Coupled Inositide Phosphorylation and Phospholipase D Activation Initiates Clathrin-coat Assembly on Lysosomes*

Lynne S. Arneson‡, Jeannette Kunz§, Richard A. Anderson§, and Linton M. Traub‡¶

From the ‡Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri 63110 and the §Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Adaptors appear to control clathrin-coat assembly by determining the site of lattice polymerization but the nucleating events that target soluble adaptors to an appropriate membrane are poorly understood. Using an *in vitro* model system that allows AP-2-containing clathrin coats to assemble on lysosomes, we show that adaptor recruitment and coat initiation requires phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) synthesis. PtdIns(4,5)P$_2$ is generated on lysosomes by the sequential action of a lysosome-associated type II phosphatidylinositol 4-kinase and a soluble type I phosphatidylinositol 4-phosphate 5-kinase. Phosphatidic acid, which potently stimulates type I phosphatidylinositol 4-phosphate 5-kinase activity, is generated on the bilayer by a phospholipase D1-like enzyme located on the lysosomal surface. Quenching phosphatidic acid function with primary alcohols prevents the synthesis of PtdIns(4,5)P$_2$ and blocks coat assembly. Generating phosphatidic acid directly on lysosomes with exogenous bacterial phospholipase D in the absence of ATP still drives adaptor recruitment and limited coat assembly, indicating that PtdIns(4,5)P$_2$ functions, at least in part, to activate the PtdIns(4,5)P$_2$-dependent phospholipase D1. These results provide the first direct evidence for the involvement of anionic phospholipids in clathrin-coat assembly on membranes and define the enzymes responsible for the production of these important lipid mediators.

An area of membrane that will give rise to a clathrin-coated bud is demarcated by the placement of adaptors at that site. This necessitates that adaptor recruitment onto membranes be controlled with good precision. The first glimpse of the real complexity of the adaptor recruitment process came from studies using the fungal metabolite brefeldin A (1, 2). When added to cells, this compound causes an extremely rapid disappearance of AP-1 adaptors and clathrin, from the *trans*-Golgi network (TGN)[1] (1, 2), the site where this heterotetrameric adap-

tor complex is usually massed to initiate the formation of clathrin-coated buds (3). The effect of brefeldin A led to the demonstration that the binding of AP-1 to the TGN is regulated by ADP-ribosylation factor (ARF) in a cycle of GTP binding and hydrolysis (4, 5).

We proposed a model in which an AP-1-specific, ARF-activated docking site initiates clathrin-coat assembly at the TGN (5). Bound to an ARF·GTP-activated docking site, AP-1 would begin to recruit clathrin (6, 7). Sustained recruitment of both AP-1 and clathrin would result in the formation of an extensive polyhedral lattice. The association between AP-1 and the presumptive docking molecule is terminated on hydrolysis of ARF-GTP to GDP (8). We envisioned that the laterally expanding lattice would be tethered to the underlying membrane by AP-1-docking protein associations, primarily occurring at the perimeter of the growing coat. The density of AP-1 within the developing bud would be sufficiently high so that even low-affinity interactions between protein-sorting signals and the μ1 subunit of the adaptor heterotetramer (9, 10) would result in preferential retention of select transmembrane proteins within the structure. If a sorting signal on a protein to be included within the clathrin coat disengaged from one μ1 subunit, it would immediately encounter another AP-1 molecule so the mobility of sorted proteins would be severely restricted over the period in which the bud grows. Brefeldin A subverts coat assembly at the TGN by interfering with earliest known step of the process, catalyzed nucleotide exchange (11, 12), blocking ARF·GTP delivery onto the membrane and all downstream events.

Unlike the clathrin-coated structures on the TGN, clathrin-mediated endocytosis is not perturbed by brefeldin A (1, 2). This clearly establishes that the recruitment of AP-1 and AP-2 adaptors is regulated differently but, currently, very little is known about the nucleating events that precede AP-2 translocation onto membranes (9, 13). Overexpression of several plasma-membrane receptors normally internalized in clathrin-coated vesicles does not alter the steady-state distribution of AP-2 (14–16), arguing that unregulated association between protein-sorting signals and AP-2 adaptors is unlikely to begin the clathrin-coat assembly process. Synaptotagmin, a calcium- and phospholipid-binding protein first identified in synaptic vesicles, has been suggested to be a high-affinity AP-2-docking molecule (17). The mild phenotype of synaptotagmin I-null animals argues against this idea, but there are at least 7 other

¶ To whom correspondence should be addressed: Div. of Hematology, Box 8125, Dept. of Internal Medicine, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-747-3711; Fax: 314-362-8813; E-mail: ltraub@im.wustl.edu.

[1] The abbreviations used are: TGN, *trans*-Golgi network; AP, adaptor protein; ARF, ADP-ribosylation factor; GroPIns, glycerophosphorylinositol; GST, glutathione *S*-transferase; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HC, heavy chain; LC, light chain; MPR, mannose 6-phosphate receptor; PI4K, phosphatidylinositol 4-kinase; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; PKCα, protein kinase Cα;

PLD, phospholipase D; PtdBut, phosphatidylbutanol; PtdCho, phosphatidylcholine; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol phosphate; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; PtdOH, phosphatidic acid; SH3, *Src* homology domain 3; ATPγS, adenosine 5′-(γ-thio)triphosphate; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; lgp, lysosomal glycoprotein; HPLC, high performance liquid chromatography; AMP-PNP, adenylyl imidodiphosphate.

synaptotagmin isoforms, each able to bind AP-2 (18). If synaptotagmin is an AP-2-docking protein, the association with AP-2 must be tightly regulated because synaptotagmin I is predominantly a synaptic vesicle protein and synaptic vesicles are not clathrin coated until they are rapidly retrieved following their fusion with the presynaptic plasma membrane.

Stage-specific assays for endocytosis show that early, but as yet uncharacterized events in clathrin-coat assembly at the cell surface require ATP hydrolysis (19). Polymerization of AP-2 and clathrin into coats on lysed synaptosomes also requires ATP (20). Recently, we showed that AP-2-containing clathrin coats can also assemble and invaginate on lysosomes in a strictly ATP-dependent fashion (21). Three-dimensional EM images reveal that the polyhedral lattice formed on the lysosome surface is identical to clathrin coats formed at the cell surface. Using this model system, we have now carefully dissected the role of ATP in initiating clathrin-coat assembly. Our results show that phosphoinositides, in particular phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), are critical regulators of AP-2 adaptor binding. Direct evidence for a positive feedback loop between type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K) and phospholipase D (PLD) is presented. Both of the lipids generated by this regulatory loop, PtdIns(4,5)P_2 and phosphatidic acid (PtdOH), appear to play important roles in initiating clathrin-coat assembly on lysosomes.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (4,500 Ci/mmol) was from ICN and 1,2-dipalmitoyl-[1-^{14}C]phosphatidylcholine (114 mCi/mmol) was from Amersham. Apyrase, ATP, creatine kinase, creatine phosphate, neomycin, phorbol 12-myristate 13-acetate, dipalmitoyl phosphatidylcholine (PtdCho), phosphatidyethanolamine, PtdOH, phosphatidylinositol 4-phosphate (PtdIns(4)P), PtdIns(4,5)P_2, phosphoinositides, *Streptomyces chromofuscus* PLD (type VII), protease inhibitors, and wortmannin were purchased from Sigma. PtdIns(4)P, and PtdIns(4,5)P_2 were also obtained from Roche Molecular Biochemicals. Synthetic dipalmitoyl PtdIns(5)P was a gift from G. Prestwich. A-3 and recombinant protein kinase Cα (PKCα) were purchased from Calbiochem. Glutathione (GSH)-Sepharose 4B, NAP-5 columns, and Percoll were from Pharmacia. Silica Gel 60 HPTLC plates were supplied by Merck and P11 phosphocellulose and the silica LK6 TLC plates were from Whatman.

Antibodies—mAb 100/2, directed against the α subunit of AP-2, was provided by E. Ungewickell. Polyclonal serum from a rabbit injected with a peptide corresponding to residues 11–29 of the mouse $\mu2$ sequence (22) was provided by J. Bonifacino. Cell lines producing the clathrin heavy chain-specific mAb TD.1 and mAb AP.6, that recognizes the α subunit of AP-2, were provided by F. Brodsky. An antibody specific for the clathrin light chain neuron-specific insert, mAb Cl57.3, was obtained from R. Jahn. A dynamin-specific mAb (clone 41) was purchased from Transduction Laboratories and the neutralizing antibody against type II PtdIns 4-kinase (PI4K), mAb 4C5G (23), was obtained from UBI. Affinity purified rabbit anti-lgp120 antibodies and mAb YA30, against lgp85, were provided by K. Akasaki. Anti-α-mannosidase II antisera were from K. Moreman. Affinity-purified antibodies against the cation-independent mannose 6-phosphate receptor (MPR) have been described elsewhere (24). The antibodies used to detect the PIP5Ks were affinity-purified anti-type I PIP5K, prepared using purified erythroid PIP5KI (25), and polyclonal anti-PIP5K type Iα and anti-PIP5K Iβ antibodies, each affinity purified using the appropriate recombinant protein (26, 27). Serum from rabbits injected with a peptide matching the carboxyl terminus of PIP5KIγ was provided by H. Ishihara and Y. Oka (28). Affinity-purified anti-type II PIP5K antibodies were prepared using recombinant PIP5KIIα (27).

Subcellular Fractionation and Protein Purification—Rat brain cytosol and rat liver Golgi membranes and lysosomes were prepared as described previously (5, 21). Lysosomes were resuspended in 20 mM Tris-HCl, pH 7.4, 250 mM sucrose with 25 μg/ml each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin A (protease inhibitors). A plasmid bearing the SH3 domain of amphiphysin I fused to glutathione *S*-transferase (GST) was provided by H. McMahon (29). Bovine ADP-ribosylation factor 1 (ARF1), with amino acids 3–7 replaced with the corresponding residues from yeast ARF2, was from S.

Kornfeld (8). Proteins were expressed in *Escherichia coli* and purified by standard procedures. For depletion of dynamin, 1-ml aliquots of cytosol (10 mg/ml) were mixed with 150 μg of either GST or GST-amphSH3 overnight at 4 °C. GSH-Sepharose was then added to collect the protein complexes and then removed by brief centrifugation. The supernatants were stored in small aliquots at -80 °C while the pellets were washed 4 times in phosphate-buffered saline before solubilization in SDS sample buffer. Immunodepletion of AP-2 from cytosol with AP.6-Sepharose was exactly as described (5).

For the separation of type I and type II PIP5Ks (25, 30, 31), 25 ml of rat brain cytosol was dialyzed into 5 mM sodium phosphate, pH 7.5, 0.25 M NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM 2-mercaptoethanol, 10% glycerol, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A) and then loaded at 1.5 ml/min onto a phosphocellulose column (1.6 $\times$ 10 cm) pre-equilibrated in buffer A. After washing with 200 ml of buffer A, bound protein was eluted with a 250-ml linear gradient of 0.25–1.25 M NaCl in buffer A, collecting 4-ml fractions. Fractions were analyzed on immunoblots after concentration by methanol/chloroform precipitation or assayed for PIP5K activity after dialysis into buffer A lacking sodium chloride. For some experiments, the PIP5KI and PIP5KII pools were concentrated with a Centricon 10. When assayed for PtdInsP production with lysosomes, the PIP5KI and PIP5KII pools were exchanged into 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 5 mM EGTA, 1 mM dithiothreitol over NAP-5 columns.

Membrane Binding Assays—Recruitment onto lysosomes was performed as outlined previously (21). Briefly, reactions were performed in 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 5 mM EGTA, 1 mM dithiothreitol in a volume of 400 μl. All assays contained 25 μg/ml of each of the protease inhibitors indicated above. Lysosomes were added to final concentrations of 30–50 μg/ml and cytosol to 2–2.5 mg/ml as indicated in the legends. ATP, an ATP regeneration system (1 mM ATP, 5 mM creatine phosphate, 10 units/ml creatine kinase), apyrase, A-3, neomycin, various alcohols, and PLD were added and mixed on ice. After 20 min at 37 °C, reactions were stopped by chilling in an ice-water bath. Variations are noted in the figure legends. After centrifugation, membrane-containing pellets and aliquots of the supernatants were prepared for immunoblotting. Details of the conditions used for SDS-PAGE and immunoblotting have been published elsewhere (5, 21). For thin-section EM analysis, glutaraldehyde was added to the chilled reactions to give a final concentration of 2% and, after 15 min on ice, the membranes were collected by centrifugation and processed as detailed elsewhere (21). The immunofluorescence-based morphological binding assay, using digitonin-permeabilized NRK cells, was carried out on glass coverslips exactly as described (21). Cells were fixed with 3.7% formaldehyde in phosphate-buffered saline for 20 min and then processed for indirect fluorescence microscopy.

Measurement of Phosphoinositide Kinase and PLD Activity—Synthesis of phosphoinositides on lysosomal membranes was assayed in 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 5 mM EGTA, 1 mM dithiothreitol in a final volume of 50 μl. Membranes were added to a final concentration of 0.5 mg/ml and cytosol, when present, to 5 mg/ml. Incubations were initiated by the addition of [γ-^{32}P]ATP (0.5–1 Ci/mmol) to a final concentration of 500 μM. After 10 min at 37 °C, the reactions were terminated by addition of 3 ml of chloroform:methanol:concentrated HCl (200:100:0.75), followed by vigorous mixing. Carrier phosphoinositides (50 μg/tube) were added and then a biphasic mixture generated by addition of 0.6 ml of 0.6 M HCl. After centrifugation at 200 $\times$ g_{ave} for 5 min, the lower organic phase was removed, transferred to a new tube, washed twice with 1.5 ml of chloroform, methanol, 0.6 M HCl (3:48:47) and then dried under a stream of N_2 gas at about 40 °C. Dried lipid films were resuspended in chloroform:methanol:H_2O (75:25:1) and spotted onto TLC plates. Formation of PtdIns(4,5)P_2 from either commercial PtdIns(4)P or synthetic PtdIns(5)P was assayed in a final volume of 50 μl in 50 mM Tris-HCl, pH 7.5, 10 mM magnesium acetate, 1 mM EDTA, 80 μM PtdInsP, 50 μM [γ-^{32}P]ATP (5 Ci/mmol), and a source of enzyme. PtdInsP micelles were prepared by resuspending the dried lipid at 5 mg/ml in 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, and stored at -80 °C in small aliquots. Reaction mixtures were equilibrated to ~25 °C for 5 min before starting the assays with the addition of ATP. After 6 min at ~25 °C, 3 ml of chloroform:methanol:concentrated HCl (200:100:0.75) was added and the lipids extracted as described above except that no carrier lipid was added and the lower organic phase was only washed once.

PLD activity was assayed in 50 mM Hepes-KOH, pH 7.5, 250 mM sucrose, 80 mM potassium chloride, 4.5 mM magnesium chloride, 3 mM calcium chloride, 3 mM EGTA, 1 mM dithiothreitol, protease inhibitors,

and 0.5% 1-butanol in a final volume of 60 μl (32). Organelles were added to a final concentration of 160 μg/ml and activators were added as follows: a final concentration of 0.85 μM ARF1 together with 100 μM GTPγS, and 0.1 μM PKCα with 1 μM phorbol 12-myristate 13-acetate. Exogenous substrate (mixed micelles of phosphatidyethanolamine, [^{14}C]PtdCho (52 mCi/mmol), PtdIns(4,5)P$_2$ at a molar ratio of 6.7:1.15:1) was added to a final lipid concentration of 132 μM, prepared as described (32). Additions were made on ice followed by incubation at 37 °C for 1 h. Assays were stopped by addition of 1 ml of chloroform:methanol: concentrated HCl (50:50:0.3) and 0.35 ml of 1 M HCl, 5 mM EDTA, followed by vigorous mixing. After centrifugation at 370 $\times$ g_{max} for 5 min, 0.4 ml of the lower organic phase was removed and dried under a stream of N$_2$ gas. Dried lipid films were resuspended in chloroform: methanol:concentrated HCl (50:50:0.3) and spotted onto TLC plates.

Lipid Analysis—Phosphoinositides were resolved on silica gel HPTLC plates that had been previously immersed in 1% potassium oxalate, 2 mM EDTA in 50% ethanol. Lipids were spotted onto the plates after activation at 105 °C for several hours. The developing solvent consisted of chloroform:acetone:methanol:acetic acid:water (160:60:52: 48:28). PtdOH and phosphatidylbutanol (PtdBut) were resolved from PtdCho on heat-activated Silica Gel LK6 plates in a solvent system of chloroform:methanol:acetic acid (13:5:1). After chromatography, lipid standards were visualized with iodine vapor and marked. Radiolabeled lipids were visualized by autoradiography. Plates containing ^{14}C-labeled lipids were sprayed with Enhance before exposure to film. Signals were quantitated by scintillation counting after scraping the relevant portions of the plate into vials. For the anion-exchange HPLC analysis, the relevant lipid spots were scraped off the plates and deacylated directly on the silica with methylamine reagent after addition of authentic [^{3}H]PtdIns(4)P or [^{3}H]PtdIns(4,5)P$_2$ (NEN Life Science Products). The water-soluble products were applied to a Partisil 10 SAX column and eluted with a gradient of 0–1 M ammonium phosphate, pH 3.8, as described elsewhere (27).

RESULTS

The Kinase Inhibitor A-3 Prevents AP-2 Recruitment onto Lysosomes—The recruitment of AP-2 onto purified lysosomes and subsequent clathrin lattice assembly is absolutely dependent on hydrolyzable ATP (21) (Fig. 1). This makes it unlikely that coat assembly is initiated simply by the direct association of AP-2 adaptors with sorting signals located on the cytosolic domain of lysosomal glycoproteins or plasma-membrane proteins which have made their way to the limiting membrane of the lysosome. When added to gel-filtered cytosol, ATP supports coat assembly with an EC$_{50}$ of approximately 100 μM (Fig. 1A), suggesting that a phosphorylation event might be involved rather than constant ATP hydrolysis to actively drive coat assembly. Indeed, a broad specificity kinase inhibitor, the naphthalenesulfonamide A-3 (33), blocks clathrin assembly on lysosomes (Fig. 1B). Compared with the recruitment of AP-2 and clathrin seen when purified lysosomes are incubated at 37 °C with brain cytosol and 500 μM ATP (*lane e*), addition of equimolar (*lane i*) or higher (*lane k*) concentrations of A-3 fully inhibits the translocation of the α and μ2 subunits of the AP-2 complex and clathrin onto lysosomes. The amount of these proteins recovered in the pellets (*lanes i* and *k*) is equivalent to that found in the pellets from incubations lacking lysosomes (*lanes h* and *j*). Coat recruitment is little changed by addition of 250 μM A-3 (*lane g*), a competitive inhibitor with respect to ATP.

Correlation between PtdIns(4,5)P$_2$ Formation and Clathrin-coat Assembly—Three lines of evidence suggest that inositide phosphorylation might be important for the initiation of clathrin-coat assembly on lysosomes. First, poorly hydrolyzable analogues of ATP, AMP-PNF, and ATPγS, do not support coat assembly (21) and neither derivative serves as a phosphate donor for phosphoinositide synthesis (34). Second, neomycin, a polyamine antibiotic that binds to PtdIns(4)P and PtdIns(4,5)P$_2$ with high affinity (35) and has been widely used to intercede in phosphoinositide-regulated processes, inhibits clathrin-coat assembly on purified lysosomes in a dose-dependent fashion. Inhibition is apparent at 100 μM and, in the pres-

ence of 300 μM neomycin, the recruitment of both the AP-2 complex and clathrin is completely blocked, indicating that the compound inhibits an early stage of the assembly reaction (data not shown). A similar observation has been made in a study of AP-2 recruitment onto endosomes (36). Third, A-3-mediated inhibition of AP-2 and clathrin recruitment (Fig. 1B) occurs together with a complete block of PtdIns(4,5)P$_2$ synthesis (Fig. 1C). Lysosomes exhibit intrinsic PI4K activity (37, 38) and mixing purified lysosomes with ATP allows this enzyme to phosphorylate endogenous PtdIns, generating PtdIns(4)P in a temperature-sensitive reaction (Fig. 1C, *lane b* compared with *lane a*). Anion-exchange HPLC analysis of the deacylated product of this lipid shows exact co-elution with an internal [^{3}H]-glycerophosphorylinositol (GroPIns) 4-phosphate standard (data not shown), verifying this lipid as PtdIns(4)P. Addition of brain cytosol to an incubation containing lysosomes and ATP results in the synthesis of PtdIns(4)P and a second phospholipid, which comigrates with an authentic PtdIns(4,5)P$_2$ standard (Fig. 1C, *lane d*). The deacylated product of this lipid elutes coincidentally with a [^{3}H]GroPIns(4,5)P$_2$ standard (data not shown). Including 1 mM A-3, which completely abrogates AP-2 and clathrin recruitment onto the lysosome surface (Fig. 1B), totally inhibits the activity of the soluble PIP5K (Fig. 1C, *lane f*) without affecting the PI4K activity. These experiments show that mixing lysosomes with cytosol and ATP allows for a robust synthesis of PtdIns(4,5)P$_2$ on the lysosome surface and suggest that this lipid might be important for the initiation of clathrin coat formation on lysosomes.

Coat Assembly on Lysosomes Begins with AP-2 Adaptor Recruitment—The pleckstrin homology domain is a modular protein fold that appears to regulate the translocation of several soluble proteins onto membranes by binding to various phosphoinositides (39, 40). Because PtdIns(4,5)P$_2$ is generated on the lysosome in our assays, and because dynamin contains a pleckstrin homology domain that specifically binds to PtdIns(4,5)P$_2$ (41), it was possible that we had reconstituted coat assembly in reverse. Dynamin translocation onto PtdIns(4,5)P$_2$-containing lysosomes could trigger the recruitment of amphiphysin (29, 42) which, in turn, could then recruit AP-2, synaptojanin and clathrin (43–45). In fact, this reverse reaction is known to occur *in vitro* (46). If dynamin recruitment does initiate clathrin-coat assembly on lysosomes, then depleting this protein from brain cytosol should abrogate coat formation *in vitro*. Dynamin was selectively removed from rat brain cytosol using a GST-amphSH3 domain fusion protein (29). For comparison, mock-depleted cytosol, treated with GST, and AP-2-depleted cytosol (5), were also used. Examination of the specificity (Fig. 2A) and extent of depletion (Fig. 2B) confirms that dynamin and AP-2 removal is virtually complete but that the resulting depleted cytosols still contain normal levels of several other major polypeptides known to participate in clathrin coat formation.

Eliminating dynamin from cytosol does not alter clathrin-coat assembly on lysosomes detectably. AP-2 recruitment is evident both from the loss of the adaptor in the soluble fraction (Fig. 2B, *lane e* compared with *lane d*) and from the simultaneous appearance of the adaptor complex in the lysosome pellet (Fig. 2C, *lane e* compared with *lane d*). Clathrin behaves similarly. This is identical to the results obtained with the mock-depleted cytosol (*lanes b* and *c*). Removing AP-2 from the donor cytosol, however, has a dramatic effect on coat formation. Very little AP-2 and clathrin are found on the pelleted lysosomes (Fig. 2C, *lane g* compared with *lanes c* and *e*) and no change in the amount of clathrin in the soluble fraction is evident (Fig. 2B, *lane g* compared with *lane f*). This verifies that AP-2 binding is necessary for subsequent clathrin recruitment. Although
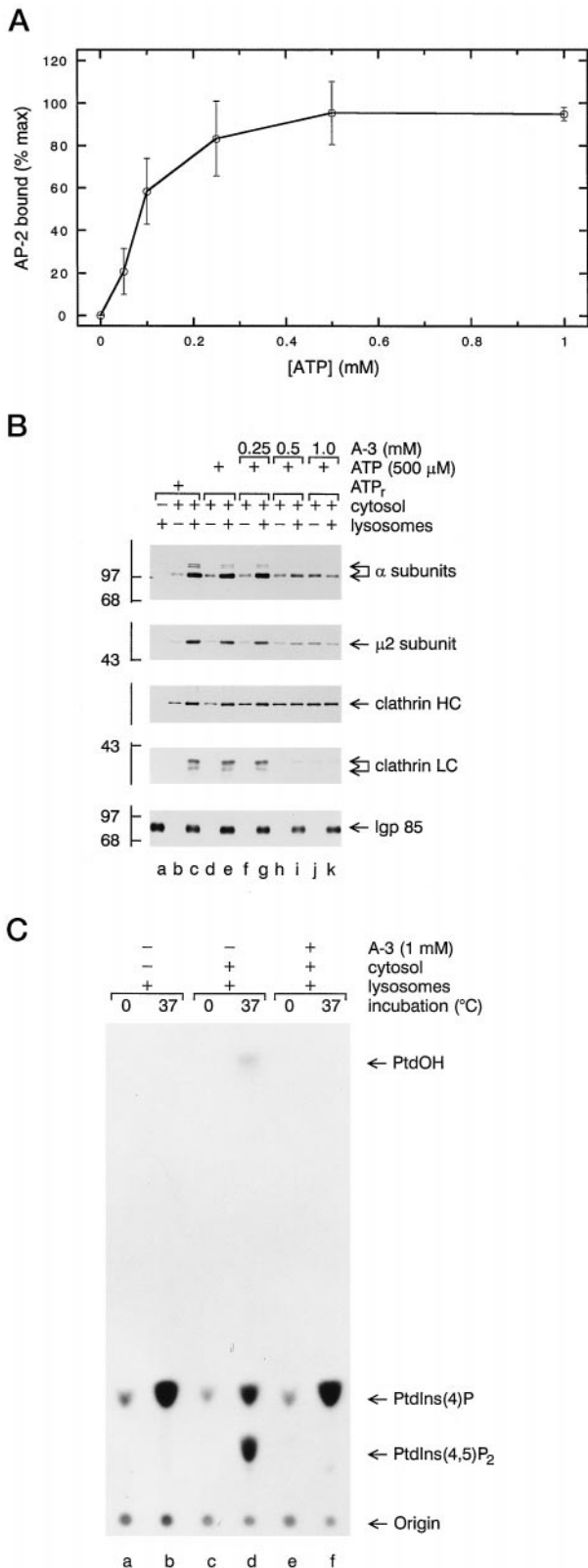
FIG. 1. **Clathrin-coat assembly and phosphoinositide synthesis on lysosomes.** *A,* reactions containing 30 µg/ml purified liver lysosomes, ~2.5 mg/ml gel-filtered rat brain cytosol, and 0–1 mM ATP were prepared as indicated. After 20 min at 37 °C, the tubes were centrifuged and the pellets analyzed by immunoblotting with anti-AP-2 α-subunit mAb 100/2. AP-2 binding is expressed as the percent of maximal obtained with an ATP-regeneration system (1 mM ATP, 5 mM creatine phosphate, 10 units/ml creatine kinase) and the mean ± S.D. of four independent determinations is shown. *B,* reactions containing 30 µg/ml purified liver lysosomes, 2.5 mg/ml gel-filtered cytosol, an ATP-regeneration system (*ATP$_r$*), 500 µM ATP, and 0–1 mM A-3 were prepared as
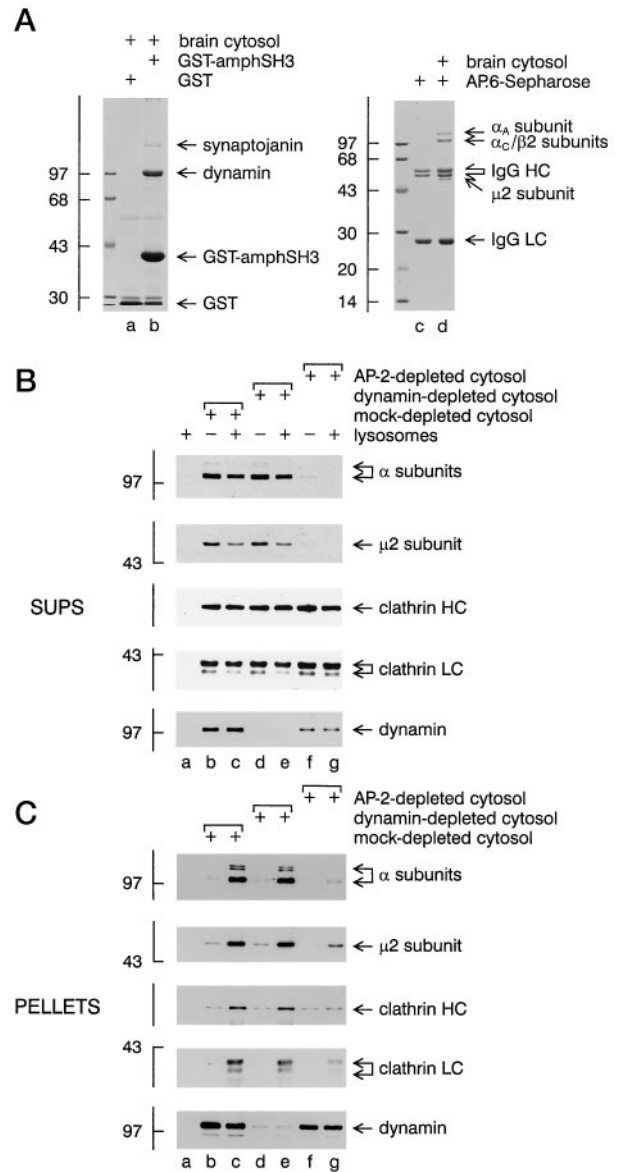

FIG. 2. **AP-2 binding initiates coat assembly.** *A,* Coomassie Blue-stained gels of the bound fraction obtained after incubating cytosol with GST (*lane a*) or GST-amphSH3 (*lane b*) and GSH-Sepharose, or with mAb AP.6-Sepharose (*lane d*) or after incubating mAb AP.6-Sepharose with buffer (*lane c*). Dynamin, synaptojanin I, and the AP-2 subunits are indicated. *B* and *C,* reactions containing 30 µg/ml lysosomes, 500 µM ATP, and 2.5 mg/ml of the soluble depleted cytosol obtained after incubation with GST, GST-amphSH3, or mAb AP.6 were prepared as indicated. After 20 min at 37 °C, membranes were recovered by centrifugation and aliquots corresponding to 1/50 of each supernatant (*B*) or 1/3 of each pellet (*C*) were analyzed by immunoblotting with anti-α-subunit mAb 100/2, anti-μ2-subunit serum, anti-clathrin heavy chain (*HC*) mAb TD.1, anti-clathrin light chain (*LC*), mAb Cl57.3, or an anti-dynamin mAb.

indicated. After 20 min at 37 °C, the tubes were centrifuged and the pellets analyzed by immunoblotting with anti-AP-2 α-subunit mAb 100/2, anti-AP-2 μ2-subunit serum, anti-clathrin heavy chain (*HC*) mAb TD.1, anti-clathrin light chain (*LC*) mAb Cl57.3 or anti-lgp85 mAb YA30. The mobility of M_r standards is indicated on the *left* and only the relevant portion of each blot is shown. Note that when ATP is limiting, light chain-free clathrin appears to aggregate and precipitate from the cytosol and is recovered in the pellet fractions (*lanes h–k*). *C,* reactions containing 0.5 mg/ml lysosomes, 5 mg/ml cytosol, 500 µM [γ-^{32}P]ATP, and 1 mM A-3 were prepared as indicated. After incubation at 37 °C for 10 min the lipids were extracted and analyzed by TLC and autoradiography. The migration positions of authentic phospholipid standards, visualized with iodine, are indicated.

the pellets from incubations with dynamin-containing cytosol do contain dynamin (Fig. 2*C, lanes c* and *g*), equivalent amounts of dynamin are also recovered in the pellets from incubations lacking lysosomes (*lanes b* and *f*). This simply reflects the propensity of dynamin to form extensive homo-oligomers, but makes it difficult to discern whether dynamin is also being actively recruited onto the clathrin-coated buds that form on the lysosomes.

These experiments establish that in our system clathrin-coat assembly follows what is considered the physiological sequence, with adaptor recruitment beginning the assembly process. The generation of PtdIns(4,5)P$_2$ may facilitate AP-2 binding and dynamin is not required for the early stages of lattice assembly. This is in agreement with our earlier results showing that GTPγS does not modulate adaptor recruitment in the lysosomal system (21).

Characterization of the Inositide Kinases Involved in PtdIns(4,5)P$_2$ Formation on Lysosomes—PI4Ks are divided into two distinct subfamilies, designated type II and type III (23). Type II enzymes are predominantly membrane-associated, whereas type III activity is mainly found in soluble extracts, although some type III activity is also associated with the particulate fraction (38, 47). The type III enzymes are also completely inhibited by micromolar concentrations of wortmannin (47–49) and, on this basis, can be distinguished from the type II PI4K, which is wortmannin insensitive. The generation of PtdIns(4)P on lysosomes is unaffected by up to 20 μM wortmannin (data not shown), suggesting that the lysosomal PI4K is most likely a type II enzyme. This conclusion is strengthened by the ability of a neutralizing anti-type II antibody, mAb 4C5G (23), to inhibit PtdIns(4)P generation in a dose-dependent fashion if the lysosomes are preincubated with the antibody prior to the addition ATP (data not shown), and is in accord with a study examining the subcellular distribution of PI4K isoforms (38). It is also important to note that the insensitivity of PtdIns phosphorylation to micromolar concentrations of wortmannin again rules out that the labeled lipid generated on lysosomes is the product of PI3K activity.

Two peaks of PIP5K activity are resolved after fractionating rat brain cytosol on phosphocellulose (Fig. 3*A*). The first and major peak, designated type I PIP5K (25, 30, 31), elutes with approximately 0.6 M NaCl and is composed of at least three distinct enzymes, PIP5K types Iα, -Iβ, and -Iγ (26, 28, 50). The different type I PIP5Ks are the products of separate genes (26, 28, 50) but, because the central kinase domains of these enzymes are about 80% identical, all these polypeptides are detected on immunoblots by affinity-purified antibodies raised against type I PIP5K purified from erythrocytes (25) (Fig. 3*B*). The identity of the ~90-kDa type Iγ, the 68-kDa type Iα, and the 67-kDa type Iβ enzymes is confirmed on duplicate blots probed with isoform-specific antibodies, however (Fig. 3*B*).

The minor peak of type II PIP5K activity elutes from phosphocellulose with about 1.1 M NaCl (Fig. 3*A*). Again, there are two main isoforms known, the ~53-kDa type IIα and type IIβ, both being detected with an affinity-purified anti-type II antibody (Fig. 3*B*). To determine whether the type I, type II, or both types of PIP5K can phosphorylate the PtdIns(4)P generated on lysosomes to form PtdIns(4,5)P$_2$, pooled fractions enriched with each activity from the phosphocellulose column were added to purified lysosomes in the presence of [γ-^{32}P]ATP (Fig. 3*C*). Only the type I PIP5K pool produces PtdIns(4,5)P$_2$ (*lane f*). The type II pool is inactive (*lane h*) although PtdIns(4)P is generated as a potential substrate. This agrees with previous data showing that the type II PIP5K does not phosphorylate intrin-

sic PtdIns(4)P in phospholipid bilayers (30). In fact, unlike the type I PIP5Ks, the type II pool shows a marked preference for PtdIns(5)P over PtdIns(4)P as a substrate (Fig. 3*D*), confirming that these enzymes appear to be predominantly PtdIns(5)P 4-kinases (51).

Because type I PIP5Ks phosphorylate not only PtdIns(4)P but 3-phosphate-containing phosphatidylinositols as well (27, 52), we again checked that the lipid produced by the type I pool is, in fact, PtdIns(4,5)P$_2$. HPLC analysis of the deacylated product also shows precise co-elution with the [^{3}H]GroPIns(4,5)P$_2$ standard (data not shown). PtdIns(4,5)P$_2$ synthesis on lysosomes catalyzed by the partially purified type I-PIP5K pool is dose dependent (Fig. 3*E, lanes c* and *e*) and, importantly, is completely abolished by including A-3 in the assay (*lanes d* and *f*), just as is seen with whole cytosol (*lanes a* and *b*). Taken together, our results show that a type II PI4K located on the lysosome membrane phosphorylates PtdIns to produce PtdIns(4)P, which, in turn, is phosphorylated by a soluble, A-3-sensitive type I PIP5K to generate PtdIns(4,5)P$_2$.

PtdOH Generation Precedes AP-2 Adaptor Recruitment—The *in vitro* activity of all known type I PIP5Ks is strongly stimulated by PtdOH (25, 28). Two main pathways for the generation of PtdOH are known. One occurs by the phosphorylation of diacylglycerol by diacylglycerol kinase while a second is via the hydrolysis of the polar head group of PtdCho catalyzed by PLD. Only trace amounts of PtdOH are generated by phosphorylation in our assays (Fig. 1*C*) so to investigate whether PLD activity is required to generate PtdOH necessary to drive the synthesis of PtdIns(4,5)P$_2$ by type I PIP5K, we checked the effect of 1-butanol on the synthesis of this lipid. PLD catalyzes a distinctive transphosphatidylation reaction producing phosphatidylalcohols in the presence of primary alcohols. Since phosphatidylalcohols are unable to substitute for the biologically active PtdOH, primary alcohols inhibit PLD-dependent reactions. When added at 1.5%, 1-butanol has a dramatic effect on PtdIns(4,5)P$_2$ formation (Fig. 4*A, lane e* compared with *lane d*) while tertiary butanol is inert (*lane f*). 1-Butanol similarly prevents the formation of PtdIns(4,5)P$_2$ catalyzed by the semi-purified type I PIP5K pool.[2] Significantly, the effect of 1-butanol on PtdIns(4)P synthesis is considerably weaker (*lanes a* and *d* compared with *lanes b* and *e*).

To determine whether PtdOH generated by PLD is important for clathrin-coat assembly, we tested the effect of adding alcohols to our recruitment assays. Adding 1-butanol to the standard assay inhibits the recruitment of AP-2 onto lysosomes in a dose-dependent manner (Fig. 4*B*). At 1.5%, inhibition is near complete while at the same concentration *t*-butyl alcohol has little effect (Fig. 4*B, lane n* compared with *lane d*). Analogous results are obtained with 1-propanol, which also abrogates coat assembly, and 2-propanol, which has no effect.[2] Taken together, these experiments confirm the existence of a positive feedback regulatory loop between PIP5KI and PLD (53) and indicate that, in our system, PIP5KI activity is largely dependent on PtdOH. The generation of PtdOH on the lysosome is a prerequisite for the initiation of coat assembly, which, based on our results, is required for the synthesis of PtdIns(4,5)P$_2$.

PLD1-like Activity Is Associated with Purified Rat Liver Lysosomes—Two forms of PtdIns(4,5)P$_2$-regulated PtdCho-specific PLD, termed PLD1 and PLD2, have been identified in mammals (54, 55). To verify that the PLD activity observed in our assay is indeed lysosome-associated, we used an exogenous substrate assay performed in the presence of 1-butanol. This

---

FIG. 3. **PtdIns(4,5)P₂ synthesis on lysosomes catalyzed by type I PIP5K.** *A,* rat brain cytosol was loaded onto a phosphocellulose column and bound proteins eluted with a linear gradient of 0.25–1.25 M NaCl. An aliquot of every second fraction was assayed for PIP5K activity using PtdIns(4)P micelles as a substrate. The two peaks of PIP5K activity resolved, type I and type II, are indicated. *B,* aliquots of every second fraction from a similar column run were analyzed on immunoblots with affinity-purified anti-erythroid PIP5KI antibodies (*a*), affinity-purified anti-PIP5KIα antibodies (*b*) affinity-purified anti-PIP5KIβ antibodies (*c*), anti-PIP5KIγ serum (*d*), or affinity-purified anti-PIP5KII antibodies (*e*). *C,* reactions containing 0.5 mg/ml lysosomes, 5 mg/ml brain cytosol, 37.5-µl aliquots of either the PIP5KI or the PIP5KII pools and 500 µM [γ-³²P]ATP were prepared as indicated. After 10 min at 37 °C, lipids were extracted and analyzed by TLC and autoradiography. *D,* reactions containing 80 µM PtdIns(4)P or synthetic PtdIns(5)P, 25-µl aliquots of either the PIP5KI or the PIP5KII pool and 50 µM [γ-³²P]ATP were prepared as indicated. After 6 min at 25 °C, lipids were extracted and analyzed by TLC and autoradiography. Note that the mobility of the PtdIns(4,5)P₂ made from the synthetic PtdIns(5)P differs from that of PtdIns(4,5)P₂ made from PtdIns(4)P because of differences in the acyl chain composition. *E,* reactions containing 0.5 mg/ml lysosomes, 5 mg/ml brain cytosol, 35-µl aliquots of either unconcentrated (*lanes c* and *d*) or 5-fold concentrated (*lanes e* and *f*) PIP5KI pool, 500 µM [γ-³²P]ATP, and 1 mM A-3 were prepared as indicated. After 10 min at 37 °C, lipids were extracted and analyzed by TLC and autoradiography. Note that the slightly aberrant migration position of PtdIns(4,5)P₂ in *lane c* of this experiment was identical to the mobility of the carrier PtdIns(4,5)P₂ in that lane visualized with iodine vapor.
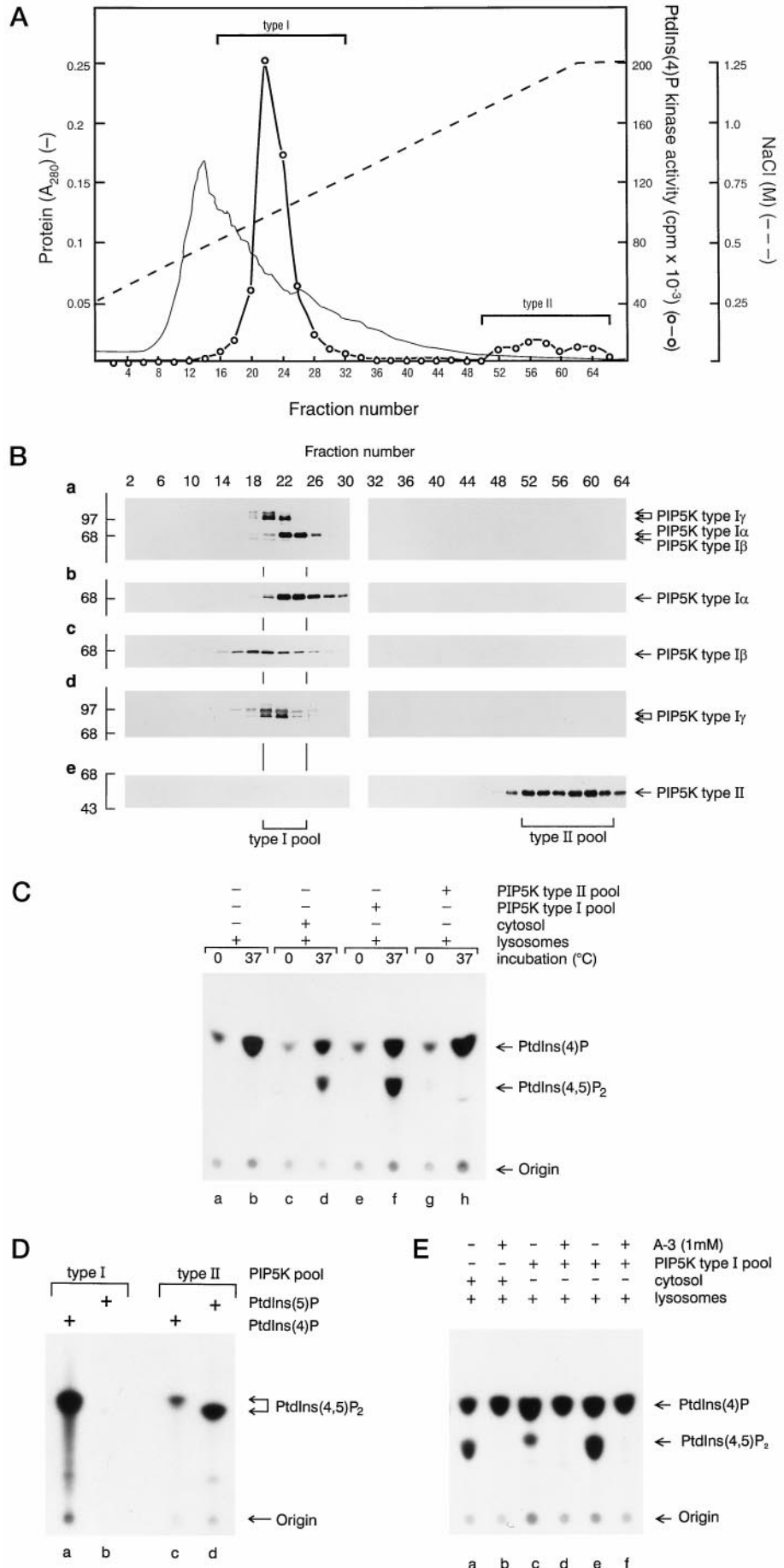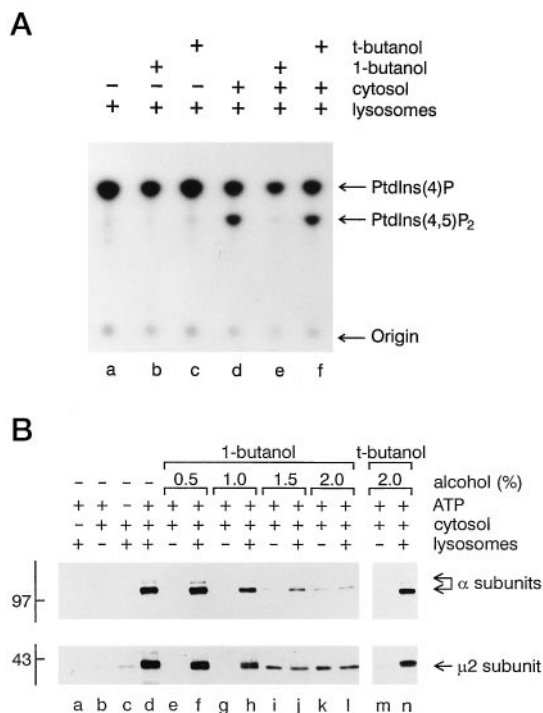
FIG. 4. **Primary alcohols inhibit PtdIns(4,5)P$_2$ synthesis and coat assembly.** *A,* reactions containing 0.5 mg/ml lysosomes, 5 mg/ml cytosol, 1.5% 1-butanol or *t*-butanol, and 500 μM [γ-^{32}P]ATP were prepared as indicated. After 10 min at 37 °C, lipids were extracted and analyzed by TLC and autoradiography. *B,* reactions containing 50 μg/ml lysosomes, 2 mg/ml cytosol, 1 mM ATP, and 0–2% 1-butanol or *t*-butanol were prepared as indicated. After 15 min at 37 °C, membranes were recovered by centrifugation and the pellets analyzed by immunoblotting with anti-α-subunit mAb 100/2 or anti-μ2-subunit serum. Note that the *t*-butanol experiment comes from a separate blot on which the immunoreactivity in the presence of 2% *t*-butanol did not differ significantly from the signal obtained in the absence of the alcohol.

enables us to follow the stable product of transphosphatidylation, PtdBut. A low level of PtdCho cleavage occurs upon mixing purified lysosomes with the exogenous reporter micelles (Fig. 5*A, column b*) indicating that PLD activity is present on lysosomes. Activators of PLD1, ARF1, and PKCα, augment the hydrolysis of the exogenous PtdCho. Added alone, ARF1·GTPγS stimulates PLD (*column c*) considerably better than PKCα and phorbol 12-myristate 13-acetate (*column d*) but, together, the two proteins synergize to stimulate PLD activity maximally (*column e*). Cleavage of PtdCho to produce PtdBut is totally dependent on PtdIns(4,5)P$_2$ and negligible PtdBut formation is seen when liposomes lacking the phosphoinositide are used as a substrate (data not shown). The production of PtdBut verifies the existence of PLD on the lysosome surface and the specific activity of the activated lysosomal enzyme ($\sim$2.5 nmol/mg/h) compares favorably with PLD1 activity measurements on other membrane preparations, although our data cannot be compared directly to assays quantitating choline head group release because 1-butanol also inhibits the catalytic activity of PLD (56).

For comparison then, we also analyzed purified Golgi membranes, which are known to contain ARF-stimulated PLD1 activity (57). The catalytic activity associated with Golgi responds to PLD1 activators very similarly to that associated with lysosomes (Fig. 5*A, columns f-i*). The relative purity of the two organelle preparations is shown on immunoblots probed with antibodies against α-mannosidase II, lgp120, and lgp85 (Fig. 5*B*). Lysosomes have no detectable mannosidase II but are heavily enriched with the lysosomal membrane proteins, as
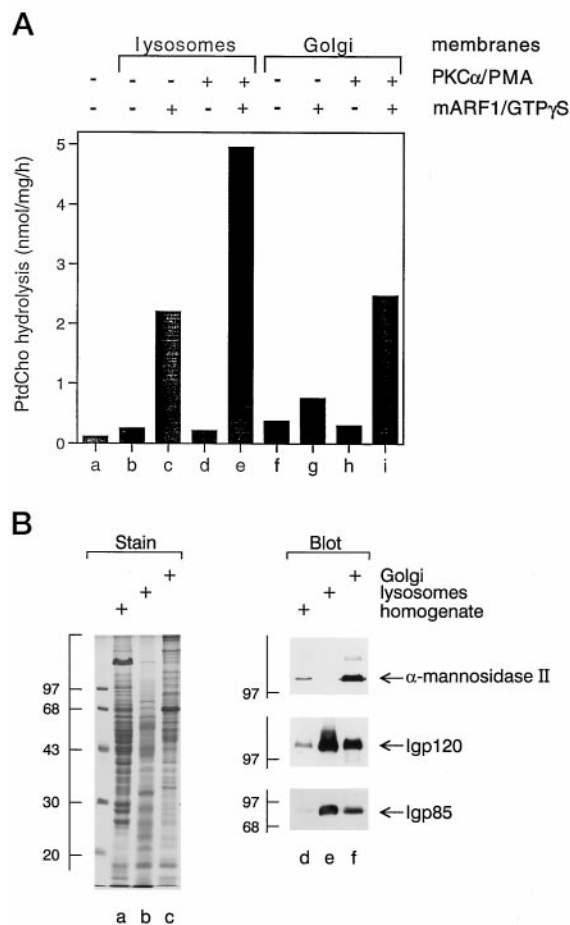

FIG. 5. **PLD1 activity associated with lysosomes and Golgi membranes.** *A,* reactions containing 0.16 μg/ml lysosomes or Golgi membranes, 0.85 μM myristoylated ARF1 with 100 μM GTPγS, 0.1 μM PKCα with 1 μM phorbol 12-myristate 13-acetate, 132 μM phosphatidylethanolamine:[^{14}C]PtdCho:PtdIns(4,5)P$_2$-containing substrate liposomes, and 0.5% 1-butanol were prepared as indicated. After 60 min at 37 °C, lipids were extracted and analyzed by TLC and fluorography. Quantitation of PtdCho hydrolysis from a representative experiment of three (in which maximal stimulated PLD activity on lysosomes (*column e*) and Golgi (*column i*) ranged from 0.46 to 4.95 and 0.64 to 2.47 nmol/mg/h, respectively) is shown. *B,* analysis of lysosome and Golgi membrane markers. Samples of 25 μg each of a total rat liver homogenate or purified liver lysosomes or liver Golgi membranes were fractionated by SDS-PAGE and either stained with Coomassie Blue (*a-c*) or transferred to nitrocellulose (*d-f*). Blots were probed with anti-mannosidase II serum, affinity-purified anti-lgp120 antibodies or anti-lgp85 mAb YA30.

expected. The gross protein profiles of the two organelle preparations are also clearly different from each other (*lanes b* and *c*) and from the total liver homogenate (*lane a*). These results show that like Golgi membranes, purified rat liver lysosomes also contain membrane-associated PLD1-like activity and the relative abundance of this enzyme on lysosomes makes it unlikely that the activity comes from contaminating Golgi or endoplasmic reticulum structures.

PtdOH Alone Drives Limited Clathrin Coat Formation— Since PtdOH is a potent biological mediator itself, the question arises whether the critical agent generated by the positive feedback loop between PLD and PIP5KI that is necessary to initiate clathrin-coat assembly is PtdIns(4,5)P$_2$, PtdOH, or both of these lipids. If PtdIns(4,5)P$_2$ is required only to stimulate PLD1-dependent formation of PtdOH, then it should be possible to promote nucleotide-independent coat assembly on lysosomes *in vitro* using exogenous PLD. When 0.5 unit/ml *S. chromofuscus* PLD, which is not dependent on PtdIns(4,5)P$_2$ for
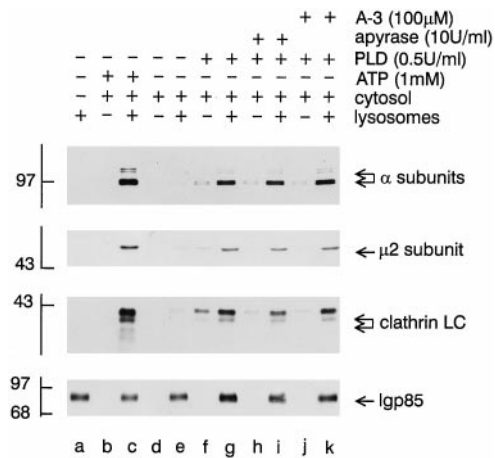
FIG. 6. **Coat-protein recruitment evoked by bacterial PLD.** Reactions containing 30 μg/ml lysosomes, 2.5 mg/ml gel-filtered cytosol, 1 mM ATP, 0.5 units/ml *S. chromofuscus* PLD, 10 units/ml apyrase, and 100 μM A-3 were prepared as indicated. After 20 min at 37 °C, membranes were recovered by centrifugation and the pellets analyzed by immunoblotting with anti-α-subunit mAb 100/2, anti-μ2-subunit serum, anti-clathrin LC mAb Cl57.3, or anti-lgp85 mAb YA30. Note some degradation of clathrin despite the protease inhibitors. Analysis of the *S. chromofuscus* PLD by SDS-PAGE shows a near homogeneous polypeptide of about 60 kDa and PLD assays confirm that the enzyme hydrolyzes PtdCho to PtdOH and choline in a PtdIns(4,5)P$_2$-independent fashion (L. S. Arneson and L. M. Traub, unpublished results).

activity,[3] is added to a mixture of gel-filtered brain cytosol and lysosomes in the absence of ATP, AP-2 does translocate onto the lysosome surface (Fig. 6, *lane g*). No recruitment is seen in the absence of PLD (*lane e*). Adaptor binding is about 3-fold less efficient with PLD than the recruitment seen in the presence of ATP (*lane g* compared with *lane c*), but is nevertheless accompanied by clathrin recruitment. Interestingly, adding up to 8 units/ml of the bacterial PLD does not increase the amount of coat components associated with the lysosomes in the absence of ATP.[3] Incubations containing PLD together with apyrase or A-3 rule out the possibility that the bacterial PLD facilitates coat recruitment by stimulating PIP5KI to generate PtdIns(4,5)P$_2$ using trace ATP remaining in the gel-filtered cytosol. Neither AP-2 nor clathrin recruitment is markedly altered by including either 10 units/ml apyrase or 100 μM A-3 (Fig. 6, *lanes i* and *k* compared with *lane g*).

Thin-section EM analysis verifies that the bacterial PLD indeed promotes the assembly of identifiable clathrin coats (Fig. 7). Our purified lysosome preparations consist predominantly of dense-core organelles and characteristic bristle-like areas of assembled clathrin can occasionally be seen on the lysosomes after incubation with gel-filtered cytosol and bacterial PLD (*panels a* and *b*). While the coat appears indistinguishable from the clathrin-coated buds that readily form on lysosomes incubated with cytosol and ATP (*panels c* and *d*), we do not find any evidence for coated bud formation in the presence of PLD alone. The PLD treatment also results in significant rupture of a proportion of the lysosomes, a phenomenon rarely seen with ATP. Empty lysosomal membrane fragments and free lumenal material are observed after the incubation with the bacterial enzyme (*panels a* and *b, arrows*). We conclude that PtdOH plays an important role in initiating AP-2 and clathrin assembly on lysosomes. Additional ATP- or PtdIns(4,5)P$_2$dependent steps are, however, required for extensive lattice formation and invagination. This is in agreement with our biochemical data showing only limited recruitment of clathrin and AP-2.
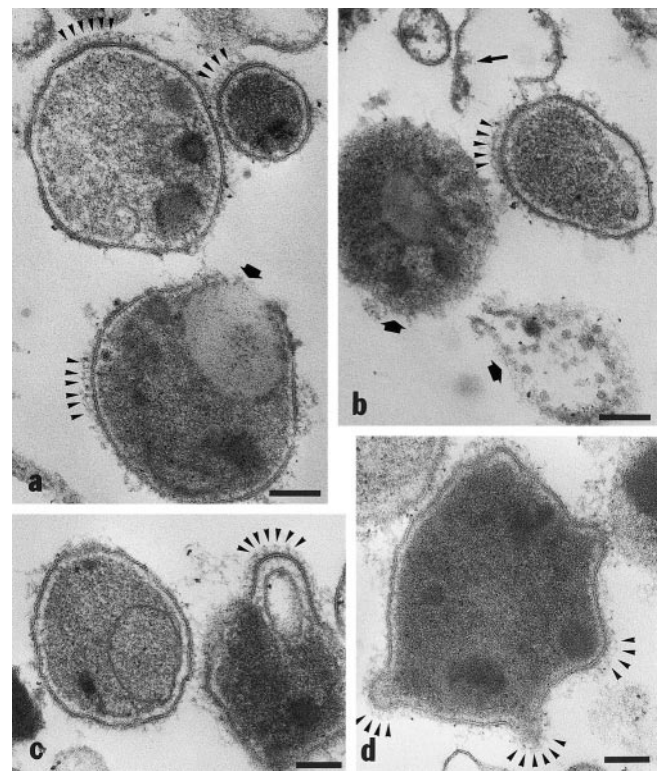
---

[3] L. S. Arneson and L. M. Traub, unpublished results.

FIG. 7. **EM analysis of clathrin-coat assembly on lysosomes.** Reactions containing 120 μg/ml lysosomes, 3 mg/ml gel-filtered cytosol, and either 0.5 unit/ml *S. chromofuscus* PLD (*a* and *b*) or an ATP regeneration system (*c* and *d*) were prepared on ice. After incubation at 37 °C for 20 min the tubes were chilled, fixed, and then processed for EM. Selected images that are typical of the coated structures (*arrowheads*) formed under each condition are shown. Note that no budding of the coated profiles (*arrowheads*) is seen after PLD treatment and significant rupture of the lysosomes, resulting in both free limiting membrane fragments (*fine arrows*) and exposed lumenal material (*bold arrows*) occurs. *Bar,* 100 nm.

Bacterial PLD Targets AP-2 to Lysosomes in Permeabilized NRK Cells—The rupture of lysosomes noted after adding PLD reiterates that this enzyme hydrolyzes PtdCho rather indiscriminately. Because PtdOH has also been linked to the assembly of COPI-coated vesicles on the Golgi (58), COPII coats at endoplasmic reticulum export sites (59, 60), protein export from the TGN (61–63) and to clathrin-coat assembly (36), we examined whether the recruitment of AP-2 in the presence of exogenous PLD shows compartmental specificity. Precise targeting of AP-2 onto lysosomes is seen in permeabilized NRK cells incubated with cytosol and ATP. An extremely high incidence of colocalization of AP-2 with the lysosomal glycoproteins lgp120 (Fig. 8, *panels a* and *b*) and lgp85[2] is evident. In this system, AP-2 and clathrin recruitment is also totally dependent on ATP. No AP-2 translocates onto lgp120-positive structures in the absence of ATP (*panels c* and *d*) unless PLD is added (*panels e* and *f*). This again suggests that PLD appears to function downstream of PIP5K. In addition, although the colocalization of AP-2 with lgp120 is less exact when PLD is used, the recruitment is still compartmentally regulated. The staining pattern of the recruited adaptor is clearly different from that of α-mannosidase II, a medial Golgi marker (*panels g* and *h*). In most cells, the adaptor complex targets onto membranes that are spatially distinct from the MPR-containing late-endosomal elements (*panels i* and *j*). While some overlap between the MPR-positive structures and the recruited AP-2 is seen in a few cells (*panels i* and *j, arrowheads*), careful examination does not reveal convincing colocalization of the discrete labeled
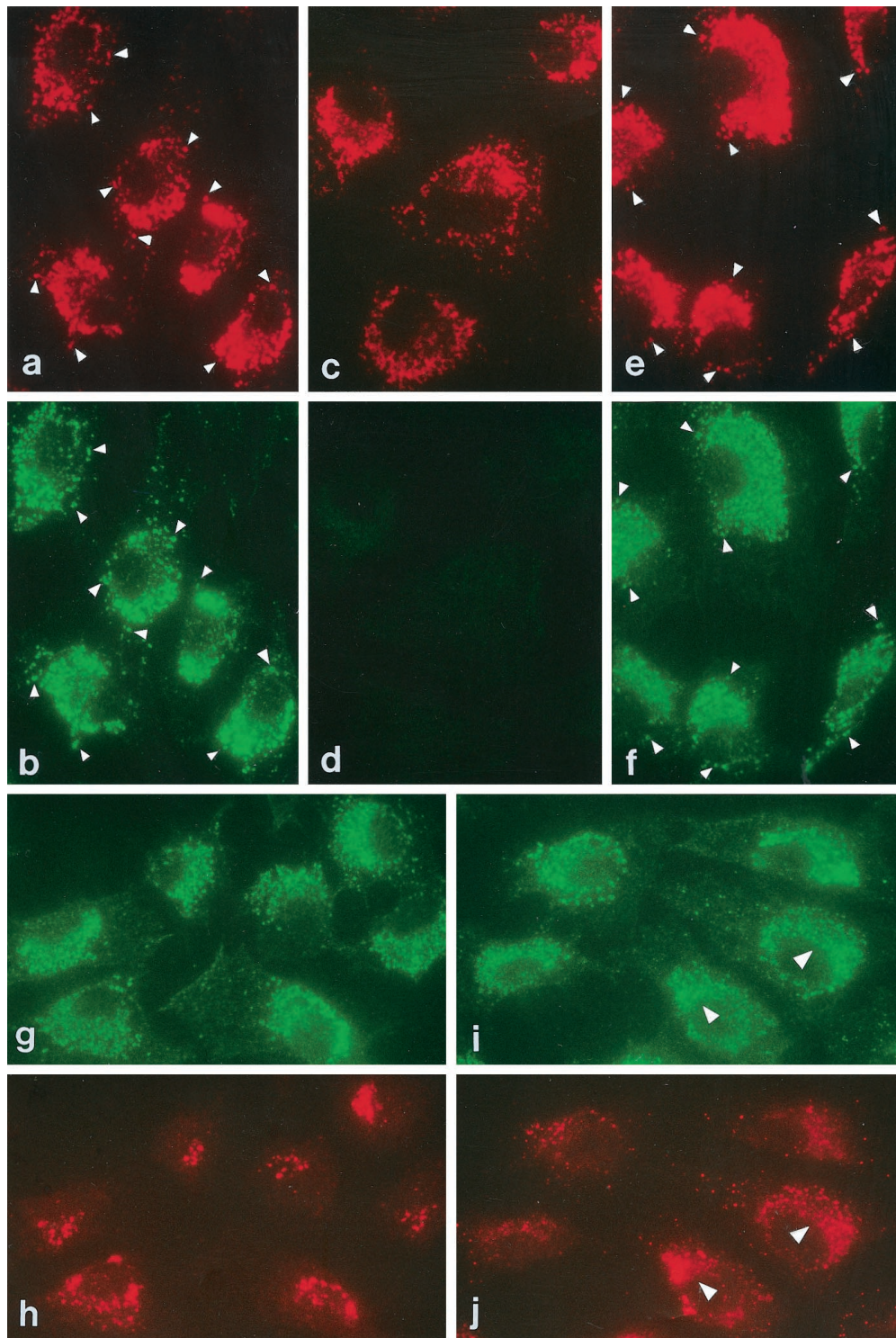
FIG. 8. **Bacterial PLD initiates adaptor recruitment onto lysosomes.** Digitonin-permeabilized NRK cells were incubated with 2.5 mg/ml gel-filtered cytosol and 1 mM ATP (*a* and *b*) or without ATP (*c-j*) and with 0.5 unit/ml *S. chromofuscus* PLD (*e-j*). After 20 min at 37 °C the cells were washed, fixed, and then processed for indirect immunofluorescence using a mixture of affinity-purified anti-lgp120 antibodies (*a, c,* and *e*) and anti-α-subunit mAb AP.6 (*b, d*, and *f*) or mAb AP.6 (*g*) and anti-mannosidase II serum (*h*) or mAb AP.6 (*i*) and affinity-purified anti-CI-MPR antibodies (*j*). The conditions for photography and printing were identical for *a-f*. The images are typical of each treatment and selected regions of colocalization of the lgp120 and AP-2 signals are indicated (*arrowheads*) as are cells in which substantial overlap between the AP-2 and MPR staining occurs.

spots. The similarity in staining may therefore reflect the close proximity of the late endosome and lysosome compartments in some of these cells. We do note that AP-2 recruitment onto remnants of the plasma membrane also becomes evident in the presence of the bacterial PLD (*panels f, g,* and *i*). Because

PtdOH is likely to be generated on all membranes in these experiments, the results establish that AP-2 does not simply associate with PtdOH-rich membranes; additional determinants at the site of coat assembly appear necessary to initiate adaptor recruitment.

FIG. 9. **Schematic model illustrating the positive feedback loop between PIP5KI and PLD1 that occurs at the lysosome surface and how the products of this regulatory loop the appear to effect clathrin-coat assembly on lysosomes.**
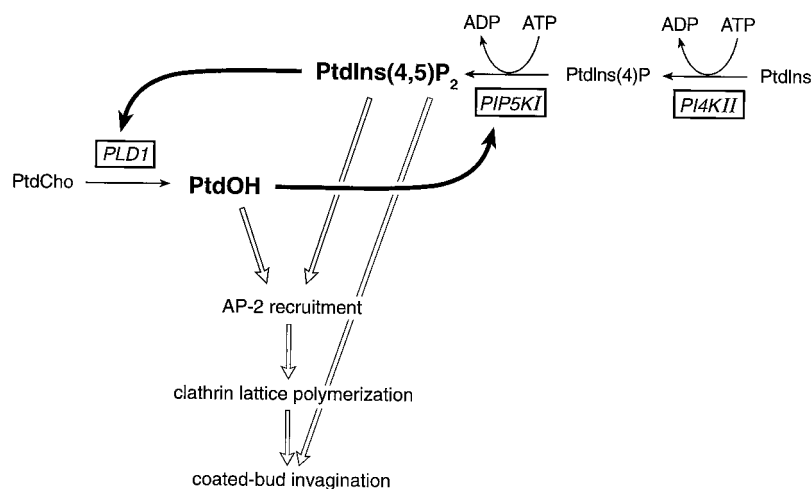
DISCUSSION

The results of this study provide a framework to begin to understand the mechanisms that regulate the precise targeting of AP-2 onto membranes in greater molecular detail (Fig. 9). We find that both PtdIns(4,5)P_2 and PtdOH are important regulators of AP-2 recruitment and clathrin-lattice assembly on lysosomes. We show that PLD1-like activity is associated with purified lysosomes, consistent with the dispersed punctate distribution of transiently overexpressed PLD1 observed by others (54, 64). Our results also demonstrate directly, in the context of a biological membrane, that a positive feedback loop exists between this enzyme and PIP5KI. PtdIns(4,5)P_2 is obligatory for PLD activity and PtdOH, the product of this activity, acts as an allosteric activator of PIP5KI activity.

PLD Activation and AP-2 Recruitment—A clear role for PtdOH in the construction of a clathrin-coated bud is evident from the ability of bacterial PLD to induce AP-2 translocation and effect limited coat assembly under conditions that avert PtdIns(4,5)P_2 synthesis. While we cannot rule out that residual PtdIns(4,5)P_2 on the limiting membrane of the lysosome contributes to AP-2 recruitment under these conditions, PtdOH must play an important role because, in the absence of ATP, no recruitment occurs without the exogenously added PLD. The data also indicate that ongoing PtdIns(4,5)P_2 formation is not required to permit adaptor binding. Superficially, some of our results appear similar to those in a recent study examining the role of PLD activation in the targeting of AP-2 onto endosomes (36). However, GTPγS is not at all required to induce coat formation in our system (21). Because the hydrolysis of exogenous PtdCho by the lysosomal PLD is potentiated by ARF·GTPγS, we assume the lysosome-associated lipase to be PLD1-like. This corroborates the recent colocalization of GFP-PLD1b, the major PLD1 isoform expressed in most rat tissues (65), with LAMP-1-positive membranes (64). Nevertheless, the minimal effect of GTPγS on clathrin-coat initiation suggests that activity of the PLD1-like enzyme toward lysosomal membrane phospholipids does not appear to be absolutely dependent on activated ARF. In fact, the precise physiological roles of the *in vitro* activators of PLD1, ARF, Rho, and PKCa, is still being debated (66).

PtdOH appears to play a general role in regulating the assembly of several types of coated membrane (36, 46, 58–63, 67), but it is unclear exactly how this is accomplished. One possibility is that direct interaction of certain phospholipids with coat proteins, in our case AP-2, initiates bud formation. It has recently been shown that clathrin-coated components can translocate onto synthetic liposomes, and commence the assembly of coated buds, in a nucleotide-independent fashion

(46). The recruitment of AP-2 and clathrin from brain cytosol onto synthetic liposomes proceeds only poorly, however, and is considerably less efficient than the recruitment of dynamin, amphiphysin, and synaptojanin onto these same liposome preparations (46). Coat assembly equivalent to that seen when cytosol is mixed with synaptosome-derived membranes is only observed on the synthetic liposomes after fortifying the donor cytosol with ~0.5 mg/ml soluble AP-2 and clathrin extracted from purified clathrin-coated vesicles (46). This indicates that the interaction between AP-2 and lipids alone is a low-affinity phenomenon and, interestingly, no dramatic effect on AP-2 and clathrin binding or coat formation occurs on adding either phosphoinositides or PtdOH to the lipid vesicles (46). In permeabilized NRK cells treated with bacterial PLD under conditions that we expect PtdOH to be generated on all PtdCho-containing membranes, we show that AP-2 targets almost exclusively onto lysosomal glycoprotein-rich lysosomal membranes. This shows that lipids alone are not sufficient to nucleate coat assembly on any biological membrane. Nonetheless, we do find that the synthesis of relatively minor but potently active acidic phospholipids is pivotal in nucleating clathrin polymerization on lysosomes and the production of these lipids promotes exceptionally efficient coat formation in the presence of dilute cytosol. Our interpretation is that there are additional factors on the membrane that raise the affinity of AP-2 for the lysosomal membrane substantially. The growing consensus is that dedicated docking molecules, restricted to the bud site, probably provide the primary binding interface for adaptors on membranes (9). We favor the idea that PtdOH activates a putative AP-2-selective docking molecule (36, 68) in a manner analogous to the way ARF activates an AP-1-specific docking site at the TGN (5, 7, 8).

PtdIns(4,5)P_2 and Clathrin-coat Assembly—In the absence of PtdIns(4,5)P_2 production, we find that AP-2 binding to lysosomes is about 3-fold lower. At least two explanations for this result can be considered. First, as the bacterial PLD compromises the integrity of the lysosomal membrane, the reduced coat assembly and the failure of the assembled lattice to invaginate might simply reflect the damage inflicted on the membrane by the enzymatic treatment. Alternatively, the data could suggests that, in addition to serving as a co-factor for PLD1, PtdIns(4,5)P_2 might also contribute to the nucleation of coated structures directly. In fact, evidence for multiple roles for PtdIns(4,5)P_2 in clathrin-mediated endocytosis has just been published (69). Our data show that the lipids formed on lysosomes are PtdIns(4)P and PtdIns(4,5)P_2, neither of which affects the affinity of AP-2 for tyrosine-based sorting signals (70). Therefore, direct modulation of tyrosine-based sorting

signals by polyphosphoinositides is unlikely to account for increased binding of AP-2 to lysosomes (70). PtdIns(4,5)P_2 could anchor AP-2 to the lysosome surface directly, since a high-affinity inositol polyphosphate-binding site is located within the amino-terminal 50 residues of the α subunit of the AP-2 heterotetramer (71). Association of AP-2 with PtdIns(4,5)P_2 might orient the molecule for optimal association with sorting signals and together, these two attachments might transiently retain an adaptor on the membrane if release from the putative docking site proceeds clathrin recruitment.

Retrograde Traffic from the Lysosome—The physiological significance of clathrin-coat formation on lysosomes is somewhat controversial. While the evidence for an outward pathway of membrane flow is only indirect in mammalian cells, we reasoned that a retrograde route from the lysosome would be important to maintain overall membrane homeostasis and speculated that this would provide a suitable mechanism for recycling regulatory molecules, like v-SNARES (21). There is now very good evidence for retrograde movement from the *Saccharomyces cerevisiae* vacuole, the yeast equivalent of the lysosome. Strikingly, a phosphatidylinositol 3-phosphate 5-kinase, Fab1p, is intimately connected with the regulation of the size of the vacuole (72, 73). Within 30 min of shifting to the restrictive temperature, the vacuole of a fab1 temperature conditional mutant more than doubles in size, prompting the conclusion that phosphoinositide synthesis is a critical regulator of the size and integrity of the vacuole, probably by modulating transport into or out of this organelle (72). Direct evidence for protein flux through the vacuole has recently been obtained (74).

It is not known yet whether clathrin and adaptors are involved in the return of proteins from the yeast vacuole and, certainly, the AP-2-dependent clathrin-coat assembly we see on lysosomes in our *in vitro* assays is an exaggerated phenomenon. Nevertheless, the yeast data, and the presence of both PI4K and PLD1 activity on the lysosome membrane suggest, but do not prove, that clathrin-coat assembly on lysosomes occurs in a tightly regulated manner (54). Some of the regulatory constraints appear to be lost in the *in vitro* systems that we use. It is important to note, however, that massive AP-2-containing clathrin-coat assembly does occur on lysosomes under some conditions *in vivo* (21, 75). More importantly, perhaps, our preliminary experiments analyzing clathrin-coat assembly on preparations of highly purified synaptic plasma membrane, and other independent studies (69), reveal that PtdIns(4,5)P_2 is also a critical regulator of AP-2 recruitment and clathrin polymerization at the cell surface. Our current interpretation then is that the biochemistry that underlies coat assembly on the lysosome is very likely to be similar to the process that occurs at the cell surface.

REFERENCES

1. Robinson, M. S., and Kreis, T. E. (1992) *Cell* **69,** 129–138
2. Wong, D. H., and Brodsky, F. M. (1992) *J. Cell Biol.* **117,** 1171–1179
3. Ahle, S., Mann, A., Eichelsbacher, U., and Ungewickell, U. (1988) *EMBO J.* **7,** 919–929
4. Stamnes, M. A., and Rothman, J. E. (1993) *Cell* **73,** 999–1005
5. Traub, L. M., Ostrom, J. A., and Kornfeld, S. (1993) *J. Cell Biol.* **123,** 561–573
6. Shih, W., Galluser, A., and Kirchhausen, T. (1995) *J. Biol. Chem.* **270,** 31083–31090
7. Traub, L. M., Kornfeld, S., and Ungewickell, E. (1995) *J. Biol. Chem.* **270,** 4933–4942
8. Zhu, Y., Traub, L. M., and Kornfeld, S. (1998) *Mol. Biol. Cell* **9,** 1323–1337
9. Kirchhausen, T., Bonifacino, J. S., and Riezman, H. (1997) *Curr. Opin. Cell Biol.* **9,** 488–495
10. Ohno, H., Aguilar, R. C., Yeh, D., Taura, D., Saito, T., and Bonifacino, J. S. (1998) *J. Biol. Chem.* **273,** 25915–25921
11. Donaldson, J. G., Finazzi, D., and Klausner, R. D. (1992) *Nature* **360,** 350–352
12. Helms, J. B., and Rothman, J. E. (1992) *Nature* **360,** 352–354
13. Pishvaee, B., and Payne, G. S. (1998) *Cell* **95,** 443–446
14. Santini, F., and Keen, J. H. (1996) *J. Cell Biol.* **132,** 1025–1036
15. Warren, R. A., Green, F. A., and Enns, C. A. (1997) *J. Biol. Chem.* **272,** 2116–2121
16. Santini, F., Marks, M. S., and Keen, J. H. (1998) *Mol. Biol. Cell* **9,** 1177–1194
17. Zhang, J. Z., Davletov, B. A., Sudhof, T. C., and Anderson, R. G. W. (1994) *Cell* **78,** 751–760
18. Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G., Brose, N., and Sudhof, T. C. (1995) *Nature* **375,** 594–599
19. Schmid, S. L., and Smythe, E. (1991) *J. Cell Biol.* **114,** 896–880
20. Takei, K., Mundigl, O., Daniell, L., and De Camilli, P. (1996) *J. Cell Biol.* **133,** 1237–1250
21. Traub, L. M., Bannykh, S. I., Rodel, J. E., Aridor, M., Balch, W. E., and Kornfeld, S. (1996) *J. Cell Biol.* **135,** 1801–1804
22. Aguilar, R. C., Ohno, H., Roche, K. W., and Bonifacino, J. S. (1997) *J. Biol. Chem.* **272,** 27160–27166
23. Endemann, G. C., Graziani, A., and Cantley, L. C. (1991) *Biochem. J.* **273,** 63–66
24. Zhu, Y., Traub, L. M., and Kornfeld, S. (1999) *Mol. Biol. Cell* **10,** 537–549
25. Jenkins, G. H., Fisette, P. L., and Anderson, R. A. (1994) *J. Biol. Chem.* **269,** 11547–11554
26. Loijens, J. C., and Anderson, R. A. (1996) *J. Biol. Chem.* **271,** 32937–32943
27. Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W., and Anderson, R. A. (1997) *J. Biol. Chem.* **272,** 17756–17761
28. Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T., and Oka, Y. (1998) *J. Biol. Chem.* **273,** 8741–8748
29. Wigge, P., Vallis, Y., and Mcmahon, H. T. (1997) *Curr. Biol.* **7,** 554–560
30. Bazenet, C. E., Ruano, A. R., Brockman, J. L., and Anderson, R. A. (1990) *J. Biol. Chem.* **265,** 18012–18022
31. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A., and Martin, T. F. J. (1995) *Nature* **347,** 173–177
32. Brown, H. A., and Sternweis, P. C. (1995) *Methods Enzymol.* **257,** 313–324
33. Inagaki, M., Kawamoto, S., Itoh, H., Saitoh, M., Hagiwara, M., Takahashi, J., and Hidaka, H. (1986) *Mol. Pharmacol.* **29,** 577–581
34. Hay, J. C., and Martin, T. F. (1992) *J. Cell Biol.* **119,** 139–151
35. Schacht, J. (1978) *J. Lipid Res.* **19,** 1063–1067
36. West, M. A., Bright, N. A., and Robinson, M. S. (1997) *J. Cell Biol.* **138,** 1239–1254
37. Collins, C. A., and Wells, W. W. (1983) *J. Biol. Chem.* **258,** 2130–2134
38. Wong, K., Meyers, R., and Cantley, L. C. (1997) *J. Biol. Chem.* **272,** 13236–13241
39. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) *Nature* **371,** 168–170
40. Harlan, J. E., Yoon, H. S., Hajduk, P. J., and Fesik, S. W. (1995) *Biochemistry* **34,** 9859–9864
41. Salim, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Gout, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996) *EMBO J.* **15,** 6241–6250
42. Shupliakov, O., Low, P., Grabs, D., Gad, H., Chen, H., David, C., Takei, K., De Camilli, P., and Brodin, L. (1997) *Science* **276,** 259–263
43. David, C., McPherson, P. S., Mundigl, O., and De Camilli, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 331–335
44. McMahon, H. T., Wigge, P., and Smith, C. (1997) *FEBS Lett.* **413,** 319–322
45. Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997) *J. Biol. Chem.* **272,** 16700–16706
46. Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998) *Cell* **94,** 131–141
47. Downing, G. J., Kim, S., Nakanishi, S., Catt, K. J., and Balla, T. (1996) *Biochemistry* **35,** 3587–3594
48. Balla, T., Downing, G. J., Jaffe, H., Kim, S., Zolyomi, A., and Catt, K. J. (1997) *J. Biol. Chem.* **272,** 18358–18366
49. Meyers, R., and Cantley, L. C. (1997) *J. Biol. Chem.* **272,** 4384–4390
50. Ishihara, H., Shibasaki, Y., Kizuki, N., Katagiri, H., Yazaki, Y., Asano, T., and Oka, Y. (1996) *J. Biol. Chem.* **271,** 23611–23614
51. Rameh, L. E., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. (1997) *Nature* **390,** 192–196
52. Tolias, K. F., Rameh, L. E., Ishihara, H., Shibasaki, Y., Chen, J., Prestwich, G. D., Cantley, L. C., and Carpenter, C. L. (1998) *J. Biol. Chem.* **273,** 18040–18046
53. Liscovitch, M., and Cantley, L. C. (1995) *Cell* **81,** 659–662
54. Ktistakis, N. T. (1998) *Bioessays* **20,** 495–504
55. Morris, A. J., Frohman, M. A., and Engebrecht, J. (1997) *Anal. Biochem.* **252,** 1–9
56. Chalifa, V., Mohn, H., and Liscovitch, M. (1990) *J. Biol. Chem.* **265,** 17512–17519
57. Ktistakis, N. T., Brown, H. A., Sternweis, P. C., and Roth, M. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4952–4956
58. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) *J. Cell Biol.* **134,** 295–306
59. Bi, K., Roth, M. G., and Ktistakis, N. T. (1997) *Curr. Biol.* **7,** 301–307
60. Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Schekman, R., and Yeung, T. (1998) *Cell* **93,** 263–275
61. Chen, Y. G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T. C., Frohman, M. A., Morris, A. J., and Shields, D. (1997) *J. Cell Biol.* **138,** 495–504
62. Siddhanta, A., and Shields, D. (1998) *J. Biol. Chem.* **273,** 17995–17998
63. Tuscher, O., Lorra, C., Bouma, B., Wirtz, K. W., and Huttner, W. B. (1997) *FEBS Lett.* **419,** 271–275
64. Brown, F. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson,

N. T., Solari, R., and Wakelam, M. J. (1998) *Curr. Biol.* **8,** 835–838

65. Katayama, K., Kodak, T., Nagamachi, Y., and Yamashita, S. (1998) *Biochem. J.* **329,** 647–652
66. Rudge, S. A., Cavenagh, M. M., Kamath, R., Sciorra, V. A., Morris, A. J., Kahn, R. A., and Engebrecht, J. (1998) *Mol. Biol. Cell* **9,** 2025–2036
67. Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R., and Orci, L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11199–11204
68. Roth, M. G., and Sternweis, P. C. (1997) *Curr. Opin. Cell Biol.* **9,** 519–526
69. Jost, M., Simpson, F., Kavran, J. M., Lemmon, M. A., and Schmid, S. L. (1998) *Curr. Biol.* **8,** 1399–1402
70. Rapoport, I., Miyazaki, M., Boll, W., Duckworth, B., Cantley, L. C., Shoelson,

S., and Kirchhausen, T. (1997) *EMBO J.* **16,** 2240–2250
71. Gaidarov, I., Chen, Q., Falck, J. R., Reddy, K. K., and Keen, J. H. (1996) *J. Biol. Chem.* **271,** 20922–20929
72. Yamamoto, A., DeWald, D. B., Boronenkov, I. V., Anderson, R. A., Emr, S. D., and Koshland, D. (1995) *Mol. Biol. Cell* **6,** 525–539
73. Gary, J. D., Wurmser, A. E., Bonangelino, C. J., Weisman, L. S., and Emr, S. D. (1998) *J. Cell Biol.* **143,** 65–79
74. Bryant, N. J., Piper, R. C., Weisman, L. S., and Stevens, T. H. (1998) *J. Cell Biol.* **142,** 651–663
75. Wang, L.-H., Rothberg, K. G., and Anderson, R. G. W. (1993) *J. Cell Biol.* **123,** 1107–1117