ARTICLE

Journal of Cellular Biochemistry 112:2250–2256 (2011)

Journal of Cellular Biochemistry

# ER-Resident $G_{i2}$ Protein Controls Sar1 Translocation onto the ER During Budding of Transport Vesicles

Hiroshi Nakagawa,[1] Haruka Umadome,[1] Shuichi Miyazaki,[2] Katsuhiro Tanaka,[3] Kazuhiko Nishimura,[1] Masayuki Komori,[3] and Saburo Matsuo[1*]

[1] *Laboratory of Toxicology, Course of Veterinary Science, Graduate School of Life and Environmental Biosciences, Osaka Prefecture University, Osaka, Japan*

[2] *The Center for Advanced Research, Graduate School of Medical Sciences, Toho University, Tokyo, Japan*

[3] *Laboratory of Cellular and Molecular Biology, Course of Veterinary Science, Graduate School of Life and Environmental Biosciences, Osaka Prefecture University, Osaka, Japan*

## ABSTRACT

In our previous study, fluoride ($[AlF_4]^-$) disturbed ER-to-Golgi transport through the activation of ER-resident heterotrimeric G protein (ER-G protein). Therefore, ER-G protein may be implicated in ER-to-Golgi transport at the early stage prior to coat protein assembly. Sar1 translocation onto the endoplasmic reticulum (ER) membrane is suppressed by non-selective protein kinase inhibitor H89, suggesting the participation of H89-sensitive kinase in this process. To investigate the involvement of ER-G protein in ER-to-Golgi transport, the effect of $G_i$ protein activator (mastoparan 7) was examined on Sar1 translocation onto the ER in a cell-free system consisting of microsome membrane and cytosol. Sar1 translocation onto the microsome membrane was induced by addition of GTPγS in the cell-free system. Translocation of Sar1 by GTPγS was suppressed significantly by both H89 and mastoparan 7. Mastoparan 7 suppressed the translocation of Sar1 onto the microsome membrane with dosage dependency, but mastoparan 17, the inactive analog of mastoparan 7, had no effect on Sar1 translocation. The suppressive effect of mastoparan 7 was recovered by treatment with pertussis toxin (IAP). Moreover, $G_{i2}$ protein was detected on the microsome membrane by western blotting for heterotrimeric $G_i$ proteins. These results indicate that ER-$G_{i2}$ protein modulated Sar1 translocation onto the ER, suggesting that ER-resident $G_{i2}$ protein is an important negative regulator of vesicular transport at the early stage of vesicle formation before coat protein assembly on the ER. J. Cell. Biochem. 112: 2250–2256, 2011. © 2011 Wiley-Liss, Inc.

**KEY WORDS:** ER-to-Golgi TRANSPORT; ER-RESIDENT HETEROTRIMERIC G PROTEIN; Sar1; MASTOPARAN; PERTUSSIS TOXIN

Transport between the cellular compartments is mediated by vesicles that perform vectorial transfer of cargo molecules. Vesicles are generated on the donor membrane by recruitment of cytoplasmic coat proteins that deform the membrane to drive vesicle formation. This specific recruitment is thought to result from direct interactions between sorting signals present on the cytosolic domains of cargo molecules and subunits of the coat machinery [Schekman and Orci, 1996]. Export of proteins from the endoplasmic reticulum (ER) is driven by the COPII coat, which consists of small G protein (Sar1), Sec23/24 complex, and Sec13/31 complex [Barlowe et al., 1994]. These three coat components form the basic machinery required for vesicle budding and cargo sorting on ER membranes [Matsuoka et al., 1998a,b]. Export from the ER is initiated by the activation of Sar1 through exchange of guanosine 5′-diphosphate (GDP) for GTP by the membrane-associated Sec12 (guanine nucleotide exchange factor, GEF). Therefore, Sar1 appears to be the master regulator of COPII coat formation. On the other hand, several lines of evidence have implicated kinases and phosphatases in regulating transport between the ER and the Golgi compartments [Davidson and Balch, 1993; Davidson et al., 1992; Jamora et al., 1999], and the budding of COPII vesicles requires ATP as well as GTP [Balch et al., 1984; Barlowe, 1997]. In particular, the non-selective protein kinase inhibitor H89 (H89) blocks several membrane trafficking steps in and around the Golgi apparatus [Lee and Linstedt, 2000] including recruitment of Sar1 to the ER membrane [Aridor and Balch, 2000]. However, the mechanisms regulating Sar1 translocation onto the ER membrane remain unknown.

Additional supporting information may be found in the online version of this article.

*Correspondence to: Saburo Matsuo, Laboratory of Toxicology, Course of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-58, Rinku-Orai-Kita, Izumisano, Osaka 598-8531, Japan. E-mail: matsuos@vet.osakafu-u.ac.jp

Received 11 November 2010; Accepted 31 March 2011 • DOI 10.1002/jcb.23142 • © 2011 Wiley-Liss, Inc.
Published online 7 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

Monomeric G proteins and heterotrimeric G proteins are presumably involved in the transport of proteins from the ER to the Golgi apparatus. However, the potential role of heterotrimeric G proteins on vesicular transport is less well known. Heterotrimeric G proteins are very likely to function in the vesicular formation of protein on the secretory pathway [Balch, 1992; Barr et al., 1992]. The heterotrimeric G protein, $G_{i3}$, was found in the Golgi complex and over-expression of $G_{i3}$ resulted in pertussis toxin-sensitive retardation of the flow of glycosaminoglycans through the Golgi compartment [Stow et al., 1991]. Recruitment of coat proteins found on clathrin-coated vesicles budding from the trans-Golgi compartment is sensitive to the G protein activator $[AlF_4]^-$ [Robinson and Kreis, 1992; Wong and Brodsky, 1992], which inhibits ER-to-Golgi transport [Beckers and Balch, 1989]. Our previous studies indicated that $[AlF_4]^-$ caused accumulation of transport vesicles around the Golgi apparatus [Matsuo et al., 1996], and that $[AlF_4]^-$ activated pertussis toxin-sensitive heterotrimeric G protein located on the ER of the secretory ameloblast [Matsuo et al., 1998]. $[AlF_4]^-$ stimulates the formation of intracisternal granules in the ER of exocrine secretory cells of the pancreas, suggesting disruption of vesicular transport from the ER [Matsuo et al., 2000]. If $[AlF_4]^-$ specifically activates only heterotrimeric G protein without activation of small G protein involved in vesicle transport, these morphological phenotypes strongly support the hypothesis that the heterotrimeric G protein (ER-G protein) and Sar1 participate in vesicle budding on the ER at the early stage of ER-to-Golgi transport. However, $[AlF_4]^-$ also activates the monomeric G proteins [Ahmadian et al., 1997; Mittal et al., 1996]. Although $[AlF_4]^-$ is well known to associate with and activate the GDP-bound α-subunits of heterotrimeric G proteins [Bigay et al., 1987; Sternweis and Gilman, 1982], the monomeric G protein, Ras, also associates with $[AlF_4]^-$ in the presence of stoichiometric amounts of its GTPase activating protein [Mittal et al., 1996]. Therefore, the participation of heterotrimeric G proteins in ER-to-Golgi transport has strong evidential support, but has not yet been conclusively established.

Peptides designed to interfere with G protein activity, such as mastoparan, have been widely used as probes to investigate the function of G proteins in protein transport. Mastoparan is a cationic amphiphilic peptide that stimulates heterotrimeric G protein GTP/GDP exchange by mimicking the interaction domain of G protein-coupled receptors [Higashijima et al., 1988, 1990]. The stimulatory effect of the peptide is largely abolished by pertussis toxin-catalyzed ADP ribosylation of $G_{\alpha i/o}$ subunits [Stow et al., 1991]. In addition to the activating effect for heterotrimeric G protein, however, mastoparan also activates the monomeric G protein through its amphiphilic property. Therefore, with appropriate negative controls such as mastoparan 17 (inactive analog of mastoparan 7) and pertussis toxin (ribosylation factor of heterotrimeric $G_{i/o}$ protein), mastoparan is useful to study the effect of heterotrimeric G proteins on vesicle transport between the cellular compartments.

To investigate the involvement of RE-resident heterotrimeric G protein (ER-G protein) in ER-to-Golgi transport at the early stage of ER budding of the transport vesicle, the effect of mastoparan and pertussis toxin on Sar1 translocation onto the ER was examined in a cell-free system consisting of microsome membrane and cytosol.

## MATERIALS AND METHODS

### ANIMALS

The experiments were carried out under the control of the Animal Research Control Committee in accordance with The Guidelines for Animal Experiments of Osaka Prefecture University. All efforts were made to minimize the number of animals used and their suffering. Eight-week-old male SD rats (Slc: SD) and rabbits (kar: NZW) were obtained from Japan SLC (Hamamatsu, Japan) and Keari (Osaka, Japan), respectively, and acclimatized for more than 1 week. The animals were kept in an air conditioned room maintained at $22 \pm 1°C$, $60 \pm 5\%$ relative humidity, and 12:12 h light/dark cycle, and were allowed free access to solid chows (CE-2 rodent chow for rats and RG-1 rabbit chow for rabbits; Clea Japan, Tokyo, Japan) and tap water.

### PRODUCTION OF RECOMBINANT RAT SAR1

Rat total RNA was isolated from the liver by using a GenElute Mammalian Total RNA Miniprep kit (Sigma, St. Louis, MO). Double strand cDNA was synthesized with a random primer (9mer) (Toyobo, Osaka, Japan). The resulting cDNA was used as the template for PCR amplification of the DNA fragment encoding full length Sar1b (accession no. NM001009622). Forward primer was 5′-GGGGATC-CATGTCCTTCATATTTGACTGG-3′ to add a *Bam*HI site immediately upstream of the first ATG. Reverse primer was 5′-GTGTCGACC-TAGTCGATGTACTGCGCCATC-3′ to add a *Sal*I site immediately downstream of the termination codon. PCR was performed under the following conditions: denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min. Thirty cycles of PCR were repeated. The amplified fragments were cloned into pBlue-Script II SK(+) (Stratagene, La Jolla, CA), and sequenced (CEQ2000, Beckman Coulter, Fullerton, CA). The PCR products were digested with *Bam*HI and *Sal*I and cloned into the pQE-30 vector (QIAGEN, Hilden, Germany). The resulting plasmid was introduced into *E. coli* (M15 harboring plasmid pREP4; QIAGEN).

*E. coli* expressing His-tagged Sarl protein was disrupted by sonication (TOMY Ultrasonic Disruptor UD-201, TOMY, Tokyo, Japan) in sonication buffer containing 100 μM GDP sodium salt. The lysate was clarified by centrifugation at 10,000*g* for 30 min, and the supernatant was loaded on a Ni-NTA agarose (Qiagen) column. The resin was washed with wash buffer (50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl, 20 mM imidazole) containing 100 μM GDP and then His-tagged Sarl protein was eluted with elution buffer (50 mM $NaH_2PO_4$ (pH 8.0), 300 mM NaCl, 250 mM imidazole) containing 100 μM GDP. After elution and dialysis against PBS, the eluted His-tagged Sar1 protein was adjusted to 2 mg/ml protein with PBS and stored at −80°C until use.

### PRODUCTION OF ANTIBODY TO SARL PROTEIN

A rabbit was injected with 100 μg purified His-tagged recombinant Sarl protein emulsified in Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI) and boosted at day 14, 28, and 56 after first immunization with emulsion of the recombinant Sarl protein and Freund's incomplete adjuvant (DIFCO Laboratories). Serum was obtained at day 66. The specificity of this anti-Sar1 antiserum was confirmed by western blotting of His-tag recombinant Sar1 protein

(10 ng), rat hepatocyte-cytosol Sar1 (40 μg cytosol total protein), and their mixture.

### MICROSOME BINDING ASSAY OF RECOMBINANT RAT SAR1

**Preparation of rat liver cytosol for microsome binding assay.** After decapitation under light anesthesia with ether, liver tissues were dissected and washed with ice-cold PBS. The livers were minced with a razor blade and homogenized in three volumes of homogenization buffer (25 mM Hepes-KOH (pH 7.2), containing 125 mM KOAc, 1 mM PMSF, 4 μg/ml pepstatin, and 4 μg/ml leupeptin with 10 strokes of a tight-fitting dounce homogenizer (Wheaton, Swedesboro, NJ). The homogenate was centrifuged at 20,000×*g* for 10 min in a RT-65 rotor (Hitachi) and the resulting supernatant further centrifuged at 150,000×*g* for 90 min in a RT-65 rotor. Contamination of Gi proteins in the supernatant was excluded by western blotting. The result of western blotting showed that the supernatant did not contained $G_{\alpha i2}$ and $G_{\alpha i3}$ (data was not shown). The supernatant was adjusted at 13.3 mg/ml protein and stored at −80°C until use as rat liver cytosol [Davidson et al., 1992]. ER and Golgi membrane fractions of rat liver was used as comparative controls to confirm the distribution patterns of heterotrimeric Gi proteins on the microsome membrane of NRK cells (RCB0043: normal rat kidney cell). ER and Golgi membrane fractions of rat liver were prepared according to the method of our previous study [Matsuo et al., 1998] modified from Balch's method [Balch et al., 1984].

**Preparation of NRK cell microsomes.** NRK cells were grown on 150-mm diameter tissue culture dishes. At confluence, cells were washed with ice-cold PBS and then scraped into sorbitol buffer (20 mM Hepes-KOH (pH 7.2), containing 0.375 M sorbitol, 1 mM PMSF, and 2 μg leupeptin) and collected by centrifugation at 500×*g* for 3 min. After resuspension in three volumes of the sorbitol buffer, the cells were homogenized using a NS-310E physicotoron (Nition, Tiba, Japan) at intensity number 6 for 1 min on ice. The homogenate was diluted with an equal volume of the sorbitol buffer and then centrifuged at 720×*g* for 5 min. The postnuclear supernatant (PNS) was adjusted to transport buffer (20 mM Hepes-KOH (pH 7.2), containing 0.25 M sorbitol, 70 mM KOAc, 1 mM $Mg(OAc)_2$, 1 mM PMSF, and 2 μg leupeptin), and centrifuged at 12,000×*g* for 2 min. After washing once with transport buffer, the pellet was re-suspended in the transport buffer at 4 mg/ml protein and stored at −80°C until use as NRK cell microsomes [Rowe et al., 1996]. For study of pertussis toxin treatment, sub-confluent monolayers of NRK cells were incubated for 24 h in the presence of pertussis toxin (100 ng/ml) (List Biological Lab. Inc., Campbell, Ca) for in vivo ADP ribosylation of Gα subunits in cells [Höltje et al., 2000; Stow et al., 1991]. Stow et al. [1991] confirmed that, on intact LLC-PK1 cell, pertussis toxin pretreatment of the cell resulted in the complete in vivo ADP ribosylation of all αi subunits present in the cells, such that no further ADP ribosylation was detected by re-exposing membranes prepared from these cells to pertussis toxin in vitro. The microsomes containing ADP-ribosylated Gα subunits were prepared from pertussis toxin pretreatment cells with the processes described above.

**Microsome binding-assay.** NRK microsome membranes (40 μg) were incubated with rat liver cytosol (200 μg) in a 60-μl final volume of reaction mix containing 36 mM Hepes-KOH (pH 7.2), 70 mM KOAc, 2.5 mM MgOAc, 250 mM sorbitol, 1.8 mM $CaCl_2$, 1.5 mM EGTA, 1 mM PMSF, 2 μg leupeptin, and 100 μM GDP in the presence or absence of 100 μM GTPγS [Aridor and Balch, 2000; Aridor et al., 1995]. H89 (200 μM), and mastoparan 7 (100, 5–100 μM) or mastoparan 17 (100 μM) were added to the reaction mixture containing GTPγS for each drug treatment. Following incubation at 32°C for 15 min, the reaction samples (60 μl) were layered on a 15% sucrose cushion (180 μl) containing 75 mM KOAc and 2 mM MgOAc in a 1,500 μl microtube, and were centrifuged at 16,000×*g* and 4°C for 15 min. The membrane-containing pellet was solubilized with Laemmli SDS-sample buffer and analyzed by quantitative immunoblotting [Aridor and Balch, 2000]. The amount of microsome-bound Sar1 was quantified by densitometry of Sar1 positive band using Multi Gauge software (FUJIFILM, Tokyo, Japan). To normalize the densitometric values of positive band in each experiment, the density values of Sar1 positive bands were corrected with the density value of calnexin positive band in corresponding lane. Amounts (density values corrected with calnexin) of Sar1 binding to the microsomes were indicated as the rate of Sar1 amount of each group to the Sar1 amount of −GTPγS group in relevant figures. For the study of pertussis toxin treatment, the microsome membranes prepared from the cells cultured in the presence of pertussis toxin were incubated in the reaction mixtures..

**Western blot analysis.** To identify the organelle-membrane properties of the fractions and for qualitative analysis of microsome-bound proteins, membrane fractions and microsome binding samples were separated by 10% or 12% SDS–PAGE, and transferred onto polyvinyl difluoride (PVDF) membranes (Biorad, Hercules, CA). After blocking with 1% bovine serum albumin or 5% skim milk, the membranes were incubated with anti-$G_{\alpha i2}$ or $G_{\alpha i3}$ polyclonal antibodies (1:2,000; Gramsch Laboratories, Schwabhausen, Germany), anti-calnexin polyclonal antibody (1:2,000; StressGen Biotechnologie, Canada or 1:15,000; Sigma), or anti-serum (Sar1; 0.4 μg/ml) overnight at 4°C. After incubation with HRP-labeled secondary antibody (1:2,000–5,000; Capple, Aurora, OH) for 1 h at room temperature, reaction proteins were detected with Fuji UX-R X-ray film by enhanced chemiluminescence (Amersham, Piscataway, NJ), or by LAS-3000 (FUJIFUILM) after enhancement with chemiluminescence.

**Statistical analysis.** Unless otherwise indicated, all results are presented as the mean ± SD of the indicated number of separate individual experiments, each performed at least in triplicate. Statistical analysis was performed by Student's paired two-tailed *t*-test with a *P*-value less than 0.05 considered significant.

## RESULTS

### CONFIRMATION OF THE PROPERTIES OF MEMBRANE FRACTIONS

The distribution patterns of heterotrimeric G proteins in the microsome membranes of NRK cells, and the ER and Golgi membranes of rat liver cells were examined by western blotting of anti-$G_{\alpha i2}$ and -$G_{\alpha i3}$ antibodies. The positive band of $G_{\alpha i2}$ protein was observed at 40 kDa in the microsome membrane fraction of NRK cells and the ER membrane fraction of rat liver, but $G_{\alpha i3}$-positive

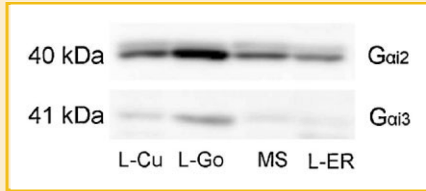


Fig. 1. Distribution patterns of heterotrimeric Gi proteins ($G_{\alpha i2}$ and $G_{\alpha i3}$) in the microsome membrane by western blot analysis. Aliquots of microsome fraction prepared from NRK cell homogenates by Rowe's method and subcellular membrane fractions prepared from rat liver homogenates by Balch's method (described in Materials and Methods) were separated by 10% SDS–PAGE, and transferred to PVDF membrane. The properties of the subcellular membrane fractions were identified by western blotting (ECL) for $G_{\alpha i2}$ and $G_{\alpha i3}$. The results shown are representative of three independent experiments. L-Cu: rat liver crude membrane, L-Go: Golgi-membrane fraction of rat liver, MS: microsome fraction of NRK cell, and L-ER: ER-membrane fraction of rat liver.

band was only negligibly or faintly observed in these membrane fractions (Fig. 1). Such negligible or faint $G_{\alpha i3}$-positive bands in the rat liver ER fraction and microsome membrane lanes probably resulted from slight contamination of the Golgi membrane fraction into these membrane fractions during the separation processes. On the other hand, the Golgi membrane fraction of rat liver contained both $G_{\alpha i2}$ and $G_{\alpha i3}$ proteins (Fig. 1). In our pilot study using ER and Golgi membrane fractions prepared from rat liver and kidney, no band of $G_{\alpha i1}$ was detected in any membrane fraction (data not shown). Therefore, the distribution of $G_{\alpha i1}$ on microsome membranes was not examined in this study.

### MICROSOME BINDING ASSAY OF RECOMBINANT RAT SAR1

The specificity of the polyclonal anti-Sar1 antiserum obtained from rabbits treated with recombinant rat Sar1 was evaluated by western blotting to rat liver cytoplasm, recombinant rat Sar1, and their mixture. The anti-Sar1 antiserum detected hepatocyte cytosolic Sar1 as the 20 kDa band in the rat liver cytoplasm and the mixture, and also detected the His-tag recombinant Sar1 as a slightly heavier band in the recombinant Sar1 solution and the mixture (Fig. 2).

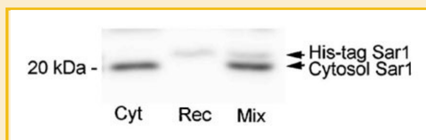


Fig. 2. Western blot analysis to confirm the specificity of anti-Sar1 antiserum. Western blotting used 40 μg rat hepatocyte cytosol protein (lane 1), 10 ng His-tag recombinant Sar1 protein (lane 2), and their mixture (lane 3). Rat hepatocyte cytosolic Sar1 and His-tag recombinant Sar1 were detected as the 20 kDa band in the hepatocyte and mixture lanes, and as the slightly heavy band in the recombinant protein and mixture lanes, respectively. The results shown are representative of two independent experiments with these protein concentrates (40 μg rat hepatocyte cytosol protein and 10 ng His-tag recombinant Sar1 protein). Cyt: Rat liver cytosol, Rec: 10 ng His-tag recombinant Sar1 protein solution, and Mix: Mixture solution of rat liver cytosol and recombinant Sar1 solution.

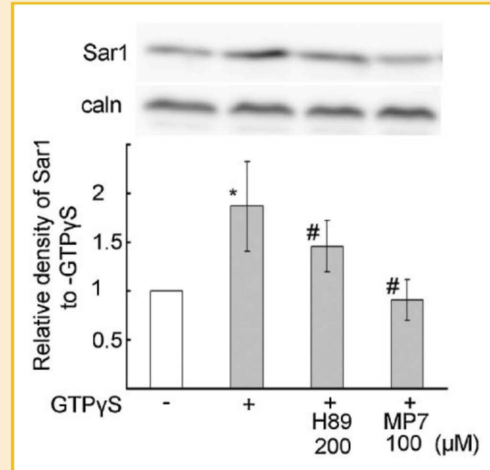


Fig. 3. Western blot analysis and densitometry for Sar1 in the microsome membrane binding assay in the presence of 200 μM H89 (H89) and 100 μM mastoparan 7 (MP7). NRK microsome membranes (40 μg) and rat liver cytosol (200 μg) were incubated in the presence or absence of 100 μM GTPγS. H89 (200 μM) or MP7 (100 μM) was added to the reaction mixture containing GTPγS as required. The membrane fractions in the reaction samples were collected by centrifugation. The membrane pellet was solubilized with Laemmli SDS-sample buffer and analyzed by quantitative western blot analysis for Sar1. Densitometry of the amount of microsome binding protein was performed using Multi Gauge software. Representative western blotting from one of three independent experiments is shown. Caln: Calnexin, value: mean ± SD, * (−GTPγS vs. +GTP-γS): $P < 0.05$, # (+GTPγS vs. H89 or MP7): $P < 0.05$.

Quantitative analysis of Sar1 translocated onto the microsome membrane was investigated by densitometry of the western blot of Sar1. The addition of GTPγS in the reaction medium increased the amount of Sar1 bound to the microsome membrane by about 1.5–2 times (Figs. 3–5, +GTPγS vs. −GTPγS). The added GTPγS apparently induced the transformation of Sar1 to the Sar1-GTP binding form, resulting in translocation of Sar1 onto the microsome membrane. The Sar1 translocation was suppressed by protein kinase inhibitor H89 with dose dependency (as shown in our previous study [Nakagawa et al., 2011]), and the amount of the Sar1 binding to the microsome membrane was significantly decreased by 200 μM H89 treatment (Fig. 3). Mastoparan 7 (100 μM), which stimulates heterotrimeric $G_{i/o}$ protein GTP/GDP exchange by mimicking the interaction domain of the G protein-coupled receptor, also significantly suppressed Sar1 translocation onto the microsome vesicles (Fig. 3), depended on the concentration of mastoparan 7 (Fig. 4, MS7 5–100 μM vs. +GTPγS). On the other hand, mastoparan 17 (100 μM, inactive analog of mastoparan) did not decrease the amount of Sar1 translocated onto the microsome membrane.

The suppression of Sar1 translocation by mastoparan 7 was largely abolished by 100 ng/ml pertussis toxin treatment (pertussis toxin-catalyzed ADP ribosylation of $G_{\alpha i2}$). The pertussis treatment restored 150% versus mastoparan single treatment of the relative Sar1 amount (from 0.5 with 50 μM mastoparan single treatment to 0.75 with 100 ng/ml pertussis toxin and 50 μM mastoparan double treatments; Fig. 5: −IAP and +IAP of MS7), although suppression of Sar1 translocation by mastoparan 7 was extremely severe compared to that of H89 treatment (Fig. 5: MS7 and H89). On the other hand,

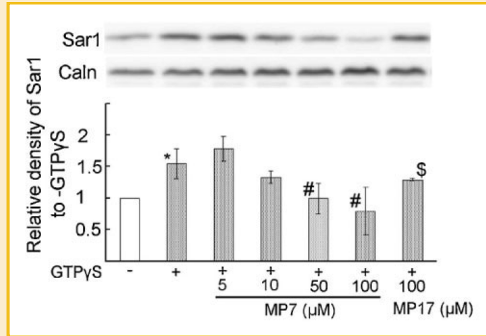


Fig. 4. Western blot analysis and densitometry for Sar1 in the microsome membrane binding assay in the presence of 5–100 μM mastoparan 7 (MP7) or 100 μM mastoparan 17 (MP17). NRK microsome membranes (40 μg) and rat liver cytosol (200 μg) were incubated in the presence or absence of 100 μM GTPγS. Mastoparan 7 (5, 10, 50, or 100 μM) or mastoparan 17 (100 μM) was added to the reaction mixture containing GTPγS as required. The membrane fractions in the reaction samples were collected by centrifugation. The membrane pellet was solubilized with Laemmli SDS-sample buffer and analyzed by quantitative western blot analysis for Sar1. Densitometry of the amount of microsome binding protein was performed using Multi Gauge software. Representative western blotting from one of three independent experiments is shown. Caln: Calnexin, value: mean ± SD,* (−GTPγS vs. +GTPγS): $P < 0.05$, # (+GTPγS vs. each dose MP7): $P < 0.05$, $ (100 μM MP7 vs. 100 μM MP17): $P < 0.05$. There is no significant difference between +GTPγS and 100 μM MP17.

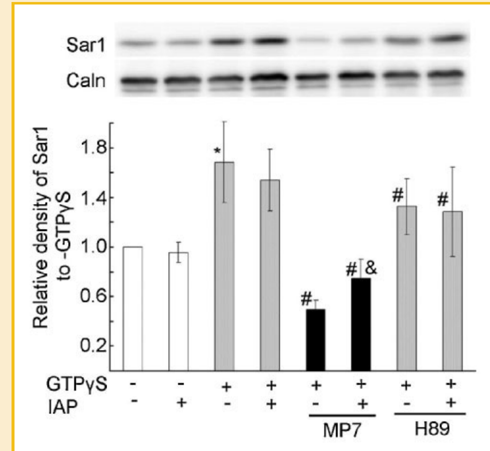


Fig. 5. Western blot analysis and densitometry for Sar1 in the microsome membrane binding assay in the presence of 50 μM mastoparan 7 (−IAP of MP7), 50 μM MP7 + 100 ng/ml pertussis toxin pretreatment (+IAP of MP7), 200 μM H89 (−IAP of H89) or 200 μM H89 + 100 ng/ml pertussis toxin pretreatment (+IAP of H89). NRK microsome membranes (without [−IAP] or with pertussis toxin pre-treatment [+IAP]; 40 μg) and rat liver cytosol (200 μg) were incubated in the presence or absence of 100 μM GTPγS. Pertussis toxin pre-treatment microsome membrane was prepared by centrifuged fractionation of NRK cells cultured for 24 h in the presence of pertussis toxin (100 ng/ml) (described in Materials and Methods). Mastoparan 7 (50 μM) or H89 (200 μM) was added to the reaction mixture containing GTPγS as required. The membrane fractions in the reaction samples were collected by centrifugation. The membrane pellet was solubilized with Laemmli SDS-sample buffer and analyzed by quantitative western blot analysis for Sar1. Densitometry of the amount of microsome binding protein was performed using Multi Gauge software. Representative western blotting from one of five independent experiments is shown. Caln: Calnexin, value: mean ± SD, * (−GTPγS vs. +GTPγS): $P < 0.05$, # (+GTPγS vs. MP7 or H89): $P < 0.05$, & (−IAP vs. +IAP of MP7): $P < 0.05$. There in no significant difference between −IAP and +IAP of H89.

pertussis toxin treatment did not have any effect to the suppression induced by H89 (Fig. 5: −IAP and +IAP of H89).

## DISCUSSION

The involvement of ER-resident heterotrimeric G protein in ER-to-Golgi transport, was studied by investigating the effect of mastoparan (G protein activator) and pertussis toxin (ADP ribosylation factor of $G_{\alpha i/o}$) on Sar1 translocation onto the ER in a cell-free system consisting of NRK microsome membrane and rat liver cytosol. GTPγS induced translocation of Sar1, resulting in increased Sar1 bound to microsome membrane. Sar1 translocation was suppressed by protein kinase inhibitor H89. Mastoparan 7 also decreased the amount of Sar1 binding to microsome vesicles with dose dependency, but mastoparan 17 did not suppress Sar1 translocation caused by GTPγS. Pertussis toxin treatment of culture cells suppressed the effect of mastoparan 7 on Sar1 translocation. These findings indicate that ER-resident $G_{i2}$ protein is an important negative regulator of vesicular transport at the early stage of vesicle formation before coat protein assembly on the ER.

### SAR1 TRANSLOCATION AS AN INDICATOR OF VESICLE TRANSPORT ACTIVITY IN THE ER

Export of proteins from the ER is facilitated by the COPII coat, consisting of the small G protein (Sar1), Sec23/24 complex, and Sec13/31 complex [Barlowe et al., 1994]. Budding of transport vesicles on the ER membrane is initiated by the assembly of Sar1 onto the membrane. Incubation of microsome membranes with ATP and GTPγS leads to COPII recruitment from the cytosol, suggesting that the recruitment of endogenous cytosolic Sar1 onto the ER membrane is dependent on GTPγS [Aridor et al., 1995]. Addition of GTPγS induces transformation of Sar1 to the Sar1-GTP binding form, resulting in translocation of Sar1 onto the microsome vesicles. In the present study, addition of GTPγS into the incubation medium containing ATP and liver cytosol also activated Sar1 and resulted in increased Sar1 translocation onto the microsome vesicles. On the other hand, protein kinase regulation on membrane transport is believed to occur at the ER-Golgi interface [Du et al., 2006; Lee and Linstedt, 2000; Nagaya et al., 2002; Palmer et al., 2005]. Specifically, the relatively non-selective kinase inhibitor H89 blocks recruitment of Sar1 to the ER membrane [Aridor and Balch, 2000]. The present study also showed that H89 suppressed the translocation of Sar1 induced by GTPγS. These findings indicate that the present in vitro system is suitable to examine the effect of ER-resident-G protein on vesicular transport at the early stage of vesicle formation before coat protein assembly on the ER.

### SAR1 TRANSLOCATION ONTO THE MICROSOME VESICLE REGULATED BY HETEROTRIMERIC G PROTEINS

Monomeric and heterotrimeric G proteins occur in the secretory pathway [Audigier et al., 1988; Chavrier et al., 1990; Melançon et al.,

1987; Stow et al., 1991]. Movement of newly synthesized proteins through the secretory pathway involves staged vesicular transport between sequential membrane compartments [Farquhar, 1985; Palade, 1975]. The requirement for G proteins at many of these steps has been confirmed [Amin et al., 2003; Bourne, 1988; Stow et al., 1991].

The present study showed that mastoparan 7 suppressed Sar1 translocation induced by GTPγS. Mastoparan, an amphiphilic tetradecapeptide obtained from wasp venom, has various biological effects, including stimulation of secretion from cells [Daniel et al., 2002; Konrad et al., 1995; Yajima et al., 2000]. Most mastoparan effects can be partly attributed to the activation of both heterotrimeric and monomeric G proteins [Loweth et al., 1996; Sukumar et al., 1997]. Mastoparan can exert its biological effects on vesicle transport in three ways: direct effect to activate heterotrimeric G protein, direct effect to activate monomeric G protein, and indirect effect related to the amphiphilic and polycationic properties. Mastoparan has two routes to activate monomeric G protein activation: a direct mechanism through GTP/GDP exchange [Amin et al., 2003] and an indirect mechanism related to its amphiphilic and polycationic properties [Chen et al., 2004]. The present study found that Sar1 translocation induced by GTPγS was suppressed significantly by mastoparan 7 but not by mastoparan 17, indicating that the suppressive effect of mastoparan may be due to the direct effect to activate key G proteins, either monomeric or heterotrimeric G proteins rather than its amphiphilic and polycationic properties. Pertussis toxin treatment of cultured cells results in complete ADP ribosylation of all $G_{\alpha i}$ subunits present in the cells [Stow et al., 1991]. $G_{\alpha i}$ subunits can be functionally uncoupled by pertussis toxin-induced ADP ribosylation. In present study, also it seems that, under pertussis toxin pretreatment, there was no $G_{\alpha i}$ protein in the microsome which could be activated by mastoparan 7 stimulation. Therefore, pertussis toxin treatment of cultured cells suppressed the effect of mastoparan 7 on Sar1 translocation (Fig. 5 MP7; −IAP and +IAP), indicating that mastoparan activates heterotrimeric $G_{i2}$ protein to reduce the amount of Sar1 translocated onto the microsome membrane.

In this study, mastoparan 7 induced greater suppression of Sar1 translocation onto the microsome than H89 (Fig. 5). Pertussis toxin treatment remained some un-recovered part in suppressive effect of mastoparan 7 on Sar1 translocation (Fig. 5: GTPγS + IAP vs. MP7 + IAP). Amphiphilic peptides, such as mastoparan, induced changes in the cisternal architecture indicative of membrane damage, and pertussis toxin treatment of the membranes appeared to increase the rate of vesicle formation through the activation of heterotrimeric G protein, but did not prevent the membrane damage induced by mastoparan [Weidman and Winter, 1994]. The membrane damage induced by mastoparan 7 may disturb co-relation of heterotrimeric G proteins and G protein coupled-protein in their geography, resulting in disturbance of their co-operative processes in ER-Golgi vesicle transport at the early stage of vesicle formation before coat protein assembly on the ER indirectly. The partial recovery by pertussis toxin (Fig. 5) may be related to the membrane damage caused by mastoparan 7. There is a possibility that suppression of Sar1 translocation in the present study resulted from the additive effects of membrane damage and G protein activation caused by mastoparan, but pertussis toxin treatment effectively restored the suppressive effect of mastoparan 7 on Sar1 translocation. Therefore, our results indicate that ER-resident $G_{i2}$ protein is important as a negative regulator of vesicular transport at the early stage of vesicle formation before coat protein assembly on the ER.

## REFERENCES

Ahmadian MR, Mittal R, Hall A, Wittinghofer A. 1997. Aluminum fluoride associates with the small guanine nucleotide binding proteins. FEBS Lett 408:315–318.

Amin RH, Chen HQ, Veluthakal R, Silver RB, Li J, Li G, Kowluru A. 2003. Mastoparan-induced insulin secretion from insulin-secreting betaTC3 and INS-1cells: Evidence for its regulation by Rho subfamily of G proteins. Endocrinology 144:4508–4518.

Aridor M, Balch WE. 2000. Kinase signaling initiates coat complex II (COPII) recruitment and export from the mammalian endoplasmic reticulum. J Biol Chem 275:35673–35676.

Aridor M, Bannykh SI, Rowe T, Balch WE. 1995. Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. J Cell Biol 131:875–893.

Audigier Y, Nigam SK, Blobel G. 1988. Identification of a G protein in rough endoplasmic reticulum of canine pancreas. J Biol Chem 263:16352–16357.

Balch WE. 1992. From G minor to G major. Curr Biol 2:157–160.

Balch WE, Dunphy WG, Braell WA, Rothman JE. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylglucosamine. Cell 39:405–416.

Barlowe C. 1997. Coupled ER to Golgi transport reconstituted with purified cytosolic proteins. J Cell Biol 139:1097–1108.

Barlowe C, Orci L, Yeung T, Hosobuchi M, Hamamoto S, Salama N, Rexach MF, Ravazzola M, Amherdt M, Schekman R. 1994. COPII: A membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 77:895–907.

Barr FA, Leyte A, Huttner WB. 1992. Trimeric G proteins and vesicle formation. Trends Cell Biol 2:91–94.

Beckers CJ, Balch WE. 1989. Calcium and GTP: Essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. J Cell Biol 108:1245–1256.

Bigay J, Deterre P, Pfister C, Chabre M. 1987. Fluoride complexes of aluminium or beryllium act on G-proteins as reversibly bound analogues of the gamma phosphate of GTP. EMBO J 6:2907–2913.

Bourne HR. 1988. Do GTPases direct membrane traffic in secretion? Cell 53:669–671.

Chavrier P, Parton RG, Hauri HP, Simons K, Zerial M. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell 62:317–329.

Chen HQ, Veluthakal R, Palanivel R, Kowluru A. 2004. GTP-binding protein-independent potentiation by mastoparan of IL-1beta-induced nitric oxide release from insulin-secreting HIT-T15cells. Apoptosis 9:145–148.

Daniel S, Noda M, Cerione RA, Sharp GW. 2002. A link between Cdc42 and syntaxin is involved in mastoparan-stimulated insulin release. Biochemistry 41:9663–9671.

Davidson HW, Balch WE. 1993. Differential inhibition of multiple vesicular transport steps between the endoplasmic reticulum and trans Golgi network. J Biol Chem 268:4216–4226.

Davidson HW, McGowan CH, Balch WE. 1992. Evidence for the regulation of exocytic transport by protein phosphorylation. J Cell Biol 116:1343–1355.

Du X, Kristiana I, Wong J, Brown AJ. 2006. Involvement of Akt in ER-to-Golgi transport of SCAP/SREBP: A link between a key cell proliferative pathway and membrane synthesis. Mol Biol Cell 17:2735–2745.

Farquhar MG. 1985. Progress in unraveling pathways of Golgi traffic. Annu Rev Cell Biol 1:447–488.

Higashijima T, Uzu S, Nakajima T, Ross EM. 1988. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). J Biol Chem 263:6491–6494.

Higashijima T, Burnier J, Ross EM. 1990. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. Mechanism and structural determinants of activity. J Biol Chem 265: 14176–14186.

Höltje M, von Jagow B, Pahner I, Lautenschlager M, Hörtnagl H, Nürnberg B, Jahn R, Ahnert-Hilger G. 2000. The neuronal monoamine transporter VMAT2 is regulated by the trimeric GTPase $G_o(2)$. J Neurosci 20:2131–2141.

Jamora C, Yamanouye N, Van Lint J, Laudenslager L, Vandenheede JR, Faulkner DJ, Malhotra V. 1999. Gβγ-mediated regulation of Golgi organization is through the direct activation of protein kinase D. Cell 98:59–68.

Konrad RJ, Young RA, Record RD, Smith RM, Butkerait P, Manning D, Jarett L, Wolf BA. 1995. The heterotrimeric G-protein Gi is localized to the insulin secretory granules of beta-cells and is involved in insulin exocytosis. J Biol Chem 270:12869–12876.

Lee TH, Linstedt AD. 2000. Potential role for protein kinases in regulation of bidirectional endoplasmic reticulum-to-Golgi transport revealed by protein kinase inhibitor H89. Mol Biol Cell 11:2577–2590.

Loweth AC, Williams GT, Scarpello JH, Morgan NG. 1996. Heterotrimeric G-proteins are implicated in the regulation of apoptosis in pancreatic beta-cells. Exp Cell Res 229:69–76.

Matsuo S, Inai T, Kuris K, Kiyomiya K, Kurebe M. 1996. Influence of fluoride on secretory pathway of the secretory ameloblast in rat incisor tooth germs exposed to sodium fluoride. Arch Toxicol 70:420–429.

Matsuo S, Kiyomiya K, Kurebe M. 1998. Mechanism of toxic action of fluoride in dental fluorosis: Whether trimeric G proteins participate in the disturbance of intracellular transport of secretory ameloblast exposed to fluoride. Arch Toxicol 72:798–806.

Matsuo S, Nakagawa H, Kiyomiya K, Kurebe M. 2000. Fluoride-induced ultrastructural changes in exocrine pancreas cells of rats: Fluoride disrupts the export of zymogens from the rough endoplasmic reticulum (rER). Arch Toxicol 73:611–617.

Matsuoka K, Morimitsu Y, Uchida K, Schekman R. 1998a. Coat assembly directs v-SNARE concentration into synthetic COPII vesicles. Mol Cell 2:703–708.

Matsuoka K, Orci L, Amherdt M, Bednarek SY, Hamamoto S, Schekman R, Yeung T. 1998b. COPII-coated vesicle formation reconstituted with purified coat proteins and chemically defined liposomes. Cell 93:263–275.

Melançon P, Glick BS, Malhotra V, Weidman PJ, Serafini T, Gleason ML, Orci L, Rothman JE. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. Cell 51:1053–1062.

Mittal R, Sharma S, Chhibber S, Harjai K. 1996. Formation of a transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate, and GTPase-activating proteins. Science 273:115–117.

Nagaya H, Wada I, Jia YJ, Kanoh H. 2002. Diacylglycerol kinase delta suppresses ER-to-Golgi traffic via its SAM and PH domains. Mol Biol Cell 13:302–316.

Nakagawa H, Miyazaki S, Abe T, Umadome H, Tanaka K, Nishimura K, Komori M, Matsuo S. 2011. H89 sensitive kinase regulates the translocation of Sar1 onto the ER membrane through phosphorylation of ER-coupled β-tubulin. Int J Biochem Cell Biol 43:423–430.

Palade G. 1975. Intracellular aspects of the process of protein synthesis. Science 189:347–358.

Palmer KJ, Konkel JE, Stephens DJ. 2005. J Cell Sci 118:3839–3847.

Robinson MS, Kreis TJ. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: Effects of brefeldin A and G protein activators. Cell 69:129–138.

Rowe T, Aridor M, McCaffery JM, Plutner H, Nuofter C, Balch WE. 1996. COPII vesicles derived from mammalian endoplasmic reticulum microsomes recruit COPI. J Cell Biol 135:895–911.

Schekman R, Orci L. 1996. Coat proteins and vesicle budding. Science 271: 1526–1533.

Sternweis PC, Gilman AG. 1982. Aluminum: A requirement for activation of the regulatory component of adenylate cyclase by fluoride. Proc Natl Acad Sci USA 79:4888–4891.

Stow JL, de Almeida JB, Narula N, Holtzman EJ, Ercolani L, Ausiello DA. 1991. A heterotrimeric G protein, G alpha i-3, on Golgi membranes regulates the secretion of a heparan sulfate proteoglycan in LLC-PK1 epithelial cells. J Cell Biol 114:1113–1124.

Sukumar M, Ross EM, Higashijima TA. 1997. A $G_s$-selective analog of the receptor-mimetic peptide mastoparan binds to Gs alpha in a kinked helical conformation. Biochemistry 36:3632–3639.

Weidman PJ, Winter WM. 1994. The G protein-activating peptide, mastoparan, and the synthetic NH2-terminal ARF peptide, ARFp13, inhibit in vitro Golgi transport by irreversibly damaging membranes. J Cell Biol 127:1815–1827.

Wong DH, Brodsky FM. 1992. 100-kD proteins of Golgi- and trans-Golgi network-associated coated vesicles have related but distinct membrane binding properties. J Cell Biol 117:1171–1179.

Yajima H, Komatsu M, Yamada S, Straub SG, Kaneko T, Sato Y, Yamauchi K, Hashizume K, Sharp GW, Aizawa T. 2000. Cerulenin, an inhibitor of protein acylation, selectively attenuates nutrient stimulation of insulin release: A study in rat pancreatic islets. Diabetes 49:712–717.