In Vitro Fusion of Rabbit Liver Golgi Membranes with Liposomes*

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Fusion of Golgi membranes isolated from rabbit liver with liposomes was studied by lipid mixing of fluorescent lipid analogues and internal content mixing and by electron microscopic observation of transfer of horseradish peroxidase from liposomes into Golgi membranes. A monoclonal antibody was used to confirm fusion of Golgi membranes but not other contaminating vesicles. Fusion was rapid and efficient, reaching about 20% of the maximum after a 5-min incubation using small or large unilamellar dioleoylphosphatidylcholine liposomes. The fusion was dependent on temperature, decreasing at lower temperatures, and becoming nearly zero below 10 °C. The addition of ATP, GTP, cytosolic factors, or N-ethylmaleimide did not affect fusion. Treatments of Golgi membranes with 0.1 M Na\(_2\)CO\(_3\) or 1 M KCl did not cause any changes in fusion. However, treatment with proteases inhibited fusion. These results suggest that Golgi integral membrane protein(s) are involved in fusion. Changing the medium to an isosmotic substance, sucrose, in place of KCl or NaCl inhibited fusion. The binding assay of fluorescent liposomes to Golgi membranes showed that lowering the temperature or replacing salts with sucrose did not affect binding. However, treatment of Golgi membranes with proteases inhibited binding. Addition of phosphatidylserine or phosphatidylethanolamine to dioleoylphosphatidylcholine liposomes caused a 2-fold increase in binding and fusion. Fusion between Golgi membranes by themselves did not occur. These results provide some information on the mechanism of intracellular vesicular transport.

Membrane fusion is essential in many biological events, such as fertilization, polykaryon formation, virus entry, exocytosis, and endocytosis. To elucidate the molecular mechanism of membrane fusion is a major interest in cell biology. So far, the mechanism has been intensively investigated especially by studying virus entry (1–3). For example, influenza virus enters target cells by endocytosis and transfers its genome through membrane fusion in endosomes at acidic pH. This fusion activity is mediated by a viral spike protein, hemagglutinin, that contains a hydrophobic fusion peptide, which, when exposed to acidic pH, causes fusion of the membranes.

In eukaryotic cells, lipids and proteins are transported by membrane vesicles (4). Membrane fusion between the vesicles and specific acceptor membranes is essential for the transport of macromolecules from one compartment to another. Recently, many approaches for studying membrane fusion have been carried out using in vitro assays (5, 6), and some factors involved in such processes have been identified (7). However, the molecular mechanism of intracellular membrane fusion is not yet fully understood, partly because of the difficulty in isolating vesicles and directly measuring the fusion event. Membrane fusion studies in semi-intact cells or in vitro assays have been published. Kobayashi and Pagano (8) studied ATP-dependent fusion of liposomes with the Golgi complex in perforated fibroblast cells. Martinez-Bazenet et al. (9) studied fusion of liposomes with microsomal membranes of Aspergillus niger.

To study the molecular mechanism of membrane fusion, we have focused on the Golgi complex, because this organelle must fuse with various membrane vesicles and thus plays a central role in lipid and protein traffic (10). We have established an in vitro system for fusion of liposomes with Golgi membranes isolated from rabbit liver and have evaluated the factors responsible for the fusion activity. We use spectroscopic assays based on the resonance energy transfer between fluorescent probes to measure the kinetics and efficiency of fusion by lipid mixing and internal content mixing (11–13). The fusion is rapid, efficient, and temperature-dependent, but does not require ATP and cytosolic proteins. We show that Golgi integral membrane proteins play an important role in the fusion process.

**EXPERIMENTAL PROCEDURES**

**Materials**—DOPC,\(^1\) bovine brain PS, and trypsin (type XIII, L-1-tosylamide-2-phenyl ethyl chloromethyl ketone-treated) were purchased from Sigma. Egg yolk PE, NBD-PE, and cholesterol were purchased from Avanti Polar Lipids. Egg yolk PC was prepared by the method of Singleton et al. (14). R18, FITC-dextran (M, 17,200), and anti-fluorescein IgG (rabbit) were purchased from Molecular Probes. Proteinase K was purchased from Boehringer Mannheim, and Ficoll was from Nacalai Tesque.

**Preparation of Liposomes**—Lipids were mixed with fluorescent probes, NBD-PE and R18, from stock solutions (lipids/NBD-PE/R18 = 97:1.5:1.5, molar ratio), dried under nitrogen, and put under vacuum for at least 12 h. For the preparation of SUV, Hepes buffer (145 mM KCl, 5 mM Hepes, pH 7.3) was added to a thin layer of the

\(^1\)The abbreviations used are: DOPC, dioleoylphosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; R18, octadecylrhodamine B; FITC, fluorescein isothiocyanate; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; PNS, postnuclear supernatant; HRP, horseradish peroxidase; PC, phosphatidylcholine; NEM, N-ethylmaleimide; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; DAB, 3,3'-diaminobenzidine; NRK, normal rat kidney; Pipes, 1,4-piperazinediethanesulfonic acid; GTP\(\gamma\)S, guanosine 5'-O-(thio)triphosphate.
dried lipids. The mixture was vigorously vortexed and sonicated under nitrogen by a Kajiya-Denki TA-52 sonicator equipped with a titanium tip. LUV were prepared by reverse-phase evaporation (15) and extruded through 0.2-μm and 0.1-μm polycarbonate membranes, successively. Electron microscopic observation with negative-staining technique showed that the average diameter was about 30 nm and 200 nm for LUV. For electron microscopic observation or internal content mixing assay, DOPC LUV was prepared in either HRP (5 mg/ml) or FITC-dextran (0.1 mM) in Hepes buffer, respectively. Uncapsulated HRP or FITC-dextran was separated by the FicollTip method.

**Preparation of Golgi Membranes**—The Golgi membranes were prepared from rabbit liver according to the procedures of Tabas and Kornfeld (17) and Balch et al. (18). Briefly, fresh rabbit liver was minced and immediately placed in 10 mM Tris-HCl (pH 7.4), containing 0.5 M sucrose and 5 mM EDTA. The liver was then homogenized with a Polytron homogenizer (Brinkman Instruments) and centrifuged at 3,500 rpm for 10 min. PNS was collected and filtered through cheesecloth, layered on top of 1.25 M sucrose (dissolved in 10 mM Tris-HCl, pH 7.4.), and centrifuged at 25,000 rpm for 90 min in a Beckman SW 27 rotor. The crude smooth-membrane fraction, above the interface with the 1.25 M sucrose layer, was removed and adjusted to 1.2 M sucrose. Then, this membrane fraction was overlaid with 1.1 M, 1.0 M, and 0.5 M sucrose, and centrifuged for 2.5 h at 25,000 rpm in the SW 27 rotor. The Golgi membrane fraction, which banded at the 0.5 M sucrose/1.0 M sucrose interface, was collected, frozen in liquid nitrogen, and stored at -80°C until use. Once thawed and unused portions were discarded. The protein concentration was adjusted to 2.0 mg/ml by the method of Lowry et al. (19).

Smooth ER, rough ER, and plasma membranes were isolated from rabbit liver according to the method of Fleischer and Kervina (20).

After fusion with DOPC LUV containing HRP, the Golgi membranes were examined by electron microscopy. One μl of liposomes was incubated with the Golgi membranes (10 μl in Hepes buffer) at 37°C for 15 min and washed by sedimentation through 0.5 M sucrose onto a cushion of 1.0 M sucrose at 25,000 rpm in a Beckman SW 27 rotor. The membranes were collected from the top of the 1.0 M sucrose layer and resuspended in 0.5 M sucrose and 5 mM EDTA. The liver was minced and immediately placed in 10 mM Tris-HCl, pH 7.4, for 30 min at room temperature, and sedimented at 2,000 rpm for 10 min. To visualize HRP, the fixed membranes were incubated with 0.25 mg/ml 3,3'-diaminobenzidine (DAB; Dojin) in 50 mM Tris-HCl, pH 7.4, for 30 min and then added with H2O2 at a final concentration of 0.0025%. After 10 min incubation in the dark, the membranes were post-fixed with 1% OsO4 in 0.2 M cacodylate buffer for 90 min, dehydrated in ethanol, and embedded in Epon.

**Monoclonal Antibody Production and Immunofluorescence**—Six-week-old BALB/c mice were injected intraperitoneally with 150 μg of Golgi membranes in Freund's complete adjuvant. After two weeks, the animals were boosted with the same amount of Golgi membranes in Freund's incomplete adjuvant. After two weeks, a final booster of 450 μg of the Golgi membranes without adjuvant was injected intra-peritoneally. Splenies were removed from three immunized mice 3 days after the booster. The spleen cells were fused with P3X63-Ag8.653 myeloma line (21). Positive clones were identified by immunofluorescence screening using normal rat kidney (NRK) cells. A hybridoma cell line secreting an antibody exhibiting a Golgi-like pattern was subcloned twice by limiting dilution. The monoclonal antibody C6A3, purified by ammonium sulfate precipitation, was of the IgG1 subclass.

NRK cells were grown on 15-mm round glass coverslips in Ham's F-12 medium containing 5% fetal calf serum for 48 h prior to use. The cells were washed twice with PBS and fixed by immersion in methanol at -20°C for 5 min. In some experiments, cells were incubated with 1% sodium citrate-phosphate buffer, pH 11.5, incubated for 30 min on ice, and rinsed by sedimentation through 0.5 M sucrose onto a 1.0 M sucrose layer. KC1-extracted Golgi membranes (25) were prepared by diluting membranes 3-fold in 3.0 M KC1, 5 mM Hepes, pH 7.3, incubated for 30 min on ice, washed, and collected. The pellets were incubated with NEM (final concentration 1 mM) for 15 min on ice with the addition of diethiothreitol (final concentration 2 mM). The control membranes were prepared by mixing the enzymes and inhibitors at the start of incubation.

Na2CO3-extracted Golgi membranes (24) were prepared by diluting membranes 3-fold in 0.3 M Na2CO3, pH 11.5, incubated for 30 min on ice, and rinsed by sedimentation through 0.5 M sucrose onto a 1.0 M sucrose layer. KC1-extracted Golgi membranes (25) were prepared by diluting membranes 3-fold in 3.0 M KC1, 5 mM Hepes, pH 7.3, incubated for 30 min on ice, washed, and collected. The pellets were incubated with NEM (final concentration 1 mM) for 15 min on ice with the addition of diethiothreitol (final concentration 2 mM). The control membranes were prepared by mixing NEM and diethiothreitol at the beginning of incubation.

**Vesicle Fusion Assay**—The binding of liposomes to Golgi membranes was assayed using UV containing NBD-PE and R18 in a similar way to the lipid mixing assay. After incubation, the assay mixture (350 μl) was laid on a discontinuous gradient consisting of 200 μl of 0.5 M, 150 μl of 1.0 M sucrose buffer containing 10 mM Tris-HCl, pH 7.4. The centrifugation was carried out at 40,000 rpm for 30 min in a Beckman SW 50.1 rotor. The membranes were collected from the top of the 1.0 M sucrose layer. KCl-extracted Golgi membranes (25) were prepared by diluting membranes 3-fold in 3.0 M KCl, 5 mM Hepes, pH 7.3, incubated for 30 min on ice, washed, and collected. The pellets were incubated with NEM (final concentration 1 mM) for 15 min on ice with the addition of diethiothreitol (final concentration 2 mM). The control membranes were prepared by mixing NEM and diethiothreitol at the beginning of incubation.

**Enzyme Assay**—The binding of liposomes to Golgi membranes was assayed using UV containing NBD-PE and R18 in a similar way to the lipid mixing assay. After incubation, the assay mixture (350 μl) was laid on a discontinuous gradient consisting of 200 μl of 0.5 M, 150 μl of 1.0 M sucrose buffer containing 10 mM Tris-HCl, pH 7.4. The centrifugation was carried out at 40,000 rpm for 30 min in a Beckman SW 50.1 rotor. The membranes were collected from the top of the 1.0 M sucrose layer. KCl-extracted Golgi membranes (25) were prepared by diluting membranes 3-fold in 3.0 M KCl, 5 mM Hepes, pH 7.3, incubated for 30 min on ice, washed, and collected. The pellets were incubated with NEM (final concentration 1 mM) for 15 min on ice with the addition of diethiothreitol (final concentration 2 mM). The control membranes were prepared by mixing NEM and diethiothreitol at the beginning of incubation.
RESULTS

Characterization of the Golgi Membrane Fraction—The Golgi membrane fraction was obtained from rabbit liver by the sucrose density gradient centrifugation as described under “Experimental Procedures.” The specific activity of galactosyltransferase and α-mannosidase (Golgi marker enzymes) in the Golgi fraction was much higher, 27- and 13-fold, respectively, than that in PNS. On the other hand, in the Golgi membrane fraction, the activity of the endoplasmic reticulum marker glucose-6-phosphatase and the plasma membrane marker 5′-nucleotidase was 1.7- and 2.6-fold higher than that in PNS, respectively.

Golgi membranes were exposed to DOPC LUV containing HRP and isolated from unbound LUV by centrifugation. HRP activity was visualized by reaction with DAB and observed by electron microscopy (Fig. 1). The Golgi membranes consisted of flattened saccular structures and vesicles with a diameter of about 0.5 μm. The HRP reaction products were predominantly confined to the internal spaces of the saccules and vesicles. There were some vesicles exhibiting no HRP activity, which probably came from fragments of other organelles and plasma membranes. To inactivate HRP that were not transferred into the lumen of the saccules, Golgi membranes fused with HRP-containing LUV were incubated with trypsin (final 100 μg/ml) for 30 min at 37 °C and reacted with DAB. The electron micrographs of these specimen were almost the same as those of untreated Golgi membranes (data not shown).

We raised a monoclonal antibody, called C6A3, against the Golgi membrane fraction. By immunofluorescence, C6A3 revealed a juxtanuclear reticular structure in NRK cells (Fig. 2A). When cells were treated with nocodazole, the structure was fragmented into large elements distributed through the cytoplasm (Fig. 2B). In cells treated with brefeldin A for 1 h at 37 °C, C6A3 did not reveal any characteristic staining pattern (data not shown). These results suggest that the monoclonal antibody bound to the Golgi complex in NRK cells. Similar specific staining for the Golgi complex by C6A3 was obtained in the acinar cells of pancreas and parotid gland, the duct cells of parotid gland, and the epithelial cells of urinary tubules and small intestines (data not shown).

The protein A-Sepharose 4B beads were coupled with C6A3 or affinity-purified mouse IgG as a control. These beads were incubated with HRP-containing Golgi membranes. HRP activity from Golgi membranes coupled with C6A3 beads was much larger (5 times) than that with normal IgG beads (Fig. 2C). These results suggest that the HRP-containing membranes have a protein recognized by C6A3 and are therefore derived from the Golgi complex.

Fusion of Golgi Membranes with Liposomes—Fusion was assayed by two independent fluorescence methods, lipid mixing and internal content mixing, as described under “Experimental Procedures.” In lipid mixing assay, DOPC vesicles containing NBD-PE and R18 were incubated with Golgi membranes. The fluorescence intensity of NBD-PE should increase on fusion because of dilution of the fluorescent probes with lipid molecules of Golgi membranes. In content mixing assay, DOPC vesicles entrapping FITC-dextran at a self-quenching concentration was incubated with Golgi membranes. The fluorescence intensity of FITC should increase on fusion because of dilution of FITC-dextran with the internal aqueous content of Golgi vesicles. The anti-fluorescein IgG was added to vesicle suspension to bind to FITC-dextran, which was probably leaked out from vesicles to the outer aqueous buffer, and quench the fluorescence.

When DOPC/NBD-PE/R18 SUV (0.04 mM) were incubated with Golgi membranes (0.3 mg/ml protein) at 37 °C for 2 h, the fluorescence intensity was 20% of the maximum after 5 min (Fig. 3A). LUV were prepared from DOPC, NBD-PE, and R18 and were
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FIG. 3. Fusion of Golgi membranes with liposomes assayed by lipid mixing. SUV (a) or LUV (c) of DOPC/NBD-PE/R18 at a molar ratio of 97:1.5:1.5 (0.04 mM) were incubated with Golgi membranes (0.3 mg/ml protein) in Hepes buffer at 37 °C. b, DOPC/NBD-PE/R18 SUV (0.04 mM) were incubated with rabbit liver PNS (0.3 mg/ml protein). The fluorescence intensity of NBD-PE was measured as described under "Experimental Procedures."

incubated with Golgi membranes. The fusion occurred similarly to that with SUV, with a little smaller efficiency (15% of the maximum after 5 min) (Fig. 3c).

When DOPC/NBD-PE/R18 SUV (0.04 mM) were incubated with rabbit liver PNS (0.3 mg/ml protein) or with non-labeled DOPC SUV (0.04 mM), the NBD fluorescence intensity did not change (Fig. 3b). These results indicate that the fluorescence probes were not released from vesicles to cytosolic lipid transfer proteins and were not nonspecifically transferred to non-labeled vesicles.

In content mixing assay, DOPC LUV containing FITC-dextran (0.3 mM) were incubated with Golgi membranes (0.3 mg/ml protein) at 37 °C. The fluorescence intensity of FITC-dextran rapidly increased. The fusion was about 25% of the maximum after 5 min (Fig. 4a). In the absence of anti-fluorescein IgG, the fluorescence intensity was a little (10%) larger than that in its presence (Fig. 4b). The results suggest that only a small leakage of FITC-dextran from vesicles to the outer aqueous phase occurs before and during fusion.

Golgi membranes were sonicated and labeled with R18 at a self-quenching concentration. The fluorescent Golgi membranes (0.3 mg/ml protein) were mixed with unlabeled Golgi membranes (0.2 mg/ml protein) and incubated at 37 °C. However, there was no increase in the fluorescence intensity of R18, indicating absence of fusions between Golgi membranes by themselves (data not shown).

Fusion of DOPC/NBD-PE/R18 SUV (0.04 mM) with other organelle membranes isolated from rabbit liver (0.3 mg/ml protein) was also studied by lipid mixing assay (Fig. 5). For

FIG. 4. Fusion of Golgi membranes with liposomes assayed by internal content mixing. FITC-dextran-containing LUV (0.04 mM) were incubated with Golgi membranes (0.3 mg/ml protein) in Hepes buffer in the presence (a) or absence (b) of anti-fluorescein IgG. The increase in FITC fluorescence was measured as described under "Experimental Procedures."

FIG. 5. Fusion of rabbit liver organelle membranes with SUV assayed by lipid mixing. DOPC/NBD-PE/R18 SUV (0.04 mM) were incubated with 0.3 mg/ml protein from smooth ER membranes (a), rough ER membranes (b), or plasma membranes (c) in Hepes buffer at 37 °C.

FIG. 6. Requirements for fusion of Golgi membranes, treated with proteases, Na2CO3 or sucrose, with SUV or LUV. The fusion efficiency, assayed by lipid mixing, after 5 min of incubation is expressed as the average percentage ± S.D. from three experiments as compared with the control.

FIG. 7. Binding of SUV to Golgi membranes. DOPC/NBD-PE/R18 SUV (0.04 mM) and Golgi membranes (0.3 mg/ml protein) were incubated for 5 min at 37 °C and centrifuged into the upper and the lower fractions. After addition of Triton X-100, the NBD fluorescence intensity of both fractions was measured. The binding percentage was calculated as explained under "Experimental Procedures." The average percentage ± S.D. from three experiments is presented as compared with the control.
smooth ER membranes, the NBD fluorescence intensity increased and became about 20% of the maximum after 5 min incubation (Fig. 5a). The fusion rate for smooth ER membranes was much slower than that for Golgi membranes (compare Fig. 5a with 3a). The NBD fluorescence intensity for rough ER membranes and plasma membranes only slightly increased after 3 min (Fig. 5, b and c).

Requirements for Fusion of Golgi Membranes with Liposomes—The fusion assay was usually carried out in Hepes buffer containing 145 mM KCl. Substitution of NaCl for KCl did not show any changes in fusion. However, when the solvent was changed to isosmotic sucrose (0.25 M) in place of KCl, the fusion was inhibited. The fusion efficiency after 5 min was reduced to 20% of the control (Fig. 6). When isotonic KCl was added to 0.25 M sucrose after 5 min of incubation, the fusion efficiency was recovered to the control level (data not shown). These results were obtained by lipid mixing as well as content mixing assays.

Treatment with ATP (1 mM), GTP (1 mM), GTPγS (10 μM), or cytosolic proteins (1 mg/ml) obtained from PNS showed the same fusion as that for the control. NEM treatment (1 mM) of the Golgi membranes did not affect fusion. Lectins (wheat germ agglutinin or concanavalin A) also did not affect fusion. CaCl₂ (1–5 mM), Tris (5 mM), Pipes (5 mM), or ionophores such as valinomycin (10 μM) and nigericin (30 μM) showed the same extent of fusion as that for Hepes buffer (data not shown).

Treatment of Golgi membranes with trypsin (10 μg/ml) or proteinase K (1 μg/ml) for 1 h on ice inhibited the fusion by about 50% of the control (Fig. 6). Extraction of Golgi membranes with 0.1 M Na₂CO₃ or 1 M KCl did not inhibit the fusion. However, when Golgi membranes were pretreated with Na₂CO₃ and then treated with trypsin, the fusion was inhibited to about 30% of the control (data not shown). These results suggest the involvement of integral proteins in the Golgi membranes for fusion.

The temperature dependence of fusion was also studied. The fusion of Golgi membranes with SUV or LUV after 5 min of incubation was reduced gradually with decreasing temperature and was essentially nonexistent below 10 °C.

Binding of Liposomes to Golgi Membranes—The binding of liposomes to Golgi membranes was assayed using DOPC/NBD-PE/R18 SUV in a manner similar to that for lipid mixing assay. After incubation and centrifugation, the NBD fluorescence intensity of the upper and the lower fractions were measured. Triton X-100 was added to both fractions to quench the energy transfer between fluorescent probes and the light scattering of Golgi membranes. In this assay, the NBD fluorescence intensity of the lower fraction corresponds to the extent of SUV bound to Golgi membranes, whether SUV were fused with membranes or not. The results are shown in Fig. 7. The binding of SUV to Golgi membranes at a lower temperature (4 °C) or in isosmotic sucrose media was nearly the same as that of the control. The fusion was, however, inhibited under these conditions. Trypsin or proteinase K treatments of Golgi membranes inhibited the binding to about 50% of the control.

Effects of Liposome Composition—The effects of addition of PS or PE to DOPC SUV on binding and fusion with Golgi membranes were studied. Equimolar addition of PS or PE to DOPC SUV caused a 2-fold increase in binding as well as fusion (Fig. 8). The addition of cholesterol to DOPC did not affect binding and fusion to a large extent. Egg PC SUV showed less efficient binding and fusion than DOPC SUV.

**DISCUSSION**

We demonstrate fusion of Golgi membranes with liposomes by fluorescent assays using lipid mixing and internal content mixing, and by electron microscopic observation using transfer of HRP from LUV into Golgi membranes. The fusion is rapid and efficient, reaching about 20% of the maximum in 5 min at 37 °C. Nearly the same results were obtained from two independent fluorescence assays, confirming the fusion event. Evidence for liposomes fused with Golgi membranes but not with the contaminating vesicles is suggested by using the Golgi-specific monoclonal antibody C6A3. The fusion occurs independent of ATP or cytosolic proteins. GTP, GTPγS, and NEM do not inhibit the fusion. Nonspecific exchange and release of the fluorescent probes from liposomes in the presence of cytosol do not occur. LUV also fused with Golgi membranes with a slightly lower efficiency.

It is most likely that the fusion process is mediated by integral protein(s) in Golgi membranes. Protease treatment of Golgi membranes inhibits both the binding to liposomes and the fusion activity. However, the extraction of peripheral proteins by 1 M KCl or 0.1 M Na₂CO₃ from Golgi membranes does not affect binding and fusion. Lower temperature (below 10 °C) or substitution of KCl with isosmotic sucrose does not affect binding but inhibits fusion. One possibility is that the putative fusion protein works well at higher temperature or at larger ionic strength. However, the binding remains active under these conditions. Another possibility is that the binding and fusion are mediated by different proteins or factors.

The protease treatment inhibited binding and fusion by 50% of the control at most. This may be due to partial digestion of the relevant protein because of a relatively low concentrations of protease and reaction incubations on ice. When Golgi membranes were first treated with Na₂CO₃ and then subjected to protease treatment, the inhibition was increased to about 70%. This may also suggest that peripheral proteins reduce the inhibition by proteases.

Addition of PS or PE to DOPC liposomes causes a 2-fold increase in binding and fusion. These lipids may increase the binding to Golgi membranes and therefore enhance fusion. The aminophospholipids, which are dominant in cytoplasmic leaflet of the ER and plasma membranes (30), may have a high affinity for the Golgi complex. However, we should consider these results carefully, because lipid composition could affect the size and stability of liposomes, and then fluidity of lipids.

Kobayashi and Pagano (8) studied fusion of fluorescent-labeled liposomes with the Golgi complex in perforated fibro-
blasts by using fluorescence microscopy. Their results, in general, agreed with the present results. However, the fusion that they saw was dependent on ATP and inhibited by NEM treatment. Addition of equimolar PS to DOPC liposomes (30–80 nm in diameter) inhibited fusion with the Golgi complex. DOPC/PE at equimolar ratio showed only faint labeling. These features are different from the present results obtained from studying in vitro fusion itself. Perforated cells contain cellular components such as microtubules, microfilaments, vesicles, organella, and cytoplasm. Protein transport from the ER to the Golgi requires free Ca**+** in penetrated cells (31), not in a cell-free system (32). In perforated cells, ATP and NEM may have the capability to act on different steps that were not reconstituted in the present system.

The intracellular protein transport in in vitro systems has been studied: the ER to the Golgi (31, 33), intra-Golgi (18, 34), endosome fusion (5), and trans-Golgi network to plasma membrane (35–37). These systems require ATP and cytosolic proteins, and are sensitive to NEM treatment and GTP7S. Especially, NEM-sensitive proteins are involved in the transport from the ER to the Golgi (38), intra-Golgi (39, 40), and endosome fusion (41). The existence of vesicular transport of lipids has been suggested in Dictyostelium discoideum (42), Accanthamoeba spp. (43) and cultured mammalian cells (44). Exogenous fluorescence-labeled phospholipids (PC and PE) could be incorporated into cells by vesicles (45, 46). Delivery of newly synthesized cholesterol to plasma membrane was also mediated by vesicular carriers (47). Recently, vesicular transport of lipids from the ER to the Golgi complex was studied in a cell-free system (48). These systems require energy and are temperature-dependent, similar to that of the protein transport. These studies involved several steps such as vesicle formation, targeting and fusion in protein, and lipid transport. Therefore, it is unclear for which step in the transport the specific factor should be required. For example, ATP and NSF are involved in only early steps in the vesicular transport between the ER to the Golgi complex (49). On the contrary, we reconstitute only the fusion process and conclude that the in vitro fusion of Golgi membranes with liposomes is independent of ATP and cytosolic proteins. These results may provide some information about the mechanism(s) of intracellular protein and lipid transport.

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