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Acetylated tubulin associates with the fifth cytoplasmic domain of Na^+/K^+-ATPase: possible anchorage site of microtubules to the plasma membrane

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We showed previously that NKA (Na^+/K^+-ATPase) interacts with acetylated tubulin resulting in inhibition of its catalytic activity. In the present work we determined that membrane-acetylated tubulin, in the presence of detergent, behaves as an entity of discrete molecular mass (320–400 kDa) during molecular exclusion chromatography. We also found that microtubules assembled *in vitro* are able to bind to NKA when incubated with a detergent-solubilized membrane preparation, and that isolated native microtubules have associated NKA. Furthermore, we determined that CD5 (cytoplasmic domain 5 of NKA) is capable of interacting with acetylated tubulin. Taken together, our results are consistent with the idea that NKA may act as a microtubule–plasma membrane anchorage site through an interaction between acetylated tubulin and CD5.

Key words: acetylated tubulin-binding domain, acetylation, cytoskeleton, P-type ATPase (P-ATPase), post-translational modification, sodium pump.

INTRODUCTION

NKA (Na^+/K^+-ATPase, also known as 'the sodium pump') is a transmembrane membrane protein that transports three Na^+ ions out of the cell in exchange for two K^+ ions, using energy from ATP hydrolysis. NKA's main function is to generate and maintain the electrochemical gradient of Na^+ and K^+ ions through the plasma membrane of the cell [1]. This gradient is essential for numerous biological functions, including regulation of internal pH, calcium concentration, cell volume, sodium-dependent transport of glucose and amino acids, transmission of nervous impulses and regulation of blood pressure [2–6]. NKA is also involved in signal transduction events, as a receptor of endogenous ouabain (reviewed in [7]). NKA is formed from two subunits, a catalytic α-subunit and a regulatory β-subunit [8–10]. α-NKA has ten transmembrane segments and presents six cytoplasmic domains (CD1–CD6), some of which interact with proteins such as ankyrin [11,12] and Src [7,13].

We have shown previously that NKA is capable of interacting with acetylated tubulin (but not with non-acetylated tubulin), and that such interaction inhibits its enzymatic activity both *in vitro* and *in vivo* [14–17].

Tubulin acetylation is a post-translational modification consisting of the reversible addition of an acetyl group on the ε-amino group of the Lys^{40} residue of α-tubulin [18]. There is little evidence about the physiological role of this modification. It is known that acetylation does not influence tubulin polymerization or microtubule stability. However, it has been recently reported that tubulin acetylation is involved in motor-based trafficking in mammals [19–21]. Our previous studies indicate that acetylation of tubulin is essential for its interaction with NKA [17].

Although the acetylated tubulin/NKA complex is very stable, it can be dissociated, and enzymatic activity consequently restored, by treatment of cells with L-glutamate or other drugs [15,16]. We therefore speculated that acetylated tubulin is involved in the regulation of NKA, but have not known whether: (i) tubulin interacts directly with NKA or through an intermediate; (ii) other partners integrate into the complex; and (iii) the tubulin molecule that constitutes the complex is in a dimeric form or forms part of a microtubule. The term 'acetylated tubulin/NKA complex' has been used for simplicity, since molecular characterization of the complex had not been performed until now.

In the present study, we investigated the molecular mass of the detergent-resistant acetylated tubulin/NKA complex, the possible interaction of microtubules with NKA and the structural domain of NKA involved in interacting with tubulin. Our results indicate that NKA is capable of interacting with microtubules and may act as an anchorage site for microtubules, connecting them to the plasma membrane, and that CD5 (cytoplasmic domain 5) of α-NKA is an acetylated tubulin-binding domain.

EXPERIMENTAL

Materials

TSA (Trichostatin A), Triton X-100, taxol, nocodazole, mouse mAb (monoclonal antibody) 6–11B-1 specific for acetylated tubulin, mouse mAb DM1A specific for α-tubulin, mouse mAb M7-PB-E9 specific for α-NKA, cyanogen bromide-activated Sepharose 4B, keyhole limpet haemocyanin and purified NKA were from Sigma–Aldrich. EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide) and NHS (*N*-hydroxysuccinimide) were from Pierce Biotechnology. IRDye 800CW goat anti-mouse

Abbreviations used: CD1–6, cytoplasmic domain 1–6; EDC, 1-ethyl-3-[3-dimethyl-aminopropyl]carbodiimide; HDAC6, histone deacetylase 6; mAb, monoclonal antibody; NHS, *N*-hydroxysuccinimide; NKA, Na^+/K^+-ATPase; TSA, Trichostatin A.

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IgG and IRDye 800CW goat anti-rabbit IgG were from Li-Cor Biosciences. The pTrcHis2-TOPO expression kit was from Invitrogen. Oligonucleotides were from Ruralex. Rabbit anti-calnexin antibody was from Santa Cruz Biotechnology.

Cell culture

CAD cells (a subclone of a cathecolaminergic cell line derived from a neuronal brain tumour in mouse) were a gift from Dr D. M. Chikaraishi (Department of Neurobiology, 427G Bryan Research Building, Duke University Medical Center, Durham, NC 27710, U.S.A.). These cells were cultured in DMEM/F12 (Dulbecco's modified Eagle's medium/Ham's F12, Sigma–Aldrich) (1:1), supplemented with 10 % (v/v) FBS (fetal bovine serum, Natocor), 10 units/ml penicillin and 100 μg/ml streptomycin (Sigma–Aldrich). Cells were maintained at 37 °C in an air/CO_2 (19:1) atmosphere with high humidity.

Cloning, expression and purification of CD1, CD2, and CD3

The coding sequences of the three cytoplasmic domains CD1–CD3 were amplified by PCR from a rat α1-NKA (NM_012504)-containing plasmid using the following primers: 5′-GGA CGA GAC AAG TAT GAG CCC-3′ and 5′-ACA GAA TTT GAC CCA CTC GGG-3′ for CD1 (amino acids 6–93); 5′-CAA GAA GCA AAA AGC TCC AAG-3′ and 5′-GTG CTC GAT TTC TTC AGC AAT-3′ for CD2 (amino acids 150–290); 5′-ACG CTC ACT GCC AAG CGC ATG–3′ and 5′-GGA TTT CTT CAA GTT ATC AAA-3′ for CD3 (amino acids 345–775). After purification, PCR products were ligated into pTrcHis2-TOPO according to the manufacturer's instructions, and sequenced by the dideoxynucleotide chain termination method (Macrogen). Constructs were expressed, and the peptides were purified using a nickel affinity column. Peptides were eluted with 50 mM sodium phosphate buffer, pH 7.5, containing 0.3 M NaCl and 250 mM imidazole.

Synthetic peptides of CD5 and CD6

Peptides were purchased from Genscript. The amino acid sequences of CD5 and CD6 are ICKTRRNSVFQQGMKNK (936–952) and LIIRRRPGGWVEKETYY (1007–1023) respectively.

Preparation of CD5-specific antibody

A polyclonal antibody specific to CD5 was raised in rabbits as described in [22]. Briefly, CD5 was bound to keyhole limpet haemocyanin using glutaraldehyde as a cross-linker. The resulting protein was used for primary injection (with complete Freund's adjuvant, 1:1), and subsequent booster immunizations (with incomplete adjuvant) every 15 days. Antisera were collected 15 days after the last injection, and tested for affinity and specificity. The CD5-specific antibody was affinity-purified by standard procedures using a Sepharose column containing covalently linked CD5. The purified antibody was eluted by pH change, neutralized, aliquoted and stored at − 20 °C.

Isolation of membrane fraction from rat brain

Rat brains were homogenized (1:10, w/v) in 10 mM Tris/HCl buffer, pH 7.4, containing 0.32 M sucrose, and centrifuged for 10 min at 10 000 ***g***. $CaCl_2$ was added to the supernatant to a final concentration of 1 mM in order to induce depolymerization of cold-stable microtubules. The preparation was sonicated and then centrifuged at 100 000 ***g*** for 20 min. The membrane-containing pellet was resuspended in 10 mM Tris/HCl buffer, pH 7.4, and NaCl and $CaCl_2$ were added to final concentrations of 2 M and 1 mM respectively. The suspension was incubated at 0 °C for 30 min, and washed with 50 mM phosphate buffer, pH 7, containing 0.15 M NaCl. When necessary, membranes were solubilized with 1 % Triton X-100, and the soluble fraction was separated by centrifugation (100 000 ***g*** for 40 min). All procedures were performed at 4 °C. 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).

Preparation of cell membranes

Confluent CAD cells from three 100-mm-diameter Petri dishes were washed three times with PBS at room temperature (23 °C), harvested with 3 ml of distilled water, and incubated for 30 min at 0 °C. This hypotonic shock helps to obtain complete cell lysis. The suspension was homogenized in a glass Dounce homogenizer, and centrifuged at 3000 ***g*** for 1 min. The supernatant was centrifuged for 25 min at 48 000 ***g***, and the membrane-containing pellet was resuspended in a sufficient amount of buffer. When necessary, membranes were resuspended in 0.30 ml of 50 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 1 % Triton X-100, centrifuged twice at 100 000 ***g*** for 20 min, and the supernatant was used immediately.

Rat brain tubulin preparation

Rat brains were homogenized in a 1:1.5 volume of 100 mM Mes buffer, pH 6.7, containing 1 mM $MgCl_2$ and 1 mM EGTA, and centrifuged at 100 000 ***g*** for 20 min. The supernatant (called 'tubulin preparation not enriched in acetylated tubulin') was collected, and the pellet was resuspended in the same buffer (2 ml per 3 g of brain) containing 5 mM $CaCl_2$, and was incubated for 30 min at 0 °C. The preparation was centrifuged twice for 30 min at 100 000 ***g***, the supernatant was collected, and EGTA was added up to a concentration of 7.5 mM. The resulting preparation was termed 'tubulin preparation enriched in acetylated tubulin'. For cross-linking experiments, Mes buffer was replaced by 0.1 M phosphate buffer, pH 7.2, containing 150 mM NaCl. Each tubulin preparation was tested by Western blot to verify the absence of NKA.

Molecular exclusion chromatography

A membrane preparation (~5 mg/ml) solubilized in 1 % Triton X-100 was subjected to molecular exclusion chromatography on an FPLC system, using a 25-ml Superdex 200 column (Pharmacia) pre-equilibrated with 50 mM sodium phosphate buffer, pH 7, containing 0.15 M NaCl and 1 % Triton X-100. The running conditions were a sample volume of 500 μl, a flow rate of 0.25 ml/min and a fraction volume of 0.5 ml at room temperature. Aliquots (16.5 μl) of collected fractions were analysed by Western blot for detection of total tubulin, acetylated tubulin and NKA.

For cytosolic tubulin preparation, a 1:3 (w/v) rat brain homogenate in phosphate buffer (without Triton X-100) was centrifuged at 100 000 ***g*** for 30 min. Triton X-100 was added to the supernatant, to a final concentration of 1 % (v/v), and 500 μl of this preparation was subjected to molecular exclusion chromatography as described above. Molecular mass standards were separated under identical conditions.

Pull-down assay

Cyanogen bromide-activated Sepharose (30 mg) was rinsed twice with 1 mM HCl, then once with 0.1 M sodium carbonate buffer, pH 8, containing 0.5 M NaCl, and was added immediately to a

solution of synthetic or recombinant peptides dissolved in the same buffer (200 μl, 0.5 mg/ml). The mixture was incubated for 2 h at room temperature with mild agitation. Sepharose beads were collected by centrifugation for 1 min at 3000 ***g***, incubated for 2 h with 0.2 M glycine, pH 8 at room temperature, and then rinsed successively with sodium carbonate buffer, pH 8, with 0.1 M sodium acetate buffer, pH 4, containing 0.5 M NaCl, and with sodium carbonate buffer again. The resin was incubated overnight at 4 °C with a tubulin preparation enriched in acetylated tubulin (~2 mg/ml) containing 0.1 M NaCl. This preparation was washed eight or more times with 25 mM sodium phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.1 % Triton X-100, and bound proteins were then eluted by three washes with 100 mM sodium carbonate buffer, pH 12 (the resulting fractions termed F1, F2, and F3). For competition and displacement experiments, a detergent-solubilized membrane preparation from CAD cells was used.

Covalent cross-linking experiments

EDC and NHS were added to 20 μl of CD5 peptide solution (1 mg/ml in 0.1 M Mes buffer, pH 6, plus 500 mM NaCl) to final concentrations of 2 mM and 4.75 mM respectively. The reaction was continued for 15 min at room temperature, and then 20 μl of tubulin preparation and 1 μl of 1 M NaOH were added. After 2 hr incubation at room temperature, the reaction was stopped by addition of 5 μl of 100 mM hydroxylamine. The reaction medium was diluted with 3× sample buffer and subjected to Western blotting.

In vitro association of NKA with microtubules

A tubulin preparation enriched in acetylated tubulin was obtained as described above, and separated into two fractions (of 100 μl and 300 μl). The 100 μl fraction was kept on ice until needed (termed the 'tubulin fraction'). Taxol was added to the 300 μl fraction, to a final concentration of 10 μM, and incubated at 37 °C for 20 min. The insoluble material was then separated by centrifugation (100 000 ***g***, 15 min), washed with 100 mM Mes buffer, pH 6.7, containing 1 mM $MgCl_2$, 1 mM EGTA and taxol, and centrifuged again. The pellet was resuspended in 100 μl buffer (termed the 'microtubule fraction'). Tubulin fraction, microtubule fraction or 100 μl of buffer were added to 100 μl of solubilized membrane preparation. Taxol, to a final concentration of 10 μM, was then added to all three tubes and these were incubated for 20 min at 37 °C. The soluble and insoluble materials were separated by centrifugation (100 000 ***g***, 15 min) and analysed by Western blotting.

Electrophoresis and Western blotting

Proteins were separated by SDS/PAGE (10 % gels) as described in [23], and transferred to nitrocellulose sheets [24]. Blots were incubated with primary antibodies, and then incubated with infrared fluorescent secondary antibodies. Sheets were scanned using an Odyssey infrared scanner (Li-Cor), and bands were quantified using Scion Image software.

RESULTS

Membrane acetylated tubulin co-elutes with NKA during molecular exclusion chromatography in the presence of detergent

We showed previously that acetylated tubulin binds to NKA, forming 'acetylated tubulin/NKA complex' [25]. To further characterize this complex, a detergent-solubilized membrane preparation was subjected to gel filtration chromatography, and eluted fractions were analysed by Western blotting.

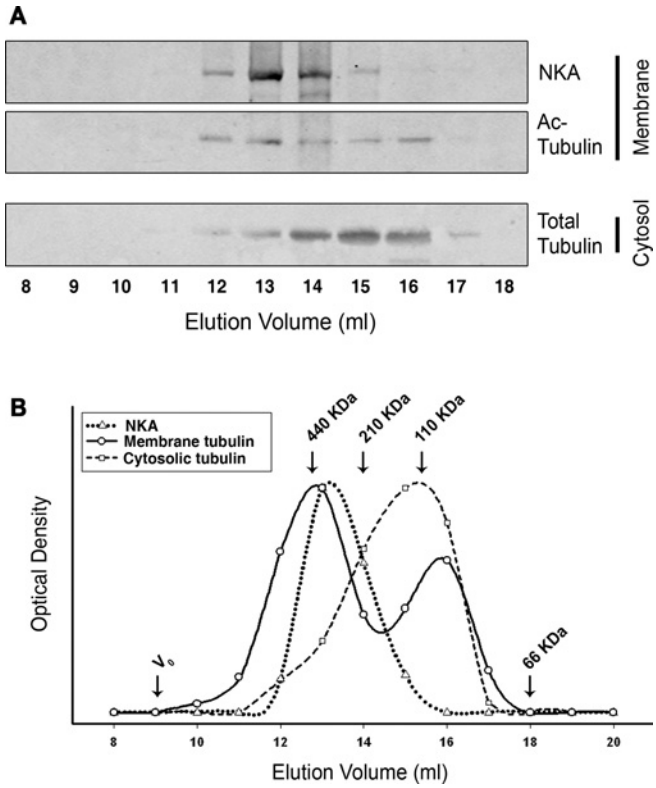


Figure 1 Characterization of tubulin/NKA complex by molecular exclusion chromatography

(**A**) Membrane and cytosolic preparations obtained from rat brain (see Experimental section) were treated with detergent and subjected to molecular exclusion chromatography. Fractions, corresponding to different elution volumes, were collected and analysed by Western blot using specific antibodies against total tubulin, acetylated tubulin (Ac-Tubulin) and NKA as indicated. (**B**) The bands indicated in (**A**) were quantified and relative optical densities were plotted as a function of elution volume. Arrows indicate elution volumes of molecular mass standards under the same conditions: ferritin (440 kDa), β-amylase (210 kDa), $\alpha\beta$-tubulin (110 kDa) and BSA (66 kDa). This experiment was repeated twice with identical results.

Acetylated tubulin present in the membrane preparation eluted in two peaks (Figure 1). The first one (~13 ml) partially overlapped with the NKA peak, and the second one eluted at a position similar to that of a cytosolic tubulin preparation run under identical conditions. The fact that tubulin from the membrane preparation eluted at a lesser volume than that from the cytosolic preparation indicates that tubulin is found in the membrane fraction because it is associated with an hydrophobic molecule. Coincidence of this peak with the elution volume of NKA suggested that this protein could be a partner of the complex.

A calibration curve with molecular mass standards (results not shown) run under identical conditions revealed that the elution volume of the first peak corresponds to a molecular mass in the range of 320–400 kDa.

Tubulin binds to CD5 but not to CD1, 2, 3, or 6

Crystallographic structural analysis of NKA [26] shows that its α-subunit includes 10 transmembrane segments and six cytoplasmic domains (CD1–6) (Figure 2A). We tested separately the abilities of these six cytoplasmic domains to interact with tubulin *in vitro*. CD1, 2, and 3 were obtained as recombinant peptides in *Escherichia coli,* and CD5 and CD6 were obtained synthetically (we tried to express CD4, but were unable to obtain significant amounts of the peptide). The peptides were

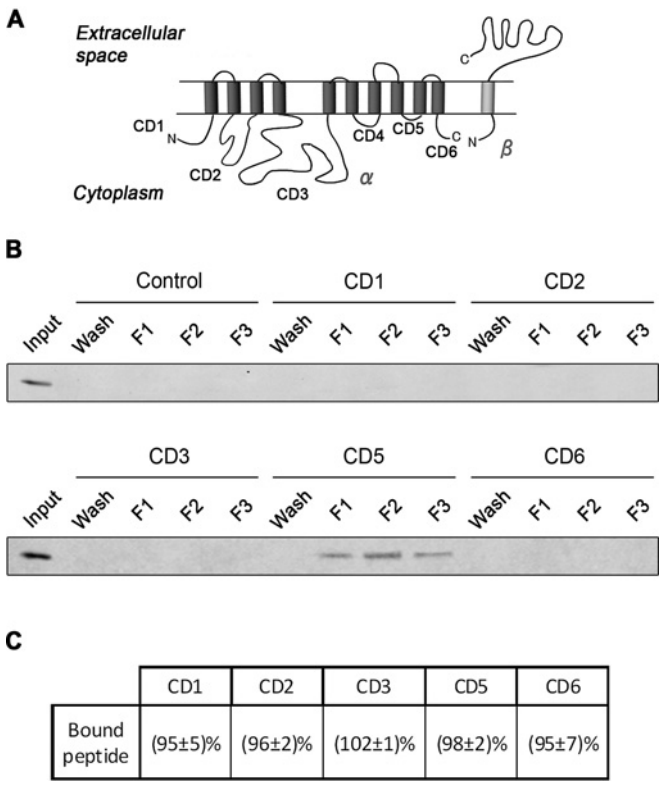


Figure 2 Only CD5 is capable of interacting with tubulin

(**A**) Schematic representation of the topological structure of NKA. CD1–6 are indicated. (**B**) Synthetic or recombinant peptides were covalently linked to Sepharose beads and incubated overnight with an acetylated tubulin-enriched preparation (see Experimental section). After several washes, the retained proteins were eluted by three more washes with 100 μl of a high-pH buffer. Aliquots of the last wash (Wash) and the three elution fractions (F1–3) were analysed by Western blot using an anti-acetylated tubulin antibody. This experiment was repeated four times with similar results. Results from a typical experiment are shown. (**C**) Aliquots of each peptide were taken from the supernatant fraction before and after binding to Sepharose beads and centrifugation. Samples were completely hydrolysed by incubation with 5 M HCl at 90 °C for 24 h and amino acids were quantified by a standard ninhydrin method. The amount of bound peptide was calculated as the difference between total peptide (before binding) and unbound peptide (after binding) and expressed as percentage of the former. Values are mean ± S.D. from three independent experiments.

covalently linked to Sepharose beads, and incubated overnight with a preparation enriched in acetylated tubulin. This preparation was verified to lack NKA by Western blot (results not shown). The resin was washed several times, and the last wash was collected. Based on our previous demonstration that the tubulin/NKA complex is dissociated by high pH [27], we performed three washes with carbonate buffer, pH 12, to elute the retained proteins. Supernatant fractions of these washes were analysed by Western blot. Only CD5 retained tubulin (Figure 2B).

In order to confirm that all peptides were efficiently linked to the Sepharose beads, we measured the amount of each peptide before (total) and after (unbound) incubation with the resin and sedimentation of the beads. Quantification was done by complete hydrolysis and subsequent determination of amino acids by a standard ninhydrin method [28]. As shown in Figure 2(C) more than 95 % of every peptide was covalently linked to the beads.

Only acetylated tubulin interacts with CD5

We showed previously that only acetylated tubulin is able to interact with NKA [17]. To determine whether this specificity also applies to CD5, we used this fragment bound to Sepharose

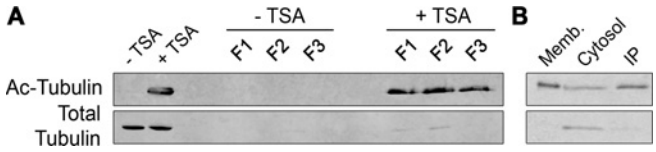


Figure 3 CD5 selectively binds acetylated tubulin

(**A**) Confluent CAD cells (in three 100-mm-diameter Petri dishes) were cultured with or without 15 μM TSA (+TSA or -TSA) for 13 h, washed twice with PBS, and harvested in 1.5 ml of 50 mM Tris/HCl buffer, pH 7.5, containing 150 mM NaCl. The suspension was sonicated and centrifuged at 100 000 ***g*** for 30 min (4 °C). The supernatant was used as input material for standard pull-down procedure using Sepharose-conjugated CD5. Identical results were obtained in three independent experiments. (**B**) A membrane preparation (Memb.), a non-enriched cytosolic tubulin preparation (Cytosol), obtained as described in the Experimental section, and an immunopurified acetylated tubulin fraction (IP) were subjected to Western blot using anti-acetylated tubulin (upper panel, Ac-Tubulin) and anti-total tubulin (lower panel, Total Tubulin) antibodies. Immunopurification of acetylated tubulin was performed as for the pull-down experiments, except that 75 μl of acetylated tubulin-specific antibody (~5 mg total protein/ml) was conjugated to the Sepharose beads. After incubation with tubulin preparation and several washes, bound proteins were eluted with 100 μl of sample buffer and analysed by Western blot.

beads and repeated the same pull-down assay shown in Figure 2, but this time with a preparation of tubulin isolated from CAD cells. This cell line has little or no acetylated tubulin, unless the culture is treated with TSA, a human HDAC6 (histone deacetylase 6) inhibitor. HDAC6 is a member of the histone deacetylase family [29–32], which is known to associate with microtubules and deacetylate tubulin [32–35]. Treatment with TSA therefore significantly increases the amount of the acetylated isoform. When tubulin from non-treated CAD cells was used in the pull-down experiment, no interaction was observed (Figure 3A). In contrast, when tubulin from TSA-treated cells was used: (i) a significant amount remained associated with CD5–Sepharose beads (Figure 3A) and; (ii) the ratio of acetylated tubulin to total tubulin in fractions F1, F2, and F3 was much higher than the ratio in the original tubulin preparation. This indicates that CD5 recognizes mainly (or exclusively) the acetylated isoform of tubulin.

The ratio of acetylated to total tubulin obtained with the fractions eluted in Figure 3(A) was very similar to that obtained when a tubulin preparation was immunopurified using an acetylated tubulin-specific antibody (Figure 3B). The relative amount of acetylated tubulin in a membrane preparation (Figure 3B) was comparable with that in the purified preparation, whereas this ratio was very low in a preparation of cytosolic tubulin (Figure 3B). Thus CD5 interacts preferentially or exclusively with acetylated tubulin, enriching that isoform to a degree similar to that of a membrane preparation or immunopurified acetylated tubulin preparation.

Association of acetylated tubulin with CD5 is prevented by the presence of full-length NKA

The question arose as to whether CD5 and full-length NKA interact with the same motif of the tubulin molecule. If this were the case it would be expected that the interaction between acetylated tubulin and CD5–Sepharose beads would be inhibited by the presence of full-length NKA during pull-down experiments. By using membranes from CAD cells, solubilized with detergent, we found that the amount of acetylated tubulin bound to CD5–Sepharose beads was significantly reduced when a detergent-solubilized membrane preparation was added to the beads following incubation with tubulin (Figure 4A). Similar results were obtained when the solubilized membrane preparation was added simultaneously with tubulin (Figure 4B).

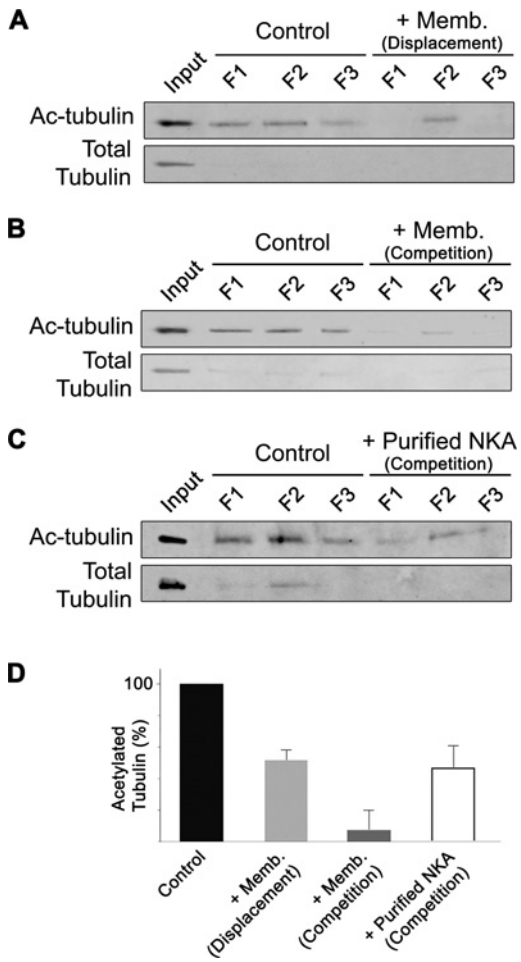


Figure 4 Interaction between CD5 and acetylated tubulin is inhibited by detergent-solubilized membranes

(**A**) Displacement experiment. CD5–Sepharose beads were incubated overnight at 4 °C in the presence of the tubulin preparation enriched in acetylated tubulin (Ac-tubulin). Then, 100 μl of detergent-solubilized CAD membranes (+Memb.) or buffer containing 1 % Triton X-100 (Control) were added and further incubated for 1 h at 0 °C. Beads were further processed for the pull-down assay as described in the Experimental section. (**B**) Competition experiment. 100 μl of tubulin preparation enriched in acetylated tubulin was incubated with an equal volume of detergent-solubilized CAD membranes (+Memb.) or with buffer plus 1 % Triton X-100 (Control) for 1 h at 0 °C. The preparation was then added to CD5–Sepharose beads and incubated overnight at 4 °C. Beads were subsequently processed as for the displacement experiment. (**C**) An experiment similar to that shown in (**B**) was performed, except that a partially-purified NKA preparation (4 mg of total protein per ml, solubilized in 1 % Triton X-100) was used instead of CAD membranes. (**D**) Quantification of bands in the displacement and competition experiments. Optical densities of the three eluted fractions were added, and expressed as percentage of the control. Values shown are mean ± S.D. from three independent experiments.

Membranes from CAD cells were used in these experiments because they lack acetylated tubulin/NKA complex due to the absence of acetylated tubulin in these cells [17]. However, as the detergent-solubilized membrane fraction is a complex, non-purified NKA preparation, we repeated the competition experiment using a partially purified, commercially available NKA preparation. Under these conditions, similar results were obtained (Figure 4C).

These results indicate that full-length NKA does not only compete with CD5 for acetylated tubulin, but also displaces CD5 from its interaction with acetylated tubulin already bound to the resin.

Acetylated tubulin interacts directly with CD5

Although CD5 does not require any other membrane component to bind acetylated tubulin, it is conceivable that other cytosolic protein(s) present in the tubulin preparation could mediate the interaction. To determine whether the association between CD5 and tubulin is direct, we performed a two-step cross-linking experiment using EDC [36]. This procedure allows activation of CD5 prior to the association step. Once CD5 is activated, the tubulin preparation is added, and the affinity interaction is established. Due to the proximity of the molecules, CD5 reacts chemically with its partner, resulting in a covalent cross-linked adduct of the two peptides.

When the reaction products were analysed by Western blot using a polyclonal CD5-specific antibody produced in our laboratory (see Experimental section), a single band was observed (Figure 5A, top panel). When a tubulin preparation enriched in acetylated tubulin was used, the same band with similar intensity was found, even though the total amount of tubulin was much lower (Figure 5A, middle and bottom panels). Thus cross-linking efficiency depends not on the amount of tubulin present in the preparation, but on the amount of the acetylated isoform. Controls without CD5 or tubulin preparation gave no visible band in Western blot using the anti-CD5 antibody. To confirm the specificity of the anti-CD5 antibody, we pre-incubated the antibody with an excess of CD5 prior to Western blotting. The immunoreactivity was greatly reduced (Figure 5B). Figure 5(C) shows the merged image when the blot in Figure 5(A) (top panel) was further incubated with the acetylated tubulin-specific antibody. The CD5 band does not overlap exactly with the acetylated tubulin band, but is located immediately above it. This is most likely due to a slight increase in the molecular mass of acetylated tubulin produced by linkage with CD5 (molecular mass = 2.04 kDa).

In order to confirm that CD5 cross-links to tubulin and not to another 55 kDa protein present in the tubulin sample, we performed an experiment under identical conditions using a 90 % purified tubulin preparation. As shown in Figure 5(E), the use of this preparation produced similar results. As a negative control, we performed the same experiment using Myc-tagged recombinant peptides corresponding to CD2 and CD3. Figure 5(F) shows that none of these peptides was covalently bound to tubulin.

Taken together, these results indicate that CD5 interacts directly with tubulin, i.e. no intermediate protein is required. To further characterize the interaction between acetylated tubulin and CD5, we repeated the cross-linking experiment shown in Figure 5(A), but this time using a tubulin preparation pre-heated for 10 min at 85 °C. The amount of cross-linked product was significantly reduced (Figure 5D). We also tested whether the interaction of CD5 with acetylated tubulin could be established after the tubulin preparation was subjected to SDS/PAGE and transferred to a nitrocellulose sheet. The nitrocellulose was then incubated with CD5 activated with cross-linking agent and washed several times. No band was observed when the nitrocellulose was reacted with the anti-CD5 antibody (results not shown). These results indicate that tubulin must be in its native state in order to interact with CD5.

NKA co-sediments with microtubules assembled *in vitro*

A question arose as to whether NKA is able to associate only with dimeric tubulin or if it can also interact with microtubules. If NKA were able to interact with tubulin that is forming part of a microtubule, cytoplasmic microtubules within the cell could conceivably interact with membranes through their association with NKA.

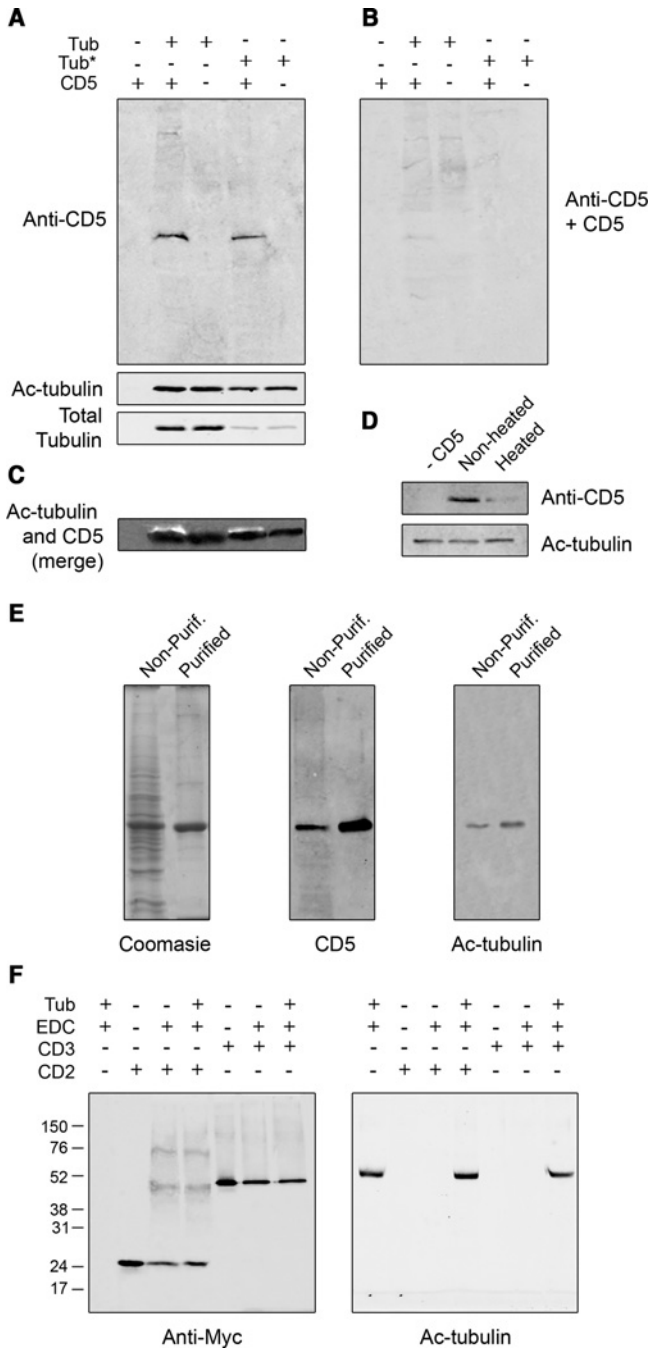
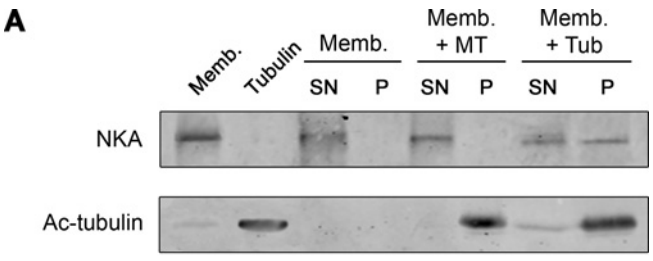


Figure 5 CD5 binds directly to acetylated tubulin

(**A**) CD5 was activated with EDC and NHS as described in the Experimental section, and added to an acetylated tubulin-enriched preparation (Tub*) or to a non-enriched tubulin preparation (Tub). After the reaction was stopped, aliquots were subjected to Western blot using antibodies against acetylated tubulin (Ac-tubulin), total tubulin or CD5. Control experiments were performed in the absence of CD5 or tubulin preparation. (−) and (+) indicate the absence and presence of each component in the reaction mixture respectively. (**B**) A Western blot was performed as in (**A**), except that the anti-CD5 antibody was pre-incubated at room temperature for 15 min with 20 μg/ml CD5. (**C**) A merge of the blot shown in (**A**). CD5 is shown in white, and acetylated tubulin in black. (**D**) The experiment was performed with a tubulin preparation incubated (Heated) or not (Non-heated) at 85 °C for 10 min. A control lacking CD5 (-CD5) is also shown. (**E**) The cross-linking reaction was repeated using a purified (Purified) or a non-purified (Non-Purif.) tubulin preparation. Products were analysed by Coomassie Brilliant Blue staining and by Western blot using anti-CD5 and anti-acetylated tubulin antibodies as indicated. (**F**) The standard reaction protocol was performed using the Myc-tagged peptides CD2 and CD3. Reaction products were subjected to Western blot using anti-Myc and anti-acetylated tubulin antibodies. The absence or presence of each reactant is indicated by (−) and (+) respectively.



B

	Memb.		Memb.+ MT		Memb.+ Tub	
	SN	P	SN	P	SN	P
NKA	(98±7)%	(6±8)%	(88±6)%	(4±3)%	(42±8)%	(51±9)%
Ac-tubulin	ND	ND	(1±4)%	(96±7)%	(13±9)%	(103±4)%

Figure 6 NKA associates with growing microtubules and not with pre-assembled microtubules

(**A**) Detergent-solubilized membranes were incubated with taxol alone (Memb.), with pre-assembled taxolated microtubules (Memb.+MT), or with soluble tubulin plus taxol (Memb.+Tub) as described in the Experimental section. After incubation, samples were centrifuged, and soluble (SN) and insoluble (P) materials were analysed by Western blot to visualize NKA and acetylated tubulin (Ac-tubulin). (**B**) Optical densities of bands from three independent experiments were quantified and expressed as percentages ± S.D. with respect to the sum of the SN and P values. ND, not detectable.

We first tested whether NKA is able to interact with microtubules assembled *in vitro*. A detergent-solubilized membrane preparation was incubated with taxol pre-assembled microtubules, and centrifuged to collect the insoluble material. Taxol is a microtubule stabilizer. Almost all NKA remained in the supernatant fraction, while microtubules were completely sedimented, indicating that NKA did not associate with microtubules (Figure 6). In contrast, when detergent-solubilized membranes were already present in the incubation system, and assembly of microtubules was initiated by the addition of taxol, nearly half of NKA was found in the sedimented fraction (Figure 6). These results indicate that NKA can associate with microtubules provided that they are in a growing process.

NKA is associated with native microtubules in rat brain tissue

Although NKA associates with microtubules *in vitro*, the same might not be true in living cells. To clarify this point, we isolated native microtubules from rat brain homogenized in the presence of Triton X-100 and tested whether this preparation contained NKA. Homogenization was performed at a high ratio of buffer volume to brain mass in order to avoid, or reduce, interaction of membranes with native microtubules during the *in vitro* procedure and to avoid *in vitro* tubulin polymerization (Figure 7A). NKA was found to be present in the sedimented native microtubule fraction (Figure 7B, top panel) suggesting its association with microtubules. Its presence in this fraction is not due to membrane contamination since calnexin (another integral membrane protein) was shown to be absent (Figure 7B, middle panel). As a positive control for the calnexin antibody, a sample of membrane fraction isolated from rat brain as described in the Experimental section was also analysed (Figure 7B).

When the pellet containing native microtubules was treated with taxol and centrifuged again, the amount of NKA in the supernatant was very low (Figure 7C). On the other hand, when

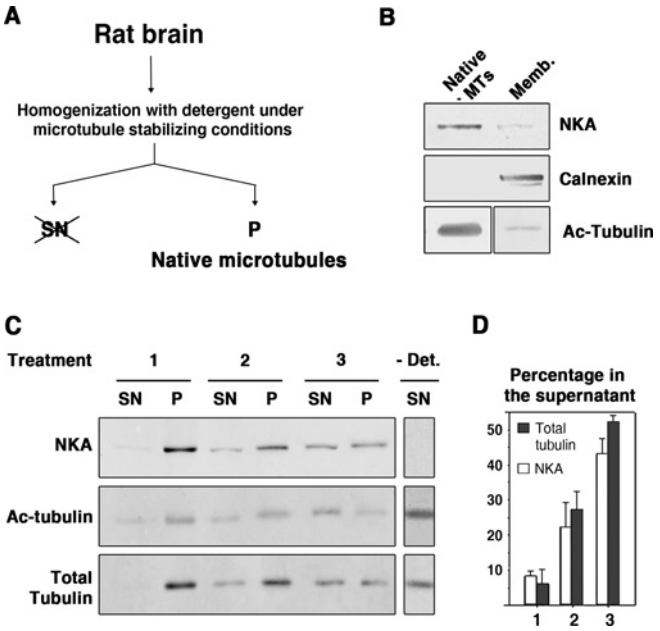


Figure 7 NKA is associated with native microtubules

(**A**) Schematic representation of the experimental protocol. Three rat brains were homogenized (1:15, weight/vol) in microtubule-stabilizing buffer (100 mM Mes buffer, pH 6.7, containing 1 mM $MgCl_2$, 1 mM EGTA, 30 % glycerol and 5 % DMSO) plus 4 % Triton X-100 at room temperature. The homogenate was centrifuged at 30 000 ***g*** for 25 min at 25 °C, and the supernatant (SN) was further centrifuged at 100 000 ***g*** for 40 min at 25 °C. The supernatant of this centrifugation was discarded and the native microtubule-containing pellet (P) was washed twice by resuspension/sedimentation with microtubule-stabilizing buffer (100 000 ***g***, 40 min, 25 °C). (**B**) Western blot analysis of native microtubule preparation (Native MTs) using anti-NKA, anti-calnexin and anti-acetylated tubulin (Ac-Tubulin) specific antibodies. As a positive control for calnexin, a sample of the membrane fraction isolated from rat brain was also analysed (Memb.). (**C**) In a separate experiment, a pellet containing native microtubules (obtained as described above) was washed by resuspension/sedimentation with 100 mM Mes buffer, pH 6.7, containing 1 mM $MgCl_2$ and 1 mM EGTA and finally resuspended in the same buffer at room temperature. The preparation was subsequently separated into three fractions and each one was treated under the following conditions: (1) 10 μM taxol, 60 min at 37 °C; (2) 60 min at 0 °C; and (3) 10 μM nocodazole, 250 mM KCl, and 10 mM $CaCl_2$, 60 min at 37 °C. After incubation, samples were centrifuged at 100 000 ***g*** for 30 min and the soluble (SN) and insoluble (P) materials were subjected to Western blot using the indicated antibodies. In a parallel experiment, native microtubules were obtained following a protocol identical to that described in (**A**), except that homogenization of brains was performed in buffer without Triton X-100. The microtubule fraction was treated as (3) and after centrifugation, a sample of the SN was analysed for NKA, and total and acetylated tubulin (-Det). (**D**) Relative optical densities of bands corresponding to NKA and total tubulin from Western blots shown in (**C**) were measured and the amount of each protein in the SN fractions was expressed as a percentage $\pm$ S.D. with respect to the total (SN plus P fractions). Results are means $\pm$ S.D. from three independent experiments.

the native microtubule fraction was treated with microtubule-depolymerizing agents, NKA was found in the supernatant fraction, similarly to tubulin (Figure 7C). Thus NKA appears to be associated with microtubules, rather than with other sedimentable structures or in another non-specific aggregated state. When native microtubules were obtained under the same conditions, except that Triton X-100 was absent from the homogenization buffer, and processed as described above, final treatment of the pellet with microtubule-depolymerizing agents did not solubilize NKA (Figure 7C). A reasonable explanation is that, in the absence of detergent during homogenization, NKA remained inserted in membranes and therefore could not be solubilized by treatment with microtubule-depolymerizing agents.

Taken together, these results indicate that, in the living cell, NKA is associated with microtubules.

DISCUSSION

As shown in Figure 1, most tubulin contained in membrane preparations behaves as an entity of discrete molecular mass and elutes at a lesser volume than cytosolic tubulin. This means that the presence of tubulin in these preparations is due to its specific association with a hydrophobic component. Furthermore, it can be seen that the tubulin and NKA peaks overlap. This observation together with other results presented in this work and our previously published findings (immunoprecipitation experiments [17], MS [25], detergent partition [14–16] etc.) means that it is highly probable that the increased molecular mass of membrane tubulin, with respect to cytosolic tubulin, is due to its association with NKA. In any case, the complex has a molecular mass of 340–400 kDa which rules out the possibility of a non-specific aggregate of proteins and/or lipids, and is consistent with the association of a tubulin dimer with the α/β-NKA dimer and, possibly, other low-molecular-mass components.

Other P-type ATPases (H^+-ATPase and Ca^{2+}-ATPase), in addition to NKA, are known to interact with acetylated tubulin ([37,38], reviewed in [39]). The fact that each type of acetylated tubulin/ATPase complex is dissociated by a different effector (L-glutamate for NKA, glucose for H^+-ATPase and ethanol or calmodulin for Ca^{2+}-ATPase) tends to rule out the possibility that these complexes are *in vitro* artifacts. Our present results provide new evidence along this line. Isolation of native microtubules from rat brain tissue by homogenization, at a high volume buffer/tissue ratio, using a microtubule-stabilizing buffer containing detergent, resulted in a significant amount of NKA associated with microtubules (Figure 7). This association is unlikely to have occurred during the isolation procedure, since a large volume of buffer was used for homogenization and NKA concentration was therefore very low. Furthermore, the microtubule stabilizing conditions used during homogenization, and the low concentration of dimeric tubulin (below the critical concentration for polymerization), precluded growth of native microtubules, which seems to be a prerequisite for NKA association (Figure 6).

We demonstrated previously that association of acetylated tubulin with NKA causes inhibition of its enzymatic activity, concomitantly with reduced potassium influx, in living cells [14]. We now show that NKA may function as an anchoring site for microtubules to the plasma membrane. This is an important finding because it allows the possibility of 'cross-talk' between the microtubule cytoskeleton architecture and dynamics, and regulation of NKA activity, i.e. microtubules may regulate NKA activity, and conversely the activation state of NKA may influence structure of the microtubule network. NKA has recently been shown to act as an ouabain-specific receptor and as an activator of signal cascades [7]. On the basis of our observations, it is possible that microtubules are involved in such signal transduction events.

Moreover, NKA has been shown to interact with other cytoskeleton-interacting proteins such as ankyrin [11,12] and cofilin [40]. The former is an adaptor protein that mediates the attachment of integral membrane proteins to the spectrin–actin-based membrane skeleton, and the latter is an actin–binding protein that regulates the assembly and disassembly of actin filaments. Based on our present findings, it is conceivable that NKA might function as a scaffolding protein providing the physical interactions necessary for the coupling of the tubulin and actin cytoskeletons and tethering them to the plasma membrane.

Another important aspect of the association between microtubules and NKA is that the interaction requires tubulin to be acetylated at the ε-amino group of Lys^{40} of the α-chain [17]. However, X-ray diffraction structural analyses suggest that this

acetyl group is located facing the microtubule lumen [41,42]. There are several possible explanations for these seemingly contradictory findings. One possibilty is that NKA can interact only with the tip of microtubules, where the lumen is exposed to the cytoplasm. This is consistent with our observation that only dynamically growing microtubules are capable of associating with NKA (Figure 6). Because previously assembled microtubules contain very few tips, there is no significant association of NKA. If assembly is initiated in the presence of NKA, this enzyme can continuously associate with the microtubules ends as they grow. A second possibility is that association occurs while tubulin is in its heterodimer form, with the acetyl group still exposed, and the complex is then able to assemble into a microtubule. A further possibility is that acetylated Lys^{40} of the α-chain is not in the interaction site with NKA, but causes a conformational change of tubulin which is necessary for the association.

Whatever the case, it is clear that CD5 acts as an acetylated tubulin-binding domain. The following observations support this idea: (i) CD5 alone, i.e. in the absence of other membrane components and NKA domains, is capable of interacting with tubulin in pull-down experiments (Figure 2); (ii) interacting tubulin consists mainly or exclusively of the acetylated isotype (Figure 3); and (iii) after covalent cross-linking and SDS/PAGE, CD5 migrates in a single band which is located slightly above the acetylated tubulin band (Figure 5). The latter observation also indicates that the interaction occurs in a direct manner. Thus CD5 is sufficient for sustaining the interaction, and acts as an acetylated tubulin-binding domain.

The fact that full-length NKA prevents the interaction between acetylated tubulin and CD5 (Figure 4) indicates that they both bind to the same site of the tubulin molecule. This observation, along with the finding that CD1, 2, 3 and 6 failed to interact with tubulin in the pull-down experiments (Figure 2) strongly support the idea that NKA interacts with tubulin through CD5. Furthermore, as shown in Figure 3, CD5 binds to tubulin only if it is acetylated at Lys^{40}, which is also required for full-length NKA to bind tubulin in cultured cells [17]. However, despite the suggestive results mentioned above, we have no direct evidence that NKA interacts with tubulin through CD5. In fact, we cannot rule out the possibility that additional interactions with other NKA domains might occur. The finding that tubulin must conserve its native conformation in order to interact with CD5 (Figure 5D) indicates that the association requires a specific structure, and it is not a mere electrostatic interaction due to the basic nature of CD5 and the acidic nature of tubulin.

Since tubulin must be acetylated in order to interact with NKA, we considered the possibility that the sequence of amino acids surrounding Lys^{40} might be involved in the interaction. However, the 11-amino acid peptide containing acetylated Lys^{40} ($QMPSDK^{acetyl}TIGGG$) did not interact with full-length NKA in pull-down experiments (results not shown), supporting the idea that a specific three-dimensional structure of tubulin is essential for the interaction.

The acetylated tubulin-binding domain on NKA (CD5) is located at some distant from the actuator, phosphorylation and nucleotide-binding domains. It is therefore unlikely that these domains could interfere with the association of tubulin and CD5. This domain, along with the C-terminal tail and transmembrane segment 5, may be involved in optimizing the binding of Na^+ [26], which would explain why binding of tubulin inhibits the enzymatic activity of the pump. The interaction could also sterically impair the conformational transition necessary for ion transport.

Given the importance of NKA activity and the structure of the microtubule network, and the possible implications of their interaction, it is not surprising that the tubulin-binding domain involved in the association is highly conserved among different species. Our group find CD5 is nearly totally conserved not only in mammals, but also in birds, amphibians, fish, and even insects (results not shown), indicating that the interaction between NKA and microtubules is presumably essential for cellular physiology, appeared early in animal evolution, and has changed little since then.

The present results support our previous finding [17] that only acetylated tubulin is capable of interacting with NKA. Thus association with and inhibition of NKA is a specific function of acetylated tubulin. This is the first well-established role of a tubulin post-translational modification, and CD5 is the first acetylated tubulin-binding domain to be identified.

AUTHOR CONTRIBUTION

Guillermo Zampar carried out most of the experiments. María Chesta contributed with her expertise on CAD cells and immunofluorescence experiments. Agustín Carbajal produced recombinant cytoplasmic fragments of the sodium pump. Natalí Chanaday and Nicolás Díaz produced the anti-CD5 antibody. César Casale contributed with his knowledge on theoretical aspects of the sodium pump and its interaction with tubulin. Carlos Arce designed the experiments, supervised the experiments and wrote the manuscript.

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