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DEMONSTRATION OF GTP-BINDING PROTEINS AND ADP-RIBOSYLATED PROTEINS IN RAT LIVER GOLGI FRACTION

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SUMMARY: A Golgi-rich fraction isolated from rat liver was found to contain GTP-binding proteins with 20-25 kDa, which were tightly bound to the Golgi membrane. The Golgi fraction also contained two species of proteins which were ADP-ribosylated by bacterial toxins. Protein(s) which was ADP-ribosylated by botulinum toxin had a similar molecular mass as those with GTP-binding activity but was easily released from the membrane. Another protein with 46 kDa which was ADP-ribosylated by pertussis toxin was tightly bound to the membrane but had no significant GTP-binding activity under conditions tested here. These proteins were much less or negligible in the plasma membrane and the endoplasmic reticulum.

There is a family of GTP-binding proteins (G proteins) with a subunit structure of $\alpha\beta\gamma$; α (39-52 kDa), β (35-36 kDa), and γ (10 kDa) (1,2). These G proteins are conceivable to regulate various effectors such as adenylate cyclase, cGMP phosphodiesterase, acetylcholine-dependent K^+ channels, voltage-dependent Ca^{++} channels, and phosphoinositide-specific phospholipase C in transmembrane signaling (1,2). In addition to this family of G proteins, there is another family of G proteins with 20-25 kDa without a subunit structure. This family includes G proteins of the ras (3), rho (4), ral (5), and ypt1 (6) gene products and other novel G proteins (7,8). G proteins in both groups are known to be ADP-ribosylated by bacterial toxins (1,2).

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Abbreviations used: BT, botulinum toxin; G proteins, GTP-binding proteins; DTT, dithiothreitol; GTP[γS], guanosine 5'-(3-O-thio)triphosphate; PT, pertussis toxin; RER, rough endoplasmic reticulum.

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Recent studies suggest that some of G proteins are involved in controlling various steps of the secretory pathway. A yeast G protein, the YPT1 gene product, was found to function early in the secretion pathway, and its mammalian counterpart was localized in the Golgi apparatus (9). The yeast sec4 protein (23.5 kDa), a ras-like G protein, was proposed to play an essential role in a late stage of transport between the Golgi apparatus and the plasma membrane (10,11). In a mammalian cell system with an *in vitro* assay, it was demonstrated that non-hydrolyzable GTP analogs inhibit the transport between Golgi subcompartments and thereby cause the accumulation of vesicles, suggesting the involvement of G proteins in transport through the Golgi stack (12,13).

In the present study we demonstrate that a Golgi fraction highly purified from rat liver indeed contains GTP-binding proteins and proteins that are ADP-ribosylated by bacterial toxins.

MATERIALS AND METHODS

Materials. [α-^{32}P]GTP (111 TBq/mmol) and [^{32}P]NAD (29.6 TBq/mmol) were obtained from Du Pont-New England Nuclear. Pertussis toxin (200 µg/ml, >5,000 units/ml) and botulinum toxin type C1 (1 mg/ml, 0.6 x 10^7 mouse LD_{50}/mg of protein) were from Seikagaku Kogyo (Tokyo, Japan) and Wako Chemicals (Osaka, Japan), respectively.

Preparation of subcellular fractions. Subcellular fractions were prepared from Wistar rat livers as described previously; total Golgi fraction (14); Golgi subfractions GF-1, GF-2 and GF-3 (15,16); plasma membrane (17); and rough endoplasmic reticulum (RER) (18)

Separation of the total Golgi fraction into membrane and soluble fractions. The total Golgi fraction was suspended in either 60 mM veronal buffer (pH 8.6) or 0.1 M Na_2CO_3 (pH 11.5) (19), sonicated, and centrifuged at 105,000 x g for 1 h. The resulting sediments and supernatants were used as the membrane and soluble fractions, respectively, of the Golgi fraction.

SDS-PAGE. Proteins in each sample (50 µg of protein) were separated by SDS-PAGE (12.5% gels) according to Laemmli (20) and electrophoretically transferred to nitrocellulose followed by determination of [^{32}P]GTP-binding activity. When [^{32}P]ADP-ribosylated proteins were analyzed, the gels were dried and subjected to autoradiography.

Binding of [α-^{32}P]GTP to proteins in subcellular fractions. GTP-binding to subcellular proteins was carried out by the procedure described by Lapetina and Reep (21). The transfer-blots were rinsed in binding buffer (50 mM Tris-HCl, 0.3% Tween 20, 5 mM $MgCl_2$ and 1 mM EDTA, pH 7.5). When the specific binding of [α-^{32}P]GTP to proteins was examined, the blots were preincubated with competing substrates (GTP, GDP, GTP[γS], or ATP) in binding buffer for 30 min at 25°C. The blots with or without preincubation were then incubated for 1 h at 25°C with [α-^{32}P]GTP (2 µCi/ml, 1 µCi=37 kBq) in binding buffer in the presence or absence of competitors. The blots were rinsed with four changes of binding buffer for 1 h, air-dried and subjected to autoradiography.

ADP-Ribosylation by pertussis toxin (PT). Each sample (50 μg of protein) of subcellular fractions was incubated for 20 min at 30°C with 2.5 μCi of [^{32}P]-NAD in 135.5 μl of a reaction mixture (20 mM Tris-HCl, 2 mM EDTA, 250 mM sucrose, 25 mM DTT, 5 mM ATP and 10 mM thymidine, pH 7.5) in the presence or absence of activated PT (3.5 μg) (22). Proteins were precipitated with trichloroacetic acid (10%), washed and dissolved in the Laemmli's sample buffer, followed by SDS-PAGE/autoradiography.

ADP-Ribosylation by botulinum toxin (BT). A reaction mixture (135 μl) containing a subcellular fraction (50 μg of protein), 100 mM Hepes-NaOH (pH 8.0), [^{32}P]NAD (2.5 μCi), 10 mM DTT, 0.1 mM $MgCl_2$ and 10 mM thymidine was incubated for 45 min at 30°C in the presence or absence of BT (15 μg) (23). Proteins were precipitated with trichloroacetic acid and analyzed by SDS-PAGE/autoradiography as above.

RESULTS AND DISCUSSION

The Golgi fraction used in the present study contained the Golgi marker galactosyltransferase with the specific activity of 470 nmol/h/mg protein, which was increased about 100-fold compared with that of the liver homogenate. Other biochemical and morphological observations also indicate that this fraction is highly enriched with the Golgi apparatus (16). Fig. 1 shows protein profiles of subcellular fractions analyzed by SDS-PAGE. The Golgi fraction (lane 1) characteristically contained a major protein corresponding to albumin, which was recovered in a soluble fraction (lanes 2 and 4) after separation from the Golgi membrane (lanes 3 and 5). Protein compositions of the plasma membrane and RER fractions are apparently different from each other and from those of the Golgi fractions.

Three subcellular fractions, Golgi, plasma membrane and RER, were analyzed for [α-^{32}P]GTP-binding. As shown in Fig. 2A, the Golgi fraction was found to contain at least four [α-^{32}P]GTP-binding proteins with 25, 23, 22 and 20 kDa, although the latter three forms (20-23 kDa) were not so clearly resolved under the conditions. In contrast, none of the (20-23)-kDa forms were detectable in both the plasma membrane and RER fractions, which contained only the 25-kDa form with much less intensity than that in the Golgi fraction. The specific binding of [α-^{32}P]GTP to these proteins was confirmed by the findings that it was blocked by preincubation of the blots with 10 μM of GTP, GDP or GTP[γS], but not with 10 μM ATP (data not shown)

One of characteristic properties of G proteins is that they are ADP-ribosylated by bacterial toxins (1,2,23). ADP-Ribosylation by BT (Fig. 2B)

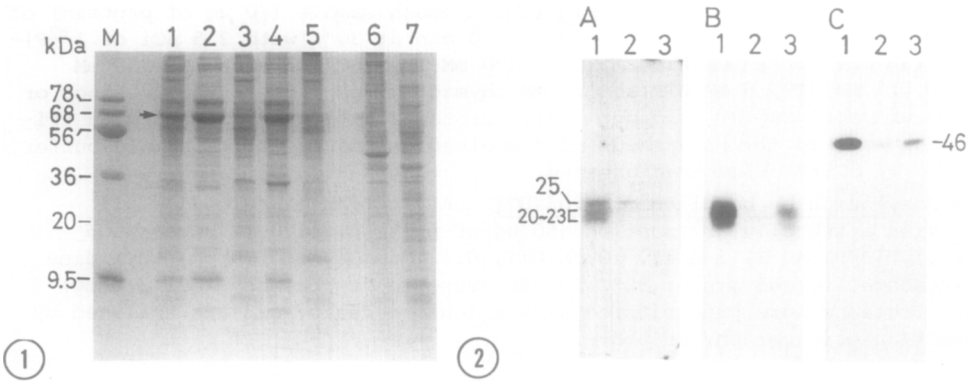

Fig. 1. SDS-PAGE of rat liver subcellular fractions. Each sample (50 μg of protein) of subcellular fractions was analyzed by SDS-PAGE (12.5% gels) and stained for protein with Coomassie brilliant blue. Lane 1, total Golgi fraction; lanes 2 and 3, membrane and soluble fractions, respectively, of the Golgi fraction obtained after sonication in 60 mM veronal buffer (pH 8.6); lanes 4 and 5, membrane and soluble fractions, respectively, of the Golgi after treatment with 0.1 M Na_2CO_3 (pH 11.5); lane 6, plasma membrane; lane 7, RER; lane M, marker proteins including transferrin (78 kDa), albumin (68 kDa), α_1-protease inhibitor (56 kDa), haptoglobin β subunit (36 kDa), α_{2u}-globulin (20 kDa), and haptoglobin α subunit (9.5 kDa). An arrow indicates the position of albumin.

Fig. 2. Analysis of GTP-binding proteins and ADP-ribosylated proteins in subcellular fractions. In (A), each sample (50 μg of protein) was separated by SDS-PAGE (12.5% gels), transferred to nitrocellulose, and subjected to [α-^{32}P]GTP-binding, as described in MATERIALS AND METHODS. In (B) and (C), samples (50 μg each) were ADP-ribosylated with [^{32}P]NAD by BT (B) or PT (C) and analyzed by SDS-PAGE (12.5% gels) followed by autoradiography. Lane 1, Golgi, lane 2, plasma membrane; lane 3, RER.

and by PT (Fig. 2C) revealed that the Golgi fraction contained two different forms of proteins; one (20-25 kDa) was ADP-ribosylated by BT and the other (46 kDa) was modified by PT. The two forms were detectable in a small amount in the RER fraction, and much less in the plasma membrane. The 46-kDa form had no apparent [α-^{32}P]GTP-binding activity under the denaturing conditions employed here. It remains to be determined whether its native form has the GTP-binding activity.

The Golgi fraction was separated into membrane and soluble fractions, which were then analyzed for GTP-binding (Fig. 3A) and ADP-ribosylation by BT (Fig. 3B) or PT (Fig. 3C). All the [α-^{32}P]GTP-binding proteins and the 46-kDa form ADP-ribosylated by PT were found exclusively in the membrane fraction, whereas the BT-dependent ADP-ribosylated form was in the soluble fraction. The results indicate that the [α-^{32}P]GTP-binding proteins are not the same molecules as that ADP-ribosylated by BT, although these proteins in both groups have similar molecular masses (20-25 kDa).

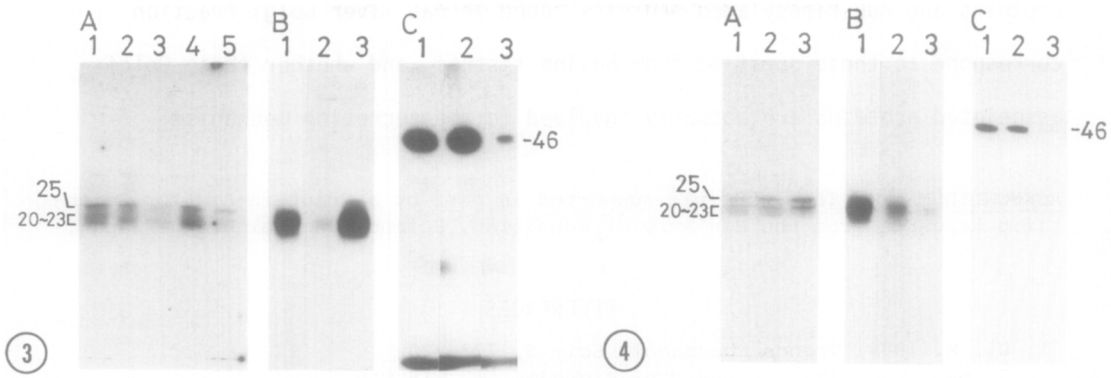

Fig. 3. Distribution of GTP-binding proteins and ADP-ribosylated proteins in the membrane and soluble fractions of the Golgi fraction. The total Golgi fraction(lane 1) was separated into membrane (lanes 2 and 4) and soluble (lanes 3 and 5) subfractions as described in MATERIALS AND METHODS, and each sample (50 µg) was subjected to GTP-binding (A) and ADP-ribosylation by BT (B) or PT (C) under the same conditions as shown in Fig. 2. Samples in lanes 2 and 3 were obtained by sonication in 60 mM veronal buffer (pH 8.6), and those in lanes 4 and 5 were obtained by treatment with 0.1 M Na_2CO_3 (pH 11.5).

Fig. 4. Distribution of GTP-binding proteins and ADP-ribosylated proteins among Golgi subfractions. Golgi subfractions, GF-1 (lane 1), GF-2 (lane 2) and GF-3 (lane 3), were isolated as described in MATERIALS AND METHODS, and each sample (50 µg of protein) was analyzed for GTP-binding (A) and ADP-ribosylation by BT (B) or PT (C), as shown in Figs. 2 and 3.

We then examined the distribution of these proteins among the Golgi subfractions GF-1, GF-2 and GF-3; GF-1 and GF-3 were composed mainly of secretory vesicles and of cisternal elements, respectively, and GF-2 was an intermediate fraction containing the two components (15,16). As shown in Fig. 4, the [α-^{32}P]GTP-binding proteins were most enriched in GF-3 (Fig. 4A), whereas both the proteins ADP-ribosylated by BT and PT were in GF-1 (Fig. 4B and 4C). Taken together, these results (Figs. 3 and 4) suggest that [α-^{32}P]GTP-binding proteins are tightly associated with the Golgi cisternal membrane and the ADP-ribosylated proteins are loosely (BT-dependent protein) and tightly (PT-dependent protein) associated with the secretory vesicles.

It has been demonstrated that there are at least six G proteins with molecular masses between 20 kDa and 25 kDa in bovine brain membranes (24). Some of them were purified from the brain membranes (24-26) and from the cytosol of adrenal glands (27). One of the purified G proteins which were ADP-ribosylated by BT was identified to be the *rho* gene product (28,29). However, subcellular localization of these G proteins has not been examined in detail. Thus, it is of interest to examine whether [α-^{32}P]GTP-binding

proteins and ADP-ribosylated proteins found in rat liver Golgi fraction correspond to those purified from bovine tissues, and whether these Golgi-associated proteins are actually involved in the secretion mechanism.

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