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SPECIFIC BINDING OF TUBULIN TO A GUANINE NUCLEOTIDE-BINDING INHIBITORY REGULATORY PROTEIN IN ADENYLATE CYCLASE SYSTEM, N_i

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SUMMARY: A protein was isolated from the soluble fraction of rat brain by affinity chromatography with Sepharose to which guanine nucleotide-binding inhibitory regulatory protein in adenylate cyclase system, N_i, was immobilized. The molecular weight of this protein, specifically bound to the N_i-affinity column, was estimated as 54,000 on sodium dodecylsulfate-polyacrylamide gel electrophoresis. Alternately prepared tubulin also bound to the N_i-affinity column. The amino acid compositions of these proteins were also identical. It is strongly suggested that this N_i-binding cytosolic protein is tubulin.

Evidence has accumulated that guanine nucleotide-binding inhibitory regulatory protein in adenylate cyclase system, (abbreviated as N_i), is involved not only in the regulation of adenylate cyclase system (1) but also in various cell-membrane functions, including phosphoinositide metabolism, Ca^{2+} influx, etc (2,3). Molecular mechanisms of the actions of N_i, however, have not been clarified except for its role in adenylate cyclase system (1).

In the present study, we prepared a N_i-affinity column in search of proteins interacting with N_i. One of major proteins in the soluble fraction of rat brain was specifically retained in the column. From the results obtained here, it is quite likely that this N_i-binding protein is tubulin.

MATERIALS AND METHODS

^{3}H-GppNHp and activated CH-Sepharose were obtained from Amersham and Pharmacia, respectively. All other chemicals were of reagent grade.

Abbreviations : EGTA, ethyleneglycol bis(β-aminoethylether)-N,N,N',N'-tetraacetate; GppNHp, guanylyl-5'imidodiphosphate; N_i, guanine nucleotide-binding inhibitory regulatory protein in adenylate cyclase system; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane.

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N_i was purified from 200 g of frozen bovine brain by a procedure of Sternweis et al. (4) except for the use of phenyl-Sepharose instead of heptylamine-Sepharose in the final step. This hydrophobic chromatography was effective for the purification of Ni. The isolated N_i was assumed to be homogenous from the results of SDS-PAGE and specific activity of GppNHp binding.

The purified N_i was concentrated with DEAE-Sephacel minicolumn (Vt=1 ml). One ml of concentrated N_i (about 30 nmol/ml) was mixed with 132 µl of GppNHp binding mixture and incubated for 2 hr at 30°C. Final concentrations of the reaction mixture were as follows ; 26.5 µM N_i, 17.7 mM Tris-HCl (pH 8.0), 0.9 mM EGTA, 0.9 mM dithiothreitol, 100 mM $MgCl_2$, and 39 µM ^{3}H-GppNHp (4 µ Ci). At the end of the incubation, reaction was stopped by ice chilling. This GppNHp-bound N_i was passed through Sephadex G-25 equilibrated with 0.1 M $NaHCO_3$, 5 mM $MgCl_2$, 0.5 M NaCl, and 0.1 % Lubrol-PX (pH8.0), to separate from Tris-Cl and free GppNHp, which are known to perturb the conjugation with activated CH-Sepharose. Then, 26 nmol of GppNHp-bound N_i were conjugated to 4 g of activated CH-sepharose .

To determine the capacity of this affinity-column, an aliquot of the prepared N_i-Sepharose gel was charged into a mini-column (Vt=0.5 ml). Successive washing of this column with an equilibration buffer consisted of 20 mM Tris-HCl (pH 8.0), 1 mM EGTA, 1 mM dithiothreitol, 11 mM $MgCl_2$, and 100 mM NaCl, eluted no ^{3}H-GppNHp. Elution with 6 M guanidium chloride, resulted in release of 1.32 nmol/ml gel of ^{3}H-GppNHp from the column. This denatured affity-gel could easily be renatured by incubation with equilibration buffer at room temperature within 30 min. Rebindable ^{3}H-GppNHp binding-sites were estimated as more than 0.94 nmol/ml gel.

Microtubule fraction was prepared from rat brain soluble fraction by an assembly-disassembly method of Shelanski et al. (5). SDS-PAGE revealed a major band (mol. wt. 54,000) of tubulin and minor bands (>200,000) of microtubule-associated proteins in this fraction. Tubulin was further purified by DEAE-cellulose column chromatography (6).

Amino acid analysis were performed with a TOYO amino acid analyzer using ortho-phthalaldehyde as the detection reagent (7). Hydrolysis of proteins was effected in 6 N of HCl (constant bioling grade) at 110°C for 20 h in vacuo.

SDS-PAGE was carried out with a 11 % polyacrylamide gel according to the method of Laemmli (8). Protein concentration was determined by the method of Lowry, et al. (9) or by dye staining method with Bio-Rad protein assay kit and by micro-biuret reaction (10) in the case of amino acid analysis.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of proteins in the soluble fraction of rat brain homogenate in the N_i-Sepharose column (Vt=11 ml) chromatography.

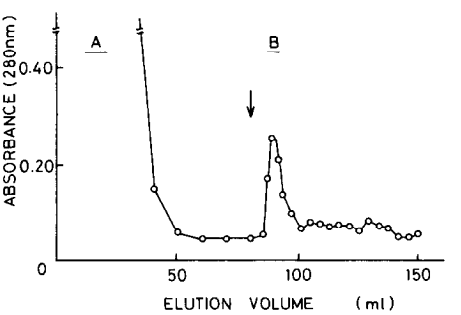

Fig. 1. N_i-affinity chromatography of rat brain soluble proteins. Approximately 120 mg of soluble protein from rat brain was put on N_i-Sepharose column (Vt=11ml). An arrow indicates that the eluant was changed to 0.2 % Lubrol-PX.

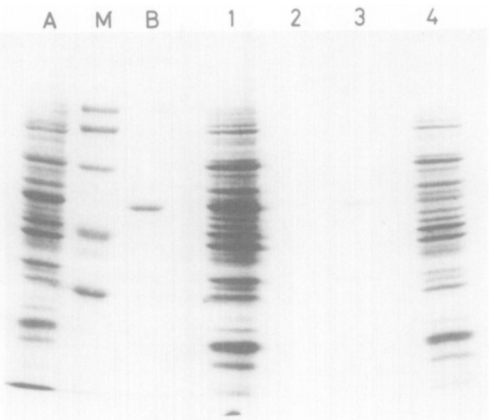

Fig. 2. SDS-PAGE of the N_i-Sepharose eluate. Lanes A and B, fractions A and B in Fig. 1. Fraction A was further rechromatographed on either Sepharose without N_i (lanes 1 and 2) or Ni-Sepharose (lanes 3 and 4). Lanes A, 1, and 4, unadsorbed fractions; and lanes B, 2, and 3, 0.2 % Lubrol-PX eluate fractions. Lane M, molecular weight markers; myosin-H chain (200,000), ß-galactosidase (116,250), phosphorylase-b (92,500), serum albumin (66,200), obalbumin (45,000), and lactic dehydrogenase (30,000)

SDS-PAGE analysis revealed that peak-B in Fig. 1, which was retained in the column and was eluted with 0.2 % Lubrol-PX, was mainly composed of a protein with a molecular weight of 54,000 (Fig. 2, lane 3). Furthermore, when the unadsorbed fraction (peak-A in Fig. 1; corresponding to lane A in Fig. 2) was rechromatographed on the same affinity gel, the protein with the molecular weight of 54,000 was decreased in the unadsorbed fraction of rechromatography (lane 4 in Fig. 2). Thus, it was likely that N_i-Sepharose was saturated with the binding protein in the first chromatography. The capacity of this affinity column was estimated to be 1 - 2 nmol protein/gel, the value being compatible with that of immobilized N_i. From abundance and the molecular weight of the binding protein, we assumed that this binding protein could be tubulin and compared this protein with tubulin prepared from rat brain. The binding protein comigrated with the prepared tubulin on SDS-PAGE(Fig. 3). In a separate experiment, 75 ml of the purified tubulin solution (0.13 µM) was put on a 10 ml of N_i-Sepharose column. Tubulin was eluted out from the column not with the buffer alone, but with 0.2 % Lubrol-PX (data not shown). For the identification of this protein, amino acid composition of this N_i-binding protein (254 ng) which has been further purified through DEAE-sephacel, and

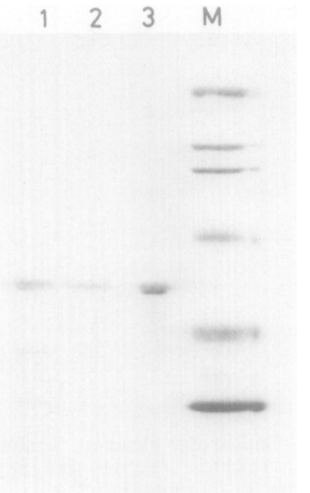

Fig. 3. Comparison of N_i-Sepharose adsorbed protein (molecular weight 54,000) and tubulin. Lanes 1 and 2, the adsorbed protein; lane 3, purified tubulin; and lane M, molecular weight markers.

that of a purified rat brain tubulin were compared (Table 1). Amino acid composition of this protein showed good agreement with that of tubulin from rat brain, and from calf brain reported previously (11). For further identification of the N_i-binding protein as tubulin, colchicine-binding to this protein was examined. But the binding was not observed probably due either to the presence of non-ionic detergent Lubrol-PX (constituent of elution buffer)

Table 1: Amino acid composition of Mr. 54,000 protein

Amino acid	Calf brain tubulin [a]	Rat brain tubulin [b]	Mr. 54,000 protein [b]
		(this study)	
	residues / 54,000		
Aspartic acid	51	50.5	50.3
Threonine	33	30.4	30.3
Serine	29	27.6	28.3
Glutamic acid	63	64.8	67.1
Glycine	39	40.8	46.8
Alanine	36	38.4	38.9
Valine	34	36.6	36.5
Isoleucine	23	24.0	24.0
Leucine	38	39.5	37.7
Tyrosine	15	15.9	16.4
Phenylalanine	19	21.4	22.9
Histidine	13	14.3	14.3
Lysine	20	22.7	24.6

a) Data from reference 11.
b) These values were means of duplicates.

or to high lability of colchicine-binding activity of tubulin. The binding could not be obtained even with the purified tubulin under the same condition. Then, we tried to bind colchicine to the cytoplasmic protein first, and then to separate the colchicine-bound protein by N_i-Sepharose column. But the trial was also unsuccessful. Since the purified colchicine-bound tubulin was neither retained to N_i-Sepharose column, the result seemed to indicate that the colchicine-binding prevented the interaction between tubulin and N_i.

It is not certain if such a binding of tubulin to N_i is actually operating in the cell. It would be of interest, however, if the interaction between the two proteins regulates the function of N_i, or vice versa.

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