A Rat Monoclonal Antibody Reacting Specifically with the Tyrosylated Form of α-Tubulin. I. Biochemical Characterization, Effects on Microtubule Polymerization In Vitro, and Microtubule Polymerization and Organization In Vivo

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ABSTRACT The antigenic site recognized by a rat monoclonal antibody (clone YL 1/2) reacting with α-tubulin (Kilmartin, J. V., B. Wright, and C. Milstein, 1982, *J. Cell Biol.*, 93:576–582) has been determined and partially characterized. YL 1/2 reacts specifically with the tyrosylated form of brain α-tubulin from different mammalian species. YL 1/2 reacts with the synthetic peptide Gly-$(Glu)_3$-Gly-$(Glu)_2$-Tyr, corresponding to the carboxyterminal amino acid sequence of tyrosylated α-tubulin, but does not react with Gly-$(Glu)_3$-Gly-$(Glu)_2$, the constituent peptide of detyrosylated α-tubulin. Electron microscopy as well as direct and indirect immunofluorescence microscopy shows that YL 1/2 binds to the surface of microtubules polymerized in vitro and in vivo. Further in vitro studies show that the antibody has no effect on the rate and extent of microtubule polymerization, the stability of microtubules, and the incorporation of the microtubule-associated proteins (MAP_2) and tau into microtubules. In vivo studies using Swiss 3T3 fibroblasts injected with YL 1/2 show that, when injected at low concentration (2 mg IgG/ml in the injection solution), the antibody binds to microtubules without changing their distribution in the cytoplasm. Injection of larger concentration of YL 1/2 (6 mg IgG/ml) induces the formation of microtubule bundles, and still higher concentrations cause the aggregation of microtubule bundles around the nucleus (>12 mg IgG/ml).

The conditions for microtubule polymerization in vitro are known in great detail. Microtubule assembly in vitro requires physiological temperature, ionic strength (0.1–0.14 M), pH 6.7–7.0, guanosine triphosphate (1 mM), Mg^{++} (1 mM) (33), and is greatly enhanced by a set of proteins called MAP[1] (i.e., microtubule-associated proteins; 8, 14, 32). In contrast the chemical mechanisms actually involved in the assembly of microtubules in vivo and in vitro are poorly understood.

Phosphorylation of MAP (7, 25) and the binding of Ca^{++}-calmodulin (12) to microtubules have been shown respectively to inhibit polymerization (7) and to promote depolymerization (12) of microtubules in vitro, but it is not known whether they are relevant in vivo. Although the posttranslational modification of proteins is an important device in the regulation of protein function, nothing is known about the role played by the phosphorylation of β-tubulin (3, 21) and the detyrosylation of α-tubulin (28) in the polymerization and function of microtubules in vivo. With respect to the removal and incorporation of tyrosine into the carboxyterminus of α-tubulin, it would be important to locate this part of the tubulin molecule in the microtubule and to determine whether or not it is involved in the assembly and function of microtubules.

[1] *Abbreviations used in this paper:* MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; and MAP, microtubule-associated proteins.

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Here we report the characterization of a monoclonal antibody that specifically recognizes the tyrosylated form of α-tubulin. By using this antibody we have found the following: that the tyrosylated carboxyterminus of α-tubulin is (*a*) exposed on the surface of microtubules assembled in vitro and of cytoplasmic, spindle, and midbody microtubules polymerized in vivo, is (*b*) not involved in the polymerization of tubulin into microtubules, is (*c*) not part of the domains of tubulin binding MAP_2 and tau, and (*d*) could play a yet unknown role in the normal distribution and organization of microtubules in the cytoplasm of interphase cells and in their function (32). Portions of this work have been presented in abstract form (31).

MATERIALS AND METHODS

Purification of Microtubule Protein and Studies on Microtubules Polymerized In Vitro: Pig and rat brain microtubule protein were purified by two cycles of temperature-dependent polymerization-depolymerization (24) using buffer A (0.1 M MES, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM β-mercaptoethanol, 1 mM guanosine triphosphate, 0.2 mM phenylmethylsulfonyl fluoride, pH 6.75), and adding 30% glycerol in the first cycle of polymerization. Homogeneous tubulin, MAP_2, and tau were prepared from microtubule protein as described (8, 22).

All purified proteins were stored under liquid nitrogen. Studies on microtubule polymerization in vitro were performed using solutions of microtubule protein prepared in buffer A without phenylmethylsulfonyl fluoride. Polymerization was induced by raising the temperature of the microtubule protein solution from 4°C to 37°C. The time course of microtubule polymerization was studied by continuously reading the turbidity developed at 350 nm using a Cary 15 spectrophotometer. The content of MAP_2 and tau in microtubules was studied by measuring the MAP_2 and tau contained in microtubule pellets obtained from steady state microtubules polymerized using microtubule protein. Protein concentrations were estimated on SDS polyacrylamide gels using a microdensitometer (Joyce Loeble 3CS). The incorporation of MAP_2 and tau into steady state microtubules polymerized using homogeneous tubulin was studied in a similar way (23). The effects of the monoclonal YL 1/2 α-tubulin antibody on microtubule polymerization and stability, the content of MAP_2 and tau in microtubules, and binding of MAP_2 and tau to microtubules were studied by preincubating different concentrations of antibody (see Results) with microtubule protein or purified tubulin at 4°C for 45 min before incubation at 37°C for 45 min or by polymerizing microtubule protein or purified tubulin to steady state for 45 min at 37°C before adding the antibody for another 45 min at 37°C.

Antibodies: The rat monoclonal YL 1/2 and YOL 1/34 α-tubulin antibodies used in our studies were a gift of Dr. Kilmartin (Medical Research Council, Cambridge). Affinity-purified rat brain tubulin antibodies were obtained as previously described (36). Rhodamine-conjugated goat anti-rabbit IgGs as well as rhodamine- and fluoresceine-conjugated goat anti-rat IgGs (Cappel Laboratories, Cochranville, PA) were purified by affinity chromatography using respectively rabbit IgG and rat IgG coupled to Sepharose 4B columns. Rhodamine-conjugated YL 1/2 antibodies containing 2 mol rhodamine/mole antibody were prepared using tetramethylrhodamine isothiocyanate as described (16). Cleavage of YL 1/2 antibodies into Fab-fragments with mercuripapain (Worthington Biomedical) was performed as described (18) using a ratio of enzyme to IgG (wt/wt) of 1:600. Digestion was performed at 37°C for 1 h with an IgG concentration of 2 mg/ml, and cleavage products were separated on a Sephacryl S-300 column.

Antibody Probes: Approximately 15% of the total α-tubulin purified from brain is normally tyrosylated (17), detyrosylated bovine brain microtubule protein was a gift of Dr. Kumar (National Institutes of Health) and was prepared as previously described (13) by treatment of microtubule protein with pancreatic carboxypeptidase A. The octapeptide Gly-$(Glu)_3$-Gly-$(Glu)_2$-Tyr and the heptapeptide Gly-$(Glu)_3$-Gly-$(Glu)_2$, constituents of the tyrosylated and detyrosylated carboxyterminus of α-tubulin, were synthesized by Penninsula Laboratories, Inc. (Belmont, CA) and were 69% (octapeptide) and 83% (heptapeptide) pure when examined by amino acid analysis.

Antibody-binding Studies: Immunoautoradiography of tyrosylated and detyrosylated brain microtubule protein resolved into the α- and β-tubulin subunits by SDS PAGE was performed as previously described (5) using YL 1/2 and YOL 1/34 as first antibodies and ^{125}I-iodinated purified rabbit anti-rat IgGs as second antibodies.

Indirect Immunofluorescence Microscopy, Electron Microscopy, and Microinjection Studies: Swiss 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. PtK_2 cells (rat kangaroo) were grown in HAM's F12 medium containing 5% fetal calf serum. All cells were grown for 2 d before being used. Microtubule cytoskeletons of PtK_2 cells grown on coverslips were prepared as follows at room temperature. The cells were briefly washed with 0.1 M MES, 1 mM $MgCl_2$, 1 mM EGTA, 4% polyethylenglycol 6000, pH 6.75 (buffer B), then extracted for 30 s with buffer B containing 0.2% Triton X-100, washed with buffer B, fixed with buffer B containing 2% glutaraldehyde for 15 min, and washed with PBS. The fixed cytoskeletons were finally treated with 1 mg/ml $NaBH_4$ in PBS as described (29). For specific removal of carboxyterminal tyrosine from α-tubulin the cytoskeletons were incubated with 10 μg/ml pancreatic carboxypeptidase A (Worthington Biomedical) in PBS for 30 min at 37°C and washed three times with PBS. Microinjection and indirect immunofluorescence microscopy were performed by procedures already described (30) using a Zeiss ICM 405 microscope. Cells were normally fixed and permeabilized with cold methanol (−20°C) for 5 min. For electron microscopy cells were grown in plastic dishes, fixed, and embedded in situ, and processed as previously described (35).

For preabsorption experiments using indirect immunofluorescence microscopy, the peptides Gly-$(Glu)_3$-Gly-$(Glu)_2$ or Gly-$(Glu)_3$-Gly-$(Glu)_2$-Tyr and either YL 1/2 or YOL 1/34 antibodies were mixed in 0.1 M MES, pH 6.75, containing 10 mg/ml BSA at final concentrations of 10 μg IgG/ml and 0.5 mg peptide/ml. After being kept for 20 min at room temperature, 20 μl of each mixture were added to PtK_2 cytoskeletons on circular 18-mm coverslips and indirect immunofluorescence microscopy was performed as described above.

RESULTS

Monoclonal YL 1/2 Antibody Reacting with α-Tubulin Specifically Recognizes the Tyrosylated Form of the Protein

Two rat monoclonal antibodies (clones YL 1/2 and YOL 1/34) raised against yeast tubulin were shown to react specifically with α-tubulin (9). In the cell α-tubulin exists in two different forms, tyrosylated and detyrosylated (2, 15, 17, 19; see also reference 26), α-tubulin is translated as the tyrosylated form (28).

Detyrosylation is performed by a specific carboxypeptidase that removes tyrosine from the carboxyterminus of the protein (1, 6, 10, 27; see also reference 13). The removed tyrosine is put back into the protein by the enzyme tubulin tyrosine ligase in a reaction requiring ATP (20; see also reference 4). Immunoautoradiography of bovine brain microtubule protein containing either tyrosylated or detyrosylated α-tubulin showed that the YL 1/2 antibody reacted exclusively with the tyrosylated form of α-tubulin (Fig. 1, compare lanes *5* and *6*). On the other hand YOL 1/34 antibody reacted with both tyrosylated and detyrosylated α-tubulin (Fig. 1, lanes *3* and *4*).

Studies on the Effects of YL 1/2 on Microtubule Polymerization and the Incorporation of MAP_2 and Tau into Microtubules Assembled In Vitro

Microtubule polymerization resulted from the longitudinal and lateral association of tubulin molecules. Although both tyrosylated and detyrosylated α-tubulin displayed identical abilities to polymerize in vitro (19), it was not known whether the carboxyterminus of α-tubulin was involved in the association of tubulin molecules needed for the polymerization of tubulin into microtubules. The specific reaction of YL 1/2 with the tyrosylated carboxyterminus of α-tubulin made it possible to study the involvement of this part of α-tubulin in the polymerization of tubulin into microtubules. For this purpose we studied the time course of microtubule polymerization in the presence and absence of YL 1/2.

As shown in Fig. 2*A*, the lag time preceding the start of

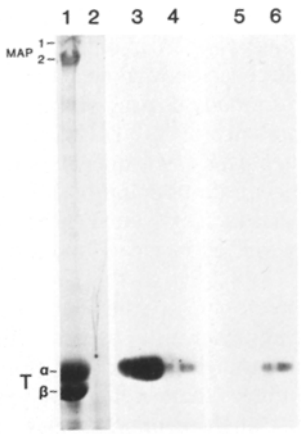


FIGURE 1 Immunoautoradiography of tyrosylated and detyrosylated brain tubulin run on SDS polyacrylamide gels and incubated with YL 1/2 and YOL 1/34 antibodies. 50 μg of detyrosylated bovine brain tubulin (lanes *1*, *3*, and *5*) and 0.1 μg of tyrosylated rat brain tubulin (lanes *2*, *4*, and *6*) were run on a 10% SDS polyacrylamide slab gel. The gel was cut in three parts. One part was stained with Coomassie Blue (lanes *1* and *2*) and the other two incubated with YOL 1/34 (lanes *3* and *4*) and YL 1/2 (lanes *5* and *6*) for immunoautoradiography (see Materials and Methods). Observe that the small amount of tyrosylated tubulin sampled on lanes *2*, *4*, and *6* was not detected by Coomassie Blue staining (see lane *2*). Note the specific reaction of YL 1/2 with the tyrosylated form of α-tubulin (compare lanes *5* and *6*) and the binding of YOL 1/34 to both tyrosylated and detyrosylated α-tubulin (compare lanes *3* and *4*). *T*, tubulin.

tubulin polymerization was identical in preparations of microtubule protein preincubated with or without YL 1/2 at 4°C for 45 min before polymerization at 37°C. These results indicated that the antibody had no effect on the rate of microtubule nucleation and therefore that the tyrosylated carboxyterminus of α-tubulin was not required for the formation of microtubule nuclei. Studies on both the rate and extent of turbidity developed during microtubule polymerization revealed slight increases in samples incubated with the antibody. The increase was larger at higher concentrations of YL 1/2 (Fig. 2*A*). Because no differences in the critical tubulin concentration for polymerization were found between preparations of microtubule protein polymerized in the presence and absence of YL 1/2 (data not shown; see Fig. 2*B*), the increases of the rate and extent of turbidity in the presence of YL 1/2 were probably due to coating of microtubules by antibody (see Fig. 3*b*). When microtubules polymerized in the presence and absence of antibody were cooled at 4°C, the turbidity decreased with identical rates to the initial basal levels (data not shown).

This result indicated that the antibody did not stabilize microtubules against cooling. Furthermore, microtubules polymerized in the absence and presence of YL 1/2 contained comparable amounts of MAP_2 and tau (Fig. 2*B*), suggesting that the two MAP do not bind to the tyrosylated carboxyterminus of α-tubulin exposed on the surface of the microtubules (see below).

Location of the Tyrosylated Carboxyterminus of α-Tubulin in Microtubules Polymerized In Vitro and In Vivo

To study the location of the tyrosylated carboxyterminus of α-tubulin in microtubules, we looked to the ability of YL 1/2 to bind to tubulin in microtubules polymerized both in vitro and in vivo.

We studied the ability of YL 1/2 to bind to tubulin in microtubules polymerized in vitro by measuring the antibody content of microtubule pellets obtained from preparations of (*a*) microtubule protein incubated with different concentrations of antibody at 4°C before raising the temperature to 37°C, and of (*b*) steady state microtubules incubated with different concentrations of antibody at 37°C. The results of the sedimentation assay shown in Fig. 2*B* demonstrated that

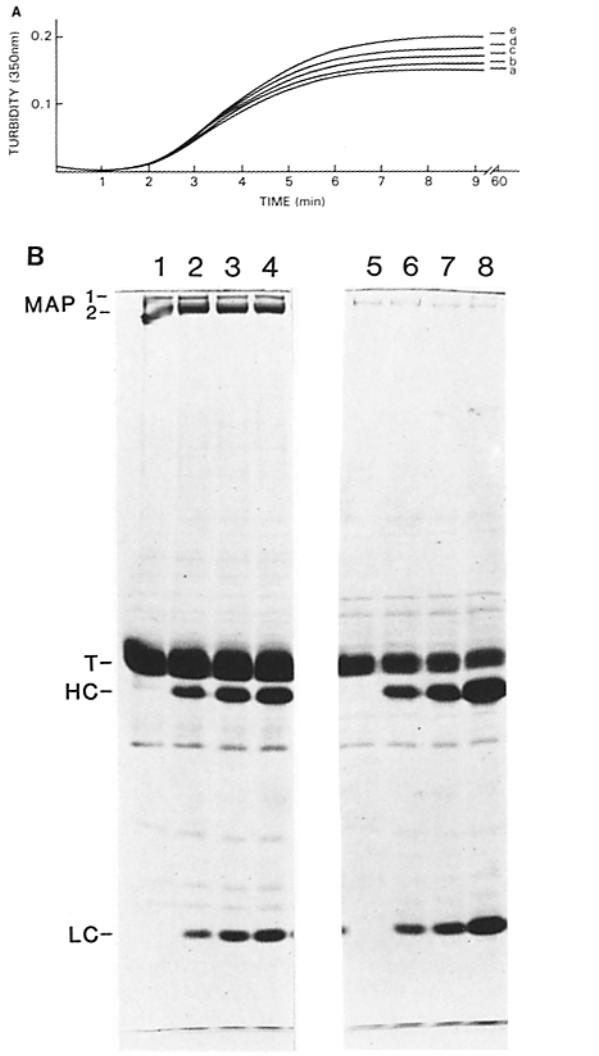


FIGURE 2 (*A*) Time course of microtubule polymerization in vitro in the absence and presence of YL 1/2 antibody. Rat brain microtubule protein was prepared in buffer A at 1 mg/ml and preincubated in the absence (*a*) and presence (*b*–*e*) of different concentrations of YL 1/2 for 45 min at 4°C. The molar ratios of tubulin/antibody used were 2.5 (*b*), 1.25 (*c*), 0.6 (*d*), and 0.125 (*e*). Polymerization was started by raising the temperature to 37°C. Observe the similar polymerization lags of the samples incubated without and with different concentrations of YL 1/2. Note the increase in the rate and total extent of turbidity developed in samples incubated with increasing concentrations of YL 1/2. (*B*) MAP_2 and tau content of microtubules polymerized in vitro in the absence and presence of YL 1/2. Rat brain microtubule protein (1 mg/ml in buffer A) was incubated without and with different concentrations of YL 1/2 for 45 min at 4°C before polymerization for 45 min at 37°C. The microtubules formed were collected by centrifugation using a Beckman Airfuge (20 psi for 20 min) and their protein content analyzed on a 12% SDS polyacrylamide slab gel (lanes *1*–*4*). The protein contained in the corresponding sample supernatants was similarly studied (lanes *5*–*8*). Sample incubated without YL 1/2 (lanes *1* and *5*), and samples incubated with a molar ratio of tubulin/antibody of 2 (lanes *2* and *6*), 1 (lanes *3* and *7*), and 0.2 (lanes *4* and *8*). *LC*, IgG-light chain; *HC*, IgG-heavy chain; *T*, tubulin.

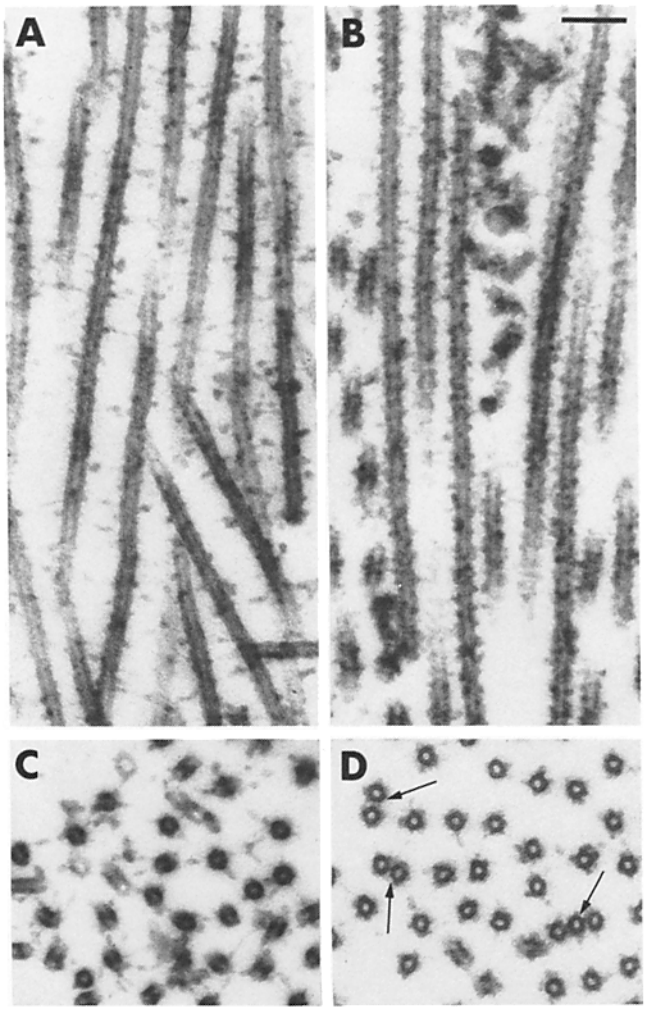


FIGURE 3 Longitudinal and cross section views of microtubules polymerized in the absence or presence of YL 1/2. (*a* and *c*) Microtubules polymerized in the absence of antibody (corresponding to sample shown in Fig. 2*B*, lane *1*). Note the arm-like projections of the microtubule walls. (*b* and *d*) Microtubules polymerized in the presence of YL 1/2 (corresponding to the sample shown in Fig. 2*B*, lane *4*). Observe the uniform antibody-coating of microtubules (compare *a* and *b*) and the frequent presence of doublets and triplets of microtubules in the samples of microtubule protein polymerized in the presence of YL 1/2 (arrows in *d*). Bar, 0.1 μm. × 88,000.

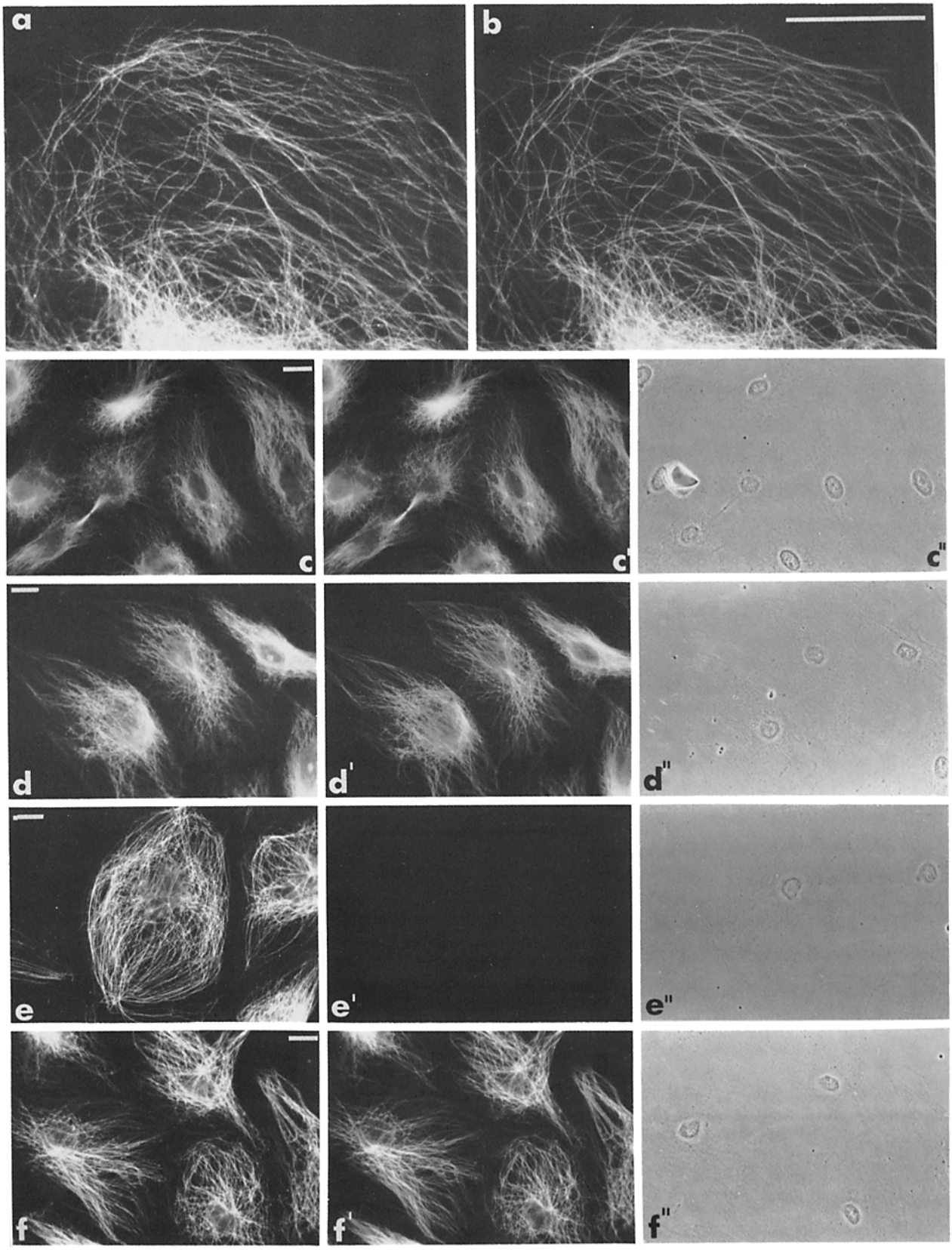
YL 1/2 was able to bind to tubulin in a concentration-dependent manner without affecting polymerization. Electron microscopy studies of microtubule pellets showed no aberrant polymerization and furthermore antibody coating of the microtubules polymerized in the presence of YL 1/2 (Fig. 3). Identical results were obtained when steady state microtubules were incubated with different concentrations of antibody (not shown). These results indicated that the tyrosylated carboxyterminus of α-tubulin was located on the surface of microtubules polymerized in vitro.

Indirect immunofluorescence microscopy studies using YL 1/2 showed that the tyrosylated carboxyterminus of α-tubulin was also located on the surface of cytoplasmic, midbody (Fig. 4, *b* and *c'*), and spindle microtubules (data not shown) as shown by the fluorescent labeling of these microtubules in cytoskeletons incubated with YL 1/2. The binding of YL 1/2 to the microtubules of cultured cells was prevented by preincubating the antibody with the synthetic peptide Gly-$(Glu)_3$-Gly-$(Glu)_2$-Tyr but not by preincubation with Gly-$(Glu)_3$-Gly-$(Glu)_2$ (data not shown). Gly-$(Glu)_3$-Gly-$(Glu)_2$-Tyr corresponds to the sequence of the eight last amino acids of the carboxyterminus of tyrosylated α-tubulin (17, 28). Gly-$(Glu)_3$-Gly-$(Glu)_2$ is constituent of the carboxyterminus of detyrosylated α-tubulin. Neither peptide affected labeling of microtubules when preincubated with YOL 1/34. Furthermore the binding of YL 1/2 to microtubules was abolished by treating microtubule cytoskeletons with carboxypeptidase A (Fig. 4*e'*), an enzyme which selectively removed carboxy-terminal tyrosine from α-tubulin (19). As expected treatment of cytoskeletons with carboxypeptidase A did not affect the binding of YOL 1/34 (Fig. 4*f'*) or the polyclonal tubulin antibody (Fig. 4, *e* and *f*) to microtubules. Comparison of labeled microtubules using a mixture of YL 1/2 and polyclonal tubulin antibody as first antibodies in indirect immunofluorescence studies revealed identical numbers and patterns of microtubule distribution (Fig. 4, compare *a* and *b*).

Effects of the Injection of YL 1/2 into Swiss 3T3 Fibroblasts on the Organization of Cytoplasmic Microtubules

Swiss 3T3 fibroblasts displayed microtubules stretching radially from the perinuclear centrosome to the plasma membrane (see uninjected cells in Fig. 5, *b* and *e*). Injection of rhodamine-conjugated YL 1/2 into cells at a concentration

FIGURE 4 Double immunofluorescence microscopy of microtubule cytoskeletons prepared from PtK_2 cells. Cytoskeletons were prepared as described in Materials and Methods. Microtubules were studied by double immunofluorescence microscopy using mixtures of antibodies recognizing different tubulin antigenic determinants. The first antibody mixture consisted of rabbit polyclonal tubulin antibody mixed with either YL 1/2 or YOL 1/34 antibody. The second antibody consisted of a mixture of fluorescein-conjugated goat anti-rat IgG and rhodamine-conjugated goat anti-rabbit IgG. Binding of the polyclonal antibody to microtubules was studied with optics selective for rhodamine, binding of the monoclonal YL 1/2 and YOL 1/34 antibodies to microtubules was studied with optics selective for fluorescein. (*a*, *b*, *c*, *c'*, *d*, and *d'*) Display of microtubules in cells studied by double immunofluorescence using either mixtures of polyclonal and YL 1/2 antibodies (*a*, *b*, *c*, and *c'*) or mixtures of polyclonal and YOL 1/34 antibodies (*d* and *d'*). (*a* and *b*) Detail of a cytoskeleton studied using polyclonal (*a*) and YL 1/2 (*b*) antibodies. (*c''*) Corresponding phase contrast to cytoskeletons in *c* and *c'*. (*d''*) Corresponding phase contrast to cytoskeletons in *d* and *d'*. Observe that microtubule patterns revealed by the polyclonal and the YL 1/2 antibodies are identical (compare *a* with *b* and *c* with *c'*), as well as microtubule patterns revealed by polyclonal and YOL 1/34 antibodies (compare *d* with *d'*). Note the labeling of microtubules in the intracellular bridge by polyclonal and YL 1/2 antibodies (compare *c* with *c'*). (*e*, *e'*, *f*, and *f'*) Effect of treatment of the microtubule cytoskeleton with pancreatic carboxypeptidase A on the ability of the polyclonal (*e* and *f*), YL 1/2 (*e'*), and YOL 1/34 (*f'*) antibodies to bind to microtubules. (*e''* and *f''*) Corresponding phase contrast to cytoskeletons in *e* and *e'* and *f* and *f'*, respectively. Observe that treatment of the microtubule cytoskeletons with carboxypeptidase A abolished the binding of YL 1/2 to microtubules (*e'*), but has no effect on the binding of the polyclonal (*e* and *f*) and YOL 1/34 (*f'*) antibodies to microtubules. Bars, 20 μm. (*a* and *b*) × 1,250. (*c–f''*) × 250.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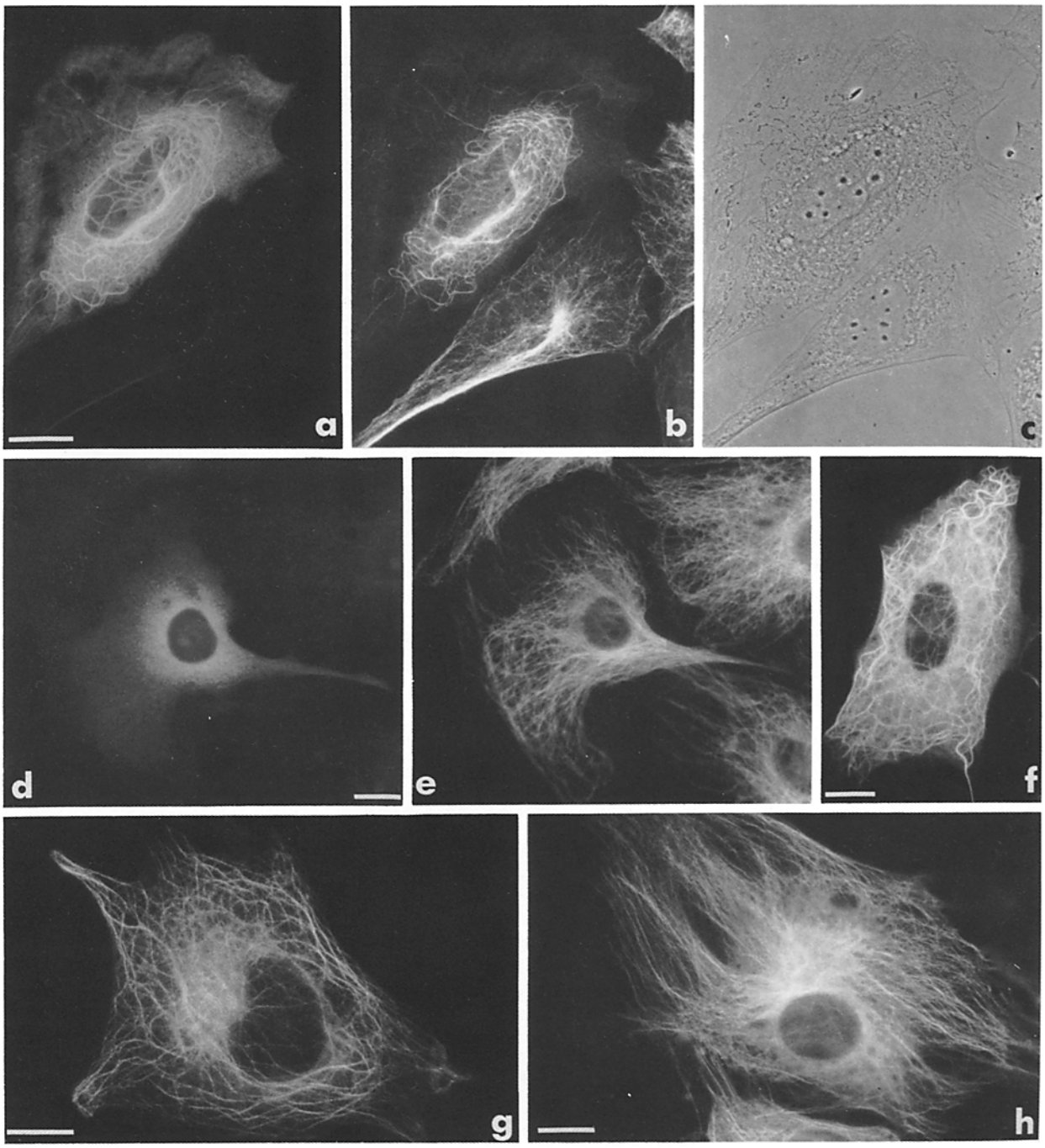

FIGURE 5 Effect of the intracellular injection of YL 1/2 and YOL 1/34 on the distribution of microtubules in the cytoplasm of Swiss 3T3 fibroblasts. (*a–e*) Double immunofluorescence of cells injected with unconjugated YL 1/2 (*a*) or unconjugated YOL 1/34 (*d*) with ~12 mg IgG/ml in the injection solution. (*c*) Corresponding phase contrast of cells in *a* and *b*. 3 h after the injection, the cells were fixed and processed for indirect immunofluorescence using polyclonal rabbit tubulin antibody followed by a mixture of fluorescein-conjugated goat anti-rat IgG and rhodamine-conjugated goat anti-rabbit IgG as second antibody. Cells in *a* and *d* were viewed with optics selective for fluorescein to study the distribution of YL 1/2 (*a*) and YOL 1/34 (*d*) injected into the cells. The same fields were viewed with optics selective for rhodamine (*b* and e) to study the distribution of microtubules in both uninjected and injected cells. Note the aggregation of microtubule bundles around the nucleus of the cell injected with YL 1/2 (*a* and *b*) but diffuse distribution of injected YOL 1/34 (*d*) without affecting the normal distribution of microtubules in the same cell (*e*). (*f*, *g*, and *h*) Direct immunofluorescence of cells injected with rhodamine-conjugated YL 1/2. Cells were injected with ~12 mg IgG/ml (*f*), 6 mg IgG/ml (*g*), 2 mg IgG/ml (*h*) of rhodamine-conjugated YL 1/2 in the injection solution and 3 h later fixed with 3% formaldehyde for 10 min. Note the normal microtubule distribution in the cell injected with the lowest concentration of YL 1/2 (*h*) and the formation of microtubule bundles in the cells injected with the two higher concentrations of antibody (*f* and *g*). Observe the higher capacity of unconjugated YL 1/2 compared to rhodamine-conjugated YL 1/2 to induce the aggregation of microtubule bundles (compare *a* with *f*). Bars, 20 μm; (*a*, *b*, and *g*) × 500. (*d*, e, and *f*) × 400.

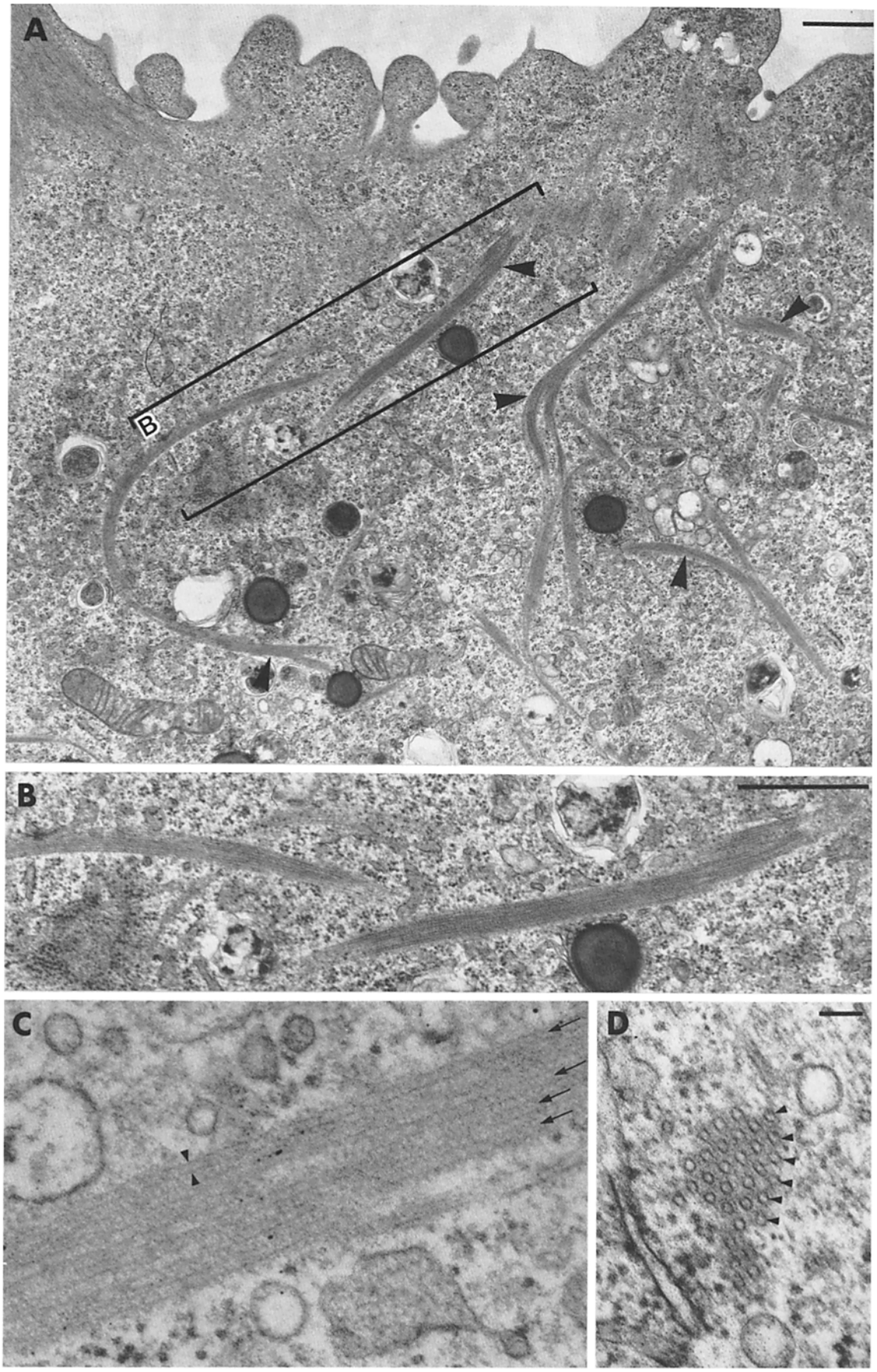

FIGURE 6 Electron microscopy of Swiss 3T3 fibroblasts injected with YL 1/2. Cells were injected with unconjugated YL 1/2 with ~6 mg IgG/ml in the injection solution and after 3 hr processed for electron microscopy. (*A*) Low magnification of the periphery of an injected cell with numerous microtubule bundles (arrowheads in *A*). (*B*) Higher magnification of *inset* in *A*. Longitudinal (*C*) and cross sections (*D*) of microtubule bundles at high magnification to show regular alignment of microtubules (arrows in *C* and arrowheads in *D*) in antibody-induced bundles. Bars, (*A* and *B*) 1 μm; (*C* and *D*) 0.1 μm. (*A*) × 12,000. (*B*) × 22,500. (*C* and *D*) × 75,000.

of 2 mg IgG/ml in the injection solution resulted in binding of the antibody to microtubules without changing the distribution of microtubules in the cytoplasm (Fig. 5*h*). Injection of larger concentrations of rhodamine-conjugated YL 1/2 resulted in formation of microtubule bundles (6–12 mg IgG/ml in the injection solution; Fig. 5, *g* and *f*). Although unconjugated YL 1/2, when injected at low concentration (2 mg/ml), did not affect the normal distribution of microtubules in the cytoplasm by binding to microtubules (data not shown), unconjugated YL 1/2 at higher concentrations was more effective than rhodamine-conjugated YL 1/2 in promoting the aggregation of microtubule bundles around the nucleus (in Fig. 5, compare *a* with *f* and *g*). On the other hand injected Fab-fragments of YL 1/2 remained diffusely distributed in the cytoplasm (data not shown), even when injected at high concentrations (10 mg/ml in the injection solution), i.e., microtubules were not decorated and their normal distribution remained unaffected when examined by double immunofluorescence. Even though Fab-fragments bound to microtubules when examined by indirect immunofluorescence (not shown), nonreactivity of microinjected Fab-fragments with microtubules might be due to a decreased affinity often found for Fab-fragments prepared from monoclonal antibodies.

After injection of YL 1/2 at intermediate concentrations (compare with Fig. 5*g*), numerous microtubule bundles were revealed in injected cells by electron microscopy (Fig. 6). At higher magnification the spacing between single microtubules in such bundles was not <20 nm (Fig. 6*D*).

DISCUSSION

We have identified and partially characterized the antigenic site of α-tubulin reacting with the rat monoclonal YL 1/2 antibody raised against yeast tubulin (9). Studies on the ability of YL 1/2 to bind to tyrosylated and detyrosylated α-tubulin indicated that the antibody reacts specifically with tyrosylated α-tubulin. The primary structure of the antigenic site is not known in complete detail but probably related to the peptide Gly-$(Glu)_3$-Gly-$(Glu)_2$-Tyr. This peptide constitutes the sequence of the eight last amino acids of the carboxyterminus of tyrosylated α-tubulin (18, 28) and reacts specifically with YL 1/2 as shown by preabsorption experiments.

With respect to this result it is important to note frequent antigenicity of the carboxyterminus of proteins and hydrophilic peptides (11). Both indirect immunofluorescence and electron microscopy studies of cytoskeletons and microtubules polymerized in vitro in the presence of YL 1/2 antibody, respectively, show that YL 1/2 binds to the surface of microtubules. Thus the carboxyterminus of α-tubulin is exposed on the microtubule surface. This location is in agreement with the removal of carboxyterminal tyrosine from α-tubulin in cytoskeletal microtubules by pancreatic carboxypeptidase A and further with the lack of effect of YL 1/2 on tubulin polymerization, a process requiring the interaction between tubulin domains presumably masked within the microtubule after polymerization.

We have also observed that YL 1/2 does not interfere with the binding of MAP_2 or tau to microtubules in vitro. This result indicates that the tyrosylated carboxyterminus of α-tubulin is not part of the microtubule binding sites for MAP_2 or tau and suggests that binding of MAP_2 and tau to microtubules is not regulated by tyrosylation of α-tubulin.

Although YL 1/2 has no effect on microtubule polymerization and stability, and the binding of MAP_2 and tau to microtubules, it produces strong concentration-dependent changes in the distribution and function of microtubules when injected into cultured cells (see accompanying paper, reference 32). It is not clear whether or not the formation of microtubule bundles in cells injected with YL 1/2 is produced by the cross-linking of microtubules by the antibody. It is important to note in this respect that the lateral distance between the microtubules packed in the cell into bundles was not smaller than 20 nm. This distance is much larger than the largest distance possible between the two antigen binding sites of an IgG molecule (10–12 nm). Therefore it is possible that the binding of YL 1/2 to the microtubule surface can instead interfere with the interaction of microtubules with unknown cell components required for the normal distribution of microtubules inside the cell. The collapse of microtubule bundles around the nucleus in cells injected with high concentrations of YL 1/2 could either result from formation of large bundles of microtubules or as indicated above from the antibody interfering in the interaction of other unknown cell components with the microtubules (see accompanying paper, reference 32).

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