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REVIEW

Utilization of Liposomes in Vesicular Transport Studies

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ABSTRACT. **Various coated vesicles are implicated in the intracellular transport between different compartments. *In vitro* reconstitution is a powerful experimental system to study molecular mechanisms involved in assembly of coat proteins from cytosol onto membranes as well as formation of coated vesicles. Liposomes have been recently utilized in the cell-free systems. In this review, we summarize studies on reconstitutions of coated vesicles or coated structures on liposomes. A novel method using dynamic light scattering (DLS) to quantify vesicle formation from liposomes also is described. Our recent study on the role of phospholipids in vesicle formation, where the DSL assay is used in combination with lipid analysis, also is introduced.**

Key words: liposome/vesicular transport/coat proteins/phospholipids/light scattering/endocytosis

Vesicular transport between different intracellular compartments is achieved by coated vesicles. To date, three types of coats have been well characterized, namely, clathrin coat, coat protein complex I (COPI), and coat protein complex II (COPII). Formation of coated vesicles is initiated by the assembly of coat proteins from cytosol onto membranes. Coated membranes generate coated buds, which are pinched off to generate coated vesicles to be transported to acceptor compartments.

It has been a focus of interest for cell biologists to understand the molecular mechanisms underlying the assembly of coats and the formation of coated vesicles. Rapid progress was made possible by a powerful experimental system developed by Rothman and colleagues (Balch *et al.*, 1984), which reconstituted vesicular traffic between Golgi cisterna *in vitro*, by incubating Golgi membrane with cytosol and nucleotides. By manipulating *in vitro* conditions, they have identified one of the non-clathrin coats, now designated as COPI coat, and characterized the sequential processes of the vesicular transport (Rothman and Orci, 1992; Rothman, 1994; Söllner and Rothman, 1996). Together with yeast genetics, the *in vitro* system has been widely employed in studies of all types of vesicular transport. As a result, the constituents of the coats and several novel molecules regulating the processes of coat assembly and vesicle formation have been identified.

At present, all three types of coated vesicles and the coated intermediates can be reconstituted *in vitro* by incubating the biological membranes with cytosol and nucleotides (Orci *et al.*, 1986; Barlowe *et al.*, 1994; Takei *et al.*, 1995). These *in vitro* systems have been modified so that the coated structures were generated on liposomes not on biological membranes (Spang *et al.*, 1998; Matsuoka *et al.*, 1998; Takei *et al.*, 1998). The reconstitution of coated structures on liposomes allows us to analyze functional roles of membrane lipids in vesicle formation as well as specific interactions between coat proteins and specific membrane lipids.

In this review, we first summarize the reconstitution of each type of coated vesicle or coated structure generated from liposomes. Next, we describe a novel method to quantify vesicles formed from liposomes by using dynamic light scattering (DLS). We also introduce our recent study on the role of phospholipids in vesicle formation, which was analyzed by the combination of DSL assay and lipid analysis (Kinuta *et al*., 2002).

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Abbreviations: AP, adaptor protein; Arf, ADP-ribosylation factor; COP, coat protein complex; DLS, dynamic light scattering; EM, electron microscopy; ER, endoplasmic reticulum; GTPγS, guanosine 5'-O-thiotriphosphate; HPLC, high-performance liquid chromatography; HPTLC, high performance thin-layer chromatography; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, phosphatidylserine; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; SLS, static light scattering.

Clathrin coats

Clathrin coats are implicated in clathrin-mediated endocytosis at the plasma membrane and at vesicle formation from the Golgi apparatus (Hirst and Robinson, 1998). Clathrin (heterohexamer of heavy chains and light chains) is attached to membrane *via* adaptor proteins, adaptor protein 1 (AP1) at Golgi, and adaptor protein 2 (AP2) at plasma membrane. Neuronal cells implicate an additional adaptor protein, AP180, at the plasma membrane (McMahon, 1999).

Clathrin-mediated endocytosis, a process common to virtually all eukaryotic cells for internalization of nutrients, antigens, recycling receptors and so on, is especially enhanced at the nerve terminals. Neurons secrete neurotransmitters at the synapse by exocytosis of synaptic vesicles, and the synaptic vesicle membranes fused to the plasma membrane by exocytosis are retrieved mainly by clathrin-mediated endocytosis and reused for the generation of new synaptic vesicles. To compensate for the high rates of synaptic vesicle exocytosis, synapse has strong activity for endocytosis, and proteins that function in clathrin-mediated endocytosis are enriched in the synapse (De Camilli *et al*., 2001; Takei and Haucke, 2001). Therefore, the nerve terminal has been considered to provide a suitable model for studies of clathrin-mediated endocytosis.

Both clathrin-coated pits and clathrin-coated vesicles are very transient structures *in vivo*. However, clathrin-coated pits can be stabilized *in vitro* by incubating synaptic membranes with brain cytosol in the presence of ATP and guanosine 5'-O-(thiotriphosphate) (GTPγS), an unhydrolyzable analogue of GTP. The presence of GTPγS inhibits the fission reaction of clathrin-coated pits by dynamin 1, a GTPase enriched in nerve terminals. As a result, clathrin-coated pits remain unpinched and dynamin 1 is polymerized, forming a washer-like ring structure around the neck of clathrin-coated pits, and often overpolymerized to form long spirals along the neck of the clathrin-coated pits (Takei *et al*., 1995).

Morphologically identical clathrin-coated pits and dynamin-coated tubules can be generated on protein-free liposomes by incubating with brain cytosol (Takei *et al*., 1998). In that study, the formation of dynamin rings preferred the presence of acidic phospholipids, such as phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylserine (PS) in liposomes. Clathrin-coat fractions alone can form clathrin-coated pits on liposomes made of total brain lipid extract (Fig. 1). However, the lipid preference for the formation of clathrin-coated pits remains to be clarified.

Incubation of liposomes with dynamin 1 alone results in the formation of dynamin-coated tubules protruding from the liposomes, and the dynamin-coated tubules are vesiculated in the presence of GTP (Takei *et al*., 1998; Sweitzer and Hinshaw, 1998). Thus dynamin's function in membrane fission can be reconstituted by using liposomes. It has been proposed that dynamin's interaction with phospholipids represents the physiological role of dynamin, because dynamin's GTPase activity is stimulated by acidic phopspholipids, such as PS, PG, and phosphatidylinositol (PtdIns) (Tuma *et al.*, 1993). It is noteworthy that PS stimulates both the dynamin GTPase activity and the formation of dynamin-coated tubules. Recently, dynamin polymers on PS liposomes were utilized to determine its three dimensional structure (Zhang and Hinshaw, 2001).

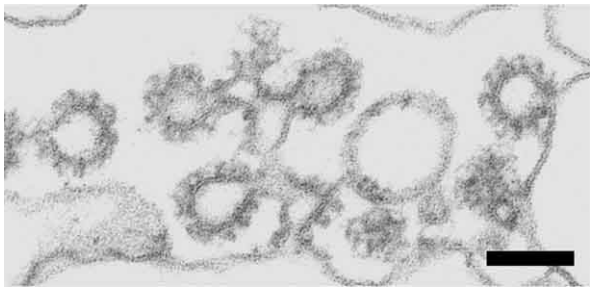

Fig. 1. Clathrin-coated buds formed on liposomes. Electron micrograph of clathrin-coated buds generated on liposomes of total brain lipids by incubating with a bovine brain fraction highly enriched in clathrin coat proteins. The coat demonstrates bristle-like structures typical of clathrin coats observed *in vivo*. Calibration bar represents 100 nm (From Takei *et al*., 1998).

Vesicle formation from dynamin-coated tubules can be monitored by measuring static light scattering (SLS) and EM observation (Sweitzer and Hinshaw, 1998; Takei *et al*., 1999; Takei *et al*., 2001). Using a combination of these assays, it has been revealed that dynamin's fission activity of lipid tubules is enhanced by amphiphysin 1, which functions corporately with dynamin 1 in clathrin-mediated endocytosis in the synapse (Takei *et al*., 1999). For functional analysis of dynamin 1, lipid nanotubules, tubular liposomes that contain non-hydroxy fatty acid galactocerebroside at high concentration, have been also used (Marks *et al*., 2001).

AP1 functions as a clathrin adaptor protein at the Golgi apparatus. The clathrin coat is implicated in vesicular transport from trans-Golgi network to endosomal/lysosomal compartments. The assembly of clathrin/AP1 coat is initiated by GTP-binding ADP-ribosylation factor 1 (Arf1), which generates high affinity binding sites for AP1 on the membrane (Zhu *et al*., 1999; Zhu *et al*., 2001). The formation of clathrin/AP1-coated vesicles can be reconstituted by the incubating membrane, isolated Golgi fractions or liposomes, with bovine liver cytosol, Arf1, and GTPγS. However, clathrin-coat fractions, Arf1 and GTP are not sufficient to recruit AP1 or clathrin on the membrane, indicating the requirement of yet unidentified cytosolic factors. PS is proposed to play a major role in AP1 recruitment (Zhu *et al*., 2001).

COPI or COPII

Non-clathrin coats are categorized into COPI and COPII. COPI-coated vesicles mediate retrograde transport from the Golgi apparatus back to the endoplasmic reticulum (ER), as well as the bidirectional transport among the Golgi stacks (Pelham and Rothman, 2000). The coatomer, the assembly unit of the COPI coat, comprises the heterooligomeric protein complex (α-COP, β-COP, β'-COP, γ-COP, δ-COP, ε-COP, and ζCOP). By incubating liposomes with coatomers, Arf1 and GTPγS, COPI vesicles can be generated from liposomes containing a variety of charged and neutral phospholipids, although the formation prefers acidic phospholipids (Spang *et al*., 1998; Nickel and Wieland, 2001). As in the case with the clathrin/AP1 coat, the recruitment of coatomer coat is initiated by Arf1-GTP, the activated form of Arf1 (Nickel and Wieland, 2001). COPII-coated vesicles mediate anterograde transport from the ER to the Golgi apparatus (Barlowe *et al*., 1994). COPII vesicles also can be formed from liposomes and the formation requires only the three coat subunits, Sar1p (GTP bound), Sec13/31p, and Sec23/24p (Matsuoka and Schekman, 2000). COPII-coated vesicle formation required phosphatidylinositol 4-phosphate [PtdIns(4)P] or phosphatidylinositol 4,5-bisphosphate [PtdIns (4,5)P_2] (Matsuoka *et al*., 1998). The COPII-coated vesicles generated from liposomes have been utilized to determine the relative distance of the coat components and the membranes, as well as EM observation of the surface structure (Matsuoka *et al*., 2001).

The role of phosphoinositides in endocytosis

The role of membrane lipids in membrane traffic is a growing focus of investigation. Accumulating evidence suggests key roles of phosphoinositides, especially PtdIns(4,5)P_2, in various vesicular traffic processes (De

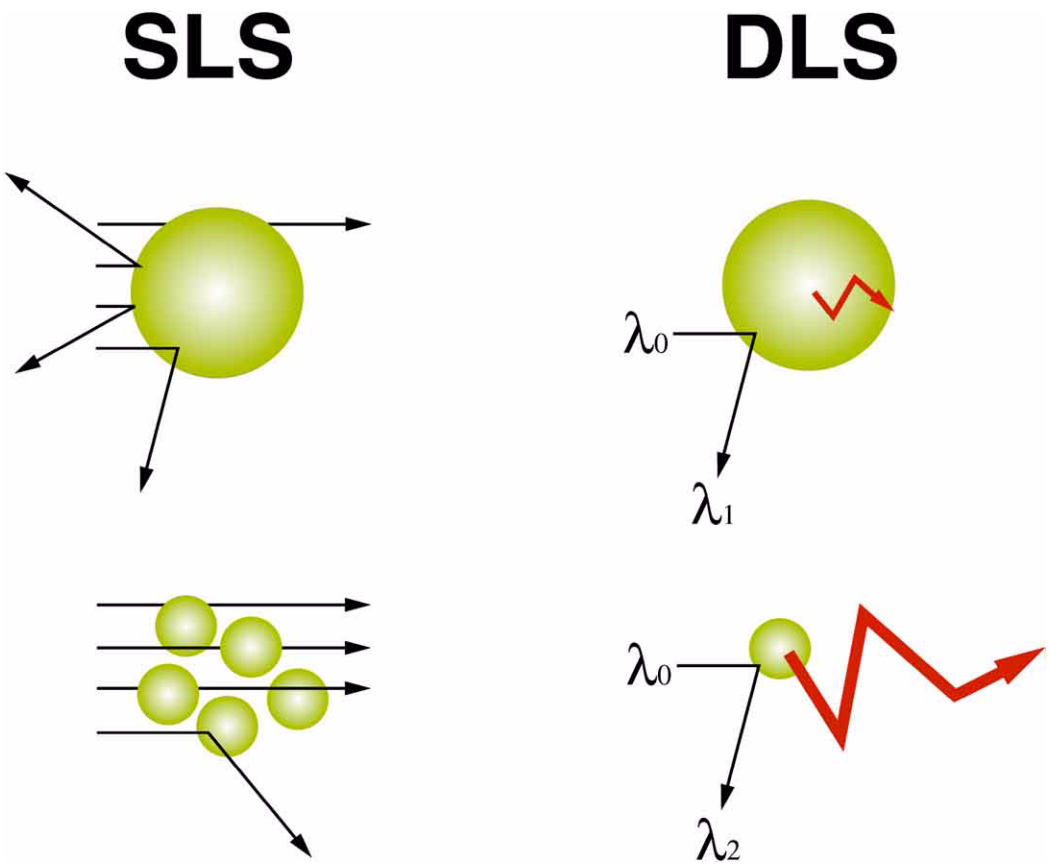

Fig. 2. Illustration of difference in principles between static light scattering (SLS) and dynamic light scattering (DLS). Microparticles in the solution generally move as in so-called Brownian movement. The rate of the movement of particles is based on their diameters; namely, in proportion to their diameter, the larger particles move more slowly than the smaller ones. Left, In SLS the smaller particles have less frequency in response to the incident light, and as a result, less intensity of the scattered light is observed for the smaller particles. Right, When light (wavelength, λ_0) is irradiated to moving particles, the wavelength of the scattered light (λ_1, λ_2) is altered by Doppler effect, which is caused by Brownian movement (irregular loci shown in arrows). In DLS assay the sizes of particles (Storkes' diameter) can be measured by analyzing "fluctuation", the qualitative change of the scattered light. DLS is a useful method for observing diameters of microparticles such as liposomes and small vesicles formed from liposomes as described in the text.

Camilli *et al*., 1996; Corvera *et al*., 1999; Takei and Haucke, 2000; Huijbregts *et al*., 2000; Cremona and De Camilli, 2001). In clathrin-mediated endocytosis, cytosolic proteins that function in endocytosis are recruited to the cytosolic surface of the membrane. The recruitment is likely to be mediated by interactions between these endocytic proteins and PtdIns(4,5)P_2. PtdIns(4,5)P_2 has been known to bind to the α-subunit of AP2 via a positively charged lysine triad at its N-terminus (Gaidarov and Keen, 1999), and also to the pleckstrin homology domain of dynamin (Lin and Gilman, 1996; Lin *et al*., 1997). Recently, another binding motif for PtdIns(4,5)P_2, termed epsin N-terminal homology (ENTH) domain, was shown to be conserved in several endocytic proteins including epsin, AP180/CALM, Hip1R (Ford *et al*., 2001; Itoh *et al*., 2001). Disruption of these interactions by mutations in the phosphoinositide binding modules or by application of neomycin, a PtdIns(4,5)P_2 binding reagent, inhibits both recruitment of these proteins to the membrane (West *et al*., 1997) and endocytosis (Jost *et al*., 1998; Lee *et al*., 1999; Achiriloaie *et al*., 1999; Itoh *et al*., 2001).

Determination of vesicle formation from liposomes by DLS assay

Formation of small vesicles *in vitro* from lipid particles has been assayed generally by a combination of EM, biochemical detection of coat proteins in the vesicle fraction and SLS method (Sweitzer and Hinshaw, 1998; Takei *et al*., 1999; Antonny *et al*., 2001). SLS method is based on the principle that larger particles can scatter the irradiated light more than smaller ones (Fig. 2 left). Since intensity of the scattered light can be measured quickly, SLS is a useful method to survey the time course of vesicle formation from liposomes. However, measuring the size of particles is difficult when samples contain heterogeneous particles. The particles in sample solution randomly move by Brownian movement and this movement depends largely on particle size (Fig. 2 right). When light is irradiated to the moving particles, the wavelength of the scattered light is altered by Doppler effect, and the qualitative changes of the reflecting light called “fluctuation” can be detected by DLS. Thus, DLS allows us to determine not only the diameters of particles ranging from 3 nm to several micrometers, but also the relative number and weight distribution at each size range of the vesicles (Berne and Pecora, 1976; Chu, 1974). Comparison of the principle of SLS with that of DLS is depicted in Fig. 2.

Recently, DLS was utilized for quantitative and qualitative analysis of vesicle formation from liposomes (Kinuta *et al*., 2002). As shown in Fig. 3, the incubation of large unilammelar liposomes (average diameters exceeding 1 μm) with brain cytosol in the presence of ATP and GTP resulted in the generation of small vesicles (average diameters smaller than 100 nm), which appeared as distinct peaks in DLS mesurement. The results obtained by the DLS assay

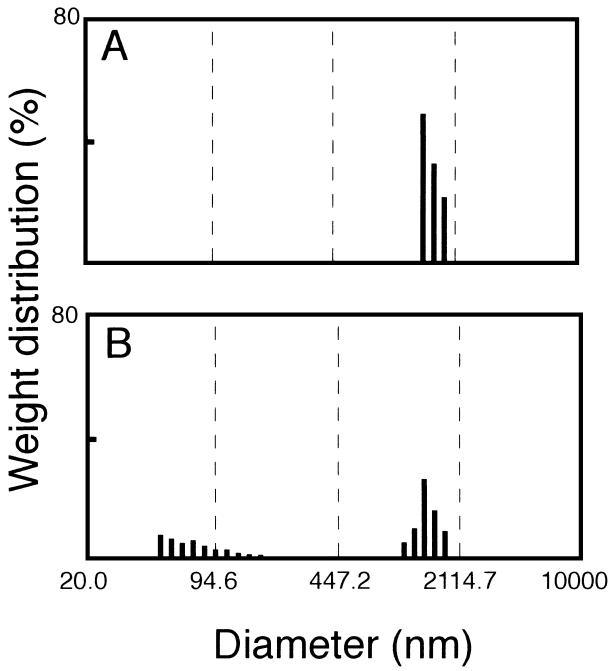

Fig. 3. Distributions of particles in weight measured by DLS. Before incubation (A) and after incubation (B) of large unilammelar liposomes containing 20% (w/w) cholesterol, 80% brain extract (Folch fraction type I) with brain cytosol in the presence of ATP and GTP for 15 min at 37°C. It is noted that small vesicles are formed in B (From Kinuta *et al*., 2002).

were consintent with the EM observations (Fig. 4). DLS assay of this cell-free system revealed that vesicle formation by brain cytosol requires both ATP and GTP. Furthermore, immuno-depletion of dynamin 1 from the brain cytosol greatly inhibits the vesicle formation, indicating a major contribution of this GTPase in the vesicle formation (Kinuta *et al*., 2002).

Because liposomes can be prepared with a variety of lipid constituents at various concentrations, liposome-based cell-free systems are useful to elucidate the role of phospholipids in vesicle formation. By using liposomes, it has been demonstrated that increasing concentrations of PtdIns(4,5)P_2 but not those of PtdIns(4)P or PtdIns in the lipid membranes enhance vesicle formation (Kinuta *et al*., 2002). This result is consistent with the proposed implication of PtdIns(4,5)P_2 in clathrin-mediated endocytsis.

Lipid analyses in the cell-free system

Another advantage of using liposomes is that lipid analysis is easily carried out compared to biological membranes. For example, membranes can be “radio labeled ” by preparing liposomes with isotope-labeled lipids. Liposomes used for *in vitro* reconstitution systems can be subjected to lipid extraction and lipid analysis. To extract phospholipids from liposomes or from the reaction mixture of liposomes and

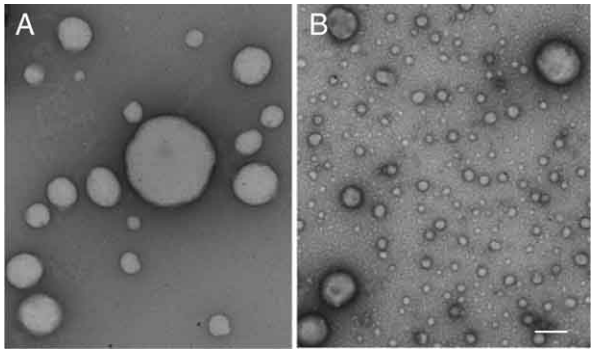

Fig. 4. Formation of small vesicles from liposomes. Electron microscopy of negatively stained large unilamellar liposomes prepared from the lipid film containing 20% (w/w) cholesterol and 80% (w/w) brain lipid extract (A), and vesicles after incubation of the liposomes with brain cytosol for 15 min at 37°C in the presence of ATP and GTP (B). Calibration bar represents 200 nm (From Kinuta *et al*., 2002).

coat proteins, a chloroform/methanol solvent system (Bligh and Dyer, 1959) is commonly used.

One of the most useful methods for the analysis of phospholipids is high performance thin-layer chromatography (HPTLC), where various kinds of solvent systems can be used (Kupke and Zeugner, 1978; Creer *et al*., 1985). Horwitz and Perlman (1987) reported an effective technique for determination of phosphoinositides as followed: a TLC plate (silica gel) is impregnated with potassium oxalate in methanol/water (2:3, v/v) and then activated by heating before spotting the sample; the lipids are chromatographed with a developing solvent system of chloroform/methanol/4 M NH_3 (45:35:10, v/v). Phosphoinositides and other phospholipids can be visualized as blue spots on the TLC-plate by spraying Ryu-MacCoss's reagent which reacts specifically with the phosphatidyl group in their molecules (Ryu and MacCoss, 1979).

High-performance liquid chromatography (HPLC) also is used for the analysis of phospholipids. Focusing on the analysis of phosphoinositides, Auger *et al*. (1990) reported a useful HPLC system where fatty acyl groups are removed from phosphoinositides (deacylation) prior to analysis, and the resulting glycerophosphoinositol phosphates molecules are separated on the basis of structural differences in their inositol groups. Recently, another useful HPLC system that requires no deacylation procedure has been developed and, by using this system, the mixture of PtdIns(4,5)P_2 and its metabolites PtdIns(4)P and PtdIns generated by the reaction of liposomes containing PtdIns(4,5)P_2 with brain cytosol are clearly separated (Kinuta *et al*., 2002).

Radioactive lipids including [^{3}H]- or [^{32}P]-labeled phospholipids also can be determined by a combination of HPTLC, an imaging plate and a bio-imaging analyzer BAS 2000 autoradiography. Using this method, the rapid degradation of PtdIns(4,5)P_2 during vesicle formation can be detected, and the kinetics can be compared with vesicle formation assessed by DLS (Fig. 5) (Kinuta *et al*., 2002). Thus the combination of DLS and lipid analysis may prove a more powerful tool to establish the relationship between the vesicle formation and the role of lipid metabolism.

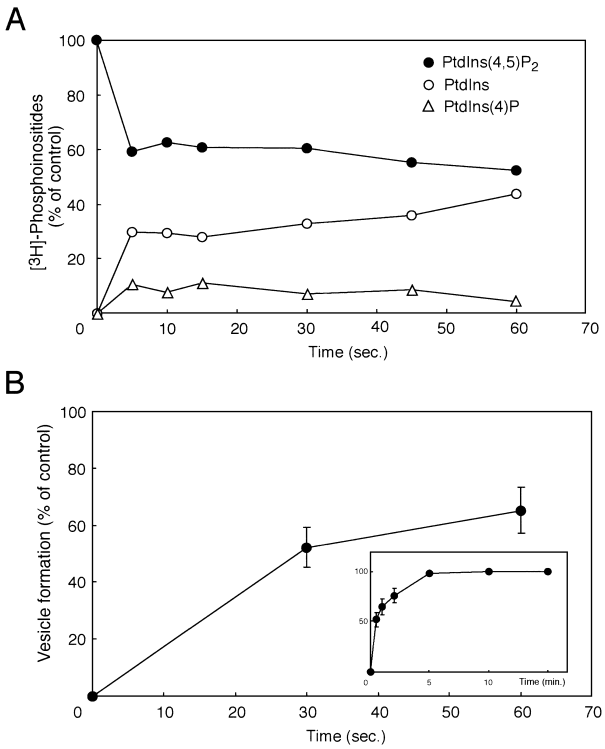

Fig. 5. Rapid PtdIns(4,5)P_2 degradation. A, Liposomes containing [^{3}H]-PtdIns(4,5)P_2 were incubated with brain cytosol in the presence of ATP and GTP for time periods indicated. Lipid extracts from the reaction mixtures were applied to HPTLC, and analyzed by BAS 2000 autoradiography. Radioactivity of the spot for [^{3}H]-PtdIns(4,5)P_2 and its metabolites, [^{3}H]-PtdIns(4)P and [^{3}H]-PtdIns, were normalized against the radioactivity obtained before the incubation and blotted in percent. B, Liposomes were incubated with brain cytosol in the presence of ATP and GTP for the time periods indicated, and the vesicle formations were quantified by DLS assay. The data obtained were normalized against the vesicle formation at 15 min as 100% (inset) (From Kinuta *et al*., 2002).

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