The intimate relationship between the IEV and the actin cytoskeleton suggests that the virus contains protein(s) that can associate directly or indirectly with actin. In *Listeria* species and *Shigella*, the proteins ActA, IactA and IcsA are critical for the nucleation of actin filaments at the bacterium surface[15,24,26]. Sequence comparisons of the vaccinia virus genome with ActA, IactA or IcsA failed to identify any ORF that contains significant homologous sequences. One possible candidate for viral interactions with actin is the A42R ORF, which is 32.1% identical to human profilin[27]. However, deletion of A42R has previously shown that it is not required for infectivity or release of mature virions[27]. Moreover, we found that vaccinia virus lacking A42R is still able to form actin tails and projections (data not shown), which is consistent with the recent observation that profilin is not required for actin-based motility of *Listeria* in the cell-free *Xenopus* system[28]. Whether profilin is involved in vaccinia motility will require further characterization, as the virus may be able to utilize cellular profilin isoforms. Given the similarities between the motility of bacterial pathogens and vaccinia virus, we suggest that intracellular pathogens have developed a common mechanism to exploit the actin cytoskeleton for their own purposes. □

Received 25 August; accepted 6 October 1995.

1. Goebel, S. J. *et al. Virology* **179,** 247–266 (1990).
2. Moss, B. in *Virology* (eds Fields, B. N. *et al.*) 2079–2111 (Raven, New York, 1990).
3. Payne, L. G. *J. gen. Virol.* **50,** 89–100 (1980).
4. Schmelz, M. *et al. J. Virol.* **68,** 130–147 (1994).
5. Morgan, C. *Virology* **73,** 43–58 (1976).
6. Stokes, G. V. *J. Virol.* **18,** 636–643 (1976).
7. Hiller, G., Weber, K., Schneider, L., Parajsz, C. & Jungwirth, C. *Virology* **98,** 142–153 (1979).
8. Hiller, G. & Weber, K. *J. Virol.* **44,** 647–657 (1982).
9. Hiller, G., Jungwirth, C. & Weber, K. *Expl Cell Res.* **132,** 81–87 (1981).
10. Krempien, U. *et al. Virology* **113,** 556–564 (1981).
11. Pollard, T. D. *Curr. Biol.* **5,** 837–840 (1995).
12. Cossart, P. *Curr. Opin. Cell Biol.* **7,** 94–101 (1995).
13. Tilney, L. G. & Portnoy, D. A. *J. Cell Biol.* **109,** 1597–1608 (1989).
14. Heinzen, R. A., Hayes, S. F., Peacock, M. G. & Hackstead, T. *Infect. Immun.* **61,** 1926–1935 (1993).
15. Bernardini, M. L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M. & Sansonetti, P. J. *Proc. natn. Acad. Sci. U.S.A.* **86,** 3867–3871 (1989).
16. Blasco, R. & Moss, B. *J. Virol.* **66,** 4170–4179 (1992).
17. Hiller, G., Eibl, H. & Weber, K. *J. Virol.* **39,** 903–913 (1981).
18. Kato, N., Eggers, H. J. & Rolly, H. *J. exp. Med.* **129,** 795–808 (1969).
19. Payne, L. G. & Kristenson, K. *J. Virol.* **32,** 614–622 (1979).
20. Schmutz, C., Payne, L. G., Gubser, J. & Wittek, R. *J. Virol.* **65,** 3435–3442 (1991).
21. Dabiri, G. A., Sanger, J. M., Portnoy, D. A. & Southwick, F. S. *Proc. natn. Acad. Sci. U.S.A.* **87,** 6068–6072 (1990).
22. Theriot, J. A., Mitchison, T. J., Tilney, L. G. & Portnoy, D. A. *Nature* **357,** 257–260 (1992).
23. Payne, L. G. & Kristensson, K. *Archs Virol.* **74,** 11–20 (1982).
24. Kocks, C. *et al. Cell* **68,** 521–531 (1992).
25. Gouin, E., Dehoux, P., Mengaud, J., Kocks, C. & Cossart, P. *Infect. Immun.* **63,** 2729–2737 (1995).
26. Domann, E. *et al. EMBO J.* **11,** 1981–1990 (1992).
27. Blasco, R., Cole, N. B. & Moss, B. *J. Virol.* **65,** 4598–4608 (1991).
28. Marchand, J.-B. *et al. J. Cell Biol.* **130,** 1–13 (1995).
29. Herzog, M., Draeger, A., Ehler, E. & Small, J. V. *Cell Biology: A Laboratory Handbook* (Academic, San Diego, 1994).
30. Rodriguez, J. F., Janeczko, R. & Esteban, M. *J. Virol.* **56,** 482–488 (1985).

ACKNOWLEDGEMENTS. We thank Y. Rivière, V. David and E. Gouin for help in preliminary experiments; R. Blasco for vRB 12 and profilin null virus; D. Klaus and M. Esteban for the S1 myosin and C3 antibody, respectively; C. Dotti for help and suggestions concerning video microscopy; I. Reckmann for vaccinia virus preparations; S. Schleich for help with electron microscopy; and A. Hyman, K. Simons, M. Zerial, R. Parton and E. Karsenti for suggestions and comments on the manuscript.

Microtubule nucleation by γ-tubulin-containing rings in the centrosome

Michelle Moritz, Michael B. Braunfeld*, John W. Sedat*, Bruce Alberts† & David A. Agard*

Department of Biochemistry & Biophysics and
* Howard Hughes Medical Institute, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, California 94143-0448, USA

THE microtubule cytoskeleton of animal cells does not assemble spontaneously, but instead requires the centrosome. This organelle consists of a pair of centrioles surrounded by a complex collection of proteins known as the pericentriolar material (PCM)[1]. The PCM is required for microtubule nucleation[2]. The minus, or slow-growing, ends of microtubules are embedded in the PCM and the plus, or fast-growing, ends project outwards into the cytoplasm during interphase, or into the spindle apparatus during mitosis. γ-Tubulin is the only component of the PCM that is so far implicated in microtubule nucleation[3–6]. Here we use immuno-electron microscopic tomography to show that γ-tubulin is localized in ring structures in the PCM of purified centrosomes without microtubules. When these centrosomes are used to nucleate microtubule growth, γ-tubulin is localized at the minus ends of the microtubules. We conclude that microtubule-nucleating sites within the PCM are ring-shaped templates that contain multiple copies of γ-tubulin.

We previously reported that the PCM of isolated *Drosophila* centrosomes that lack microtubules (MTs) contains hundreds of ring-like structures that are 25–30 nm in diameter and 10–13 nm thick. In addition, we found that the minus ends of MTs in centrosomes with regrown asters appear to be blunt and arise abruptly, inasmuch as they are not associated with any discernible structure[7]. We suggested then that the ring structures are MT-nucleating sites. Because their diameter is similar to that of MTs, they could be invisible at the minus ends of MTs in centrosomes with regrown asters. If these suggestions are correct,

TABLE 1 Distribution of gold particles in PCM of centrosomes with and without microtubules

	Number of gold particles (% total particles)		
(*a*) Centrosomes with MTs	At (−) ends of MTs	Ambiguous position	Total
All gold	77 (65%)	41 (35%)	118 (100%)
Single gold particles	2 (2%)	7 (6%)	
Clusters of 2	12 (10%)	8 (7%)	
Clusters of 3	15 (13%)	12 (10%)	
Clusters of 4	24 (20%)	8 (7%)	
Clusters of >4	24 (20%)	6 (5%)	
(*b*) Centrosomes without MTs	At ring structures	Ambiguous position	Total
All gold	68 (69%)	30 (31%)	98 (100%)
Single gold particles	3 (3%)	6 (6%)	
Clusters of 2	12 (12%)	8 (8%)	
Clusters of 3	12 (12%)	3 (3%)	
Clusters of 4	36 (37%)	0 (0%)	
Clusters of >4	5 (5%)	13 (13%)	

The gold particles were randomly chosen in each reconstruction and their association with identifiable structures (MTs or rings) was then determined by stepping through the relevant projections from the reconstruction, which reveals the three-dimensional structure of objects.

† Present address: National Academy of Sciences, 2101 Constitution Avenue NW, Washington, DC 20418, USA.

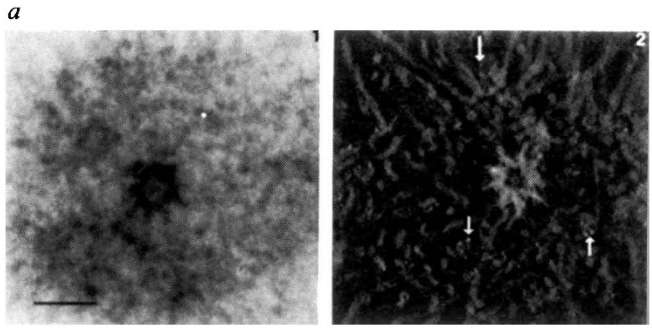
we expect gold-labelled antibodies against γ-tubulin to stain the rings in centrosomes without MTs and to stain the minus ends of MTs in centrosomes with MTs. In this study, we have tested this idea, determining the three-dimensional structure of the gold-labelled centrosomes by immuno-electron microscopic (EM) tomography[7-9].

For the three-dimensional reconstructions, purified *Drosophila* centrosomes were stained with gold-labelled antibodies, embedded, cut into semi-thick (0.7 μm) sections and examined in the EM (Fig. 1). Because these sections can contain entire *Drosophila* centrosomes (which vary in diameter from ~0.2 to 1 μm[7]), a complete, high-resolution (~6–8 nm) three-dimensional image of an intact centrosome can be reconstructed by EM tomography. In some cases, individual MTs can then be followed through such reconstructions to their minus ends, allowing the origins of MTs to be identified unambiguously[7].

We have used this approach to determine the location of γ-tubulin in the centrosome under two different conditions: first, in the centrosome, as isolated, without any MTs, and second, after the same centrosomes have been mixed with pure tubulin and used to grow asters composed of hundreds of MTs. We present the results for centrosomes with asters in Fig. 1: the centrosomes are abundantly and specifically labelled with gold throughout the PCM (Fig. 1*a*, white dots, arrowed). In enlarged views of selected regions of the PCM, it can be seen that the minus ends of MTs are labelled with clusters of gold, which are often arranged in semicircles or rings which follow the shape of the MT minus end (Fig. 1*b*). When we analysed 100 randomly chosen gold particles, we found that 65% of them were associated with MT minus ends; the remaining 35% could not be localized unambiguously because of the close and complicated arrangement of MTs within the PCM. In addition, the majority (62%) of the gold particles that were associated with MT minus ends were in clusters of four or more (Table 1*a*). In no case was the gold seen along the length of MTs, even in regions where the MTs were embedded in the PCM. As controls, centrosomes were incubated with the gold-labelled secondary antibodies alone, or with a primary antibody (a gift from C. Field) that recognizes the non-centrosomal protein, peanut (a contractile-ring component), followed by these antibodies. Sections of 0.2 μm were prepared from control samples and scanned by conventional transmission EM for gold particles. In both cases, the number of gold particles found to label the control sample was less than 1% of the number found in samples that had been incubated with antibodies recognizing γ-tubulin. In addition, the gold particles on control samples were never found in clusters (data not shown).

The presence of multiple gold particles at the ends of single MTs suggests that there are multiple γ-tubulin molecules at these positions, in support of a model that postulates that a γ-tubulin molecule sits at the minus end of each of the 13 protofilaments in a MT[3]. In addition, the semicircular or circular arrangement of the gold particles closely follows the shape of the MTs. However, our current results do not allow us to ascertain the number of γ-tubulin molecules in the nucleating structure (13 are predicted).

The results for centrosomes without MTs are presented in Fig. 2. In unlabelled centrosomes, numerous (~400 to 800) ring structures are visible throughout the PCM[7]. When centrosomes without MTs were immuno-gold-labelled to detect γ-tubulin, the gold particles were distributed in clusters throughout the PCM (Fig. 2*a*, white dots, arrowed). In enlarged views, we found that approximately 60% of the rings that are visible in the reconstruction are associated with at least one gold particle. More-



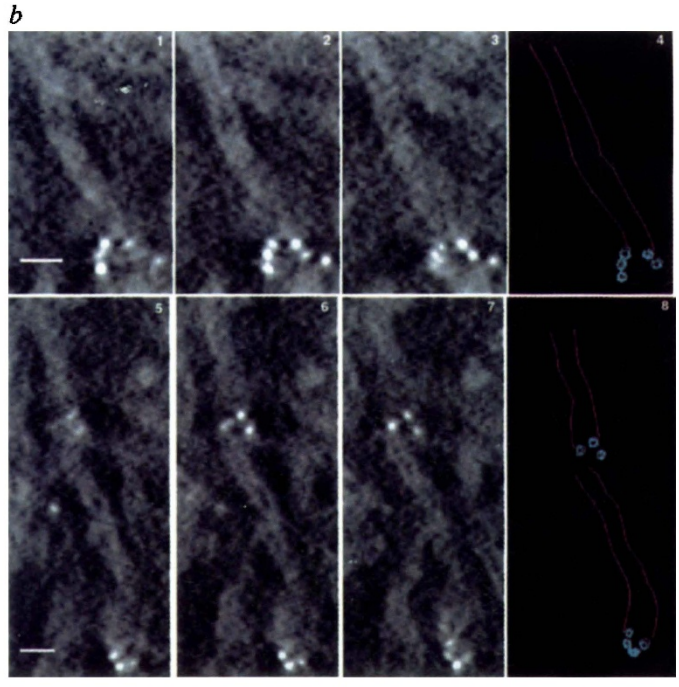


FIG. 1 Tomographic reconstruction of a centrosome with MTs after immuno-gold labelling to detect γ-tubulin. *a*, Panel 1 shows an example of raw data used to generate the reconstruction shown in panel 2 (Magnification, ×10,400; scale bar, 200 nm); panel 2 shows a selected projection (representing a slice 2.75 nm thick) from the computer-generated reconstruction (out of a total of 179). White dots (arrowed) are 6-nm gold particles. *b*, γ-Tubulin is localized in semicircles and rings at the minus ends of MTs. Panels 1–3 and 5–7: enlarged views of two examples of gold-labelled MT minus ends taken from the reconstruction in *a*. Selected projections stepping through the three-dimensional structure are shown (2.75 nm per step). Bar, 30 nm. Panels 4 and 8: models of the relevant MTs and gold particles seen in panels 1–3 and 5–7. Red, MT outlines; aqua, 6-nm gold particles.

METHODS. Centrosomes were purified from 0–3.5-hr *Drosophila* embryos, MTs were regrown on them, and the resulting asters were then fixed and sedimented onto coverslips as described in Ref. 7. For immuno-gold labelling of γ-tubulin, nonspecific binding sites were saturated by a 20-min incubation in Block, consisting of PBS[7] plus 0.5% fish gelatin, 0.8% bovine serum albumin, 0.1% Tween-20, and 0.5 M NaCl. Samples were then incubated for 3 h with an affinity-purified rabbit polyclonal antibody specific for γ-tubulin, diluted 1:500 in Block, which was raised against a peptide corresponding to the unique 17-amino-acid C terminus of the maternal form of *Drosophila* γ-tubulin and affinity-purified on the same peptide (Y. Zheng, personal communication). Samples were washed quickly in Block three times and then three times for 5 min in PBS plus 0.1% Tween-20. They were then incubated for 3 h, with gentle rocking, in 6-nm-gold-labelled, affinity-purified goat-anti-rabbit-IgG (EM grade, Jackson Immunological Laboratories) diluted 1:25 in Block. Washes were repeated, and samples were then washed into PBS and fixed in 1% glutaraldehyde in PBS for 15 min. Further preparation of samples for EM was as described in ref. 7. The tomographic data set consisted of 99 views covering a tilt range of −63.75° to +58.75° at 1.25° intervals. Images were recorded as 480 × 480 pixel arrays (2 × 2 binning) on a Gatan photometric-UCSF CCD, giving a final magnification of 2.75 nm per pixel. Data were collected automatically using our EMACT data collection system and processed using the EMCAT suite of software[13] as in ref. 7.

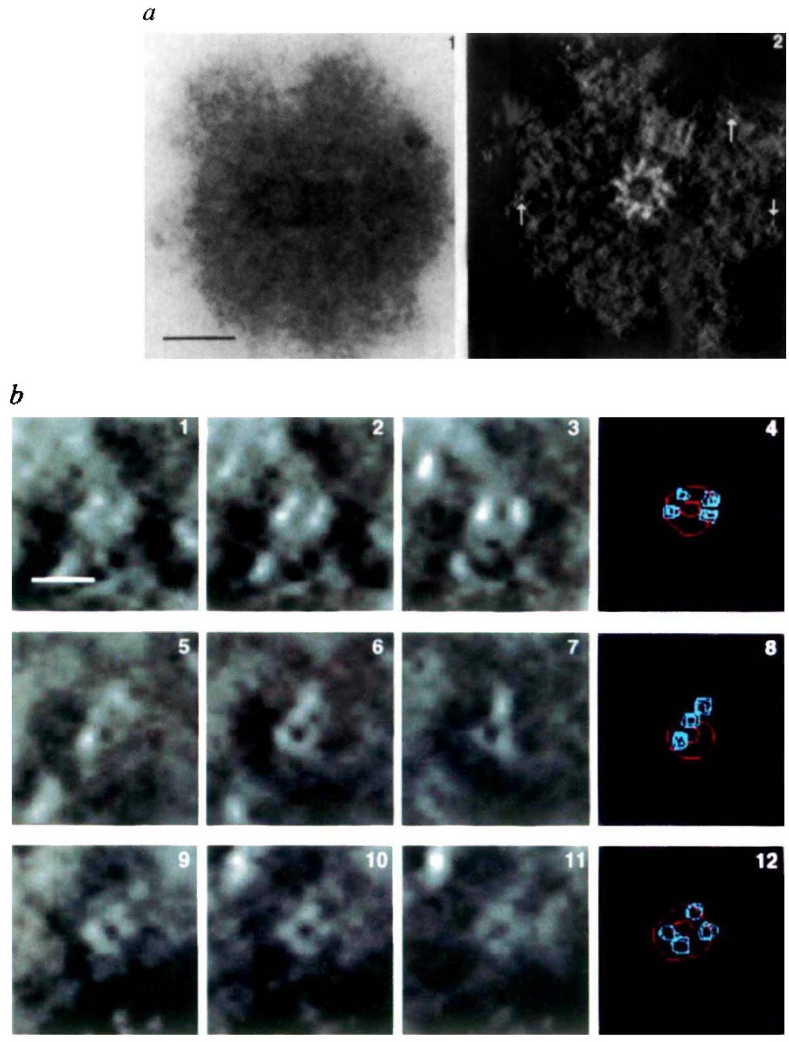


FIG. 2 Tomographic reconstructions of purified centrosomes without MTs after immuno-gold labelling to detect γ-tubulin. The γ-tubulin is localized to ring structures scattered throughout the PCM. *a*, Panel 1: An example of the raw data used for generating the reconstruction shown in panel 2 (magnification, ×10,400; bar, 200 nm); panel 2: a selected projection (representing a slice 2.75 nm thick) from the computer-generated reconstruction (out of a total of 143). White dots (arrowed) are 6-nm gold particles. *b*, The γ-tubulin is found throughout the PCM in ring structures 25–30 nm in diameter and 10–13 nm thick. Panels 1–3, 5–7 and 9–11: enlarged views of three examples of gold-labelled ring structures taken from the reconstruction in *a*. Selected projections stepping through the three-dimensional structure are shown (2.75 nm per step). Panels 4, 8, 12: models showing the relevant rings and gold particles in panels 1–3, 5-7 and 9–11. Red, ring structure; aqua, 6-nm gold particles. Bar, 30 nm.
METHODS. Samples were prepared as described for Fig. 1 and in ref. 7, except that MTs were not regrown on the purified centrosomes. The tomographic data set consisted of 105 views covering a tilt range of −65° to +65° at 1.25° intervals.

over, 69% of the gold particles were clearly associated with the ring structures, and most (60%) of this gold is in clusters of 4 or more (Fig. 2*b* and Table 1*b*). The clusters of gold associated with the rings are often arranged in semicircles and circles that correspond to the shape of the rings. We conclude that MT-nucleating sites consist of ring-shaped structures containing multiple copies of γ-tubulin. The general idea that some sort of ring could serve as the template for MT growth has been around for some time[10,11].

The conclusion that the MT-nucleating site in the centrosome is a ring complex is strongly substantiated by work described in an accompanying paper[12], in which a ring-shaped complex of proteins containing γ-tubulin (the γTuRC) has been purified from *Xenopus* oocytes and shown to have MT-nucleating activity[12]. The *Xenopus* complex contains multiple copies of γ-tubulin as well as at least 6 other proteins, of which only α- and β-tubulin have been identified. Preliminary biochemical evidence suggests that a similar complex may exist in *Drosophila* (Y. Zheng, personal communication). The fact that the nucleating ring exists in organisms as divergent as *Xenopus* and *Drosophila* suggests that its complex structure is evolutionarily very old.

The similarity of the purified γTuRC structure[12] to the ring structures seen in centrosomes suggests that MT-nucleating complexes are at least partially assembled in the cytoplasm before associating with the centrosome. Other centrosomal proteins presumably constitute the 'scaffolding' onto which the γTuRC assembles at the centrosome. *In vivo*, the γTuRC must become activated for MT nucleation only after it associates with the centrosome, because MTs do not appear randomly in the cytoplasm; perhaps, therefore, activation requires the binding of the ring structure to one or more PCM proteins. □

Received 29 August; accepted 9 November 1995.

1. Kellogg, D. R., Moritz, M. & Alberts, B. M. *A. Rev. Biochem.* **63,** 639–674 (1994).
2. Gould, R. R. & Borisy, G. G. *J. Cell Biol.* **73,** 601–615 (1977).
3. Oakley, B. R. *Trends Cell Biol.* **2,** 1–5 (1992).
4. Joshi, H. C., Palacios, M. J., McNamara, L. & Cleveland, D. W. *Nature* **356,** 80–83 (1992).
5. Felix, M.-A., Antony, C., Wright, M. & Maro, B. *J. Cell Biol.* **124,** 19–31 (1994).
6. Stearns, T. & Kirschner, M. *Cell* **76,** 623–637 (1994).
7. Moritz, M. *et al. J. Cell Biol.* **130,** 1149–1159 (1995).
8. Koster, A. J., Chen, H., Sedat, J. W. & Agard, D. A. *Ultramicroscopy* **46,** 207–227 (1992).
9. Koster, A. J. *et al. Microsc. Soc. Am. Bull.* **23,** 176–188 (1993).
10. Tilney, L. G. *et al. J. Cell Biol.* **59,** 267–275 (1973).
11. Evans, L., Mitchison, T. & Kirschner, M. *J. Cell Biol.* **100,** 1185–1191 (1985).
12. Zheng, Y., Wong, M. L., Alberts, B. & Mitchison, T. *Nature* **378,** 578–583 (1995).
13. Fung, J. C. *et al. J. struct. Biol.* (in the press).

ACKNOWLEDGEMENTS. We thank Y. Zheng and C. Field for antibodies, J. Fung and H. Chen for help with computer programs, M. L. Wong for help with controls, and Y. Zheng and T. Mitchison for discussions and for comments on the manuscript. M.M. was supported by postdoctoral fellowships from the American Cancer Society and the National Cancer Institute, and an NIH institutional training grant. This work was supported by the Howard Hughes Medical Institute (J.W.S. and D.A.A.), and by separate NIH grants to B.A., D.A.A. and J.W.S.