

# A novel method for purification of polymerizable tubulin with a high content of the acetylated isotype

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Tubulin can be acetylated/deacetylated on Lys<sup>40</sup> of the  $\alpha$ -subunit. Studies of the post-translational acetylation/deacetylation of tubulin using biochemical techniques require tubulin preparations that are enriched in AcTubulin (acetylated tubulin) and (for comparison) preparations lacking AcTubulin. Assembly–disassembly cycling of microtubules gives tubulin preparations that contain little or no AcTubulin. In the present study we demonstrated that this result is owing to the presence of high deacetylating activity in the extracts. This deacetylating activity in rat brain homogenates was inhibited by TSA (Trichostatin A) and tubacin, but not by nicotinamide, indicating that HDAC6 (histone deacetylase 6) is involved. TSA showed no effect on microtubule polymerization or depolymerization. We utilized these properties of TSA to prevent deacetylation during the assembly–disassembly procedure. The effective inhibitory concentration of TSA was

3  $\mu$ M in the homogenate and 1  $\mu$ M in the subsequent cycling steps. By comparison with immunopurified AcTubulin, we estimated that ~64 % of the tubulin molecules in the three cycled preparations were acetylated. The protein profiles of these tubulin preparations, as assessed by SDS/PAGE and Coomassie Blue staining, were identical to that of a preparation completely lacking AcTubulin obtained by assembly–disassembly cycles in the absence of TSA. The tyrosination state and *in vitro* assembly–disassembly kinetics were the same regardless of the degree of acetylation.

**Key words:** acetylated tubulin purification, histone deacetylase 6 (HDAC6), microtubules, tubulin acetylation/deacetylation, Trichostatin A (TSA).

## INTRODUCTION

Microtubules, together with intermediate and actin filaments, comprise the cytoskeletons of all eukaryotic cells. They are cylindrical hollow dynamic structures, ~25-nm diameter and comprise  $\alpha/\beta$ -tubulin dimers [1]. The microtubules of living cells continuously explore the environment via alternating phases of growth and shrinking, a process called dynamic instability [2]. They perform a variety of functions including the transportation of organelles, formation of the mitotic spindle during mitosis and meiosis, determination of cell shape, regulation of cell migration, and formation of the axonemes of cilia and flagella. This wide range of functions is made possible by the variety of  $\alpha/\beta$ -tubulin dimers that can form microtubules. This variety arises from different  $\alpha$ - and  $\beta$ -tubulin isotypes and from different post-translational modifications of tubulin, particularly detyrosination,  $\Delta 2$  modification, polyglycylation, polyglutamylation and acetylation [3]. Acetylation consists of the addition of an acetyl group to the  $\epsilon$ -amino group of Lys<sup>40</sup> of the  $\alpha$ -subunit of tubulin by the acetyltransferase  $\alpha$ -TAT ( $\alpha$ -tubulin *N*-acetyltransferase) 1 or its paralogues Mec-17 (mechanosensory abnormality protein 17) and  $\alpha$ -TAT2 [4,5]. This acetyl group can be removed by the action of HDAC6 (histone deacetylase 6) [6,7] or Sirt2 (sirtuin-2) [8]. The role of this post-translational modification remains unclear in spite of numerous studies. Some of the functions proposed for AcTubulin (acetylated tubulin) are association with Na<sup>+</sup>/K<sup>+</sup>-ATPase [9,10], neuronal polarization,

neurite branching, proper neuronal functioning [11–13] and ciliogenesis [4]. Most of the findings on which these proposals are based are from studies using molecular and cell biological techniques; very few biochemical studies have been performed. AcTubulin is essentially absent in preparations purified by traditional polymerization–depolymerization cycling [14]. The strategies employed to obtain AcTubulin for biochemical studies have included its extraction from cell cultures treated with TSA (Trichostatin A; an inhibitor of HDAC6) [15], chemical synthesis of a peptide with the sequence surrounding Lys<sup>40</sup> of the  $\alpha$ -subunit [7], incubation of tubulin with recombinant  $\alpha$ -TAT [16], chemical acetylation of tubulin with acetic anhydride [17] and the use of a tubulin preparation containing a low percentage of AcTubulin [6]. Each of these approaches has limitations and/or disadvantages, e.g. extraction from TSA-treated cells yields a very small amount of non-pure tubulin, the use of a synthetic peptide consisting of a few amino acids rather than native tubulin severely constrains the conclusions that can be drawn from experiments, the incubation of tubulin with  $\alpha$ -TAT is expensive and time-consuming, chemical acetylation produces various alterations of tubulin structure, and the use of tubulin preparations with a low content of the acetylated isotype limits the types of experiments that can be performed. In the present paper we describe a simple method, analogous to the traditional cycling method, for large-scale purification of tubulin with a high content of the acetylated isotype. The method is based on the presence of TSA in the brain homogenization buffer and the subsequent purification steps, and

Abbreviations used: AcTubulin, acetylated tubulin; Glu-tubulin, non-tyrosinated tubulin; HDAC6, histone deacetylase 6; mAb, monoclonal antibody; RT, room temperature; Sirt2, sirtuin-2;  $\alpha$ -TAT,  $\alpha$ -tubulin *N*-acetyltransferase; TSA, Trichostatin A; Tyr-tubulin, tyrosinated tubulin.

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will facilitate biochemical studies of AcTubulin and its associated proteins.

## EXPERIMENTAL

### Chemicals

TSA, tubacin, Triton X-100, mouse mAb (monoclonal antibody) 6-11B-1 specific against AcTubulin, mouse mAb Tub-1A2 specific against Tyr-tubulin (tyrosinated tubulin) and mouse mAb DM1A specific against  $\alpha$ -tubulin (or specific to total tubulin because it does not discriminate isotypes) were from Sigma-Aldrich. IRDye® 800CW goat anti-(mouse IgG) and IRDye® 800CW goat anti-(rabbit IgG) were from Li-Cor Biosciences. Recombinant Protein G–Sepharose 4B conjugate was from Invitrogen. Nicotinamide was from ICN Biomedicals. Rabbit antibodies specific against Glu-tubulin (non-tyrosinated tubulin) and  $\Delta 2$ -tubulin were produced in-house.

### Purification of microtubule-protein-depleted AcTubulin

Brains excised from Wistar rats were homogenized in 1.5 volumes of cold (0°C) MEM buffer [100 mM Mes buffer (pH 6.7), containing 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM PMSF and 1 mM phenanthroline] and centrifuged (100 000 *g*) at 4°C for 30 min. The supernatant fraction (termed SN<sub>1</sub>) was added with glycerol (final concentration 40%) and incubated at 37°C for 30 min. The mixture was centrifuged (100 000 *g*) for 30 min at 27°C, and the supernatant (SN<sub>2</sub>) and sedimented microtubules (P<sub>2</sub>) were separated. Microtubules were resuspended in cold MEM buffer (1/5 of the original volume), kept at 0°C for 30 min and then centrifuged (100 000 *g*) for 30 min at 4°C. The supernatant (SN<sub>3</sub>; microtubule protein purified by one cycle) was collected and added with GTP (final concentration 0.3 mM) and glycerol (final concentration 40%). The mixture was kept at 37°C for 30 min and centrifuged. The pelleted microtubules (P<sub>4</sub>) were resuspended in cold MEM buffer containing 1.5 mM ATP, kept at 0°C for 30 min and then centrifuged (100 000 *g*) for 30 min at 4°C. The supernatant fraction (SN<sub>5</sub>; microtubule protein purified by two cycles) was treated in the same way as SN<sub>3</sub> to obtain SN<sub>7</sub> (microtubule protein purified by three cycles).

Animal handling was performed according to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and approved by the local animal care committee (Faculty of Chemistry, Universidad Nacional de Córdoba, Córdoba, Argentina).

### Purification of microtubule protein enriched in AcTubulin

Rat brains were homogenized and processed to purify microtubule protein by three cycles of assembly–disassembly as described above except that the homogenization buffer contained 5  $\mu$ M TSA and the buffer used in the following assembly–disassembly steps contained 1  $\mu$ M TSA.

### SDS/PAGE and Western blotting

Proteins were separated by SDS/PAGE (10% gels) [18] and transferred on to nitrocellulose sheets [19]. After blocking with 5% non-fat dried skimmed milk powder in PBS, the sheets were separately incubated with different primary antibodies for 4 h at RT (room temperature) as follows. Anti-(total tubulin), anti-AcTubulin and anti-Tyr-tubulin (1:5000 dilution) and anti-Glu-tubulin and anti- $\Delta 2$ -tubulin (1:500 dilution) in 1% non-fat dried skimmed milk powder in PBS. After three washing steps the sheets

were incubated with infrared fluorescent secondary antibodies for 1 h at RT (1:25000 dilution), washed and then scanned by an Odyssey infrared scanner (Li-Cor). Bands were quantified using the Scion Image software program (Scion Corporation).

### Monitoring of microtubule assembly

Tubulin polymerization/depolymerization were followed by turbidimetry at 350 nm. Microtubule protein (2.5 mg of protein/ml) was incubated at 37°C in the presence of 0.5 mM GTP. Polymerization was monitored using a UV–visible recording spectrophotometer with a temperature-controlled cell holder (model UV-2401PC; Shimadzu). Depolymerization was achieved by setting the cell holder temperature to 10°C. Absorbance was measured every 30 s.

### Estimation of the amount of AcTubulin

Several aliquots containing different amounts of the protein to be analysed and of immunopurified AcTubulin (100% AcTubulin) were immunoblotted and stained with an anti-(total tubulin) antibody. Samples of the two preparations containing equal amounts of tubulin (i.e. similar optical densities of tubulin bands) were selected for immunoblotting and stained with an anti-AcTubulin antibody. The percentage of AcTubulin relative to total tubulin was calculated as (absorbance of AcTubulin band corresponding to analysed sample)/(absorbance of AcTubulin band corresponding to immunopurified AcTubulin)  $\times$  100.

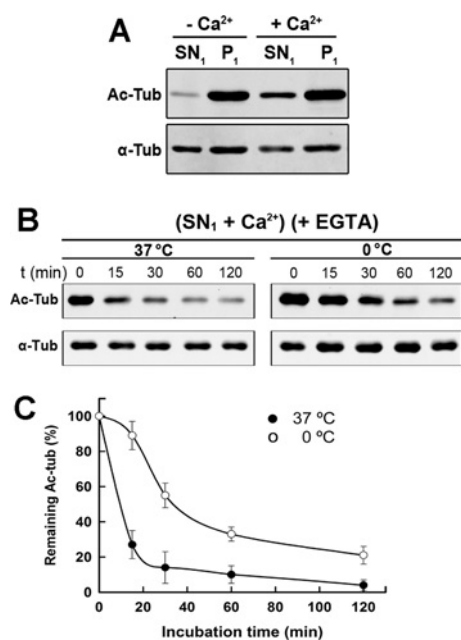
### Immunopurification of AcTubulin

Anti-AcTubulin was linked to Protein G–Sepharose beads (100  $\mu$ g IgG, 200  $\mu$ l beads) following the manufacturer's instructions. The 6-11B-1-linked beads were incubated for 2 h at RT with a preparation enriched in AcTubulin (600  $\mu$ l of 1.5 mg/ml SN<sub>5</sub>) and washed five times with 10 bed volumes of PBS supplemented with 0.5% Triton X-100. Bound proteins were eluted twice with 400  $\mu$ l of 100 mM phosphate buffer (pH 12), containing 9  $\mu$ M TSA for 5 min at RT with mild agitation. The two eluted fractions were combined and stored at –20°C. As a control, 6-11B-1-linked beads were incubated with a preparation depleted of AcTubulin and processed as above.

## RESULTS AND DISCUSSION

### Tubulin-deacetylating activity in rat brain

Our initial attempts to purify AcTubulin were based on previous reports by other laboratories indicating that the presence of millimolar-order Ca<sup>2+</sup> during tissue homogenization helps disintegrate 'stable' microtubules, which were assumed to be highly acetylated. We observed dramatic differences in AcTubulin content of SN<sub>1</sub> obtained from homogenates prepared in the presence compared with the absence of millimolar-order Ca<sup>2+</sup> and therefore presumed that calcium somehow promoted the disintegration of highly acetylated microtubules. In effect, following homogenization of rat brains with a buffer lacking Ca<sup>2+</sup>, the supernatant fraction (SN<sub>1</sub>), obtained by the procedure described in Experimental section and the legend to Figure 1, contained a very low amount of AcTubulin, whereas the SN<sub>1</sub> fraction obtained following homogenization with a buffer containing 5 mM Ca<sup>2+</sup> contained a substantial amount of AcTubulin (Figure 1A). Two possible explanations of this finding are: (i) Ca<sup>2+</sup> allows extraction of AcTubulin from the sedimentable fraction [20]; and (ii) Ca<sup>2+</sup> inhibits a tubulin-deacetylating activity present in the SN<sub>1</sub> fraction. The latter



**Figure 1** Tubulin-deacetylating activity in rat brain extracts

Brains were homogenized in cold buffer with or without 5 mM  $\text{Ca}^{2+}$  and centrifuged at 100 000 *g* for 30 min at 4 °C, as described in the Experimental section. (A) Samples of the supernatant ( $\text{SN}_1$ ) and pellet ( $\text{P}_1$ ) fractions were subjected to Western blotting and immunostained with the anti-AcTubulin (Ac-Tub) mAb 6-11B-1 and the anti- $\alpha$ -tubulin ( $\alpha$ -Tub) mAb DM1A. The loaded samples contained equal amounts of total tubulin. (B) Samples of  $\text{SN}_1$  from the homogenate obtained with  $\text{Ca}^{2+}$  were added with EGTA (final concentration 10 mM) and incubated at 0 and 37 °C. At the indicated times, aliquots were subjected to Western blotting and stained with 6-11B-1 and DM1A. Result from a representative experiment is shown. (C) Optical densities of bands corresponding to AcTubulin in (B) were determined, and values were expressed as a percentage of the value at time zero. Results are means  $\pm$  S.D. from three independent experiments.

explanation was supported by the observation that incubation (at 0 or 37 °C) of the  $\text{SN}_1$  fraction, with 10 mM EGTA to chelate  $\text{Ca}^{2+}$ , led to a rapid decrease of AcTubulin (Figure 1B). Quantification of this process is shown in Figure 1(C). These findings indicated that the increased amount of AcTubulin in the soluble extracts obtained in the presence of millimolar  $\text{Ca}^{2+}$  is not due to disassembly of stable acetylated microtubules, but mainly to inhibition (by  $\text{Ca}^{2+}$ ) of an enzyme activity present in  $\text{SN}_1$  that removes the acetyl group from Lys<sup>40</sup> of  $\alpha$ -tubulin. Therefore it was clear that obtaining a tubulin preparation enriched in the acetylated isotype requires inhibition of the deacetylating activity during the homogenization (even when performed at 2–4 °C) and subsequent purification steps. However, because  $\text{Ca}^{2+}$  interferes with tubulin polymerization, it is necessary to chelate it during the polymerization steps to allow the deacetylating enzyme to act. Therefore we needed another substance that inhibited deacetylating activity, but did not interfere with tubulin polymerization or depolymerization.

#### Identification of HDAC6 as the tubulin deacetylase activity in rat brain extracts

Two deacetylases have been reported to remove the acetyl group from Lys<sup>40</sup> of the  $\alpha$ -subunit of tubulin: NAD<sup>+</sup>-dependent Sirt2 (class III histone deacetylase [21]) and HDAC6 (class IIb histone deacetylase [22]). Various inhibitors of these enzymes were tested for their ability to inhibit the deacetylase activity present in the  $\text{SN}_1$  fraction obtained with  $\text{Ca}^{2+}$  buffer and added with

EGTA (Figure 2). TSA (an inhibitor of all HDAC classes except class III [23–25]) and tubacin (a specific inhibitor of HDAC6, [26]) completely inhibited the deacetylating activity (Figure 2A), whereas nicotinamide (an inhibitor of Sirt2 [23]) had no effect. These results indicate that HDAC6 was responsible for most of the deacetylating activity present in the  $\text{SN}_1$  fraction. The TSA concentration added to the homogenate required to inhibit 50% of the deacetylating activity was  $\sim$ 700 nM (Figure 2B, left-hand plot). The  $\text{IC}_{50}$  value for TSA when  $\text{SN}_1$  was used as the source of enzyme and substrate was  $\sim$ 150 nM (Figure 2B, right-hand plot).

Tubulin purified from one assembly–disassembly cycle in the absence of  $\text{Ca}^{2+}$  was incubated in the absence or presence of 1  $\mu\text{M}$  TSA under microtubule-assembly conditions, and microtubule formation was monitored as the increase of solution turbidity. TSA did not interfere with microtubule formation or with the disassembly of microtubules induced by lowering the temperature to 10 °C (Figure 2C). Therefore for developing a purification procedure we considered TSA as an excellent candidate because it completely inhibited the deacetylase activity (Figure 2B), did not interfere with tubulin polymerization or depolymerization (Figure 2C), and was commercially available at a reasonable cost.

#### Protocol for isolation of a tubulin preparation enriched in the acetylated isotype

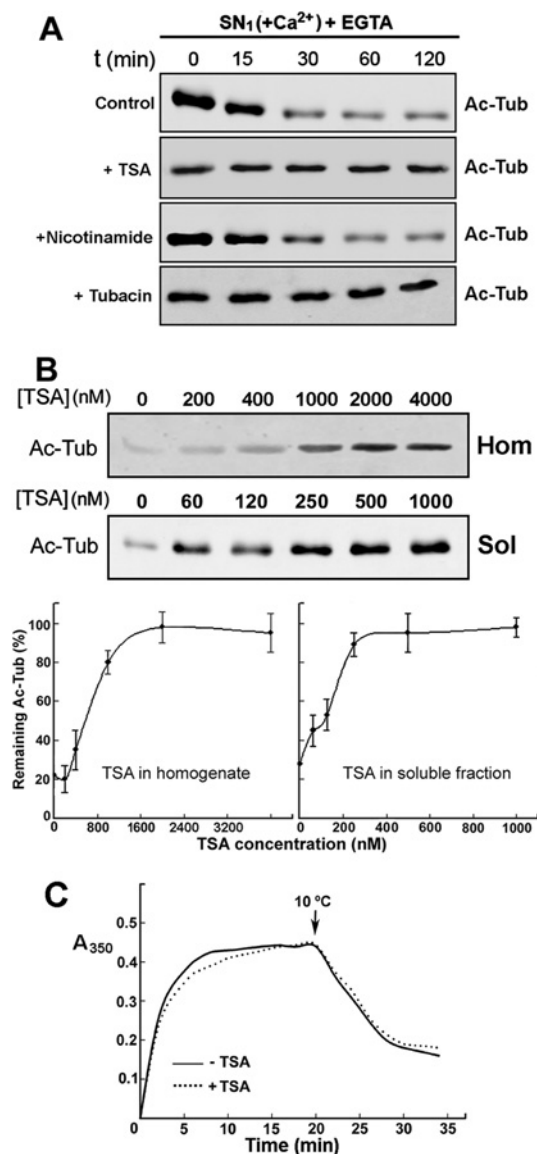
Rapid homogenization of brains in cold buffer containing TSA was necessary to prevent high HDAC6 activity. To completely prevent tubulin deacetylation, the homogenization buffer was added with 5  $\mu\text{M}$  TSA. Centrifugation of the homogenate yielded  $\text{SN}_1$  that theoretically contained 3  $\mu\text{M}$  TSA. The presence of TSA during the first and subsequent microtubule-assembly steps was necessary to prevent deacetylation and had no effect on the assembly or disassembly of microtubules (Figure 2C). The steps of this purification method are shown schematically in Figure 3(A). The bands from Western blots corresponding to AcTubulin and total tubulin in the main fractions from a three-cycle purification procedure are shown in Figure 3(B). In the  $\text{SN}_7$  fraction, which corresponds to a microtubule protein preparation purified by three assembly–disassembly cycles, the acetylation state of tubulin was approximately the same as that in  $\text{SN}_1$  and the homogenate as judged by direct visual observation, suggesting that the acetyl group on tubulin was adequately preserved during the purification process.

#### Quantification of AcTubulin

We used a tubulin preparation enriched in AcTubulin ( $\text{SN}_5$  obtained in the presence of TSA) to further immunopurify the acetylated isotype, as described in the Experimental section. AcTubulin, but not non-acetylated tubulin, was bound to and then eluted from 6-11B-1-linked beads (Figure 4A), indicating that AcTubulin was efficiently purified by this procedure. This preparation was therefore considered to be 100% AcTubulin and was used to estimate the amount of AcTubulin in the various microtubule protein preparations shown in Figure 3(B), as described in the Experimental section. The percentages of AcTubulin relative to total tubulin are shown in Figure 4(B).

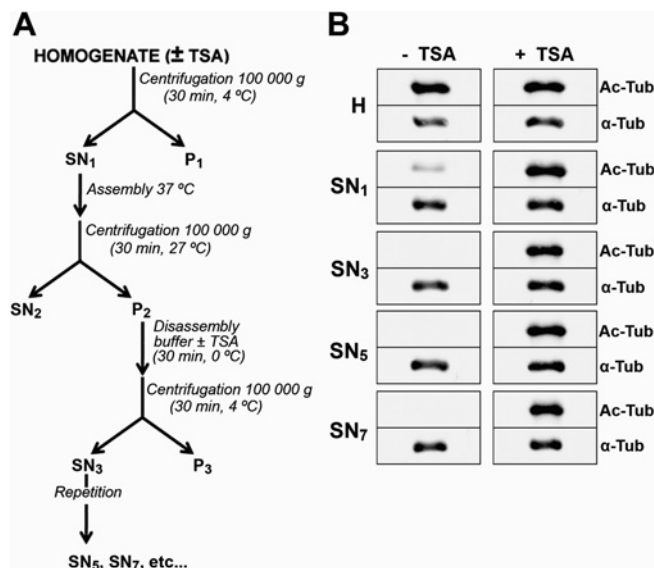
#### Comparison of microtubule protein preparations lacking or enriched in AcTubulin

Microtubule protein preparations purified by three cycles of assembly–disassembly ( $\text{SN}_7$  fraction), lacking or enriched in AcTubulin, were obtained by the procedure illustrated in Figure 3(A). Comparison of the two preparations following



**Figure 2** Identification of tubulin-deacetylating activity in rat brain

(A) Brains were homogenized with buffer containing 5 mM  $\text{Ca}^{2+}$  and centrifuged. The resulting  $\text{SN}_1$  fraction was added with EGTA (final concentration 10 mM) and incubated at 37°C in the absence (control) and presence of 5  $\mu\text{M}$  TSA, 1 mM nicotinamide or 5  $\mu\text{M}$  tubacin. Aliquots were taken at the indicated times and subjected to Western blotting and staining with anti-AcTubulin (Ac-Tub) mAb. (B) Brain homogenates were obtained in buffer without  $\text{Ca}^{2+}$  containing the indicated concentrations of TSA, incubated for 30 min at 2–4°C and centrifuged (100 000  $g$  for 30 min at 4°C) The resulting supernatant fractions were subjected to Western blotting and stained with anti-AcTubulin mAb. The blot from a typical experiment is shown at the top (Hom). In separate experiments, brains were homogenized in buffer with  $\text{Ca}^{2+}$  and  $\text{SN}_1$  was obtained.  $\text{SN}_1$  was added with EGTA (final concentration 10 mM) and incubated for 60 min at 37°C in the presence of the indicated concentrations of TSA. Aliquots were taken and subjected to Western blotting and staining with anti-AcTubulin mAb. The blot from a typical experiment is shown (Sol). The absorbance of the bands from the two experiments were determined and values are shown in the graphs at the bottom as the percentage of remaining AcTubulin relative to the value for a non-incubated control at time zero. Results are means  $\pm$  S.D. from three independent experiments. (C) An  $\text{SN}_1$  fraction obtained without  $\text{Ca}^{2+}$  was incubated under microtubule-assembly conditions as described in the Experimental section. Microtubule formation was monitored as turbidity ( $D_{350}$ ). Incubation was performed in the absence (control) or presence of 1  $\mu\text{M}$  TSA. After the plateau was reached (time = 20 min), the temperature was lowered to 10°C (arrow) and the measurement of absorbance continued. This experiment was repeated three times with similar results.



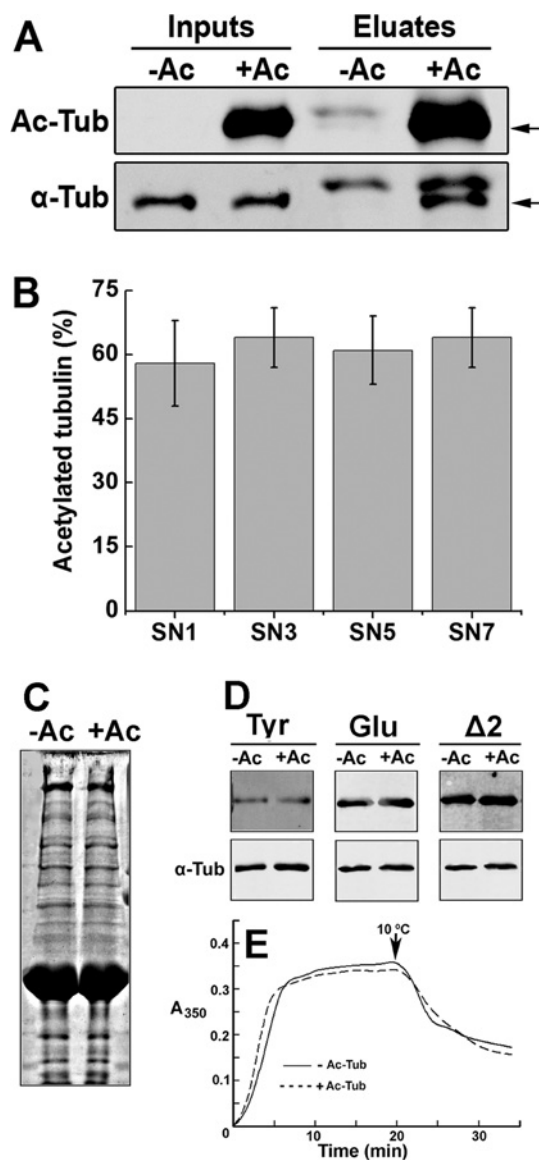
**Figure 3** Isolation of tubulin preparations lacking or enriched in the acetylated isotype

(A) Schematic purification procedure for the two types of tubulin preparations as described in the Experimental section. – TSA:  $\text{Ca}^{2+}$  and TSA were absent during the purification process. + TSA: TSA was present during the process. Steps are described until the obtaining of  $\text{SN}_3$ , which corresponds to a microtubule protein preparation purified by one cycle. The procedures for subsequent purification cycles are the same as for the first cycle. (B) Samples were taken from homogenate (H),  $\text{SN}_1$ ,  $\text{SN}_3$ ,  $\text{SN}_5$  and  $\text{SN}_7$  and subjected to Western blotting and staining with anti-AcTubulin (Ac-Tub) and anti- $\alpha$ -tubulin ( $\alpha$ -Tub) mAbs. To allow direct comparison, identical amounts of total tubulin ( $\alpha$ -Tub) were run.

SDS/PAGE and Coomassie Blue staining did not reveal any obvious differences (Figure 4C). Other aliquots were subjected to analysis of the tyrosination state of  $\alpha$ -tubulin, that is, the proportion of ‘Tyr-tubulin’ and ‘Glu-tubulin’ (so named because glutamic acid is now the C-terminal amino acid) and  $\Delta 2$ -tubulin (because it lacks the two amino acids from the C-terminus, tyrosine and glutamic acid). Results from the Western blot analysis show that the tyrosination state in the two preparations was identical (Figure 4D). The *in vitro* abilities of the two preparations to form microtubules (at 37°C) and to depolymerize (at 10°C), as measured by turbidimetry, were also the same (Figure 4E).

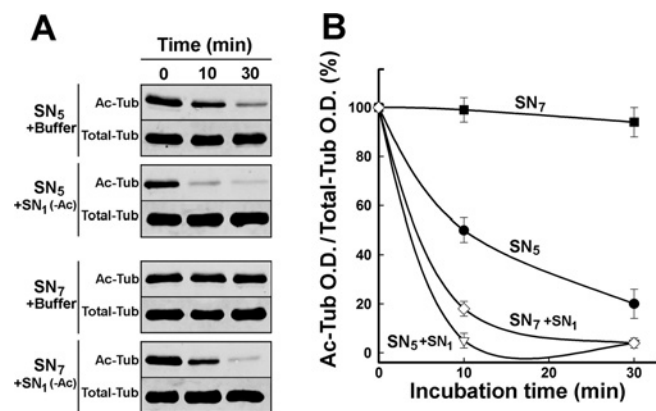
### Deacetylase activity is gradually lost during assembly–disassembly cycling

HDAC6 has been reported previously to be associated with microtubules [5,6,26]. We therefore determined whether HDAC6 activity remained constant in relation to tubulin level during microtubule assembly–disassembly cycling. HDAC6 activity was present in the soluble fraction ( $\text{SN}_1$ ) of brain homogenate (Figure 1), but AcTubulin was essentially absent if the homogenate was obtained in the absence of TSA (Figure 3). It is therefore impossible to determine the presence of HDAC6 activity in microtubule protein preparations purified by two cycles ( $\text{SN}_5$ ) or three cycles ( $\text{SN}_7$ ) because of the presence of TSA in the buffers used to obtain AcTubulin-enriched preparations. To overcome this problem, we resuspended the microtubule pellet of the preceding cycle in buffer without TSA when the deacetylase activity of  $\text{SN}_5$  and  $\text{SN}_7$  was to be measured. Aliquots of  $\text{SN}_5$  and  $\text{SN}_7$  obtained in this way were mixed with one volume of  $\text{SN}_1$  obtained in the absence of TSA (as a source of HDAC6 without significant effect



**Figure 4** Quantification of AcTubulin and comparison of properties of acetylated and non-acetylated tubulins

(A) To obtain 100% AcTubulin (Ac-Tub), a sample of SN<sub>5</sub> enriched in AcTubulin (+Ac) was subjected to immunoaffinity chromatography using mAb 6-11B-1 linked to Protein G–Sepharose beads. As a control, an SN<sub>5</sub> sample of non-acetylated tubulin (–Ac) was run in parallel. Inputs and fractions that were bound to and then eluted from the Sepharose beads were analysed by Western blotting and staining by anti-AcTubulin (arrow) and anti-(total tubulin) mAbs (arrow). (B) The immunopurified AcTubulin was used as a 100% acetylated standard, and the amounts of AcTubulin in SN<sub>1</sub>, SN<sub>3</sub>, SN<sub>5</sub> and SN<sub>7</sub> were determined as described in the Experimental section. Results are means ± S.D. from three independent experiments. (C) Samples of SN<sub>7</sub> from the two types of preparation as described in Figure 3, containing equal protein amounts, were subjected to SDS/PAGE and stained with Coomassie Blue. (D) Samples of SN<sub>7</sub> from the two types of preparation were subjected to Western blotting and stained with anti-Tyr-tubulin, anti-Glu-tubulin and anti-Δ2-tubulin mAbs. Total tubulin (α-Tub) was determined as a loading control. (E) The assembly–disassembly properties of preparations lacking or containing AcTubulin. Aliquots of SN<sub>7</sub> from both types of preparation were incubated at 37 °C under assembly conditions and microtubule formation was monitored at 350 nm. After the plateau was reached (time = 20 min), the temperature was lowered to 10 °C (arrow) and the measurement of absorbance continued. This experiment was repeated two times with similar results.



**Figure 5** Determination of deacetylase activity in microtubule preparations purified by two or three cycles of assembly–disassembly

Aliquots of microtubule protein preparations enriched in AcTubulin (Ac-Tub) purified by two cycles (SN<sub>5</sub>) or three cycles (SN<sub>7</sub>) were mixed with 1 volume of freshly prepared SN<sub>1</sub> depleted of AcTubulin (–Ac), and incubated at 37 °C. Incubation systems in which SN<sub>1</sub> was replaced by MEM buffer were run in parallel. (A) Aliquots were incubated for the indicated times and analysed by Western blotting with staining by anti-AcTubulin and anti-total tubulin (Total Tub) mAbs. (B) The absorbance of the bands in (A) were measured, and those corresponding to AcTubulin were standardized relative to those corresponding to total tubulin. The data shown are means from two independent experiments.

on AcTubulin) or with one volume of buffer. Incubation of SN<sub>5</sub> even in the absence of SN<sub>1</sub> resulted in the partial deacetylation of tubulin, indicating that microtubule protein purified by two cycles contains a significant deacetylase activity (Figure 5). In contrast, incubation of SN<sub>7</sub> without SN<sub>1</sub> did not result in deacetylation. The possibility that the absence of deacetylation was due to the loss of the ability of AcTubulin to act as a substrate was ruled out because deacetylation was rapid in the presence of SN<sub>1</sub>. Collectively, these findings indicate that microtubule protein purified by three cycles (SN<sub>7</sub>) did not contain significant deacetylase activity.

The acetylated and non-acetylated tubulin preparations obtained by the method described in the present study have several beneficial properties: (i) the two preparations have the same ability to assemble into microtubules without the need for exogenous chemicals (Figure 4E); (ii) the non-acetylated preparation contains no AcTubulin, whereas in the acetylated preparation AcTubulin comprises ~64% of the total tubulin (Figure 4B). These preparations are therefore useful for *in vitro* comparisons of the effect of the presence of the acetyl group on Lys<sup>40</sup> on various biochemical or physiological parameters; (iii) preparations of AcTubulin purified by three or more cycles of assembly–disassembly contain no deacetylase activity (Figure 5) and are therefore appropriate substrates for studies of HDAC6 or Sirt2; (iv) SN<sub>1</sub> obtained in the absence of TSA has a very low AcTubulin content (Figure 3), but high HDAC6 activity (Figures 1 and 5) and is a useful source of HDAC6.

Many laboratories are engaged in intensive studies of the physiological relevance of the acetylation/deacetylation of tubulin. Most of the published studies have been based on cellular and molecular approaches and have provided interesting results that form a basis for future investigations. In some cases, apparently conflicting conclusions have arisen. For example, the conclusion that kinesin-1 binds preferentially to microtubules enriched in AcTubulin and thereby increases kinesin-1 velocity [17,27] was challenged by the observation of Walter et al. [16] that acetylation alone is not sufficient to explain this phenomenon. In the Walter et al. [16] study, kinesin-1 properties were assayed *in vitro* using microtubules made with purified porcine tubulin that

was extensively acetylated with recombinant  $\alpha$ -TAT to obtain 'AcTubulin' or extensively deacetylated with a recombinant HDAC6 to obtain deacetylated tubulin. In this example and many others, acetylated and non-acetylated tubulin preparations are required to test or confirm results obtained from *in vivo* or cell culture experiments using biochemical approaches. The simple and easy method described in the present paper for obtaining acetylated and non-acetylated tubulin preparations will be helpful for this purpose.

Even if AcTubulin preparations obtained by our method are not 100% acetylated, microtubules formed from the preparations will be densely acetylated. Given an AcTubulin content of 64% and the presence of 13 protofilaments in microtubules (i.e. 13 tubulin dimers per turn), it is calculated that there will be at least eight AcTubulin per tubulin turn; therefore, microtubules formed in this way will be useful for comparisons with microtubules formed in preparations that do not contain AcTubulin.

### AUTHOR CONTRIBUTION

Agustín Carbajal and María Eugenia Chesta performed most of the experiments and drew the Figures. Gastón Bisig supervised the research and collaborated with some biochemical experiments. Carlos Arce supervised the general orientation of the work and wrote the paper with contributions of all authors.

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