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Identification of a 48-kDa prenylated protein that associates with microtubules as 2′,3′-cyclic nucleotide 3′-phosphodiesterase in FRTL-5 cells

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Abstract In an effort to study the nature of tubulin attachment to membranes, we have previously observed that after blocking prenylation in FRTL-5 thyroid cells, the microtubules become disconnected from the plasma membrane region [Bifulco M. et al. (1983) J. Cell. Physiol. 155, 340–348]. In this study we show that several [^{3}H]mevalonate labeled proteins in FRTL-5 cells associate with membrane and cytoskeleton and, among these, we describe the presence of a 48-kDa prenylated protein, identified by immunoprecipitation as 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), that associates with microtubules. This latter association persists through several polymerization/depolymerization cycles, whereas other prenylated proteins are lost. It is suggested that CNP can be a novel microtubule-associated protein (MAP) and a promising candidate as a membrane anchor for microtubules.

Key words: 2′,3′-Cyclic nucleotide 3′-phosphodiesterase; Microtubule-associated proteins; Prenylation; FRTL-5 thyroid cells

1. Introduction

The cytoskeleton is involved in numerous cellular functions such as secretion, shape changes and migration, ingestion in simple eukaryotes, mitosis and cytokinesis, and cell-cell contacts. In many cases these functions require anchorage of the cytoskeleton to the membrane. This could occur by direct insertion of the microtubules into the membrane, by association of microtubules with an intrinsic membrane protein, or association of microtubules to the plasma membrane through a linker protein. While the mechanisms for such interactions are known for actin microfilaments, the details of microtubule-membrane interactions are less clear [1–4]. Ample cytological evidence exists for lateral and end-on microtubule contacts with plasma and other cellular membranes [5–7]. In some cases contacts appear to be very close, whereas in others, a clear-cut space between microtubules and membranes can be observed containing bridges or linkers that appear to attach the two structures. It is reasonable to suppose that these bridges contain connector proteins attached at one end to microtubules and at the other to the membrane or the membrane cytoskeleton as occurs with actin fibers and its linkers. Dyneins and kinesins connect intracellular membranes with microtubules, but few, if any, linker proteins to cellular membranes have been identified and the nature of the forces leading to attachment is not known.

A possible clue was provided in a recent study [8], wherein it was shown that after blocking prenylation of proteins with lovastatin in FRTL-5 thyroid cells, microtubules become disconnected from the plasma membrane region and retract towards the cytosol. Because prenylation often facilitates protein attachment or translocation to the membrane [9–11], we reasoned that prenylation might be involved in the attachment of microtubules to the cell periphery or membrane. Because tubulin does not contain the carboxyl-terminal 'CaaX' (cys-aliphatic-aliphatic-any amino acid) sequence motif required for prenylation, this would presumably have to be mediated by a prenylated linker protein that does. In the present study we describe the presence of a 48-kDa prenylated protein in FRTL-5 cells that associates with membranes and microtubules, and that it is the well known enzyme 2′,3′-cyclic nucleotide 3′-phosphodiesterase (E.C.3.1.4.37) or CNP.

2. Materials and methods

2.1. Cells

FRTL-5 cells (ATCC CRL-8305) are a strain of rat thyroid cells, cloned and characterized in our laboratory by Dr. F.S. Ambesi-Impiombato, whose characteristic and culture conditions have been extensively described [8,12]. They were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and a six-hormone mixture of thyrotropin, insulin, hydrocortisone, transferrin, somatostatin, and glycil-L-histidyl-L-lysine acetate. This mixture will be referred as 6H. C6 Glioma cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

2.2. Materials

Lovastatin was a gift from Dr. Alberts of the Merck, Sharp and Dohme Inst. Taxol was provided by the National Cancer Institute, USA. (RS)-[5-^{3}H]Mevalonate (MVA) (35.0 Ci/mmol) was from NEN DuPont. The rabbit autoantibody against CNP1 was a generous gift from Dr. D. Jacobowitz, NCI Bethesda Md, USA [13]. Rat brain microtubule protein was prepared by sequential polymerization as previously described [14].

2.3. [^{3}H]Mevalonate labeling of prenylated proteins

FRTL-5 cells were preincubated with 10 μM lovastatin for 5 h, washed, and incubated with 30 μCi/ml [5-^{3}H]mevalonate for 16 h.

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Abbreviations: CNP1 and 2,2′,3′-cyclic nucleotide 3′-phosphodiesterase 1 and 2; MAP, microtubule-associated proteins; MVA, mevalonate; pCNP7, CNP1 cDNA; PBS, phosphate buffered saline; Pipes, piperazine *N*,*N*-bis(2-ethane sulfonic acid); PMSF, phenylmethyl sulfonyl fluoride; DTT, dithiothreitol

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Cells were then washed 3 times in PBS, scraped from the dish and lysed in hypotonic buffer. Equal amounts of each protein extract were analysed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described [15,16].

2.4. Cell fractionation

Total cellular homogenate was centrifuged at 100 000×*g* at 4°C for 1 h to obtain a supernatant (S100) and a pellet (P100) fraction.

Cells were extracted at room temperature with 1% (v/v) Triton X-100 in a microtubule-stabilizing buffer (0.1 M Pipes, 1 mM $MgCl_2$, 2 M glycerol, 2 mM EGTA, 1 mM PMSF and 1mM DTT, pH 6.9) to obtain a detergent soluble (SOL) and an insoluble cytoskeletal fraction (CSK) as described [17].

2.5. Microtubule purification

2.5.1. Taxol procedure. FRTL-5 cells labeled with [^{3}H]MVA were scraped from the washed dishes, collected and centrifuged at 4°C, 2000 rpm resuspended in 0.1 M Pipes, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, pH 6.6 and homogenized and processed with 20 μM taxol as described in [18].

2.5.2. Copolymerization with carrier brain microtubule protein. Total cellular lysate from [^{3}H]MVA-labeled FRTL-5 cells was mixed with unlabeled rat brain microtubule protein [19] and carried through cycles of temperature-dependent polymerization/ depolymerization [18]. In each cycle the microtubule pellets were analyzed by 12% SDS-PAGE. The gels were fluorographed or sliced into 2-mm sections, and the distribution of the major proteins-associated radioactivity was determined by scintillation counting following digestion of each slice as previously described [19].

2.6. Immunoprecipitation and SDS-PAGE

[^{3}H]MVA labeled third cycle of polymerization, obtained from labeled FRTL-5 cell homogenate mixed with rat brain microtubule protein as described before, was solubilized in RIPA buffer [20 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet P40, 0.5% (w/v) Na deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Trasylol, 0.2 mM PMSF, pH 7.4] and immunoprecipitated with preimmune rabbit serum or rabbit autoantibody against CNP1 at 4°C for 18 h, followed by incubation with Protein A-Sepharose (Pharmacia). Immunoprecipitates were washed five times with RIPA buffer and then dissolved in Laemmli loading buffer with 1 mM DTT prior to be electrophoresed in a 12% SDS-PAGE [20]. Gels were then permeated with Amplify fluorographic enhancer (Amity), dried and autoradiographed at −80°C.

2.7. Total RNA preparation and Northern blot analysis

Total cellular RNA was extracted by the guanidinium thiocyanate-acid phenol procedure and quantitated by spectrophotometry as previously described [21]. Twenty micrograms of total RNA from each cell strain were loaded and separated on 1% agarose gels containing 2% formaldehyde and blotted onto nylon membranes (Hybond-N Amersham). Prehybridization (1 h at 65°C), hybridization (16–18 h at 65°C) and high stringency washes (15 min at 37°C, 1 h at 60°C, 1 h at 55°C) were carried out as already reported [21]. The cDNA, pCNP7 (kindly provided by Dr. Braun and De Angelis, Montreal, Canada) was used as radiolabeled probe [22]. Normalization was accomplished using radiolabeled glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr. V.E. Avvedimento, Napoli, Italy) as reference probe.

2.8. Other assays

Protein concentration was determined using a Bio-Rad kit; recrystallized bovine serum albumin was the standard.

3. Results

3.1. [^{3}H]MVA-labeled proteins in FRTL-5 cells

When quiescent control FRTL-5 cells (NoH, i.e. cells maintained for 72 h in a medium from which the hormone supplement was removed) are labeled with [^{3}H]MVA for 24 h in the presence of 10 μM lovastatin to prevent dilution of the label by endogenous mevalonate, about 12 labeled bands can be identified on a 10% SDS gel. Six of these are in the low molecular weight range here labeled p21, which contain members of the Ras family. Prominent bands also occur at approximately 48 and 56 kDa (Fig. 1). We investigated the association of these prenylated bands with membrane and cytoskeletal fractions. Homogenates from [^{3}H]MVA-labeled FRTL-5 cells were partitioned into a 100 000×*g* pellet (P100) and supernatant solution (S100). This revealed similar distributions of the p21 group and 48-kDa prenylated proteins, whereas the 56-kDa protein was found primarily in the supernatant fraction (Fig. 1). Extraction of the whole homogenate with 1% Triton in a microtubule stabilizing buffer yielded a soluble and insoluble cytoskeleton fraction whose prenylated proteins resembled, respectively, that of S100 and P100 fractions (Fig. 1).

3.2. 48-kDa prenylated protein association with microtubules

Attempts were made to determine if any of the prenylated proteins might associate with tubulin or microtubules. We purified microtubules from homogenates of [^{3}H]MVA-labeled FRTL-5 cells by repeated cycles of polymerization/depolymerization in the presence of 20 μM taxol [18]. In this procedure a microtubule-associated protein (MAP) is defined as copolymerizing with microtubules through several such cycles. As shown in Fig. 2A, the overall specific activity (^{3}H/μg protein) decreased to an approximately constant level, indicating a stoichiometric relation between a labeled protein and tubulin. This suggests that some label proteins remained with the microtubule pellet, whereas most other proteins were lost during repeated cycles of polymerization [19]. If prenylated proteins behave like MAPS, they should co-cycle with brain mi-

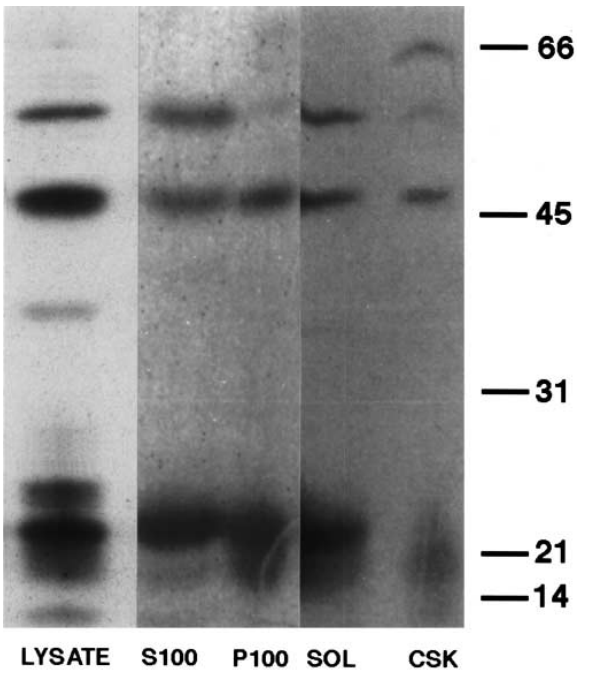

Fig. 1. Pattern of [^{3}H]MVA-labeled proteins in total cellular lysate, S-100 and P-100 fractions, cytoskeleton (CSK) and soluble (SOL) fractions from FRTL-5 thyroid cells. Cells were preincubated with 10 μM lovastatin for 5 h, washed, incubated with 30 μCi 5-[^{3}H]MVA for 16 h and then processed as described in Section 2. A portion of each fraction (90 μg of proteins) was subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Molecular weight standards are shown on the right (in kDa). The autoradiogram was exposed for 15 days.

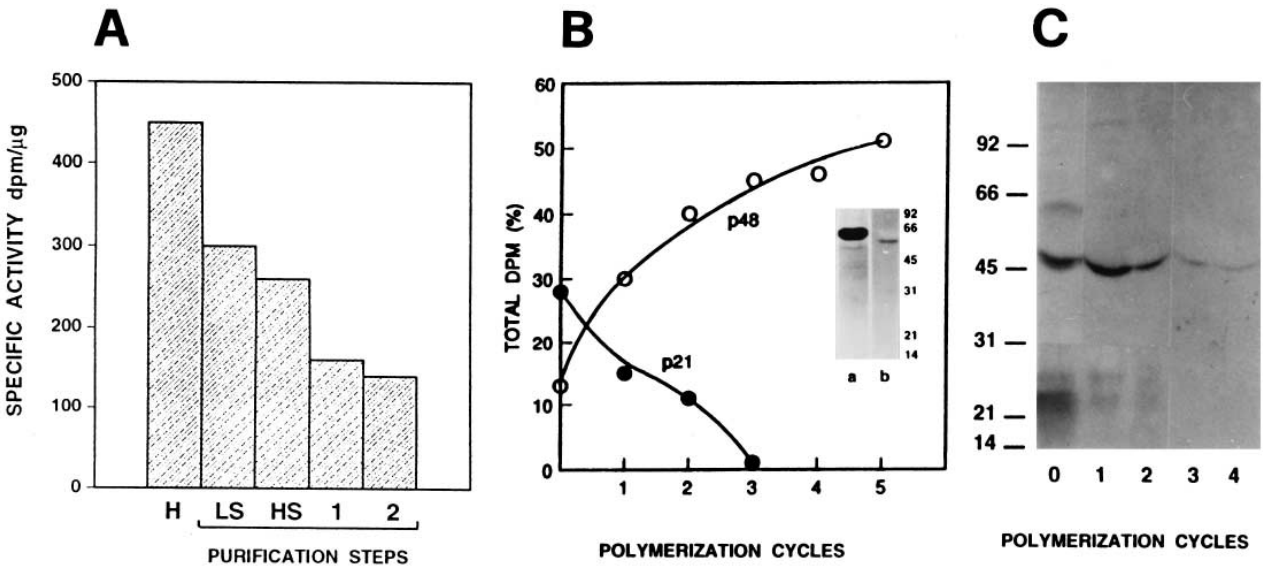

Fig. 2. Purification of microtubules from FRTL-5 cells. (A) [^{3}H]MVA-labeled proteins from FRTL-5 cells were processed as for the isolation, by taxol procedure, of tubulin and microtubule-associated proteins. An aliquot of each fraction was measured for radioactivity and protein content, and results are expressed as dpm/μg total protein. H, homogenate; LS, low speed supernatant; HS, high speed supernatant; numbers 1 and 2 refer to polymerization cycles. Bars represent the average of triplicate experiments. (B) and (C) Copolymerization of [^{3}H]MVA labeled proteins from FRTL-5 cells with carrier rat brain microtubule protein. Total cellular lysates prepared from [^{3}H]MVA labeled cells were mixed with unlabeled rat brain microtubule protein (MTP, 18 mg/ml) and carried through several cycles of polymerization/depolymerization. Equal amounts of proteins (50 μg) were analyzed by 12% SDS-PAGE. (B) The percent of the radioactivity in gel slices of the major bands, 48-kDa and 21-kDa group, which contains members of the Ras family, is listed as a fraction of the total counts applied. The insert show the Coomassie stained tubulin (a) and the corresponding autoradiograph (b) from the third cycle of polymerization. (C) Autoradiograph of the pattern of the [^{3}H]MVA-labeled proteins obtained from four consecutive copolymerization cycles, depicted in panel B, exposed for 20 days. Molecular weight standards are shown on the left. These data are representative of three independent experiments.

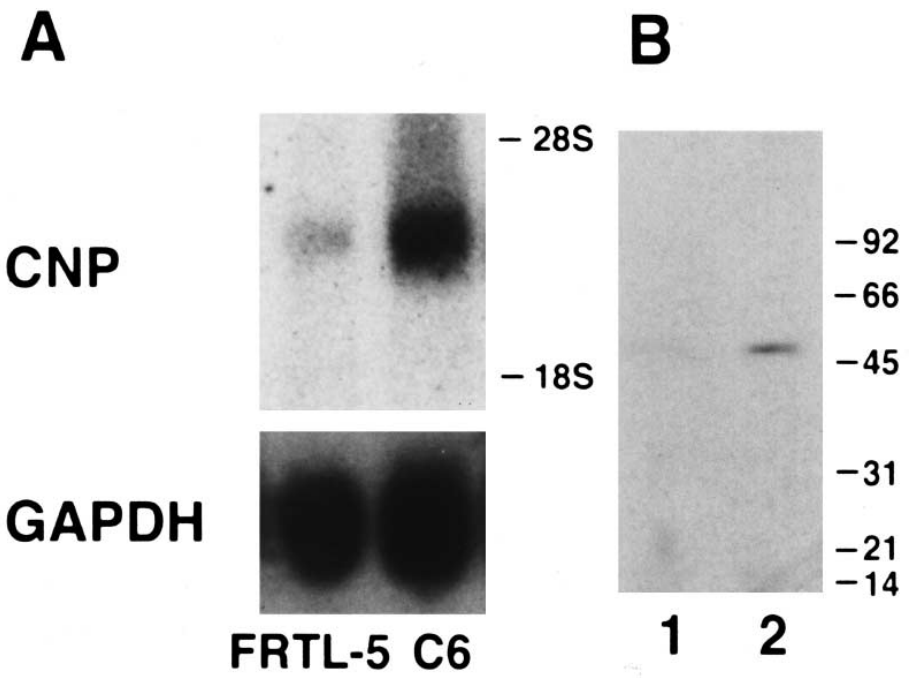

Fig. 3. Expression and immunoreactivity of CNP in FRTL-5 cells. (A) Northern blot analysis of CNP1 mRNA expression in FRTL-5 and C6 cells. Twenty micrograms of total RNA were used in each lane. Top panel, the blot was probed with a CNP1 cDNA. Bottom panel, the blot was stripped and reprobed with a rat glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe, performed as a control that similar amounts of RNA were loaded in each lane. The positions for 28S and 18S are indicated on the right. (B) Immunoprecipitation of the [^{3}H]MVA-labeled 48-kDa protein present in the third cycle of polymeryzation, obtained from labeled FRTL-5 cell homogenate mixed with unlabeled rat brain microtubule protein as described in Fig. 2, with an anti-CNP1 antibody. The immunoprecipitates were subjected to SDS-PAGE. Lanes: 1, rabbit preimmune serum; 2, rabbit anti-CNP1 antibody. Molecular weight standards are shown on the right. Autoradiogram was exposed for 30 days. These data are representative of two independent experiments.

crotubule proteins. To test it, [^{3}H]MVA-labeled FRTL-5 cell homogenate was mixed with unlabeled rat brain microtubule protein and repeatedly carried through five cycles of polymerization, in absence of taxol. As shown in Fig. 2B and C, the ^{3}H associated with the 48-kDa protein present in the microtubule increased progressively, as measured in 12% SDS gels, with each successive cycle of polymerization until the fifth cycle, whereas the p21 proteins decreased progressively and became undetectable after the third one. In the third and fourth polymerization/depolymerization cycles (Fig. 2C), while there was loss of protein, 48-kDa band was the only labeled protein remaining. This suggests that the 48-kDa protein behaves like a MAP, showing a significant association with tubulin [19].

3.3. *Identity of 48-kDa protein as 2′,3′-cyclic nucleotide 3′-phosphodiesterase*

The association of the 48-kDa prenylated protein with the particulate/membrane fraction on the one hand, and with microtubules on the other, led to attempts at identification of this protein. On the basis of prenylation and its gel molecular mass, it seemed possible that it might be 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP), a prenylated and palmitoylated enzyme of uncertain function in the cell that was first described in the liver, but is most abundant in neural tissue [20,23–26]. It is a plasma membrane-associated protein which has previously been suggested to be related in an unspecified manner to the cytoskeleton [17,27–29]. There are two isoforms, CNP1 and CNP2 (46 and 48 kDa), resulting from alternative splicing [25]. In the rat CNP1 is larger in size (~48 kDa) and substantially more abundant than CNP2 [13,30]. However, FRTL-5 cells express significant levels of mRNA for CNP1 in comparison to those present in C6 glioma cells (Fig. 3A), that possess a relatively high rate of CNP synthesis [20].

Finally we confirmed the hypothesis that the 48-kDa prenylated protein could be the CNP1 by an immunoprecipitation experiment [20]. The [^{3}H]MVA-labeled 48-kDa protein comigrating with CNP1, present in the third cycle of polymerization, obtained from labeled FRTL-5 cell homogenate mixed with unlabeled rat brain microtubule protein and processed as described above, was specifically immunoprecipitated with a rabbit anti-CNP1 antibody (Fig. 3B).

4. Discussion

In this study we describe the presence of a 48-kDa prenylated protein in FRTL-5 cells that has the characteristics of CNP. On the basis of the reaction with an antibody that recognizes exclusively CNP1, and the larger size of rat CNP1, we believe that the 48-kDa protein labeled with [^{3}H]mevalonate in these rat thyroid cells is CNP1. Moreover, FRTL-5 thyroid cells express significant levels of CNP1 mRNA. Like many other proteins whose residence in the plasma membrane is determined by post-translational lipid modifications, the partition of CNP between membrane and cytoplasm has been ascribed to isoprenoid modification [20]. CNP is modified by both types of prenyl groups, farnesyl and geranylgeranyl [31], and it is also palmitoylated [26]. Moreover, CNP has an isoelectric point >9 [13,25] which could favor interaction with acidic regions in the membrane or microtubule, although there is no distinct polybasic domain. The prerequisites for association with both membranes and microtubules are, therefore, present in CNP.

We show evidences that CNP associates with microtubules in FRTL-5 cells. Our findings in no way rule out CNP interactions with other proteins of cytoskeleton. In fact, it has been recently reported that CNP binds to actin-based cytoskeletal elements [29]. However CNP, according to our data, behaves like a MAP, showing a significant association with tubulin. Colocalization of CNP and tubulin has also been previously investigated in oligodendroglial membrane sheets [28]. The precise nature of the association of CNP with microtubules and membrane remains to be determined. The prenylated CNP can be the target of lovastatin effect in severing the connection between microtubules and the plasma membrane. Thus CNP can represent an anchor protein involved in the attachment of microtubules to the membrane.

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