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

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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$_2$, 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, 2), the site where this heterotetrameric adaptor 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 polyanalytic 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 periphery 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 $\mu$1 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 $\mu$1 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

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; ATP$\gamma$S, adenosine 5'-($\gamma$-thio)triphosphate; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; IgG, 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)P2), are critical regulators of AP-2 adaptor binding. Direct evidence for a positive feedback loop between type I phosphatidylinositol 4-phosphate 5-kinase (PIPK5) and phospholipase D (PLD) is presented. Both of the lipids generated by this regulatory loop, PtdIns(4,5)P2 and phosphatidic acid (PtdOH), appear to play important roles in initiating clathrin-coat assembly on lysosomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (4,500 Ci/mmol) was from ICN and 1,2-di-palmitoyl-[1-14C]phosphatidylcholine (114 mCi/mmol) was from Amer sham. Apyrase, ATP, creatine kinase, creatine phosphate, neomycin, phorbol 12-myristate 13-acetate, dipalmitoyl phosphatidylcholine (PtdCho), phosphatidylethanolamine, PtdOH, phosphatidylinositol 4-phosphate (PtdIns(4)P), PtdIns(4,5)P2, phosphoinositides, Streptomyces chromofuscus PLD (type VII), protease inhibitors, and Wortmannin were purchased from Sigma. PtdIns(4)P, PtdIns(4,5)P2, and PtdIns(5)P were also obtained from Roche Molecular Biochemicals. Synthetic dipalmitoyl PtdIns(5)P was a gift from G. Prestwich. A-3 and recombinant protein kinase Ca (PKCα) were purchased from Calbiochem. Glutathione (GSH)-Sepharose 4B and NAP-5 columns, and Parocel 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 subunit of AP-2, were provided by F. Brodsky. An antibody against the cation-independent mannose 6-phosphate receptor (MPR) has been described elsewhere (24). The antibodies used to detect the PIP5Ks were affinity-purified anti-type I PIP5K, prepared using recombinant PIP5KIIα obtained from UBI. Affinity purified rabbit anti-lgp120 antibodies and mAb YA30, against lgp85, were provided by K. Akasaka. 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 PIP5K1 (25), and polyclonal anti-PIP5K type Iα and anti-PIP5K type Iγ antibodies, each affinity purified using the appropriate recombinant protein (26, 27). Serum from rabbits injected with a peptide matching the carboxyl terminus of PIP5KIV was provided by H. Ishiihara 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 μM Mg2+ 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 that correspond to 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-amphiphysin SH3 overnight at 4 °C. GST-Phosphosearch was then added to collect the protein complexes and then removed by brief centrifugation. The supernatant was stored at −20 °C. Aliquots of each were then washed 4 times in phosphate-buffered saline before solubilization in SDS sample buffer. Immunodepletion of AP-2 from cytosol with AP-6-Phosphosearch 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 sucrose, 5 μg/ml each of antipain, leupeptin, and pepstatin A (protease inhibitors), 5 mM glycerol, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A) and then loaded at 1.5 ml/min onto a phosphocellulose column (1.6 × 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 mM 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 PIP5K1 and PIP5KII pools were concentrated with a Centricon 10. When assayed for PtdInsP production with lysosomes, the PIP5K1 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 diethiothreitol 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 diethiothreitol 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 digoxigenin-permeabilized, formaldehyde-fixed glass cover slips 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 diethiothreitol in a 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-32P]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 × g 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 (3:4:8:47) and then dried under a stream of N2 gas at about 40 °C. Dried lipid films were resuspended in chloroform:methanol:H2O (75:25:1) and spotted onto TLC plates. Formation of PtdIns(4,5)P2, 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-32P]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 contained membranes at 5 to −25 °C for 5–20 minutes 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 diethiothreitol, 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 substate (mixed micelles of phosphatidyether, \(^{14}C\)PtdCho (52.5 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:acetic acid (13:5:1). After chromatography, lipid products 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 0–1M 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 c), 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 l), 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 inositol 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). Second, neither plasma or neuraminide serves as a phosphate donor for phosphoinositide synthesis (34). Third, 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 presence 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-aphmphSH3 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 h and e). 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 pellet lysosomes (Fig. 2C, lane g compared with lanes e and c) 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
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 β2-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 Mr 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-32P]ATP, and 1 mM A-3 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-β2-subunit serum, anti-clathrin heavy chain (HC) mAb TD.1, anti-clathrin light chain (LC), mAb Cl57.3, or an anti-dynamin mAb.
the pellets from incubations with dynamin-containing cytosol do contain dynamin (Fig. 2C, 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 homooligomers, 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)P2 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 Inositol Kinases Involved in PtdIns(4,5)P2 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 reincubated with the antibody prior to the addition ATP (data not shown), and is in accord with a study examining the subcellular distribution of PtdIns(4,5)P2 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 P13K activity.

Two peaks of PIP5K activity are resolved after fractionating rat brain cytosol on phosphocellulose (Fig. 3A). 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. 3B). 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. 3B).

The minor peak of type II PIP5K activity elutes from phosphocellulose with about 1.1 M NaCl (Fig. 3A). 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. 3B). 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)P2, pooled fractions enriched with each activity from the phosphocellulose column were added to purified lysosomes in the presence of [γ-32P]ATP (Fig. 3C). Only the type I PIP5K pool produces PtdIns(4,5)P2 (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-
FIG. 3. PtdIns(4,5)P$_2$ 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-PIP5KIa antibodies (b), affinity-purified anti-PIP5KIIb antibodies (c), anti-PIP5KIb 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)P$_2$ made from the synthetic PtdIns(5)P differs from that of PtdIns(4,5)P$_2$ 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)P$_2$ in lane c of this experiment was identical to the mobility of the carrier PtdIns(4,5)P$_2$ in that lane visualized with iodine vapor.
enables us to follow the stable product of transphosphatidyl-
lation, PtdBut. A low level of PtdCho cleavage occurs upon mix-
ing purified lysosomes with the exogenous reporter micelles
(Fig. 5A, 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)P2 and negligible PtdBut for-
mation is seen when lysosomes 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
(−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 chlo-
line head group release because 1-butanol also inhibits the
catalytic activity of PLD (56).

For comparison then, we also analyzed purified Golgi mem-
branes, which are known to contain ARF-stimulated PLD1
activity (57). The catalytic activity associated with Golgi re-
sponds to PLD1 activators very similarly to that associated
with lysosomes (Fig. 5A, 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. 5B). Lysosomes have no detectable mannosidase II but are
heavily enriched with the lysosomal membrane proteins, as
expected. The gross protein profiles of the two organelle pre-
parations 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 un-
likely 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)P2, PtdOH, or both
of these lipids. If PtdIns(4,5)P2 is required only to stimulate
PLD1-dependent formation of PtdOH, then it should be possible
to promote nucleotide-independent coat assembly on lysos-
omes in vitro using exogenous PLD. When 0.5 unit/ml S.
chromofuscus PLD, which is not dependent on PtdIns(4,5)P2 for

Fig. 4. Primary alcohols inhibit PtdIns(4,5)P2 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-32P]ATP were pre-
pared 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, mem-
branes were recovered by centrifugation and the pellets analyzed by
immunoblotting with anti-α-subunit mAb 1002 or anti-m2-subunit se-
rum. 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.

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 phosphati-
dylethanolamine[14C]PtdCho-PtdIns(4,5)P2-containing substrate lipo-
somes, 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 homoge-
nate or purified liver lysosomes or liver Golgi membranes were frac-
tioned by SDS-PAGE and either stained with Coomassie Blue (a-c) or
transferred to nitrocellulose (d-f). Blots were probed with anti-manna-
sidase II serum, affinity-purified anti-lgp120 antibodies or anti-lgp85
mAb YA30.
activity, is added to a mixture of gel-filtered brain cytosol and lysosomes in the absence of ATP. PLD 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. 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)P₂ 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 granules 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 luminal 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)P₂-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.

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 COPII-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 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 membranes.

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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 Ab100/2, anti-μ2-subunit serum, anti-clathrin LC mAb CI57.3, 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)P₂-independent fashion (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 luminal material (bold arrows) occurs. Bar, 100 nm.
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. 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 (a-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.
Phospholipid-regulated Clathrin-coat Assembly

**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 PtdOH. 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, GTP$_\gamma$S 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 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 suggest 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

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**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 appear to effect clathrin-coat assembly on lysosomes.
signals by polyphosphoinositides is unlikely to account for increased binding of AP-2 to lysosomes (70). PtdIns(4,5)P₂ 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)P₂ 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.

**Reproductive 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 condition some-
