 mM ATPγS for 10 min at room temperature, diluted threefold with cold motor buffer (100 mM K-Pipes, pH 7.0, 0.5 mM EGTA, 2.5 mM Mg-Acetate, 1 mM DTT) containing protease inhibitors (0.1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml pepstatin) and centrifuged at 180,000 g for 90 min. Taxol was then added to a final concentration of 20 μM to the supernatant. After a 20-min incubation at room temperature, 2 mM 5-adenylylimido dipiphosphate (AMP-PNP) and 0.1 mg/ml hexokinase + 10 mM glucose, or 1 U/ml apyrase were added for a further 20 min incubation at room temperature. The mixture was then loaded on top of a 15 % sucrose cushion in the motor buffer supplemented with 5 μM taxol and centrifuged at 22°C for 20 min at 30,000 g. The pellet was washed once and resuspended in the same buffer containing 5 μM taxol and 10 mM MgATP. After a 30-min incubation at room temperature, the ATP-eluted material was separated from the microtubules by a 15-min centrifugation at 30,000 g at 22°C. 200 μl of the ATP-eluted fraction were loaded on a 4-ml, 5-25 % sucrose gradient and centrifuged at 100,000 g for 16 h in a SW 60 rotor. 200-μl fractions were collected from the bottom of the tube and analyzed by SDS-PAGE.

Results

Taxol-induced Asters Form Only in Metaphase Extracts

We have previously shown that concentrated extracts (protein concentration ~30–40 mg/ml) prepared from Xenopus eggs arrested in metaphase II of meiosis or from eggs arrested in interphase, can be used to study the interphase to metaphase transition of microtubule dynamics (Verde et al., 1990). In interphase extracts, spontaneous microtubule assembly occurred ~3 min after beginning of the incubation at room temperature and was extensive after 30 min. In metaphase extracts no spontaneous microtubule assembly occurred. Fig. 1 a shows that adding taxol (0.1 μM) to interphase extracts did not affect significantly the pattern of microtubule bundles. In contrast, in metaphase extracts, taxol induced the assembly of numerous asters (Fig. 1 b). The central region of the asters was strongly stained by the MPM2 anti-
Figure 1. Taxol induced microtubule assembly in frog egg extracts. Taxol (0.1 μM) was added to the extracts at 4°C and microtubules were fixed and observed after a 30-min incubation at room temperature. (a) Microtubule assembly in an interphase extract prepared from eggs sampled 90 min after activation by an electric shock and incubated in cycloheximide. (b) Microtubule assembly in a metaphase extract prepared from nonactivated eggs arrested in second metaphase of meiosis. (c) The center of the asters shown in b contains material recognized by the MPM2 antibody directed against mitotic phosphoproteins. The level of histone H1 kinase activity measured in the extract is shown in the lower right corner of the pictures. Bar, 20 μm.

body (Fig. 1 c). This antibody recognizes phosphorylated epitopes in spindle poles (Centouze and Borisy, 1990; Vandre et al., 1984). The taxol asters assembled in vitro contained also in their center a centrosomal antigen recognized by an mAb raised against human lymphoid cell centrosomes (Buendia et al., 1990). During mitosis, this antibody stained the minus end of the mitotic spindle microtubules. These results strongly suggest that the taxol asters produced in vitro in the egg extracts are similar to those produced in vivo in mitotic cells. They also suggest that in the asters produced in vitro, microtubules are oriented with their plus end away from the center. To see if structures, perhaps centrioles, were present in the center of the asters, we analyzed their structure by EM after flat embedding on glass coverslips. We did not find centrioles. Microtubules seemed to end in an electron-dense granular material similar to pericentriolar material (Fig. 2).

Figure 2. EM of a metaphase taxol aster. The sample was prepared from a metaphase extract incubated at room temperature for 30 min in the presence of 0.1 μM taxol. Asters were spun onto a glass coverslip and sectioned parallel to the plan of the coverslip. Bar, 5 μm.

Taxol Aster Assembly in Mitotic Extracts Requires Phosphorylation

We had previously shown that 6-dimethyl aminopurine (6-DMA), a compound reported to inhibit spindle assembly and phosphorylation in Starfish oocytes (Néant and Guerrier, 1988a,b), also suppressed cdc2-kinase–dependent phosphorylation in egg extracts as well as cdc2-kinase–dependent changes in microtubule dynamics (Félix et al., 1989; Verde et al., 1990). Addition of 0.5 mM 6-DMA to mitotic extracts induced spontaneous microtubule assembly in the absence of taxol (not shown) and inhibited the taxol-dependent formation of asters (Fig. 3 a). At this concentration, 6-DMA did not inhibit overall ATPase activities in the extract (as determined according to Seals et al., 1978, data not shown). To determine if the effect of 6-DMA was indeed due to inhibition of phosphorylation reactions, a mitotic extract was preincubated with ATPγS (to thiophosphorylate irreversibly the proteins of the extract) before adding the 6-DMA. Under these conditions, 6-DMA did not induce spontaneous microtubule assembly (not shown) and taxol asters assembled normally (Fig. 3 b). This showed that a phosphorylated factor was required for the assembly of taxol asters in mitotic extracts. Operationally, this experiment is also important because it shows that thiophosphorylation of a mitotic extract allows it to maintain its mitotic state even after inhibition of the endogenous kinases. As expected, the capacity of mitotic extracts to produce taxol asters is due to the activity of cdc2 kinase, since adding this purified enzyme (Labbé et al., 1989) to an interphase extract results in the formation of taxol asters (not shown).

1. Abbreviation used in this paper: 6-DMA, 6-dimethyl aminopurine.
Microtubule Stability in Mitotic Taxol Asters

It was important to determine the stability of microtubules in these asters for several reasons. First, previous data suggested that in vivo, similar asters were dynamic (De Brabandere et al., 1986). Second, we used relatively low doses of taxol in these experiments. 0.1 μM is way below the amount necessary to fully stabilize microtubules in vitro at tubulin concentrations equivalent to those present in our extracts (10–20 μM). Finally, the dynamic state of these microtubules may be important for the mechanism of assembly of the asters. We therefore looked at the rate of depolymerization of taxol asters after the addition of a large excess of nocodazole. This drug is useful to study microtubule half-life in general because it interferes poorly with already formed microtubules but completely inhibits association of new subunits to already assembled microtubules (for a discussion on the use of nocodazole in this way, see Wadsworth and McGrail, 1990). As shown in Fig. 4, microtubules were surprisingly stable but not irreversibly stabilized. Although it was difficult to quantify microtubule disappearance, it seemed that both the number and the mean length of microtubules per aster decreased slowly with time to reach a very small size by 45 min. The number of asters in a given volume of extract started to decrease around 15 min after nocodazole addition. During the period of aster disappearance (between 15 and 50 min) tiny asters were visible (Fig. 4, 45 min). We also saw figures that looked like disaggregating aster centers. The general impression is that microtubule stability is very heterogeneous in these conditions. Probably some microtubules are very unstable whereas others last for more than 30 min. It is also possible that the rate of depolymerization of microtubules is slowed down. Once more, real time video microscopy would be very informative to determine exactly which parameter of dynamic instability is affected. The response of these microtubules to nocodazole is certainly very different from that of centrosome-nucleated microtubules in mitotic extracts. Indeed the later microtubules remain stable for a few minutes after nocodazole addition (without changing length appreciably) and suddenly disappear altogether in a few seconds (Verde et al., 1990 and unpublished results).

Figure 3. Taxol aster assembly in metaphase extracts requires phosphorylation. A metaphase extract was pretreated with 500 μM 6-DMAP for 10 min at room temperature, and incubated with 0.1 μM taxol for 30 min (a). Taxol did not induce aster assembly, only long microtubule bundles. When the same extract was pretreated with 100 μM ATPγS for 10 min at room temperature, and then incubated with 6-DMAP and taxol for a further 30 min (b), the asters did assemble. Histone H1 kinase activity measured in the extracts is shown in the lower right corner of the pictures. Bar, 20 μm.

Figure 4. Sensitivity of taxol asters to nocodazole treatment. A metaphase II extract was incubated for 20 min in the presence of 0.1 μM taxol. Then 20 μM nocodazole was added, and aliquots taken at various time points for microtubule measurements and histone kinase assay (indicated in the lower right corner). Bar, 10 μm.
Figure 5. Taxol aster assembly is inhibited by vanadate. (a) A mitotic extract was brought to room temperature after the addition of taxol only (−Vn) or taxol and 50 μM vanadate (+Vn). At the indicated time, samples were fixed and microtubule assembly analyzed by immunofluorescence. The histone kinase activity detected in the sample remained stable during the incubation, at 8 pmoles/min/μl. (b) Samples of extract (10 μl) were incubated in the presence of 32P-γ-ATP (10 μCi) at room temperature and the reaction was stopped by addition of electrophoresis sample buffer at the indicated times. 10 μg of proteins were loaded on a 10% SDS polyacrylamide gel that was dried and autoradiographed on XAR film exposed overnight without screen. (c) A mitotic extract was incubated for 10 minutes in the presence of 1 mM ATPγS at room temperature, cooled down on ice, and taxol was added in the presence (+Vn) or absence (−Vn) of 50 μM vanadate; the extract was brought back to room temperature and fixed 20 min later. Bar, 20 μm.
Processes. The effect was specific and reversible because norepinephrine restored taxol aster assembly in a vanadate-by vanadate (Fig. 5 c). This showed that vanadate did not inhibit aster formation by interfering with phosphorylation.

The process of taxol aster formation required phosphorylation, it was important to check whether vanadate interfered with phosphorylation reactions in the mitotic extracts. As shown in Fig. 5 b, vanadate at 50 μM did not affect significantly the pattern of protein phosphorylation or rate of phosphate incorporation into proteins in metaphase extracts and the kinase activity measured after dilution in the histone H1 kinase assay buffer remained constant at 8 pmol/min/μl of extract. Moreover, irreversible phosphorylation of a metaphase extract with ATPγS did not prevent inhibition of aster formation by vanadate (Fig. 5 c). This showed that vanadate did not inhibit aster formation by interfering with phosphorylation processes. The effect was specific and reversible because norepinephrine restored taxol aster assembly in a vanadate-treated extract (not shown). Since norepinephrine reduces vanadate to oxyvanadate and inhibits its interaction with dynein (Lye et al., 1987), the vanadate effect suggested an involvement of cytoplasmic dynein in taxol aster assembly during mitosis. AMP-PNP, started to inhibit aster assembly at concentrations higher than 2 mM, probably by interfering with phosphorylations in the extract (at this concentration, 32P incorporation is strongly inhibited). This suggests that kinesin is not involved in aster assembly since this drug interferes with kinesin activity around 10 μM (Schroer and Sheetz, 1989).

Low Vanadate Concentrations Prevent Taxol Aster Assembly in Metaphase Extracts

As shown in Fig. 5 a top, the normal pathway of aster assembly in metaphase extracts involved first the polymerization and bundling of microtubules and then microtubule reorganization into asters. This suggested that microtubules could slide along each other during the formation of the asters, implying the potential involvement of microtubule motors. We therefore tested the effect of known inhibitors of motor-dependent movements on the assembly of taxol asters in mitotic extracts. Two well-characterized cytoplasmic ATPases, kinesin and dynein, behave as microtubule motors and are probably involved in organelle transport in vivo. Kinesin promotes plus end-directed motility (Porter et al., 1987; Vale et al., 1985), whereas dynein promotes minus end-directed movement (Paschal and Vallee, 1987; Schroer et al., 1989). Kinesin is not inhibited by low concentrations of vanadate (10–20 μM) whereas dynein is (see Schroer and Sheetz, 1989 for discussion). As shown in Fig. 5, vanadate at 50 μM completely abolished the formation of taxol asters in mitotic extracts and the inhibitory effect was already clear, although not complete at 10 μM (not shown). Vanadate did not interfere with microtubule bundling, but rather with the reorganization of the bundles into asters (Fig. 5 a, bottom). Moreover, addition of vanadate to already formed asters resulted in their disaggregation (not shown), suggesting that vanadate interfered with an activity required not only to form asters but also to maintain their existence once assembled. Since taxol aster formation required phosphorylation, it was important to check whether vanadate interfered with phosphorylation reactions in the mitotic extracts. As shown in Fig. 5 b, vanadate at 50 μM did not affect significantly the pattern of protein phosphorylation or rate of phosphate incorporation into proteins in metaphase extracts and the kinase activity measured after dilution in the histone H1 kinase assay buffer remained constant at 8 pmol/min/μl of extract. Moreover, irreversible phosphorylation of a metaphase extract with ATPγS did not prevent inhibition of aster formation by vanadate (Fig. 5 c). This showed that vanadate did not inhibit aster formation by interfering with phosphorylation processes. The effect was specific and reversible because norepinephrine restored taxol aster assembly in a vanadate-treated extract (not shown). Since norepinephrine reduces vanadate to oxyvanadate and inhibits its interaction with dynein (Lye et al., 1987), the vanadate effect suggested an involvement of cytoplasmic dynein in taxol aster assembly during mitosis. AMP-PNP, started to inhibit aster assembly at concentrations higher than 2 mM, probably by interfering with phosphorylations in the extract (at this concentration, 32P incorporation is strongly inhibited). This suggests that kinesin is not involved in aster assembly since this drug interferes with kinesin activity around 10 μM (Schroer and Sheetz, 1989).

Cytoplasmic Dynein in Interphasic and Mitotic Extracts

Cytoplasmic dynein was purified from egg extracts by ATP elution from microtubules followed by fractionation on a sucrose density gradient (Lye et al., 1987; Schroer et al., 1989). The ATP-eluted fraction contained a high molecular weight polypeptide that comigrated with calf brain cytoplasmic dynein on a 6% acrylamide-urea gel (Fig. 6, open arrowhead, lanes 1 and 3). The major 205-kD polypeptide is unknown but could correspond to myosin (Schroer et al., 1989) or X-MAP (Gard and Kirschner, 1987). Dynein heavy chain exposed to UV light in the presence of ATP and vanadate is cleaved into two polypeptides with Mr of 230,000 (HUV) and 200,000 (LUV) (Gibbons et al., 1987; Lye et al., 1987; Paschal et al., 1987). Exposure of crude Xenopus egg motor preparations to UV light resulted in a diminished band at 400 kD and yielded two polypeptides at ~230 and 200 kD (Fig. 6, lane 2). This strongly suggested that the 400-kD polypeptide was indeed the frog egg version of cytoplasmic dynein heavy chain. This molecule was further purified on a 5–25% sucrose gradient (Fig. 7 a). The heavy chain peaked in 20% sucrose together with two minor polypeptides of ~150 and 70 kD. This is characteristic of dynein since other dyneins contain intermediate and light chains of various sizes (Johnson, 1985; Schroer et al., 1989; Vale, 1990). Our crude motor preparation also contained a doublet polypeptide with a Mr, 120,000 (Fig. 6). This doublet peaked in 15% sucrose after overnight centrifugation at 100,000 g (Fig. 7 a). This fits with the known native molecular weight (Mr≈400,000) and sedimentation coefficient (9.5 S) of kinesin (Bloom et al., 1989). This protein attached to microtubules in the presence of AMP-PNP, another characteristic of kinesin (Dabora and Sheetz, 1988). We therefore believe that the 120-kD doublet polypeptide found in ATP eluates of frog egg microtubules is equivalent to kinesin heavy chain. Cytoplasmic dynein was purified from interphase and mitotic extracts. Both extracts were pretreated with ATPγS at room temperature before preparation of the dynein in order to maintain the phosphorylation of the proteins in their native state. Fig. 7 a shows the polypeptide composition of interphasic dynein after purification on the sucrose gradient. Dynein purified from mitotic extracts had the same composition and similar sedimentation properties (not shown).

We were interested to know whether the phosphorylation state of dynein and kinesin was different in interphasic and mitotic extracts. This was investigated by labeling interphasic and mitotic extracts with 32P. The level of 32P incorporation in total proteins was ~10 times higher in the mitotic...
Figure 7. Purification of Xenopus egg cytoplasmic dynein from mitotic and interphase extracts. (a) The ATP-eluted fraction obtained from interphase extracts was further purified on a 5–25% sucrose gradient by overnight centrifugation at 100,000 g. The fractions were analyzed on a 7.5% polyacrylamide gel. Silver-stained gel. (b) Phosphorylation pattern of the microtubule pellet after motor elution (MT) and of the eluted motors (Mot) from mitotic and interphase extracts. 10 μl of undiluted extract was incubated with 10 μCi P32 ATP for 20 min, then diluted in 2 vol of STOP buffer (50 mM NaF, 40 mM β-glycerophosphate, 10 mM EDTA, 10 mM sodium pyrophosphate, pH 7.2) to stop all phosphorylation-dephosphorylation reactions. AMPNP, hexokinase, and glucose were added and motors were then prepared as in Materials and Methods.

extract as usual (not shown, but see Fig. 5 b and Verde et al., 1990). Yet, the overall level of 32P incorporation into the microtubule pellet obtained from interphase or mitotic extracts after motor elution was similar (Fig. 7 b). However, the pattern of protein phosphorylation was clearly different. Some microtubule binding proteins not eluted by ATP were clearly phosphorylated only in mitosis (40, 80, 130 and 230 kD, dots in Fig. 7 b). One of them (80 kD) seemed to be visible by Coomassie staining only in the mitotic microtubules (arrowhead). Other microtubule binding proteins were phosphorylated only in interphase at 90 and 190 kD (Fig. 7 b, stars). We could not detect any 32P incorporation into mitotic or interphase Dynein heavy chain although it was clearly visible by Coomassie blue staining (Fig. 7 b, arrow). It is difficult to tell whether any of the dynein heavy chains or kinesin are phosphorylated. It might be worth investigating this point further, but at the moment there is no reason to believe that these motors are phosphorylated in any meaningful manner.

Purified Cytoplasmic Dynein Restores Taxol-Aster Assembly in Dynein-inactivated Extracts

Asters assembled normally in a mitotic extract exposed to UV light in the presence of vanadate and norepinephrine...
Figure 8. Taxol aster assembly in metaphase extracts requires cytoplasmic dynein. Mitotic extracts were UV irradiated in a final volume of 50 µl in the presence of vanadate. Vanadate was reduced by norepinephrine before (a) or after (b) the UV treatment. In c, purified *Xenopus* egg dynein was added (~2 ng/µl) after UV treatment. In d the same experiment was performed with *Xenopus* dynein which had been previously cleaved by UV treatment (see Fig. 6). Kinesin could not substitute for dynein, when added to the UV cleaved extract (e). Brain dynein could substitute for frog dynein (f). In all cases, the extracts were incubated for 20 min at room temperature before fixation and analysis of microtubule assembly. In Fig. 8, g, the effect of UV cleavage is shown on the *Xenopus* extract and on the purified *Xenopus* dynein. The arrow indicates the position of the dynein band before UV cleavage, and the dots indicate the cleavage products after UV treatment. Bar, 10 µm.

(Fig. 8 a). This showed that exposure to UV light did not denature proteins in the extract. However, when the extract was first exposed to UV light in the presence of vanadate and then treated by norepinephrine, aster assembly was inhibited (Fig. 8 b). This strongly suggested that the cytoplasmic dynein of the extract was cleaved and required for taxol aster assembly. The dynein was indeed cleaved in the extract as shown in Fig. 8 g (second lane; the cleaved dynein is shown by an arrowhead and the cleavage products by dots). Adding back the purified *Xenopus* egg dynein shown in Fig. 8 g (third lane), to the UV-cleaved mitotic extract, restored taxol aster assembly (Fig. 8 c). If UV-cleaved egg dynein was
used instead, (shown in Fig. 8 g, fourth lane), aster formation was not restored (Fig. 8 d). Kinesin from the same gradient used to purify the dynein shown in Fig. 8 g (third lane) did not restore aster assembly in a UV-treated extract (Fig. 8 e). Finally, the bovine brain dynein shown in Fig. 6 (lane 3), also restored taxol aster assembly in a UV-treated extract (Fig. 8 f). The minimum amount of cytoplasmic dynein required for restoring aster assembly was in the order of 5 nM, and we estimate that the total dynein concentration in the extracts is at the very least 10 times higher. These are very rough estimates based on the known sensitivity of the silver staining method we use. More accurate determination will require the use of antibodies.

**Discussion**

**Microtubule Dynamics and Taxol Aster Assembly**

In this paper, we report that in *Xenopus* egg cell-free extracts that closely mimic in vivo conditions, taxol induces the formation of microtubule asters only in mitotic cytoplasm. What is required in the mitotic cytoplasm is the phosphorylation of some factor, under direct or indirect control of the cdc2 kinase. These asters behave very similarly to those originally described by De Brabander et al. (1981) in vivo and we have previously shown that they share striking similarities to taxol asters formed in vivo in MDCK cells (Buendia et al., 1990). We think they are interesting to study, because in vivo their assembly is mutually exclusive with mitotic spindle assembly, suggesting that their formation requires molecules also necessary for spindle assembly. The transition from interphase to metaphase is characterized by a dramatic change in microtubule dynamics. Microtubule turn over increases from several minutes in interphase to seconds during mitosis (Wadsworth and Salmon, 1986). This change requires phosphorylation and is induced by the activation of the mitotic cdc2-kinase (Verde et al., 1990). The original results of De Brabander et al. (1981, 1986) showed that microtubule bundles formed in interphasic cells in the presence of 100 μM taxol were disassembled and replaced by small asters at the onset of metaphase. The tubulin concentration in the cell is in the order of 20 μM (Hiller and Weber, 1978). Therefore, in the presence of 100 μM taxol, microtubules should have been extensively stabilized since maximum stabilization is achieved at equimolar ratios of taxol and tubulin dimers (Horwitz et al., 1986). Disruption of the interphasic bundles and reassembly into asters suggests, therefore, that even in the presence of taxol, microtubules were destabilized at the onset of mitosis and that microtubule destabilization was essential for aster assembly. In our case, it seems that 0.1 μM taxol stabilizes to some extent microtubules in mitotic extracts. Indeed, after nocodazole addition, asters disappear in ~40 min, whereas centrosome-nucleated microtubules in the absence of taxol in the same extracts disappear in 2 min. What seems to be affected by taxol, is the rate of depolymerization. Indeed, both the length and number of microtubules in each aster decrease slowly with time after nocodazole addition. In any case, they are not completely stabilized and this partially dynamic state may be required for aster formation by producing relatively short microtubules. In interphase extracts the same taxol concentration completely stabilized microtubules for at least 30 min after nocodazole addition.

**Phosphorylated Factor(s) and Dynein**

We have provided evidence that one important event in taxol aster assembly is the phosphorylation of some factor(s). Many mitotic factors are likely to be phosphorylated by cdc2-kinase, directly or indirectly (Moreno and Nurse, 1990). Some of them may be involved in microtubule destabilization. However, we have also found that cytoplasmic dynein is required to assemble taxol asters in mitotic frog egg extracts. It is possible that dynein activity or its targeting to mitotic microtubules is regulated by phosphorylation and under the control of cdc2-kinase. From the results reported in this article, dynein heavy chain is not phosphorylated either in mitosis or in interphase, at least in our extracts. It does not seem likely that the light chains are either, but we do not know if our labeling conditions were optimum. It is possible that the mode of interaction of dynein with microtubules is regulated by other molecules that are phosphorylated during mitosis. One function of these molecules could be to alter the oligomerization state of dynein and, therefore, its mode of interaction with microtubules or its interaction with other structures.

**Mechanism of Taxol Aster Formation**

The specific assembly of microtubules into radial structures in metaphase extracts in the absence of added centrosomes, could be explained in two ways. One explanation says that in metaphase extracts, the microtubule-nucleating material aggregates by itself (for example because it is phosphorylated). Since the critical tubulin concentration for assembly is very high, this material cannot nucleate microtubules. However, upon the addition of taxol, the critical tubulin concentration for assembly is lowered and microtubules start to grow from these foci leading to the formation of asters. This is in fact what seems to occur, at least in part, in mouse oocytes (Maro et al., 1985), and this interpretation was proposed by Endo et al. (1990) for the assembly of ethylene-glycol microtubule asters in sea urchin eggs. This cannot be generalized however. Firstly, in our extracts which are stably arrested in metaphase, this is clearly not how it works. Indeed, after taxol addition, microtubules first grow randomly, assemble into bundles, and finally reorganize into asters. Moreover, the whole process requires dynein activity. Sec-
ondly, in mouse oocytes, taxol induces the formation of many more microtubule asters than there were foci of microtubule nucleating material before taxol addition as if taxol treatment increased the number of microtubule nucleating foci (Maro et al., 1985). Finally, De Brabander et al. (1986) have shown by video intensification polarization microscopy of individual tissue culture cells that mitotic taxol asters grow and vanish successively in the same cell following a chaotic behavior. Moreover, by EM, these authors did not find pericentriolar material or "granules" in the center of most of the asters although they did find calmodulin as in spindle poles. Only two of the numerous asters found in a given cell had centrioles (De Brabander et al., 1981, 1986).

We propose another mechanism for taxol aster assembly in metaphase extracts of *Xenopus* eggs and we think that it is compatible with most observations in other systems. It is outlined in Fig. 9. In the presence of taxol, microtubules start to grow, maybe from dispersed small nucleation sites, or simply because the critical tubulin concentration for assembly is sufficiently lowered (Fig. 9 A). These microtubules are relatively stable but not hyperstable, because of the mitotic state of the cytoplasm. Therefore, they never grow very long. Several cytoplasmic dynein, probably associated with vesicles or centrosomal material, bind to different microtubules and migrate towards their minus ends (Fig. 9, C and D). There are reasons to think that in the extracts we use, dynein is associated to vesicles. Although our extracts are 100,000 g supernatants, they are still strongly contaminated by vesicles and particulate material because of the high protein concentration that prevents normal sedimentation. Preliminary experiments suggest that the same extracts depleted of particulate material by further centrifugation do not support aster assembly. Also when undiluted extracts are recentrifuged on a sucrose gradient, at least part of the dynein sediments much faster than expected from its known sedimentation coefficient and recovers a normal behavior after Triton X-100 extraction. Finally, soluble dynein added to pure microtubules stabilized with taxol does not induce the formation of asters. All this calls for a careful analysis of the native state of dynein in interphase and mitotic extracts. One requirement of the model shown in Fig. 9 is preferential accumulation of microtubule crosslinking material at the microtubule minus end. Otherwise only microtubule bundles would form. One obvious question is why asters do not form in interphase extracts where dynein is probably also active. It is possible that in interphase extracts microtubules are too long and stable to allow a reorganization into asters. Also, minus end crosslinking material may not interact with dynein or may be in a different state.

**Relevance to De Novo Assembly of Centrioles**

The assembly of microtubule asters in the cytoplasm of early embryos has been observed in many species after treatment applied to induce parthenogenesis (Dirksen, 1961; Kallenbach, 1985; Morgan, 1896; Wilson, 1901). Most of the work along these lines is cited in Kallenbach (1985) and Endo (1990). It was difficult to understand how these asters were formed because the treatments were complicated and the relationship with the cell cycle unclear. However, in some cases, new centrioles were found in the center of the asters (Dirksen, 1961; Kallenbach, 1985), raising the question of de novo assembly of centrioles (Bornens and Karsenti, 1984). In this work, we do not find centrioles in the center of mitotic taxol asters. Also, most of the asters described by De Brabander et al. (1986) and Endo (1990) do not contain new centrioles. Therefore, mitotic taxol asters may not be entirely similar to those described in parthenogenetically activated sea urchin eggs. Nevertheless, the finding that cytoplasmic dynein is involved in the assembly of mitotic taxol asters and that centrosomal material migrates towards the center of these asters (Buendia et al., 1990), raises interesting questions concerning the de novo assembly of centrioles in parthenogenetically activated eggs. Kallenbach (1985) did an extensive description of cytaster and centriole assembly in sea urchin eggs at the electron microscope level. Centriole assembly apparently occurred over a long period of time extended over at least one cell cycle and was observed in regions of microtubule aster assembly highly enriched in ER and Golgi vesicles. It is tempting to think that cytoplasmic dynein, by reorganizing microtubules in an astral configuration and transporting centrosomal material towards the center of the asters, helps to produce, in this region, a critical concentration in centriolar material that is required to initiate centriole assembly. Centrioles may not form in the center of taxol asters in somatic cells because excess centriolar components are not stored in the cytoplasm of these cells. In our experiments, the incubation times may have been too short. Clearly the mechanism of de novo centriole assembly in eggs should now be reinvestigated in the light of our new knowledge of the cell cycle and cell motility at the molecular level.

**Relevance to Spindle Pole Assembly**

It is largely accepted that spindle poles originate from the pericentriolar material that nucleates microtubules, the centriole having the function of concentrating this material in one point. However, the situation is much more complex and many questions remain unanswered. First, it is totally unclear how the nucleating activity of the pericentriolar material increases at the onset of metaphase although phosphorylation reactions seem to be involved (Vandre et al., 1985). Second, cytoplasmic centrosomal antigens seem to migrate towards the poles between prophase and metaphase (Buendia et al., 1990; Sager et al., 1986), suggesting a role for motor molecules in the concentration of centrosomal antigen at spindle poles. This idea is further supported by the recent observation by two groups that during mitosis, cytoplasmic dynein is concentrated at the kinetochores and on spindle microtubules especially near the poles (Pfarr et al., 1990; Steuer et al., 1990). Third, there are many examples of acen-


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