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TISSUE AND SUBCELLULAR DISTRIBUTIONS OF AN INHIBITORY GDP/GTP EXCHANGE PROTEIN (GDI) FOR THE *RHO* PROTEINS BY USE OF ITS SPECIFIC ANTIBODY[1]

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We have recently purified from bovine brain cytosol to near homogeneity a GDP/GTP exchange protein for the *rho* proteins, named *rho* GDI, that inhibits the dissociation of GDP from and the subsequent binding of GTP to the *rho* proteins. In the present study, we made a monoclonal antibody against *rho* GDI and studied its tissue distribution in rat and its subcellular distribution in rat cerebrum by use of this antibody. *rho* GDI was found in most rat tissues as described for the *rho* proteins. In rat cerebrum, *rho* GDI was mostly found in the cytosol of neuron body and synaptosome. In synaptosome, it was mainly found in the synaptic cytosol.

A *rho* gene family, composed of three members (-A, -B, -C), belongs to a *ras* p21/*ras* p21-like small G protein superfamily (1-4, for reviews, see Refs. 5,6). Although the functions of the *rho* proteins have not been defined, several lines of evidence suggest that they are involved in the regulation of cell proliferation and differentiation (3,7). Moreover, the purified *rho* proteins have been shown to be ADP-ribosylated by a botulinum ADP-ribosyltransferase C3 (8-11) which has been shown to affect cytoskeletal systems (12-14).

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The abbreviations used are: G protein, GTP-binding protein; GDI, GDP dissociation inhibitor; GDS, GDP dissociation stimulator; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; BSA, bovine serum albumin.

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The *rho* proteins have GDP-bound inactive and GTP-bound active forms. The conversion from the GDP-bound to GTP-bound form and the reverse reaction are regulated by GDP/GTP exchange proteins and GTPase activating protein (GAP) specific for the *rho* proteins, respectively (15-19). We have isolated two types of GDP/GTP exchange protein for the *rho* proteins: one is an inhibitory GDP/GTP exchange protein named GDI and the other is the stimulatory one named GDS (15-17). We have purified GDI to near homogeneity and have determined its primary structure (16,20), but partially purified GDS (17). We have also clarified an additional action of *rho* GDI: it inhibits the binding of the *rho* proteins to membranes and induces the dissociation of the prebound *rho* proteins from the membranes (21).

The *rho* protein mRNAs have been detected in various mammalian tissues (1-4, for reviews, see Refs. 5,6) and the proteins ADP-ribosylated by C3 have been found in various mammalian tissues (8-11). However, since an antibody against the *rho* proteins has not been available, their tissue and subcellular distributions have not been studied. We have previously studied the tissue distribution of the *rho* GDI mRNA and detected it in various rat tissues, but the tissue distribution of the protein molecule has not been studied.

In this paper, we first made a monoclonal antibody against bovine brain *rho* GDI and then studied its tissue distribution in rat and its subcellular distribution in rat cerebrum by use of this antibody.

Materials and Methods

Materials and Chemicals——Female BALB/c mice and adult male rats (200-250 g weight) of a Sprague-Dawley strain were used for the present studies. *rho* GDI was purified from bovine brain cytosol as described (16) and used for immunization and detection of an anti-*rho* GDI antibodies. Mouse hybridoma screening kit, mouse isotyping kit, horseradish peroxidase-labeled goat anti-mouse immunoglobulin, and ^{125}I-labeled anti-mouse IgG (3.7 MBq/ml) were from Amersham Corp. Nitrocellulose sheets (BA-85, pore size: 0.45 μm) were from Schleicher and Schuell. Other materials and chemicals were obtained from commercial sources.

Generation of an Anti-*rho* GDI Monoclonal Antibody—— An anti-*rho* GDI monoclonal antibody was made by a routine method. Briefly, female BALB/c mice were immunized intrafootpadly with 20 μg of the *rho* GDI purified from bovine brain cytosol in complete Freund adjuvant. At three-days intervals, two booster injections of 10 μg of *rho* GDI were given. Three days after the last booster injection, lymphocytes in inguinal lymphnodes from mice were fused with P3U1 myeloma cells. Standard fusion, screening, and cloning procedures were followed (22). In this way, one hybridoma cell line secreting the monoclonal antibody highly specific for *rho* GDI, named JK-5 (IgG2a), was obtained.

Preparation of the Homogenates of Various Rat Tissues——Various tissues of adult male rats (about 0.1 g of wet weight each) were homogenized as described (23). The homogenates were solubilized with the Laemmli's sample buffer, boiled, and subjected to SDS-PAGE followed by immunoblotting as described below.

Subcellular Fractionation of Rat Cerebrum——Subcellular fractionation of rat cerebrum was carried out as described (24). Electron microscopic analysis of all subcellular fractions indicated that the ultrastructural characteristics of these fractions obtained by our procedures were similar to those reported previously (24). The validity for the subcellular fractionation of synaptic vesicles was furthermore checked by measuring the distribution of synaptophysin, a synaptic vesicle marker protein (24). The distribution of this protein in the fractions obtained was similar to that described previously (24).

SDS-PAGE and Immunoblotting——SDS-PAGE was performed by the method of Laemmli's (25) using 12% polyacrylamide gels. Proteins on the gels were transferred electrophoretically to nitrocellulose sheets as described (26). The sheets were incubated with TBS containing 5% BSA for 2 h at room temperature. For the tissue and subcellular distributions of *rho* GDI, the sheets were first incubated for 1 h at room temperature with a 1/200 dilution of JK-5 in TBS containing 5% BSA and washed four times for 15 min each with TBS containing 0.05% Tween 20. For the detection of the attached antibody on the sheets, the sheets were incubated for 1 h at room temperature with ^{125}I-labeled goat anti-mouse IgG (92.5 kBq/ml) in TBS containing 5% BSA and washed eight times for 15 min each with TBS containing 0.05% Tween 20. The sheets were dried and autoradiographed with Kodak X-OMAT AR films using intensifying screens at -80°C. The radioactive band was cut and the radioactivity was counted as described (24).

Determination——Protein concentrations were determined with BSA as a standard protein as described (27).

Results

When the total homogenate of rat brain was immunoblotted with an anti-*rho* GDI antibody, a single band was detected (**Fig. 1**). This band migrated at the same position as that of *rho* GDI. The Mr value of this band was about 27,000. This band was not observed when the electroblotted sheet was incubated with the antibody preincubated with an excess amount of *rho* GDI.

Various tissues were obtained from rat and the amount of *rho* GDI was quantified in each tissue by immunoblotting with the anti-*rho* GDI antibody. Figure 2 shows the result of the immunoblot analysis. *rho* GDI was detected in various tissues and was abundant in cerebrum, cerebellum, thymus, lung, small intestine, and spleen, but very poor in heart and skeletal muscles. The amount of *rho* GDI in the original homogenate of rat cerebrum was quantified from the radioactivity of the ^{125}I-labeled anti-mouse IgG on the nitrocellulose sheet compared with that of the known amount of *rho* GDI as an internal control. In this assay, the radioactivity bound was proportional to the amount of *rho* GDI present (data not shown).

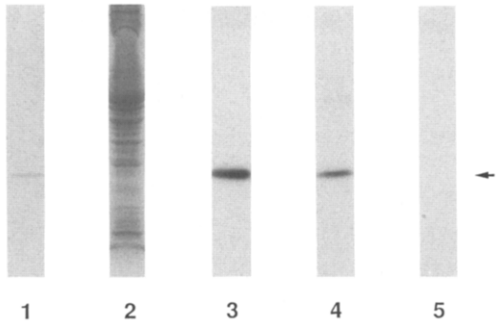

Fig. 1. Specificity of the anti-*rho* GDI antibody. The purified preparation of *rho* GDI and the homogenate of rat cerebrum were separately subjected to SDS-PAGE (12% polyacrylamide gel). After SDS-PAGE, the proteins on the gels were either stained with Coomassie Brilliant Blue or subjected to immunoblot analysis with the anti-*rho* GDI antibody. *Lanes 1* and *2*, protein staining. *Lane 1*, 1 μg of *rho* GDI; *Lane 2*, the homogenate (100 μg of protein). *Lanes 3* to *5*, immunoblot analysis. *Lane 3*, 100 ng of *rho* GDI; *Lane 4*, the homogenate (100 μg of protein); *Lane 5*, the same immunoblotted sheet as in the *Lane 4* except that the anti-*rho* GDI monoclonal antibody preincubated with an excess amount of *rho* GDI was used. The results shown are representative of three independent experiments. The arrow indicates the position of *rho* GDI.

The amounts of *rho* GDI were quantified in each subcellular fraction of rat cerebrum and the result is summarized in **Table 1**. The homogenate of rat cerebrum was first fractionated into the P_1 fraction containing nuclei and cell debris, the P_2 fraction containing synaptosomes, mitochondria, and myelin, the P_3 fraction containing microsomes, and the S fraction containing soluble cytosol. *rho* GDI was mainly present in the S, P_1, and P_2 fractions. Approximately one thirds of *rho* GDI was recovered in the S fraction

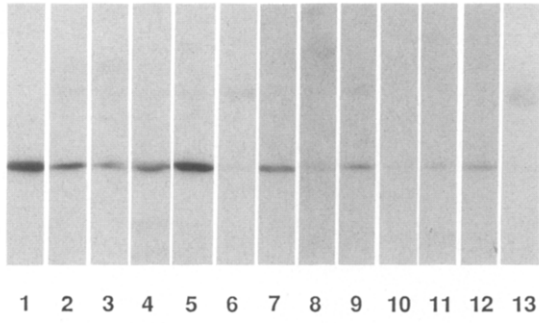

Fig. 2. Immunoblot analysis of *rho* GDI in various rat tissues. The homogenates of various rat tissues (100 μg of protein) were immunoblotted with the anti-*rho* GDI antibody. *Lane 1*, 100 ng of *rho* GDI; *Lane 2*, cerebrum; *Lane 3*, cerebellum; *Lane 4*, thymus; *Lane 5*, lung; *Lane 6*, heart; *Lane 7*, spleen; *Lane 8*, liver; *Lane 9*, small intestine; *Lane 10*, adrenal gland; *Lane 11*, kidney; *Lane 12*, testis; *Lane 13*, skeletal muscle. The results shown are representative of three independent experiments. The arrow indicates the position of *rho* GDI.

Table 1. Distribution of *rho* GDI in the subfractions of rat cerebrum

	Protein (mg/g tissue)	Specific content (μg/mg protein)	Total content (μg/g tissue)	Distribution (%)
Starting material (Homogenate)	112.4	0.63	70.8	
P_1	26.0	0.49	12.8	23.6
P_2	35.1	0.46	15.9	29.4
P_3	14.0	0.45	6.4	11.8
S	18.0	1.06	19.1	35.2
Total (P_1+P_2+P_3+S)	93.1		54.2	100
Starting material (P_2)	35.1	0.46	15.9	
P_2A	7.6	0.37	2.9	30.2
P_2B	2.6	0.36	1.0	10.4
P_2C	11.0	0.35	3.9	40.6
P_2D	6.5	0.28	1.8	18.8
Total (P_2A+P_2B+P_2C+P_2D)	27.7		9.6	100
Starting material (P_2C)	11.0	0.35	3.9	
CSM	3.6	0.14	0.5	28.9
CSV	0.2	0.13	0.03	1.7
SS	1.1	1.16	1.2	69.4
Total (CSM+CSV+SS)	4.9		1.73	100

Amounts of *rho* GDI in rat cerebrum and subcellular fractions were quantified. The results are the means of three independent experiments.

with the highest specific content. The amount of *rho* GDI in the S fraction was calculated to be about 0.11 % of the total proteins. This amount of *rho* GDI is in agreement with our previous result that about 0.08% of the soluble protein is calculated to be *rho* GDI on the basis of the yield of *rho* GDI through the purification procedures (16). The P_2 fraction was further fractionated into four fractions: the P_2A fraction containing myelin and some contaminations of membrane components in the P_2B fraction, the P_2B fraction containing a mixture of endoplasmic reticulum, Golgi complex, and plasma membranes, the P_2C fraction containing mainly synaptosomes, and the P_2D fraction containing mainly mitochondria. *rho* GDI was recovered in the four fractions. About two fifths of *rho* GDI was recovered in the P_2C fraction with the highest specific content and three fifths of *rho* GDI was in the other fractions. Since the relatively large total content and high specific content of *rho* GDI were observed in the P_2C fraction, this fraction was further separated into three fractions: the CSM fraction containing crude synaptic membranes, the CSV fraction containing crude synaptic vesicles, and the SS fraction containing synaptosomal soluble substances. About two thirds of *rho* GDI in the P_2C fraction was recovered in the SS fraction with the highest specific content and the rest of *rho* GDI was mostly recovered in the CSM fraction.

Discussion

We have shown here that *rho* GDI is found in most rat tissues. We have previously shown by Northern blot analysis that the *rho* GDI mRNA is present in many rat tissues and that *rho* GDI activity is detected in various rat tissues (16,20). The amounts of the protein molecule of *rho* GDI shown in this paper are roughly parallel with the mRNA levels and the *rho* GDI activity described earlier (16,20). It has been shown that the *rho* protein mRNAs are present in most mammalian tissues and that the proteins ADP-ribosylated by botulinum ADP-ribosyltransferase C3 are found in most mammalian tissues (8-11). The present result is consistent with these earlier observations and indicates that *rho* GDI could regulate the GDP/GTP exchange reaction of the *rho* proteins in various tissues.

We have also shown here that *rho* GDI is found in the cytosol fraction of both neuron body and synaptosome. In synaptosome, it is mainly localized in the cytosol. It may be noted that *rho* GDI is detected in the CSM fraction. However, when the CSM fraction was separated into the SM1-4 fractions, *rho* GDI was recovered in the SM1-3 fractions containing mainly synaptic plasma membranes with the very low specific content (data not shown). *rho* GDI in these membrane fractions may be a contaminant of the soluble protein. The intrasynaptosomal distribution of *rho* GDI is different from that of the *rho* proteins that are mainly localized in synaptic membranes (28). However, the proteins ADP-ribosylated by C3 are found in both the membrane and cytosol fractions depending on cell types (8-11). The reason why the subcellular distribution of the *rho* proteins differs from tissue to tissue is not known, but one reason may be related to the conditions for the homogenization of each tissue. We have previously shown (1) that *rho* GDI makes a complex with the GDP-bound form of the *rho* proteins at a molar ratio of 1:1 (16,21); (2) both the GDP- and GTP-bound forms of the *rho* proteins bind to any type of membranes including synaptic membranes, synaptic mitochondria, erythrocyte ghosts even after the membranes are digested with trypsin or boiled (21); (3) that *rho* GDI inhibits the binding of the GDP-bound form of the *rho* proteins to membranes, and (4) that *rho* GDI induces the dissociation of the GDP-bound form of the *rho* proteins from the membranes (21). Since the affinity of the *rho* GDI for the *rho* proteins is not so high (16,21), the complex of the GDP-bound form of the *rho* proteins with the *rho* GDI may be disrupted by dilution during the homogenization of each tissue. The state of the complex may be also affected by the ratio of the GDP- and GTP-bound forms of the *rho* proteins when

the cells are homogenized. We have previously shown that *rho* GDI does not bind to membranes under the conditions where the *rho* proteins bind to them (16,21). It is evident from present and previous results that *rho* GDI is localized in the cytosol fraction. However, the real intracellular distribution of the *rho* proteins still remains to be clarified.

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