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Isolation of Circumferential Microtubules From Platelets Without Simultaneous Fixation

By James G. White, E. Radha, and Marlys Krumwiede

Circumferential bands of microtubules (MT) support the discoid shape of resting platelets and participate with the contractile apparatus in shape change and internal contraction following activation. Elucidation of interactions between the circumferential coils and proteins of the stable and contractile cytoskeleton is essential for understanding MT function in platelet physiology. A previous investigation demonstrated that the circumferential rings can be isolated intact from resting platelets following simultaneous exposure to glutaraldehyde and Triton X-100. However, the use of fixation prevented the characterization of protein interactions. The present study has circumvented this problem by developing a procedure for isolating intact microtubule coils from detergent-treated platelets without the use of fixative agents. Incubation of the platelets for intervals of 30 to 60 minutes with the microtubule-stabilizing agent taxol preserved the circumferential bundle after extraction with Triton X-100 even after washing five times. The procedure has made it possible to carry out protein studies on isolated microtubule rings and associated proteins.

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THE CIRCUMFERENTIAL microtubule (MT) is an important constituent of the platelet cytoskeleton. It supports the discoid shape of resting platelets[1-3] and participates in the process of internal contraction when the cells are stimulated.[4,5] The precise mechanisms through which circumferential MT exert these functions, however, are unclear.[6]

To evaluate the role of the circumferential MT in platelet physiology, we have recently developed a procedure for isolating intact MT coils from platelets in suspension.[7] The method has been useful for studying the fate of MT in activated platelets,[8] but biochemical and physiologic experiments have been limited because glutaraldehyde was needed in the preparative procedure. In recent investigations we have found a way to avoid the presence of a fixative during the separation of MT coils from suspended platelets. The addition of a relatively large concentration of taxol,[9] an MT-stabilizing agent, obviated the need for glutaraldehyde and permitted the recovery of large numbers of MT coils in a nearly native state.

MATERIALS AND METHODS

General. After informed consent, blood was obtained from healthy adult donors whose platelets had been evaluated in many previous studies from our laboratory.[4,10,11] Samples obtained by venipuncture were mixed immediately with citrate-citric acid, pH 6.5 (93 mmol/L sodium citrate, 70 mmol/L citric acid, and 140 mmol/L dextrose), in a ratio of nine parts blood to one part anticoagulant. Platelet-rich plasma (PRP) was separated from whole blood by centrifugation at 100 g for 20 minutes at 23 °C and maintained at room temperature until used in specific experiments.

Concentration of platelets. Adenosine (5 mmol/L) and theophylline (3 mmol/L) were added to the citrate anticoagulant described previously to inhibit platelet activation.[12] Five milliliters of PRP were combined with an equal volume of the citrate anticoagulant and centrifuged at 1,500 g for 30 minutes to obtain pellets. The supernatant was decanted and the platelets gently resuspended in 1 to 2 mL of PRP. Concentrated samples were maintained in suspension at room temperature. The procedure resulted in a three- to four-fold concentration of platelets, and a substantial proportion of the cells were discoid after resuspension in PRP.

Samples of concentrated PRP, 0.5 mL in volume, were combined with taxol,[9,13,14] an MT-stabilizing agent, at a final concentration of 2×10^{-4} mol/L. Taxol was obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Md. The agent was dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L. The platelets were incubated with taxol for one hour before lysis.

Platelet lysis. A buffer designed by Small[15] to preserve cytoskeletal elements (NaCl, 127 mmol/L; KCl, 5 mmol/L; Na_2HPO_4, 1.1 mmol/L; KH_2PO_4, 0.4 mmol/L; $NaHCO_3$, 4 mmol/L; glucose, 5.5 mmol/L; $MgCl_2$, 2 mmol/L; ethylene glycol tetra-acetic acid, 2 mmol/L; piperazine-N,N^1-Gis (2-ethanesulfonic acid) (PIPES), 5 mmol/L; pH 6.0 to 6.1) was combined with Triton X-100 (Sigma, St Louis) at a final concentration of 10%. A 100-μL volume of this solution was combined with 0.5 mL of taxol-treated, concentrated platelets. The detergent caused almost immediate clearing of the cloudy cell suspension.

Preparation of whole mounts. Single drops of the extracted platelets were placed on formvar-coated, carbon-stabilized grids for 2½ minutes, rinsed with cytoskeleton buffer, and examined unfixed or after postfixation for two or more hours in 2.5% glutaraldehyde in the same buffer.[16,17] Grids were then rinsed twice with distilled water and drained briefly on the edge with filter paper. Finally, the grids were passed through four drops of 3% sodium silicotungstate, drained of excess stain, and allowed to air dry.[15]

Preparation for scanning electron microscopy. Large drops of the supernatant from extracted platelets were mounted on circular glass disks. In addition, the lysate and samples of washed, resuspended MT were deposited on millipore filters. After contact with surfaces for 2½ minutes, they were rinsed with cytoskeletal buffer and postfixed in 2.5% glutaraldehyde as previously described for whole mount preparations. The coils were then dehydrated in a graded series of alcohols, substituted with freon, and dried by the critical point method of Anderson in a Bomar apparatus (Bomar Instruments, Tacoma, Wash).[18] After coating with a 50 Å layer of carbon and gold, the coils were examined in a Cambridge S4D-10 scanning electron microscope (Cambridge Instruments, Cambridge, England).

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Preparation for polyacrylamide gel electrophoresis (PAGE). Six to eight detergent-extracted, 0.5-mL platelet samples were combined and centrifuged to obtain a pellet. Clear supernatant was discarded and the pellet resuspended in Hanks' balanced salt solution (HBSS) containing taxol at 2×10^{-4} mol/L. The washing procedure was repeated five times and the final pellet resuspended in 0.5 mL of HBSS with taxol. The protein content of the platelet concentrate and isolated MT coils was measured by the method of Lowry et al.[19] Sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli.[20] A gradient slab of 8% to 15% was used for analyzing the protein profile.[21] The samples were dissolved in an SDS sample buffer and boiled for two minutes. Protein standards were electrophoresed for molecular weight estimations. Gels were stained with brilliant Coomassie blue R and scanned with a Kratos spectrodensitometer (Kratos Analytical Instruments, Westwood, NJ) connected to a Hewlett-Packard integrator (Hewlett-Packard, Palo Alto, Calif).

Immunoblot preparation. PAGE gels of the isolated MT coils prepared as previously described were transferred to nitrocellulose electrophoretically according to the method of Towlin et al.[22] After blotting, the nitrocellulose was incubated with gelatin to block nonspecific protein-binding sites. The blot was then incubated with a monoclonal antibody to β-tubulin of mouse origin (Cooper Biomedical, Malvern, Pa). The immune complex was detected with peroxidase-labeled antibody to mouse IgG after incubation in 4-chloro-l-napthol.

RESULTS

Morphology. MT coils recovered by detergent lysis from platelets incubated for an hour with a high concentration of taxol were essentially identical in appearance to the coils isolated from platelets after simultaneous exposure to detergent and glutaraldehyde fixative. Examination of glass disks (Figs 1A and 1B) and millipore filters (Figs 2A and 2B) in the scanning electron microscope revealed fields that were virtually free of any other structures but MT coils. Whole-mount preparations viewed in the transmission microscope were also nearly devoid of cellular debris. MT coils consisted of several concentric rings, ranging from three to 12 with a median of eight. Two free ends were observed extending from some coils, but multiple free ends were also common (Figs 3A and 3B). MT coils were more loosely arranged in the absence of glutaraldehyde fixation during isolation or mounting, but the basic circular configuration of the loops was well maintained in most examples.

Biochemistry. Depending on the amount of protein applied to the gel, SDS-PAGE of proteins solubilized from multiply washed MT rings revealed a dense single or double band migrating with the same mobility as the tubulin standard (Fig 4). In addition to tubulin, several other proteins were evident on the gels. A band at approximately 210 K may represent an MT-associated protein (MAP). Two or three additional protein bands with apparent molecular

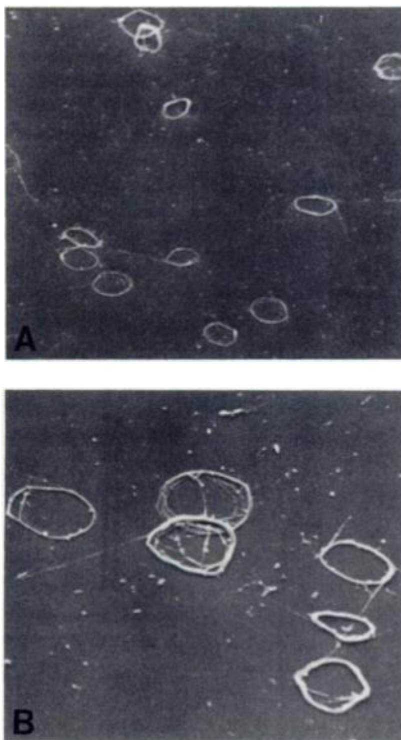

Fig 1. Low- and high-magnification scanning electron micrographs of isolated marginal band MT. The MT coils have retained their concentric organization throughout the isolation procedure and multiple washings and appear randomly dispersed on the surface of the disks. Membranes and cytoplasmic constituents are virtually absent from the coiled MT. Original magnifications (A) ×2,600; (B) ×6,500; current magnifications (A) ×1690; (B) ×4225.

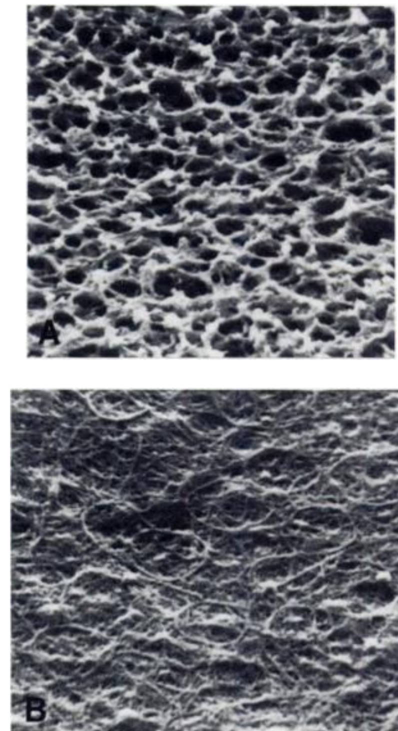

Fig 2. Scanning electron photomicrographs of nucleopore filters. (A) The filter has not been exposed to MT. (B) The surface has been exposed to the multiply washed and resuspended MT obtained from eight samples of detergent treated platelet concentrate, each 0.5 mL in volume. The surface is buried in coils. Original magnifications ×6,000; current magnifications ×3900.

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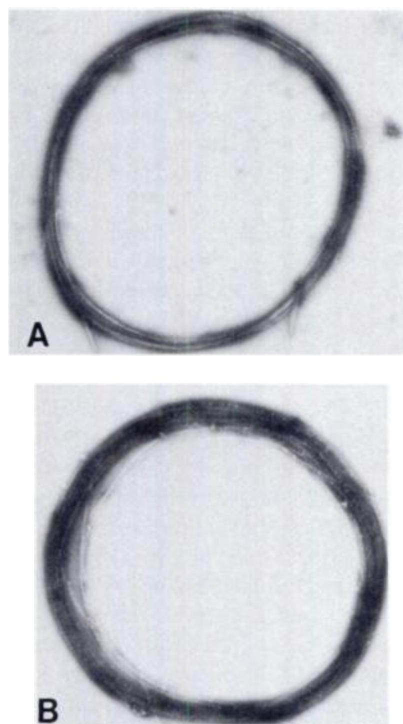

Fig 3. Negative-stain whole mounts of MT coils prepared without glutaraldehyde fixation from detergent-treated platelets after repeated washing and resuspension. The tightly coiled appearance is well preserved in the two examples. More than two loose ends are visible on each coil, but the increase may be due to fracture during preparation. Original magnifications ×18,000; current magnifications ×11,700.

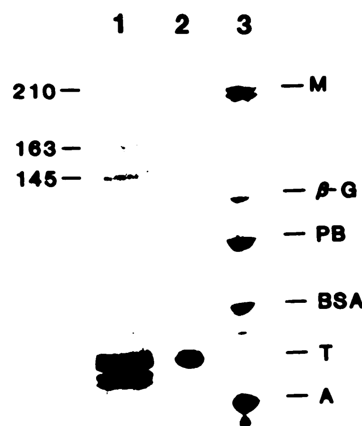

Fig 4 SDS-PAGE (5% to 15% gradient) separation of proteins solubilized from isolated MT coil preparation after five washings and resuspensions in HBSS (channel 1). The gels were stained with brilliant Coomassie blue R. Channel 2 contains partially purified brain tubulin (T), and channel 3 contains several standard proteins for molecular weight estimation, including myosin (M), β galactosidase (3G), phosphorylase B (PB), bovine serum albumin (BSA) and actin (A). α- and β-tubulin are not well separated in the standard or experimental samples because of the high protein concentration. A protein band at approximately 210,000 mol wt on the experimental gel may represent an MT associated protein like MAP.[2] The nature of the other HMW proteins is not known. The actin band is usually more prominent in the experimental gels. Several protein bands between actin and tubulin remain with isolated coils throughout isolation and washing procedures. They may represent LMW MAPs.

weights above 100,000 may represent breakdown products of high–molecular weight (HMW) proteins since proteolytic inhibitors were not included during lysis or washing. A low–molecular weight (LMW) band with the mobility of the actin standard was constantly present. Between tubulin and actin were two or three bands that may represent LMW MAPs. Immunoblots prepared from the isolated MT coils, incubated with a monoclonal antibody to tubulin, and stained with a peroxidase labeled second antibody revealed a specific reaction with platelet tubulin (Fig 5).

Densitometry of PAGE gels revealed that tubulin constituted 43% to 48% of the protein on the gels. Measurement of the protein content of the concentrated platelet suspension and multiply washed MT coils, adjusted on the basis of the densitometric observations, revealed that tubulin constituted about 3.3% to 3.8% of the total platelet protein.

DISCUSSION

The present report has described a relatively simple way to stabilize the circumferential MT supporting the discoid shape of resting platelets during isolation and purification. Taxol, an MT-stabilizing agent,[9] was added to platelet suspensions at twice the concentration used in previous studies.[13,14] Platelets were incubated for an hour and then lysed in a cytoskeletal buffer containing Triton X-100. The presence of taxol prevented disassembly and disorganization of MT coils during rupture of the cell membranes by detergent. Examination of glass disks prepared for scanning electron microscopy after contact with drops of the lysed platelet suspension for 2½ minutes revealed MT coils evenly distributed on the surface. Millipore filters on which MT coils isolated from 4 mL of cleared suspension had been deposited were completely covered by coils. Thus, the procedure appears to provide a simple way to obtain susbstantial numbers of MT coils for evaluation without requiring the use of glutaraldehyde or other fixatives.[7]

The morphology of unfixed MT coils was generally well preserved by the pretreatment with taxol. The loops making up the MT coils had separated in some examples, but in most the resemblance to a lariat was well preserved. The number of free ends of MT extending from the coils was variable. Fracture of the delicate structures during attachment to grids and glass coverslips or in the process of air or critical-point drying may have caused some disruption.[7] However, survival of the basic concentric configuration suggests that cohesive forces may be involved in holding loops of the coiled MT together. The nature of these forces is not known but may involve one or several types of MAPs.[23-25]

SDS-PAGE proteins solubilized from the multiply washed MT coils revealed a prominent tubulin band identical in

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Fig 5. An immunoblot (channel 1) transferred from a PAGE gel of isolated MT coils prepared as described in the text and shown in Fig 4. The blotted proteins were stained with a monoclonal antibody to tubulin followed by a peroxidase-coupled second antibody to the immune complex (IC). Only the protein band (channel 2) identified as platelet tubulin (PT) by its comigration with standard MT protein in the previous Figure is stained by the peroxidase-coupled antibody.

mobility to the brain-derived standard. Immunoblotting and staining with a monoclonal antibody to tubulin confirmed that the band was authentic MT protein. Despite the multiple washings and resuspensions in buffer, several other protein remained associated with MT coils. Both HMW and LMW proteins, including one with a mobility identical to the actin standard, were present in the gels. One of the HMW proteins had a mobility similar to an MAP described previously in platelets and other cell types.[23-25] Two or three protein bands lying between actin and tubulin may also represent MAPs. Studies to characterize these proteins are continuing.

Analysis of the gels by densitometry revealed that tubulin constituted approximately 40% to 50% of the total. Based on protein determinations on the concentrated platelet suspension and the multiply washed coils, the amount of tubulin recovered constituted about 3.3% to 3.8% of the total platelet protein. The recovery obtained in this study agrees well with the figure of 3.1% reported by Steiner and Ikeda.[26]

Taxol has been used previously to facilitate isolation of MT and MAPs from other cell types.[27] However, the procedure involving taxol was used primarily to separate MAPs from MT reassembled after earlier isolation of tubulin from calf brain. The present investigation appears to be the first study to use taxol for maintenance of MT organization observed in the intact cell during isolation and multiple washing. As a result, the proteins remaining in an insoluble state with isolated MT during washing may be very important to MT function in intact platelets. Studies to identify and characterize these proteins by immunoelectron microscopy are in progress.

NOTE ADDED IN PROOF

Following acceptance of this manuscript, a report using a similar approach to isolating the platelet circumferential microtubule has appeared (Kenney DM, Linck RW: The cytoskeleton of unstimulated platelets: Structure and composition of the isolated marginal microtubular band. J Cell Sci 78:1, 1985).

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