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Quantification of Acetylated Tubulin

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Running title: Quantification of AcTubulin

Abbreviations used: AcTubulin, acetylated tubulin; mAb, monoclonal antibody; TSA, Trichostatin A

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Abstract

The acetylation/deacetylation of Lys^{40} of the α-subunit is an important post-translational modification undergone by tubulin during the life of a cell. Many previous studies have addressed the physiological role of this acetylation process using various approaches based on changes of acetylated tubulin (AcTubulin) content. In most of these studies, however, the actual amounts of AcTubulin were not known and it was difficult to draw conclusions. We present here a simple method to estimate the percentage of AcTubulin relative to total tubulin. The method is based on acetylation of the tubulin sample with acetic anhydride, Western blotting stained by anti-AcTubulin antibody, and comparison of the optical density of the AcTubulin band with that of a corresponding sample that was not chemically acetylated.

Key words: acetylated tubulin, microtubules, quantification method, chemical acetylation

Introduction

Microtubules are dynamic fibrous structures present in the cytoplasm of all eukaryotic cells. The main constituent protein of microtubules, tubulin, is subject to many post-translational modifications including acetylation/deacetylation, tyrosination/detyrosination, polyglutamylation, polyglycylation, and phosphorylation [Janke and Bulinski, 2011]. The functional roles of these tubulin modifications have not been precisely defined in spite of intensive studies. It is increasingly clear that acetylation of tubulin plays an important role in various cell functions including cell migration, division, and differentiation [Perdiz et al., 2011], although conflicting results have been obtained in some studies. Our previous studies have shown that acetylated tubulin (AcTubulin)is specifically associated with Na^+,K^+-ATPase (the sodium pump) in membranes of neural and non-neural cells and that this association results in inhibition of the ATPase activity [Alonso et al., 1998; Casale et al., 2001; Casale et al., 2003; Casale et al., 2005; Santander et al., 2006; Zampar et al., 2009]. Specific acetylation catalyzed by MEC-17 occurs on the ε-NH_2 group of Lys^{40} of the α-tubulin chain [Akella et al., 2010; Shida et al., 2010], and the acetyl group can be removed by NAD-dependent Sirtuin2 (Sirt2, an analog of yeast Sir2) [North et al., 2003] and by cytoplasmic histone deacetylase 6 (HDAC6) [Hubbert et al., 2002; Zhang et al., 2003]. The acetyl group can also be added to Lys^{40}(and to other reactive amino acid residues of tubulin and of contaminant proteins) by chemical acetylation with acetic anhydride [Piperno and Fuller, 1985]. To avoid nomenclatural confusion, we use the term “AcTubulin” in this paper to refer to tubulin that contains an acetyl group bound to the ε-NH_2 of Lys^{40} of the α-chain and “chemically acetylated tubulin” to refer to tubulin in which acetyl groups are bound nonspecifically to not only Lys^{40} but also other Lys

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residues and other sites that can be acetylated. The monoclonal antibody (mAb) 6-11B-1 is specific to the acetylatedLys^{40} of α-tubulin [Piperno and Fuller, 1985] and can therefore be used to distinguish this epitope from all acetyl groups bound to other sites on α-tubulin as a result of chemical acetylation. In most published studies regarding the role of tubulin acetylation, conclusions have been drawn on the basis of cell behavior following an increase or decrease in the amount of AcTubulin in cultured cells by inhibiting/silencing or overexpressing the acetylating or deacetylating enzymes [Hubbert et al., 2002; Li et al., 2012; Tran et al., 2007]. The amount of AcTubulin in cultured cells is generally very low. Even when treatment with Sirt2 or HDAC6 inhibitors produces a seemingly large increase of AcTubulin as visualized by immunofluorescence microscopy or Western blotting, the resulting amounts of AcTubulin relative to total tubulin are uncertain. In most biochemical experiments using partially purified tubulin, the investigators have not taken into account the proportion of tubulin molecules that are acetylated on Lys^{40} of α-tubulin with respect to total tubulin. We must therefore be cautious in drawing conclusions from this type of experiment. Data regarding the amount of AcTubulin in each experimental system are essential for designing protocols and for drawing reliable conclusions. Conclusions or assumptions from cellular and molecular experiments should be corroborated using biochemical systems with purified proteins. We recently described a method for purifying tubulin with a high acetylated isotype content from rat brain and determined that approximately 64% of such tubulin molecules are acetylated [Carbajal et al., 2013]. We now describe a simple method to estimate the amount of AcTubulin as a percentage of total tubulin. This method can be used to quantify AcTubulin in purified tubulin preparations or in crude extracts from cells.

Materials and Methods

Chemicals

MES (2-(N-morpholino)ethanesulfonic acid), TSA (Trichostatin A), Taxol, Triton X-100, BSA (bovine serum albumin), SDS, EGTA, PMSF, phenanthroline, mouse mAb 6-11B-1 specific for AcTubulin, culture media and antibiotics were from Sigma-Aldrich (St. Louis, MO, USA). IRDye 800CW goat anti-mouse IgG was from Li-Cor Biosciences (Lincoln, NE, USA). Recombinant Protein G-Sepharose 4B conjugate was from Invitrogen Corp. (Grand Island, NY, USA) and Alexa Fluor® 488 Donkey Anti-Mouse IgG (H+L) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA**).** FluorSave reagent was from Calbiochem/ EMD Biosciences (La Jolla, CA, USA). Fetal bovine serum was from Natocor (Córdoba, Argentina). Boric acid was from PlusOne, Pharmacia Biotech (Uppsala, Sweden). Acetic anhydride was from J. T. Baker (Phillipsburg, NJ, USA).

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Cells

CAD cells (a subclone of a catecholaminergic cell line derived from a mouse neuronal brain tumor) were cultured in DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12). CHO (Chinese Hamster Ovary) and COS cells were grown in DMEM. All cell lines were cultured in their respective media supplemented with 10 % (v/v) FBS, 10 units/ml penicillin, and 100 µg/ml streptomycin and maintained at 37 ºC in an air/CO_2 (19:1) atmosphere with high humidity.

Purification of microtubule protein depleted or enriched in AcTubulin

Purification of two tubulin preparations was performed as described previously [Carbajal et al., 2013]. The same protocol was used for purification of tubulin depleted or enriched in the acetylated isotype except that TSA, an HDAC6 inhibitor, was added to the buffers for the AcTubulin-enriched preparation and absent for the AcTubulin-depleted preparation. Briefly, Wistar rat brains were homogenized in 1.5 volumes of cold (0 ºC) MEM buffer (100 mM MES buffer, pH 6.7, containing 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, and 1 mM phenanthroline) supplemented with 5 µM TSA for the AcTubulin-enriched preparation and centrifuged at 100,000 x *g* for 30 min at 4 ºC. The supernatant fraction (termed SN_1) was added with glycerol (final concentration 40%) and incubated at 37 ºC for 30 min. The mixture was centrifuged at 100,000 x *g* for 30 min at 27 ºC, and the supernatant (SN_2) and sedimented microtubules (P_2) were separated. The microtubules were resuspended in cold MEM buffer (1/5 of the original volume) containing 1µM TSA for the AcTubulin-enriched preparation, kept at 0 ºC for 30 min, and centrifuged at 100,000 x *g* for 30 min at 4 ºC. The supernatant (SN_3; microtubule protein purified by one cycle) was collected.

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, Argentina).

Immunopurification of AcTubulin

Anti-AcTubulin mAb 6-11B-1 was linked to Protein G-Sepharose beads (100 µg IgG: 200 µl beads) following the manufacturer's instructions. The 6-11B-1-linked beads were mixed with 600 µl (1.5 mg/ml) of an AcTubulin-enriched preparation (SN_3), incubated for 2 h at room temperature and washed 5 times with 10 bed volumes of PBS supplemented with 0.5% Triton X-100. Bound proteins were eluted twice by mild agitation for 5 min at room temperature with 400 µl of 100 mM sodium carbonate buffer, pH 12, containing 9 µM TSA. The two eluted fractions were combined and stored at -20 ℃. As a control, 6-11B-1-linked beads were incubated with an AcTubulin-depleted preparation and processed in the same way.

SDS-PAGE and Western blotting

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Proteins were separated by SDS-PAGE (10% gels) [Laemmli, 1970] and transferred to nitrocellulose sheets [Towbin et al., 1979]. The sheets were incubated with primary antibody for 4 h at room temperature (anti-AcTubulin: 1:5,000 dilution; 1% non-fat milk in PBS as blocking medium), washed, incubated with infrared fluorescent secondary antibody for 1 h at room temperature (1:25,000 dilution; blocking medium as above), washed, and scanned by an Odyssey infrared scanner (Li-Cor). Bands were quantified using the Scion Image software program (Scion Corporation; Frederick, Maryland, USA).

Immunofluorescence

Cells were cultured on coverslips and fixed with anhydrous methanol at -20 ºC for 10 min. The samples were washed with PBS, incubated with 5% (w/v) BSA in PBS for 1 h, and incubated with the primary antibody (anti-AcTubulin mAb 6-11-B1, 1:1,000 dilution; 1% BSA in PBS as blocking medium) for 4 h at 37ºC. The cells were washed 3 times with PBS, incubated for 1 h at 37ºC with secondary antibody (anti-mouse IgG-Alexa^{488} diluted 1:1000), washed 3 times with PBS, and the coverslips were mounted in FluorSave reagent. Images were collected using an FV1000 Olympus confocal spectral microscope (Olympus Latin America, Miami, FL, USA) equipped with an argon/helium/neon laser at the appropriate wavelength and the software (Olympus FV Viewer) provided by the manufacturer, and processed using the Image J program 1.46r (Wayne Rasband; NIH, USA).

Chemical acetylation of tubulin

*Partially purified preparations:*A 100-µl sample of SN_1 (15 mg/ml protein) or of a microtubule protein preparation purified by one cycle (2-4 mg/ml protein) was mixed with 125 µl of boric acid (500 mM, pH 9) and then with 50 µl of 1% (v/v) freshly prepared cold acetic anhydride. After incubation at 4 ºC for 20 min, 100 µl of 5x Laemmli sample buffer was added. The samples were diluted with 1x Laemmli sample buffer to obtain tubulin concentrations adequate for SDS-PAGE and Western blotting. Two samples (containing 1x and 2x tubulin amounts) of each sample were electrophoresed in triplicate. A control (non-acetylated) sample in which acetic anhydride was substituted by distilled water was processed in parallel.

Cell extracts: Cells from a 10-cm diameter culture dish (80% confluence) were washed with 10 ml of warm (37 ℃) PBS and harvested in 2 ml of ext raction buffer (280 mM boric acid, pH 9, 1% SDS, 3 µM TSA, 1 mM PMSF, and 1 mM EGTA). The cell extract was sonicated 3 times for 30 sec at position 40 in a Cole Parmer Ultrasonic Homogenizer and centrifuged at 10,000 x *g* for 10 min at 15 ℃. The supernatant fraction was collected and d ivided into 2 fractions of 900 µl each. One fraction was added with 110 µl of water and the other with 110 µl of 2% (v/v) freshly prepared acetic anhydride. Both aliquots were kept at 4 ℃ f or 20 min and then subjected to protein

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precipitation with methanol-chloroform (see following section). Protein pellets were resuspended in 300 µl of Laemmli sample buffer with 5% 2-mercaptoethanol and sonicated 3 times for 30 sec. Aliquots (approximately 20 µl) of each preparation were subjected (in triplicate) to Western blotting with mAb 6-11B-1 to analyze AcTubulin.

Methanol-chloroform protein precipitation

The method of Wessel and Flügge [1984] was used. Briefly, 1 volume of aqueous protein solution was mixed with 1.25 volumes of methanol-chloroform (4:1) by vortexing for approximately 30 sec. The mixture was centrifuged at 10,000 x *g* for 10 min at room temperature, and the upper phase was carefully removed without removing the interphase. Methanol (0.75 volumes relative to the original volume) was added, and the mixture was centrifuged at 10,000 x *g* at room temperature for 10 min. The solvent was removed, and the pellet was dried at room temperature and then resuspended in Laemmli sample buffer and sonicated.

Protein measurement

Protein content was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Results and Discussion

The basic concept of the method

The method described here is based on the increase in the amount ofAcTubulin produced by chemical acetylation of a tubulin sample relative to that of an identical amount of non-treated sample, assuming that Lys^{40} of α-tubulin is 100% acetylated by the procedure. A sample of the tubulin preparation to be analyzed is extensively acetylated with acetic anhydride in a one-step procedure and then subjected to Western blotting and staining with specific anti-AcTubulin mAb 6-11B-1("chemically acetylated"). A sample containing an identical amount of protein is processed in the same way but with substitution of acetic anhydride by vehicle ("non-chemically acetylated"). It should be noted that the non-chemically acetylated sample may contain a certain amount of endogenous acetylated tubulin. The optical densities (O.D.) of the AcTubulin bands are determined. The percentage of AcTubulin relative to total tubulin is calculated by the following formula:

% AcTubulin = (O.D. non-chemically acetylated / O.D. chemically acetylated) x 100%

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This formula is applicable provided that: (i) the optical density valuesare directly proportional to the protein amounts; (ii) identical amounts of total tubulin in the "acetylated" and "non-acetylated" samples are compared. To satisfy these criteria, identical volumes of the two samples must be processed in parallel.

Conditions for maximal tubulin acetylation on Lys^{40} of the α-subunit

Chemical acetylation of a crude rat brain preparation (SN_1, obtained in the absence of TSA) resulted in a large increase of AcTubulin as assessed by Western blotting with specific mAb 6-11B-1 (Fig. 1A). A slight decrease in the electrophoretic mobility of the chemically acetylated tubulin band was observed. This decrease may have resulted from an alteration in the molecular weight and/or spatial structure of the tubulin molecule and/or in the affinity for SDS induced by acetylation of protein sites other than Lys^{40} of α-tubulin. The effect of chemical acetylation on an AcTubulin preparation considered to be 100% acetylated because it was immunopurified from an AcTubulin-enriched preparation is shown in Fig. 1A (right panel). A similar shift in the electrophoretic mobility was more clearly observed. Chemical acetylation did not significantly increase the intensity of the immunopurified tubulin sample. This finding suggests that chemical acetylation does not significantly interfere with the affinity of mAb 6-11B-1. The fact that in both cases (with SN_1 and with immunopurified tubulin) the shift in electrophoretic mobility affected most (perhaps all) of the tubulin molecules suggests that the acetylation was complete. To confirm that a single acetylation step was sufficient to acetylate 100% of Lys^{40}, a SN_1 preparation (obtained in the absence of TSA) was subjected to chemical acetylation 1, 2, and 3 times. Following the analysis of various amounts of protein by Western blotting, no significant increase in AcTubulin content was observed (Fig. 1B) when this procedure was repeated once or twice on a tubulin sample that was previously chemically acetylated. This finding was confirmed statistically (Fig. 1C). Taken together, these results indicate that one cycle of chemical acetylation under the conditions described is sufficient for extensive and maximal acetylation of Lys^{40} of the α-chain of tubulin.

Because chemical acetylation is nonspecific, we considered the possibility that the reaction generated new epitopes in the tubulin molecule that were recognized by mAb 6-11B-1. However, following acetylation of a crude soluble brain extract (SN_1) in which more than 84% of the proteins were contaminants of tubulin (Fig. 2A), approximately 94% of the 6-11B-1 staining coincided with the tubulin band (Fig. 2B) and the remaining 6% with other protein bands, indicating a very low probability that chemical acetylation could generate new epitopes recognized by 6-11B-1 in sites other than Lys^{40}. This finding is consistent with the observation that chemical acetylation of a 100% pure AcTubulin sample did not increase the intensity of the AcTubulin band (Fig. 1A, right panel). We conclude that most (perhaps all) of the staining of the tubulin band following chemical acetylation was due to the reactivity of the acetyl group on Lys^{40} of the α-subunit.

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Factors that might alter the basic concept of the method

After chemical acetylation and subsequent SDS-PAGE and staining with Coomassie Blue, we observed a lower intensity in the staining that coincided with the tubulin band, in comparison with an equivalent aliquot of the non-acetylated sample. This finding was surprising because we loaded equal amounts of acetylated and non-acetylated samples. This observation was presumed to reflect a staining artifact rather than an actual loss of tubulin because silver staining did not reveal such a difference. This conclusion was supported by the finding that after nitrocellulose blotting and staining with an anti-Glu-tubulin antibody the optical densities of the two samples were identical (results not shown). These are important controls because the method is based on the comparison of equal amounts of total tubulin before and after chemical acetylation. We presume that the above difference was due to a lower affinity of Coomassie Blue for proteins whose structures were altered by the incorporation of acetyl groups in several sites.

The determination of the amount of AcTubulin relative to total tubulin is dependent on the optical density of the AcTubulin band (as detected using mAb 6-11B-1) before vs. after chemical acetylation. It was therefore important to determine whether the optical density values were directly proportional to the AcTubulin amounts and to determine the range of linearity. This function was linear up to 0.8 µg protein (equivalent to approximately 440 ng AcTubulin because in this preparation tubulin accounts for approximately 55% of total protein) (Fig. 3). When this method is applied to unknown samples, it is therefore convenient to load various amounts of the samples for the corresponding Western blot analysis and to use data collected within the linear range.

Experimental validation of the method

To test the accuracy of the method, we prepared 3 tubulin preparations with known AcTubulin concentrations and applied the chemical acetylation procedure to determine experimentally the AcTubulin concentration in each preparation. The AcTubulin standards were prepared by mixing different proportions of an AcTubulin-enriched preparation (SN_3, obtained in the presence of TSA) that contained 64% AcTubulin with another tubulin preparation (of the same concentration) lacking the acetylated isotype [Carbajal et al., 2013]. The 3 AcTubulin standards contained 7.5, 27.5, and 60% AcTubulin, respectively. The AcTubulin amounts determined by our method were in good agreement (within 10% standard error) with the values corresponding to the standards (Fig. 4). The method is therefore useful for estimation of the percentage of acetylated tubulin molecules relative to total tubulin.

Quantification of AcTubulin in cultured cells

The measurement of AcTubulin content in cultured cells using the chemical acetylation method requires the use of buffers containing SDS or Triton X-100 to dissolve the cells and, in

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some cases, to isolate the soluble and cytoskeletal fractions. We examined the effects of these detergents on the degree of acetylation obtained by chemical acetylation of a crude soluble rat brain preparation (SN_1, obtained in the absence of TSA). Various amounts of the detergents were mixed with the tubulin preparation, and the mixtures were subjected to chemical acetylation and subsequent Western blot analysis and staining with mAb 6-11B-1. No differences in the optical densities of tubulin bands were observed among any of the detergent samples or the sample without detergent (Fig. 5A). This finding indicates that the SDS or Triton X-100 used to dissolve cultured cells did not interfere with our quantification method. We then applied the method to estimate the amount of AcTubulin in 3 cell types treated or not with TSA. The AcTubulin content in non-treated cells was generally very low (Fig. 5B). The effect of TSA treatment was variable depending on the cell type. The AcTubulin content increased approximately 4-fold in CHO cells and approximately 20-fold in COS cells. CAD cells showed a very low (or zero) AcTubulin content, and this content increased to 25% of total tubulin after TSA treatment. A comparison of immunofluorescence images revealed with anti-AcTubulin of similarly treated vs. non-treated cells (Fig. 5C) showed a general consistency with Western blot values (Fig. 5B). Taxol is another well-known compound that induces an increase in the amount of acetylated microtubules in living cells because of its microtubule-stabilizing effect. A further experiment showed that the increase of AcTubulin in CHO and COS cells induced by taxol after 2 and 6 h of treatment and visualized by immunofluorescence microscopy (Fig. 6A), although less than the increase produced by TSA, could be detected and quantified by the chemical acetylation method (Fig.6B).

These findings indicate that our method is sufficiently sensitive to detect modest variations of AcTubulin content in cells. This quantification method, in combination with immunofluorescence microscopy, will be useful for further studies of the physiological role of tubulin acetylation. Immunofluorescence microscopy is a useful method for revealing the intracellular localization and eventual changes of acetylated tubulin, while the quantification method described here is a useful complement for measuring the magnitude of the phenomenon.

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The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Chemical acetylation of tubulin. (A) Two types of tubulin preparation were chemically acetylated: a SN_1 fraction (obtained in the absence of TSA) and an immunopurified AcTubulin preparation (see Materials and Methods). The 2 preparations were chemically acetylated (+) or not (-) and subjected to Western blot analysis and staining with anti-AcTubulin mAb 6-11B-1. Following chemical acetylation, the AcTubulin band in both cases showed a lower electrophoretic mobility (arrow) in comparison with the preparation that was not chemically acetylated (arrowhead). **(B)** A microtubule protein (3 mg/ml) preparation purified by 1 cycle of assembly/disassembly was incubated for 20 min at room temperature in the presence of 1% acetic anhydride, pH 9. An aliquot was removed for subsequent analysis (Western blotting with mAb 6-11B-1), and the mixture was acetylated again under the same conditions. Another aliquot was removed, and a third acetylation step was performed. (**C**) Various protein amounts from each of the 3 acetylation steps as above were immunoblotted and stained with mAb 6-11B-1. AcTubulin bands from 3 independent experiments were scanned and the optical density values (mean ± S.D.)are shown.

Fig. 2. Immunoreactivity of mAb 6-11B-1 with proteins of a crude brain extract following chemical acetylation. The soluble extract of a rat brain homogenate was acetylated with acetic anhydride and subjected to SDS-PAGE and immunoblotting with mAb 6-11B-1. **(A)** Chromatogram of the soluble extract stained with Coomassie Blue and the corresponding densitogram showing that tubulin (arrowhead) accounts for 16% of total proteins. **(B)** Immunoblot stained with mAb 6-11B-1 and the corresponding densitogram. Staining that coincides with the tubulin band accounted for 94% of the total immunoreactivity.

Fig. 3. Standard curve for AcTubulin. An AcTubulin-enriched preparation (microtubule protein preparation purified by 1 cycle) was chemically acetylated to obtain 100% AcTubulin. In this preparation, tubulin representedapproximately 55% of total proteins (results not shown). Various amounts of protein were subjected to Western blotting and visualized for AcTubulin. The bands were scanned and optical density values were calculated as a function of protein amount. The values are presented as the means ± S.D. from quadruplicate determinations.

Fig. 4. Quantification of AcTubulin in partially purified preparations. An AcTubulin-enriched preparation (microtubule protein purified by 1 cycle; 2.5 mg/ml) was mixed in various proportions with an AcTubulin-depleted preparation (microtubule protein purified by 1 cycle; 2.5 mg/ml). The resulting mixtures consisted of 7.5, 27.5, and 60% AcTubulin. Aliquots of each preparation (100 µl)

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were used to determine AcTubulin content by our quantification method (see Materials and Methods). The values shown are the means ± S.D. from 3 independent experiments.

Fig. 5. Quantification of AcTubulin in cultured cells. (A) Effects of the presence of the detergents SDS and Triton X-100 on chemical acetylation. Aliquots of a soluble tubulin preparation (SN_1, obtained in the absence of TSA), after the addition of SDS or Triton X-100 (final concentration 0%, 1%, or 3% in both cases) were chemically acetylated and subjected to Western blotting and subsequent staining for AcTubulin (AcTub). **(B)** CHO, COS, and CAD cells were cultured in 10-mm dishes and treated with (+TSA) or without (-TSA) 5 µM TSA for 6 h, and the AcTubulin content was measured as described in Materials and Methods. The values shown are the means ± S.D. from 4 independent experiments. **(C)** The same cell types as in (B) were grown on coverslips and processed in the same way (+TSA or -TSA; 6 h), and acetylated microtubules were visualized by immunofluorescence. Scale bar = 20 µm.

Fig. 6. Quantification of AcTubulin in CHO and COS cells before and after taxol treatment. (A) Cells were grown on coverslips and treated with 5 µM taxol for 0, 2, or 6 h, and microtubules were visualized by immunofluorescence microscopy using anti-AcTubulin mAb 6-11B-1. Scale bar = 20 µm. **(B)** Cells were grown in 10-cm dishes and treated as in (A) in parallel experiments. The AcTubulin content was measured by the chemical acetylation method as described in Materials and Methods. The values shown are the means ± S.D. from 3 independent experiments.

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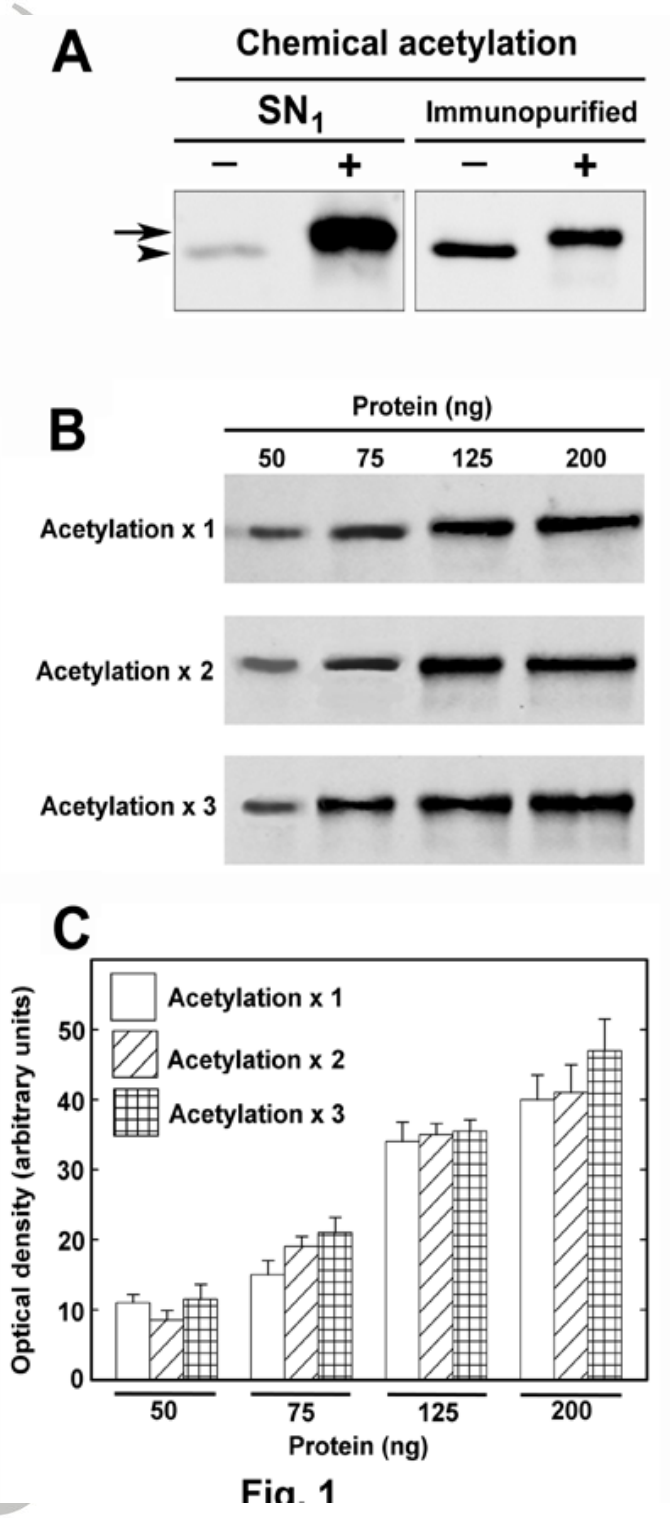

Fig. 1

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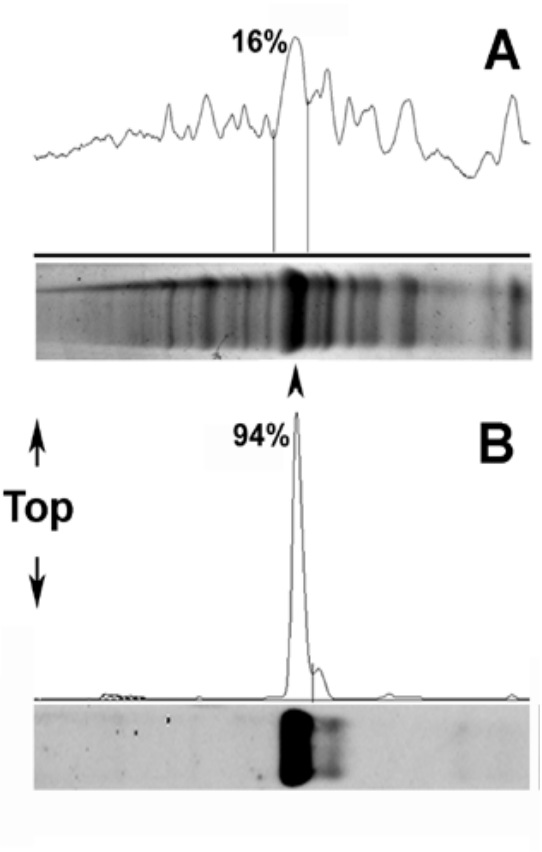

Fig. 2

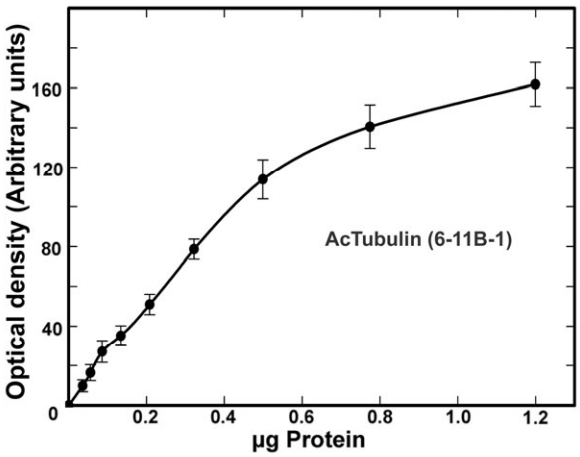

Fig. 3

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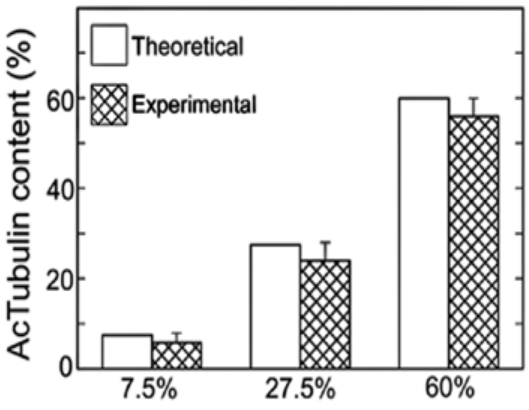

Fig. 4

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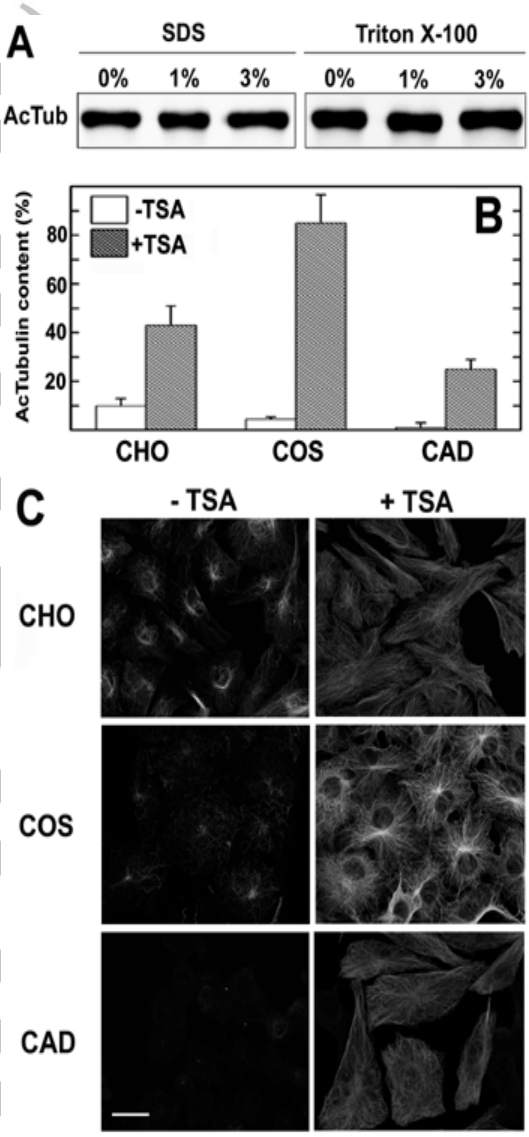

Fig. 5

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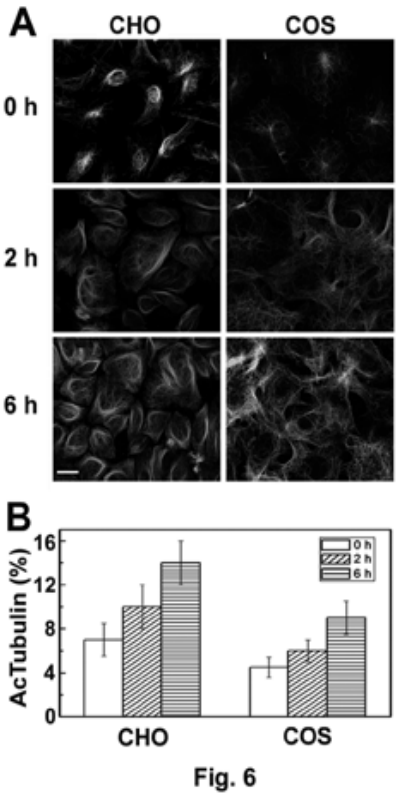

Fig. 6