AP-2–containing Clathrin Coats Assemble on Mature Lysosomes

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Abstract. Coat proteins appear to play a general role in intracellular protein trafficking by coordinating a membrane budding event with cargo selection. Here we show that the AP-2 adaptor, a clathrin-associated coat–protein complex that nucleates clathrin-coated vesicle formation at the cell surface, can also initiate the assembly of normal polyhedral clathrin coats on dense lysosomes under physiological conditions in vitro. Clathrin coat formation on lysosomes is temperature dependent, displays an absolute requirement for ATP, and occurs in both semi-intact cells and on purified lysosomes, suggesting that clathrin-coated vesicles might regulate retrograde membrane traffic out of the lysosomal compartment.

Lysosomes are acidified hydrolase-rich organelles that function as the terminal degradative compartment of the cell (14). They receive materials, such as low density lipoproteins, which have been transported from the cell surface via the endocytic pathway, and degrade them into reusable components. In addition, lysosomes are continually replenished with newly synthesized acid hydrolases and select membrane proteins via the biosynthetic pathway (36, 57). These two pathways, the biosynthetic and endocytic pathways, merge at or before the prelysosomal compartment (PLC)[1], which then fuses with lysosomes to allow the transfer of both soluble contents and membrane components. During this delivery process, the lysosomes receive a constant input of membrane components, and, consequently, there must be a mechanism for recycling phospholipids and certain proteins to maintain the characteristic steady-state composition and dimensions of this organelle. At least three possible mechanisms, which are not necessarily mutually exclusive, might regulate membrane dynamics within the lysosomal compartment. The incoming membrane lipids and proteins could first be internalized into the lumen of the lysosome, as intraluminal vesicles and whorls often seen by EM, and then be degraded. A second possibility is that the fusion between the PLC and the lysosome is a highly specialized event (23), which allows only very limited membrane exchange although it permits the efficient delivery of luminal contents, while a third possibility is that active recycling of membrane out of the lysosome occurs in the form of vesicular intermediates.

A variety of evidence lends support for the last of these possibilities (3–5, 16, 29, 34, 39, 40, 52, 71). In several cell types, antibodies against lysosomal glycoproteins (lgps) or lysosome-associated membrane proteins (lamps) are continually taken up and delivered to the lysosome despite incubation in cycloheximide for many hours, implying traffic of the existing lamps through the cell surface (3, 4, 22, 39). In addition, measurements of the distribution of lamp1 at steady state reveal that ~40% of the protein is present in endosomes rather than in lysosomes (24, 27, 28, 56). The size of this nonlysosomal population, together with the biosynthetic kinetic data (5, 26, 39), makes it unlikely that these are newly synthesized molecules en route to the lysosome. Rather, this suggests that lamps constantly exit the mature lysosome and recycle through the cell surface and endocytic pathway. The mechanism for this is unknown.

Additional evidence for constant recycling of lamps comes from experiments using wortmannin, a fungal metabolite that appears to retard protein flux through the endocytic compartments (61). Within 15 min of adding this agent to cells, large vacuoles begin to accumulate within the cell (10, 52). In normal rat kidney (NRK) cells in particular, both the mannose 6-phosphate (man-6-P)/insulin-like growth factor II receptor (10, 52) and lgp120 (52) relocate into the drug-induced vacuoles, although the two proteins show minimal colocalization (52; our unpublished data). Cathepsin L, a soluble lysosomal protease, does not redistribute along with lgp120 but remains within the lysosomal

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1. *Abbreviations used in this paper*: AMP-PNP, adenylyl-imidodiphosphate; ARF, ADP-ribosylation factor; ATPγS, adenosine 5′-O-(3-thiotriphosphate); BFA, brefeldin A; CAD, cationic amphiphilic drug; GTPγS, guanosine 5′-O-(3-thiotriphosphate); HC, heavy chain; lamp, lysosome-associated membrane protein; lgp, lysosomal glycoprotein; man-6-P, mannose 6-phosphate; NRK, normal rat kidney; PLC, prelysosomal compartment.

compartment, quite distinct from the distended lamp-positive vacuoles (52). The simplest explanation for these observations is that retrograde movement of lgp120 from the lysosome continues while traffic in the anterograde direction to the lysosome is severely constrained in the presence of wortmannin. The end result is that the steady-state distribution of lgp120 changes within the cell, now accumulating within swollen endocytic vacuoles. Again, these results strongly suggest that a mechanism of selective protein export operates at the mature lysosome.

Here we present evidence that clathrin-coated vesicles can assemble on dense lysosomes, identifying for the first time a possible intermediate for regulated protein traffic out of mature lysosomes. Furthermore, we show that these coated vesicles contain the AP-2 adaptor complex, which was previously believed to function exclusively in the formation of clathrin-coated vesicles on the plasma membrane.

Materials and Methods

Materials

Adenosine 5′-O-(3-thiotriphosphate) (ATPγS), creatine phosphate, and guanosine 5′-O-(3-thiotriphosphate) (GTPγS) were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Adenylyl-imidodiphosphate (AMP-PNP), apyrase, aprotinin, ATP, chlorpromazine, creatine kinase, DTT, D-glucose, imipramine, leupeptin, pepstatin A, and soybean trypsin inhibitor were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin was from Cooper Biomedical Inc. (Malvern, PA), and digitonin was obtained from Wako Bioproducts (Richmond, VA). Nitrocellulose membranes were purchased from Schleicher & Schuell, Inc. (Keene, NH); the ProLong mounting medium was from Molecular Probes (Eugene, OR); and Percoll, PD-10 columns, and the molecular weight markers for electrophoresis were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). ECL reagents for chemiluminescent detection were obtained from Amersham Corp. (Arlington Heights, IL). All other reagents were the highest grade available.

Antibodies

The different anti-adaptor antibodies used in this study were the α subunit–specific antibodies mAb AP.6 (11) from Frances Brodsky (University of California, San Francisco) and mAb 100/2 (1) from Ernst Ungewickell (Washington University School of Medicine, St. Louis, MO); the affinity-purified polyclonal anti-a_c antibody C619-656 (7) from Margaret Robinson (University of Cambridge, UK); the β subunit–specific antibody mAb 100/1 (1) from Ernst Ungewickell; and the γ subunit–specific antibody AE/1 (67). RY/1 serum is from a rabbit immunized with the synthetic peptide RYITQNGDYQLRTQ, corresponding to the carboxyl terminus of the murine μ1 subunit sequence. Two antibodies directed against the clathrin heavy chain, mAb TD.1 (47) and mAb X22 (9), were also obtained from Frances Brodsky, while the antibody directed against the rat clathrin LCa neuronal-specific insert CVADEAFYKQPFAD (44) was from Reinhard Jahn (Yale University School of Medicine, New Haven, CT). A hybridoma (Ly1C6) secreting an antibody that recognizes rat lgp120 (38) was provided by Ira Mellman (Yale University School of Medicine), and affinity-purified polyclonal rabbit anti–lgp120 antibodies were obtained from Kenji Akasaki (Fukuyama University, Hiroshima, Japan) (5). The polyclonal goat anti–mouse β-glucuronidase antiserum was obtained from Roger Ganschow (Children's Hospital Research Foundation, Cincinnati, OH); the polyclonal rabbit anti–rat α-mannosidase II antiserum was from Kelley Moreman (University of Georgia, Athens, GA); and the anti–cathepsin D antibodies have been described in detail previously (35). An mAb, 1D9, which recognizes ARF1, 3, 5, and 6, was supplied by Richard Kahn (Emory University School of Medicine, Atlanta, GA). The HRP-conjugated secondary antibodies were purchased from Amersham Corp. or Sigma Chemical Co., and the affinity-purified fluorochrome-coupled secondary antibodies were obtained from Cappel Laboratories (Malvern, PA). Gold-labeled secondary reagents were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Subcellular Fractionation

Rat liver Golgi-enriched membranes and cytosol were prepared from fresh liver as we have described previously (66, 67). Rat brain cytosol was prepared similarly, although the buffer used for the preparation of all cytosol preparations has been modified to include 2 mM EDTA and 2 mM EGTA. Routinely, aliquots of cytosol were gel filtered over PD-10 columns equilibrated in 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT, and then were centrifuged in a TLA-100.3 rotor at 85,000 rpm for 10 min to remove insoluble material. Dense lysosomes were prepared from fresh rat liver by Percoll density gradient centrifugation (48). Purified lysosomes were resuspended in 20 mM Tris-HCl, pH 7.4, and 250 mM sucrose and supplemented with 10 μg/ml of both leupeptin and pepstatin A. All protein concentrations were estimated using the Bradford Coomassie blue method.

Immunofluorescence-based Morphological Binding Assay

NRK cells, grown on 12-mm glass coverslips in DME supplemented with 10% FCS and 2 mM L-glutamine (complete medium), were used for the cell-based recruitment assay. The assay has been modified from our previous studies (67), with freeze-thaw lysis replaced with permeabilization using 25 μg/ml digitonin in 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, and 1 mg/ml D-glucose (permeabilization buffer) on ice for 5 min. Coverslips were then transferred to individual wells of a 24-well tray, each containing 1 ml of cold permeabilization buffer without digitonin, and incubated for 15 min on ice to deplete cytosolic components (51). The permeabilization buffer was then aspirated, and gel-filtered rat brain or rat liver cytosol was added to a concentration of ~2.5 mg/ml or ~6.0 mg/ml, respectively. Where stated, the complete ATP regeneration system was added to give final concentrations of 1 mM ATP, 5 mM creatine phosphate, and 10 U/ml creatine kinase. The ATP and/or GTPγS (100 μM) were added last, bringing the volume of each well to 200 μl, and, after gentle mixing, the tray floated in a 37°C water bath for 20 min. After two 5-min washes in permeabilization buffer on ice, the coverslips were processed for indirect immunofluorescence microscopy (1, 67). When using the affinity-purified AE/1 antibodies for immunofluorescent detection of AP-1, we found that fixing the cells with Bouin's fixative (0.9% picric acid, 9% formaldehyde, 5% acetic acid) at room temperature for 20 min produces the best results. We have also performed recruitment assays in buffers modified by either the addition of 5 mM EGTA and 1.8 mM calcium chloride to buffer free calcium to ~100 nM, or by the addition of 5 mM EGTA. Neither buffer alters the recruitment of AP-2 and clathrin onto the lgp120-positive lysosomal compartment (data not shown). To generate sucrosomes, NRK cells attached to coverslips were cultured for 16 h in complete medium containing 30 mM sucrose. The cells were washed twice with warm PBS, and then incubated at 37°C for an additional 60 min in medium lacking sucrose to chase the solute out of the endocytic compartment (34). For the experiments with chlorpromazine and imipramine, the drugs were added in complete medium at 40 and 50 μM, respectively, and incubation continued for an additional 30–60 min at 37°C before fixation in 4% paraformaldehyde in PBS.

Membrane-binding Assays

The binding assays were performed as we have described previously (66) in a buffer of 25 mM Hepes-KOH, pH 7.2, 125 mM potassium acetate, 5 mM magnesium acetate, and 1 mM DTT in a final volume of 400 μl. Purified lysosomal membranes and Golgi-enriched membranes were added to final concentrations of 5–30 μg/ml and 50 μg/ml, respectively, as indicated in the individual figure legends. 10 U/ml apyrase, either 1 mM ATP or the ATP regeneration system, 250 μM ATPγS, 250 μM AMP-PNP, or 100 μM GTPγS, were added last, and, after a 20-min incubation at 37°C, reactions were terminated by chilling on ice. After centrifugation, the membrane-containing pellets were prepared for immunoblotting. Routinely, pellets were resuspended in 20 μl of SDS sample buffer and 10 μl loaded per lane. Conditions for electrophoresis and immunoblotting have been outlined previously (66, 67). For quantitative analysis, autoradiographs were analyzed using a densitometer equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Tryptic digestion of isolated lysosomes was carried out as we have de-

scribed previously for the Golgi-enriched membrane fraction (67). Briefly, lysosomes, prepared in the absence of protease inhibitors and added to give a final concentration of 2.0 mg/ml, were mixed with 0–50 μg/ml trypsin with or without excess soybean trypsin inhibitor on ice. After incubation at 25°C for 15 min, the tubes were returned to ice, and trypsin inhibitor was added to those tubes not already containing the inhibitor. Aliquots of the pretreated lysosomes were then added to binding reactions containing gel-filtered rat brain cytosol and ATP, giving a final lysosome concentration of 30 μg/ml. For the freeze-etch analysis, purified lysosomes were first immobilized on poly-L-lysine–coated glass coverslips at a concentration of ~0.3 mg/ml (69). Binding reactions were prepared by covering the coverslip with an aliquot of cytosol containing the ATP regeneration system. After a 20-min incubation at 37°C, the cytosol was aspirated, and the membranes were fixed with glutaraldehyde and then prepared for replication (69).

Electron Microscopy

For thin-section analysis, pelleted membranes were first fixed in 2% glutaraldehyde in PBS. For cryosections, the pellets were fixed in 2% fresh paraformaldehyde in PBS for 60 min followed by extensive washing in PBS. Fixed pellets were then prepared for conventional thin-section or cryosection analysis as described previously (6). Techniques for preparing three-dimensional replicas have been described in detail (32, 69).

Results

Recruitment of Clathrin and Adaptors in Permeabilized Cells

We used indirect immunofluorescence to determine the distribution of clathrin in intact NRK cells and in digitonin-permeabilized NRK cells that had been incubated together with cytosol and ATP (51). In the intact cells, clathrin is typically observed as a dispersed array of fine punctate structures superimposed upon a more compact, often crescent-shaped, perinuclear structure (Fig. 1 *a*). These are believed to correspond to clathrin coats present on the plasma membrane and the TGN, respectively (1, 54). In the permeabilized cells, the pattern of clathrin staining is quite different. Incubation with cytosol and ATP results in numerous brightly stained perinuclear structures (Fig. 1 *b*), which replace the pattern observed in intact cells. The dimensions of the stained membranes are larger than the AP-2–containing structures on the cell surface, and often they appear ring like. If rat brain cytosol is used, the recruitment of soluble clathrin can be unambiguously followed using an antibody that detects the neuronal-specific, type a light chain (LCa) insert (44). This antibody does not recognize endogenous clathrin in either untreated (Fig. 1 *c*) or digitonin-permeabilized NRK cells incubated with cytosol alone (not shown). However, after incubating permeabilized NRK cells with brain cytosol and an ATP regeneration system, intense perinuclear clathrin staining (Fig. 1 *d*), analogous to the punctate staining seen with the anti–clathrin heavy chain (HC) antibody (Fig. 1 *b*), is observed.

Since all clathrin-coated structures within the cell are assembled from a single pool of soluble clathrin, the location

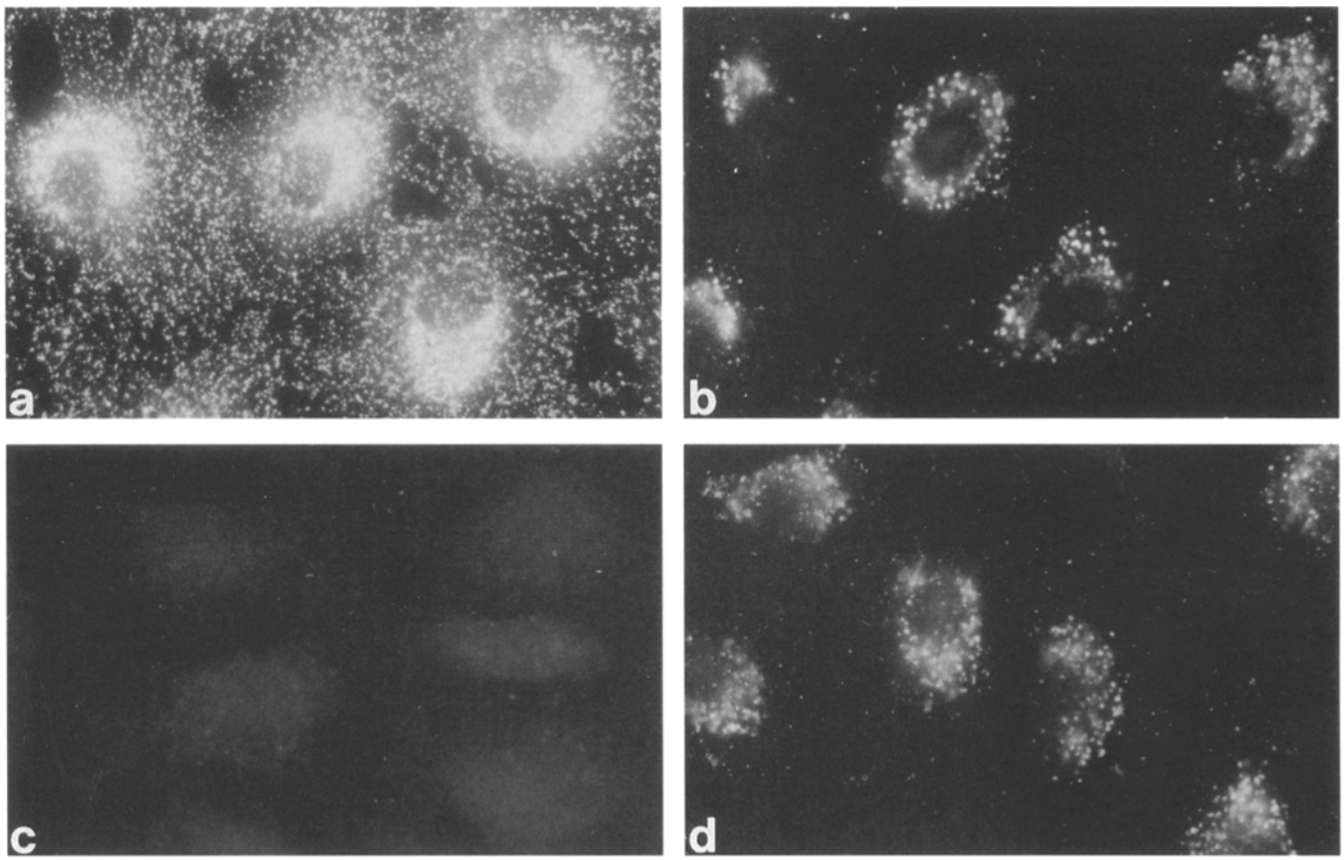

Figure 1. Subcellular localization of clathrin in intact and permeabilized cells. Intact (*a* and *c*) or digitonin-permeabilized NRK cells, incubated at 37°C for 20 min with ~2.5 mg/ml gel-filtered rat brain cytosol and an ATP regeneration system (*b* and *d*), were fixed, and then prepared for indirect immunofluorescence using either the anti–clathrin heavy chain mAb X22 (*a* and *b*) or the brain-specific anti–clathrin light chain mAb Cl 57.3 (*c* and *d*).

of the bud site is determined by another component of the coat, the adaptor complex (50, 59). To determine which adaptor population was driving the coat assembly in the permeabilized cells, the intracellular location of AP-1 and AP-2 was determined. AP-1, a heterotetramer composed of γ, β1, μ1, and σ1 subunits, cycles primarily between the TGN and a cytosolic reservoir pool and, at steady state, distributes between these pools (Fig. 2 *a*). The AP-2 adaptor complex composed of α, β2, μ2, and σ2 subunits, on the other hand, seems to be primarily localized to the cytoplasmic face of the plasma membrane (Fig. 2 *d*), although there is usually some AP-2 staining surrounding the nucleus, which, being out of the focal plane, appears as a diffuse halo. In permeabilized cells incubated with cytosol and ATP, only the AP-2 staining (Fig. 2 *e*) resembles that of clathrin and colocalizes with recruited clathrin. In fact, AP-1 does not translocate from cytosol onto intracellular membranes in the presence of an ATP regeneration system (Fig. 2 *b*) and is only efficiently recruited onto the TGN in permeabilized cells incubated with brain cytosol and the poorly hydrolyzable analogue of GTP, GTPγS (Fig. 2 *c*) (55, 67, 72). Under these conditions, minimal recruit-

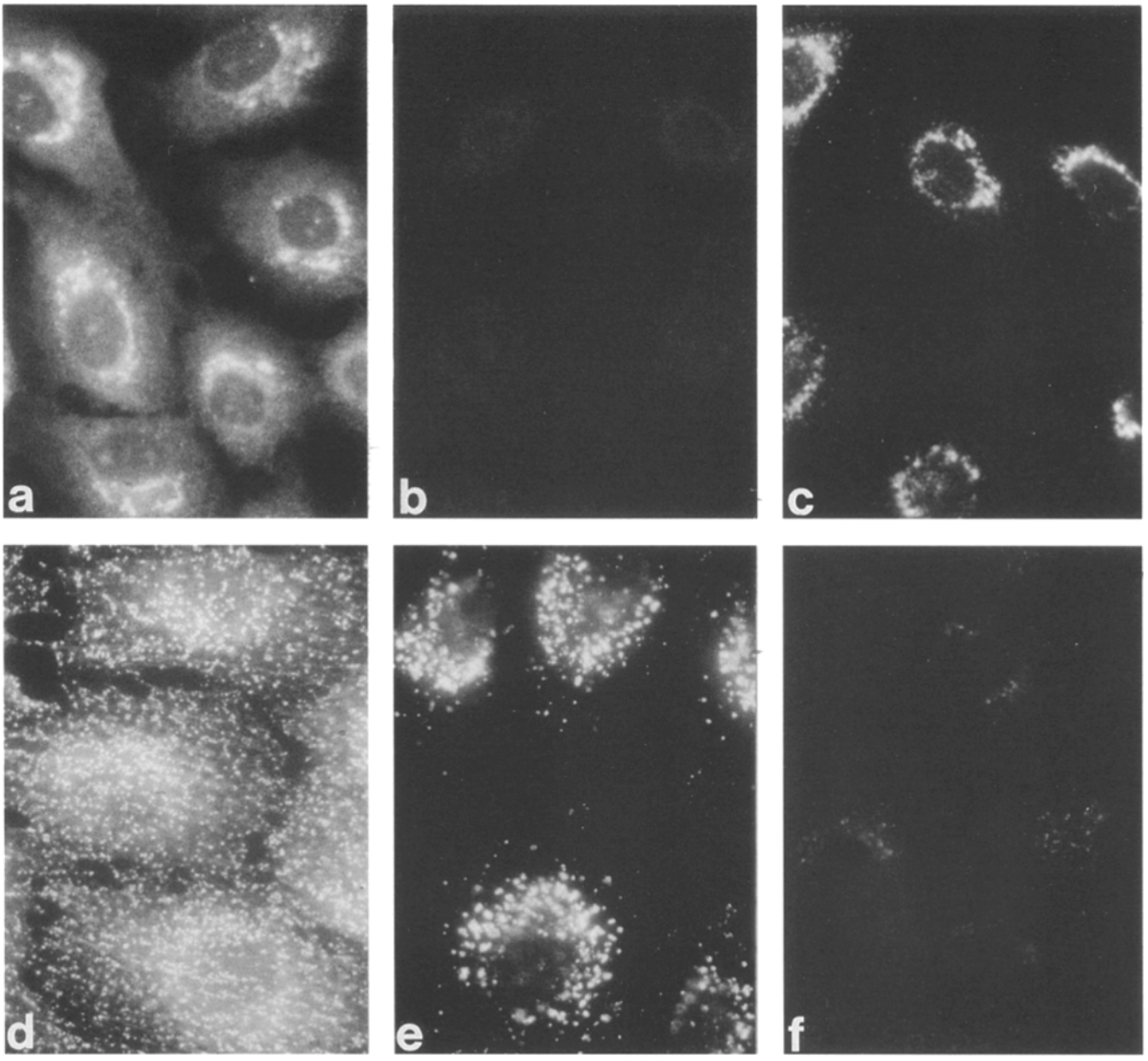

Figure 2. Intracellular localization of AP-1 and AP-2 adaptors in intact and permeabilized cells. Intact (*a* and *d*) or digitonin-permeabilized NRK cells, incubated at 37°C for 20 min with ~2.5 mg/ml gel-filtered rat brain cytosol and either an ATP regeneration system (*b* and *e*) or 100 μM GTPγS (*c* and *f*), were fixed, and then prepared for indirect immunofluorescence using the anti–AP-1 adaptor γ subunit antibody AE/1 (*a–c*) or the anti–AP-2 α subunit mAb AP.6 (*d–f*). Note that the fixation conditions required for detection of AP-1 result in the cross-linking of the cytosolic adaptor pool as well as the membrane-bound pool on the TGN in intact cells (*a*). In the permeabilized cells, the washing before fixation removes the soluble pool, resulting in only the TGN-associated adaptors being visible (*c*). Conditions for photography were identical for *b*, *c*, *e*, and *f*.

ment of AP-2 is seen (Fig. 2 *f*). We conclude that the ATP-dependent clathrin coat assembly observed on the perinuclear structures in permeabilized cells is likely to require AP-2.

One striking feature of both AP-2 and clathrin recruitment in the digitonin-permeabilized NRK cells is the meager number of clathrin-coated structures seen at the cell surface (Figs. 1 *b* and 2 *e*). This is due to the fragmentation of the plasma membrane in digitonin-permeabilized cells (18, 25, 46) (Fig. 3). On ice, digitonin disrupts the integrity of the cholesterol-rich cell membrane, allowing cytosolic components to diffuse from the punctured cells. Under these conditions, the intracellular staining pattern of AP-2 (Fig. 3 *b*) remains very similar to intact, untreated NRK cells (Fig. 3 *a*). After warming the digitonin-treated cells to 37°C, a dramatic alteration in the structure of the plasma membrane occurs. First, large holes can now be seen within each cell and AP-2 is no longer concentrated in the brightly stained structures that correspond to coated pits (Fig. 3 *c*). Rather, the overall intensity of the AP-2 staining decreases, and the distribution of the remaining AP-2 appears more reticular after the incubation at 37°C (compare Fig. 3 *c* with 3 *b*). Because digitonin converts the plasma membrane into an extensive array of tubular membrane projections (18, 25, 46), these most likely represent AP-2–containing structures that remain within cylindrical membrane fragments. In reactions that contain added cytosol as a source of coat components, the translocation of AP-2 (Fig. 3 *d*) and clathrin (not shown) onto perinuclear membranes proceeds efficiently, but the plasma membrane remnants show only limited capacity to initiate new rounds of clathrin coat assembly. Gross disruption of the plasma membrane by digitonin can also be visualized using antibodies directed against a cell surface marker protein (data not shown). Here we have taken advantage of this property of digitonin to examine AP-2–dependent clathrin assembly within the cell in the absence of lattice assembly at the cell surface.

Assembly of Clathrin Coats on lgp120-Positive Membranes

To begin to understand the functional significance of the AP-2 recruitment, it was important to determine the intracellular membrane compartment with which the adaptor associates. Based on morphology, this compartment was unlikely to be either the ER reticulum or Golgi cisternae. Furthermore, although partly overlapping, the AP-2–containing membranes could be discerned morphologically from the AP-1–labeled TGN. Compared with the somewhat scattered punctate structures that recruit AP-2 (Fig. 2 *e*), the TGN displays a more compact reticular morphology (Fig. 2, *a* and *c*), and double-labeling experiments showed that recruited AP-2 and clathrin do not colocalize with the protein TGN38 (42, 64) excluding the TGN (not shown). We therefore investigated the extent of colocalization with late endosomes and lysosomes using an antibody against the lysosomal membrane glycoprotein, lgp120 (38). We found a striking coincidence of AP-2 and lgp120 staining in permeabilized NRK cells incubated with rat brain

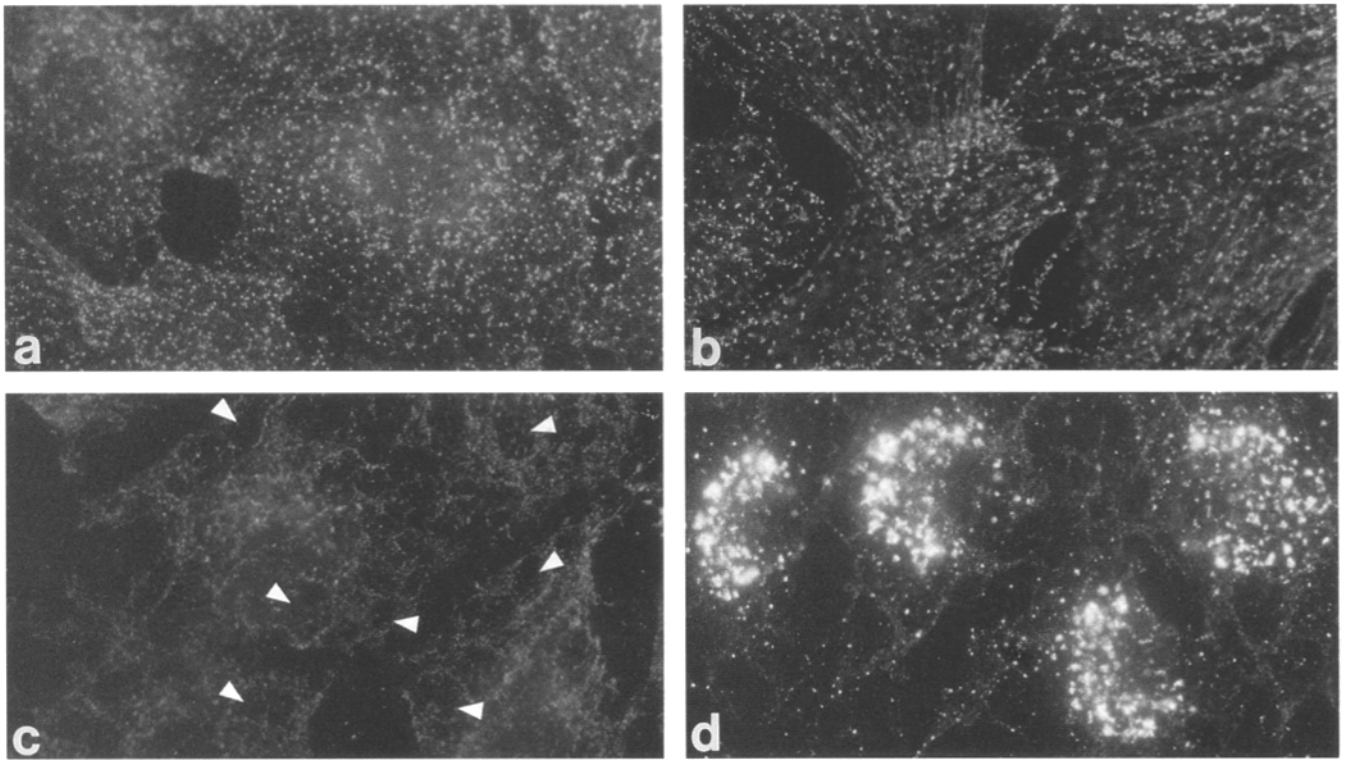

Figure 3. Disruption of the plasma membrane on warming digitonin-permeabilized cells to 37°C. NRK cells were either fixed immediately (*a*) or permeabilized with 25 μg/ml digitonin on ice for 5 min (*b–d*). Individual coverslips containing the permeabilized cells were then incubated for 20 min in either permeabilization buffer supplemented with an ATP regeneration system at 0° (*b*) or 37°C (*c*), or in ~2.5 mg/ml gel-filtered rat brain cytosol and an ATP regeneration system at 37°C (*d*). After washing, the cells were fixed, and then prepared for indirect immunofluorescence using the anti–AP-2 α subunit mAb AP.6 (*a–d*).

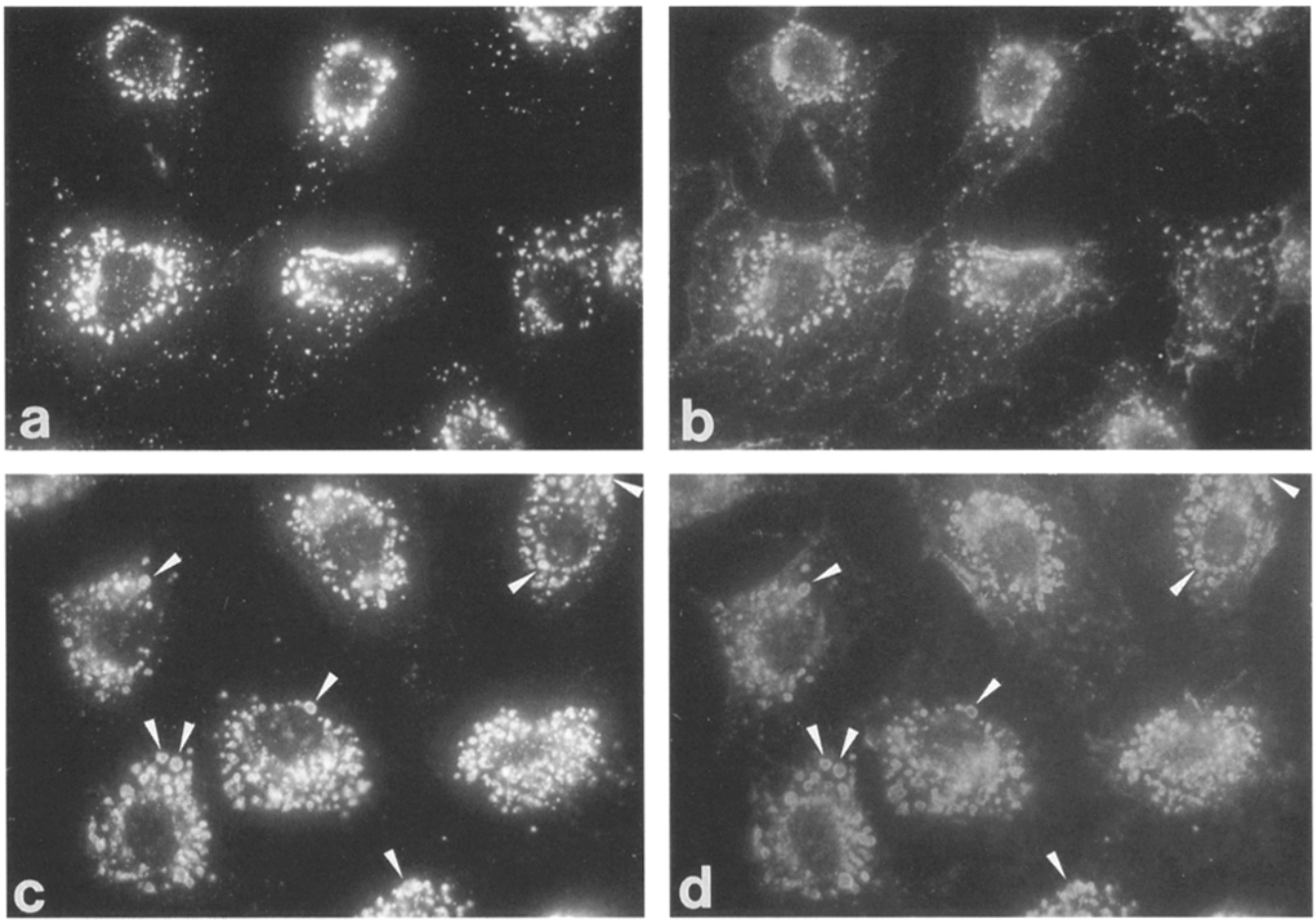

Figure 4. Recruitment of AP-2 onto lgp120-positive structures. NRK cells were cultured for 16 h in the absence (*a* and *b*) or presence (*c* and *d*) of 30 mM sucrose, and then chased for an additional 60 min in culture medium without sucrose. The cells were then permeabilized with digitonin and incubated at 37°C for 20 min with ~2.0 mg/ml rat brain cytosol and an ATP regeneration system before fixation. Fixed cells were double labeled with the lgp120-specific mAb Ly1C6 (*a* and *c*) and affinity-purified antibody C619-656, directed against the α_c subunit of the AP-2 adaptor complex (*b* and *d*). Membrane structures labeled for lgp120 clearly colocalize with the recruited AP-2 staining in both untreated NRK cells and on lysosomes swollen after sucrose treatment (*arrowheads*), although some plasma membrane–associated AP-2 is also evident.

cytosol and ATP (Fig. 4, *a* and *b*), and comparable results were also obtained using rat liver cytosol as a source of soluble AP-2 and clathrin (not shown).

A distinction between the late endosome/PLC and mature lysosomes cannot be made on the basis of lgp120 immunoreactivity alone. In NRK (27, 28) and other cells (15, 24, 34), the PLC contains high levels of both lgp120 and the man-6-P receptors at steady state. Lysosomes are essentially devoid of man-6-P receptors, but the density of lgp120 is reported to be similar to that found in membranes of the PLC (28). Therefore, in an attempt to determine whether AP-2 is recruited onto the PLC, lysosomes, or both in the presence of ATP, we treated NRK cells with sucrose to generate sucrosomes (15, 34). These distended vacuoles arise because mammalian cells do not contain the enzyme invertase within lysosomes, and, in NRK cells, it is known that sucrosomes correspond to mature, lgp120-positive, man-6-P receptor–negative lysosomes (34). The morphology of the PLC appears to be largely unaffected by the sucrose treatment (15, 34). Comparison of immunofluorescence images of control (Fig. 4 *a*) and sucrose-treated (Fig. 4 *c*) cells, after permeabilization and staining with the anti-lgp120 antibody, shows the increased volume of the sucrosome lumen. Cytosolic AP-2 is clearly recruited onto the same distended, limiting membrane of the sucrosome in the presence of ATP (Fig. 4 *d*), thus identifying the perinuclear AP-2 and clathrin-binding site as the mature lysosome.

Direct Recruitment of AP-2 and Clathrin onto Purified Dense Lysosomes

Next, we prepared dense lysosomes from rat liver (48) to assay the recruitment of soluble AP-2 and clathrin onto these organelles directly (66). The polypeptide composition of the purified lysosome fraction is distinct from that of either a rat liver homogenate or a Golgi membrane preparation (Fig. 5 *A*), and immunoblots show that two lysosomal hydrolases, β-glucuronidase and cathepsin D, are greatly enriched in the lysosomes compared with the start-

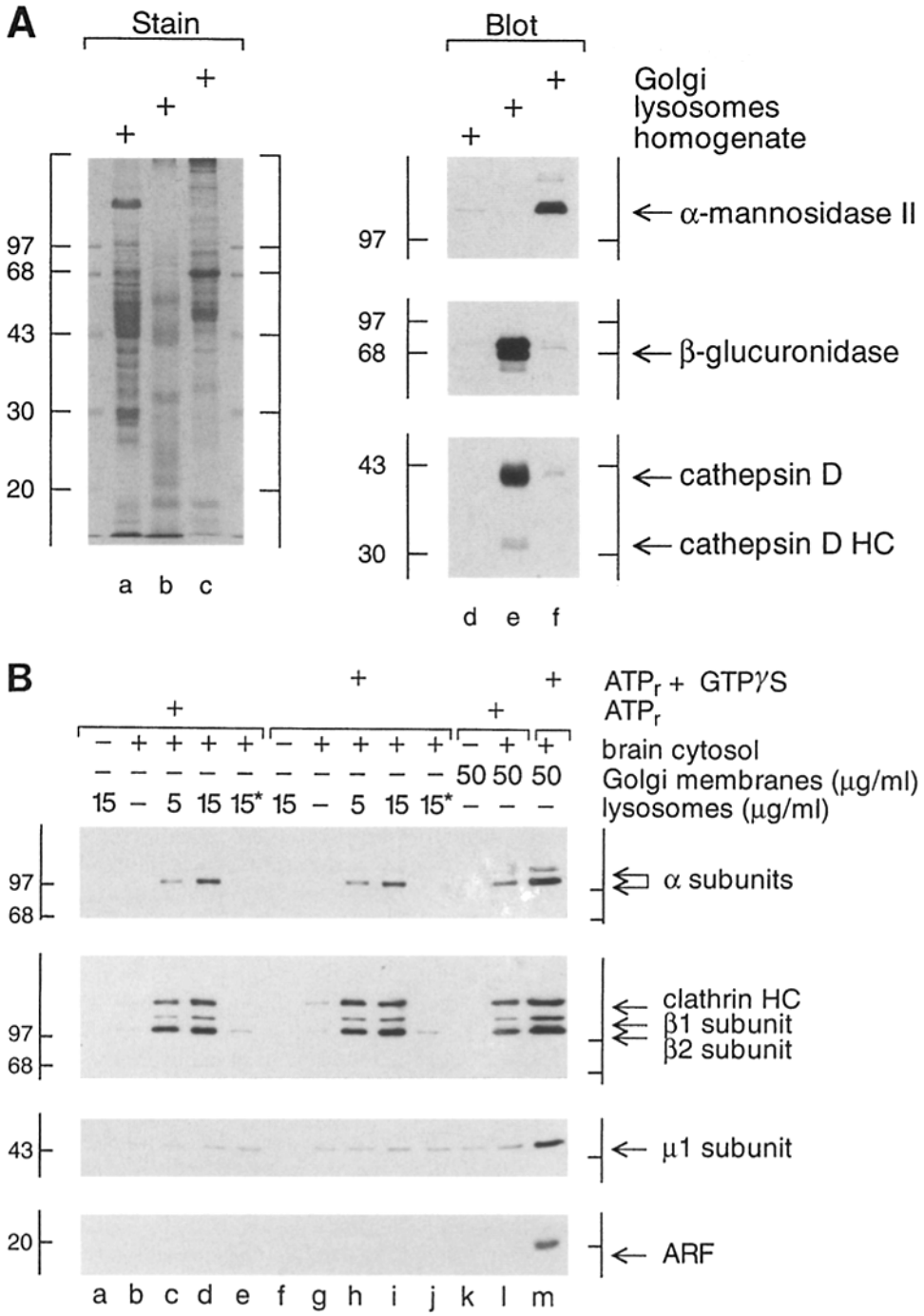

Figure 5. Clathrin coat assembly on purified dense lysosomes. (*A*) Aliquots of 25 μg protein of a rat liver homogenate (*a* and *d*), Percoll-purified rat liver dense lysosomes (*b* and *e*), and rat liver Golgi-enriched membranes (*c* and *f*) were separated on polyacrylamide gels and either stained with Coomassie blue (*Stain*) or transferred to nitrocellulose, and then probed with anti–α-mannosidase II, anti–β-glucuronidase, or anti–cathepsin D antibodies (*Blot*). The positions of the markers (in kD) are indicated at left and only the relevant portion of each blot is shown. (*B*) Tubes containing either 5 or 15 μg/ml rat liver lysosomes or 50 μg/ml rat liver Golgi and ~2.5 mg/ml gel-filtered rat brain cytosol were prepared on ice as indicated in the figure. An ATP regeneration system (*ATP*$_r$; *a–e, k,* and *l*) or both the ATP$_r$ and 100 μM GTPγS (*ATP*$_r$ + *GTPγS*; *f–j* and *m*) were added, and then the tubes were incubated at 37°C for 20 min or retained on ice (*asterisk*; *e* and *j*). The membrane pellets were then recovered and analyzed on replicate immunoblots with either the anti–AP-2 α subunit mAb 100/2, a mixture of the anti–clathrin HC mAb TD.1 and the anti–β subunit mAb 100/1, the anti–AP-1 μ1 subunit RY/1, or the anti-ARF mAb 1D9. Only the relevant portion of each blot is shown and the positions of the relevant markers (in kD) are indicated at left. Notice that in rat brain cytosol, AP-2 complexes contain both the faster migrating β2 subunit as well as lower amounts of the AP-1–specific β1 subunit (lanes *c, d, h, i,* and *l*). The promiscuity of β subunit incorporation into AP-1 and AP-2 complexes has also been noticed by others (49, 62).

ing homogenate, while the Golgi marker α-mannosidase II is absent from lysosomes but substantially enriched in the Golgi membrane preparation.

When the purified lysosomes are mixed together with cytosol in the presence of ATP, both AP-2 and clathrin associate with the lysosomes in a dose-dependent manner (Fig. 5 *B*, lanes *c* and *d* compared with *a*). Recruitment of the coat components occurs at 37°C (lanes *c* and *d*) but not on ice (lane *e*). Since the control lysosomes do not contain detectable amounts of either AP-2 or clathrin (lane *a*), the membrane-associated coat must be recruited from cytosolic components. For comparison, an equivalent level of AP-2 adaptor recruitment is only obtained with roughly 10-fold more of the Golgi-enriched membranes (lane *l*), consistent with contaminating lysosomes being an acceptor compartment for the ATP-dependent recruitment we have observed previously in this fraction (66). The Golgi-associated AP-1 adaptor complex does not bind to either membrane preparation under these conditions, as judged by the presence of the AP-1–specific μ1 subunit. Interestingly, no increase in clathrin coat recruitment onto lysosomes is seen when GTPγS is added to the ATP regeneration system (lanes *f–j*), although adding GTPγS to a reaction containing the Golgi-enriched membranes results

in both AP-1 recruitment and a three- to fivefold stimulation in membrane-associated AP-2 (lane *m*). In other experiments, we have established that both the AP-1 binding and the synergistic recruitment of AP-2 seen in the Golgi-enriched fraction are due to the action of ADP-ribosylation factor (ARF), and both are potently inhibited by brefeldin A (BFA) (data not shown). However, this ARF-dependent recruitment of additional AP-2 to the Golgi-enriched membrane preparation is unrelated to the ATP-dependent clathrin coat assembly observed on lysosomes, which is BFA insensitive.

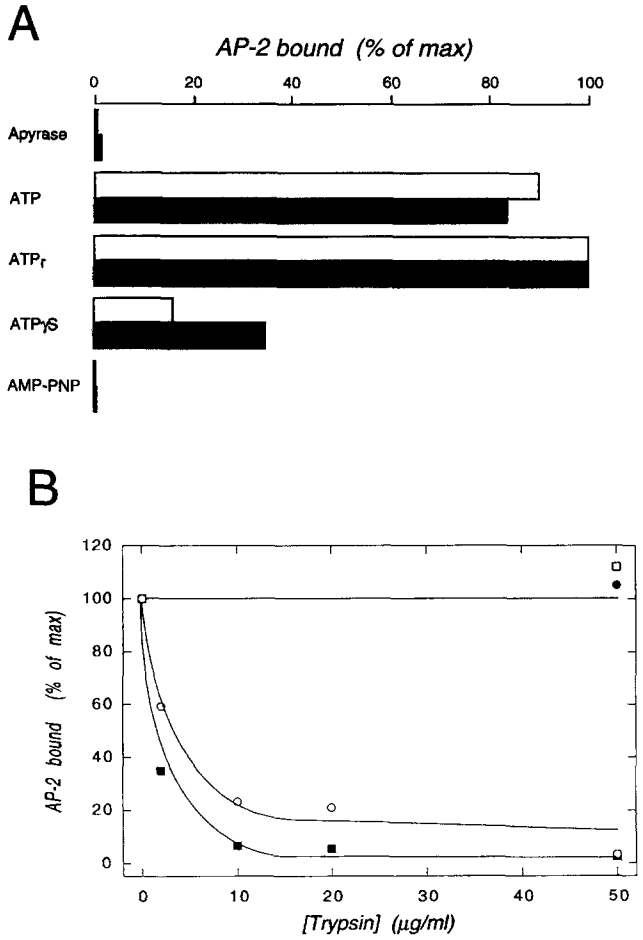

Figure 6. Requirements for coat–protein binding to isolated lysosomes. (*A*) Tubes containing 30 μg/ml rat liver lysosomes and ~2.5 mg/ml gel-filtered rat brain cytosol were supplemented with either 10 U/ml apyrase, 1 mM ATP, an ATP regeneration system (*ATP$_r$*), 250 μM ATPγS, or 250 μM AMP-PNP, and then incubated at 37°C for 20 min. The membrane pellets were recovered and analyzed on replicate immunoblots with either the anti–α subunit mAb 100/2, or the anti–β subunit mAb 100/1. The amount of AP-2, measured as either the α subunit (*open bars*) or β subunit (*solid bars*), was determined by densitometry, and the data are presented as a percentage of maximal binding in the presence of the ATP$_r$. (*B*) Purified lysosomes were incubated at 25°C in the presence of 0–50 μg/ml trypsin or 200 μg/ml soybean trypsin inhibitor together with 50 μg/ml trypsin for 15 min. After adding excess trypsin inhibitor, the pretreated lysosomes were then added to binding reactions containing ~2.5 mg/ml gel-filtered rat brain cytosol and an ATP regeneration system to give a final membrane concentration equivalent to 30 μg/ml undigested membranes. After incubation at 37°C for 20 min, the membrane pellets were recovered and analyzed on replicate immunoblots with either the anti–AP-2 α subunit mAb 100/2, or the anti–β subunit mAb 100/1. The amount of AP-2, measured as either the α subunit (*solid squares*) or β subunit (*open circles*), was determined by densitometry, and the data are presented as a percentage of maximal binding in the absence of trypsin. The amount of AP-2 recruited onto mock-digested lysosomes is indicated by the open square for the α subunit, and the solid circle for the β subunit. The difference in the quantition of AP-2 when using either the anti–α subunit or anti–β subunit antibodies probably reflects differences in affinity.

In reactions containing cytosol, recruitment of AP-2 and clathrin onto dense lysosomes requires ATP or an ATP regeneration system (Fig. 6 *A*). Consuming ATP with apyrase or adding poorly hydrolyzable analogues of ATP, ATPγS, or AMP-PNP diminishes the coat assembly on lysosomes substantially. Together with the temperature dependence, this argues that ATP hydrolysis is required for the assembly process. Coat recruitment also depends on cytosol-oriented lysosomal protein cofactor(s). Prior proteolysis of isolated lysosomes with trypsin decreases the recruitment of both AP-2 (Fig. 6 *B*) and clathrin (not shown) in a dose-dependent manner. If trypsin inhibitor is added before the trypsin, adaptor and clathrin binding is unaffected. The purified lysosomes remain intact under these digestion conditions (13), there are no gross changes in the overall protein profiles after proteolysis, and there is no detectable alteration in the electrophoretic mobility of lgp120 (data not shown).

Visualization of Lysosomal Clathrin Coats

To establish whether characteristic polygonal clathrin lattices assemble on lysosomes, sections prepared from a recruitment assay containing cytosol and ATP were examined by transmission EM (Fig. 7). In conventional thin-sections, the purified vacuoles, a mixture of multivesicular lysosomes and dense-core lysosomes with relatively little contamination by other intracellular organelles, clearly show identifiable bristle-like clathrin lattices assembled on both types of lysosomes. Buds at several different stages of curvature are visible, and numerous discrete patches of assembled polymeric lattice are also evident, presumably grazing views of budding structures out of the plane of the section. What appear to be portions of several small, free, coated vesicles are also visible. Again, the coated regions do not occur in the absence of cytosol or after incubation on ice (not shown). Double labeling of thawed cryosections with antibodies against the α subunit of AP-2 and lgp120 (Fig. 7, *f–i*) confirms that the AP-2 adaptor complex accompanies the recruitment of clathrin onto the surface of the dense, lgp120-positive lysosomes.

In freeze-etch EM images of the purified lysosome preparation, isolated lysosomes appear as roughly spherical organelles, the limiting membranes exhibiting a roughly textured surface (Fig. 8, *upper left*). Vacuolar-type proton pumps, the peripheral sector of which is easily visible as a peg like protrusion (32), are found scattered over the lysosomal surface. After incubation with brain cytosol and ATP, several discrete regions of assembled clathrin lattice

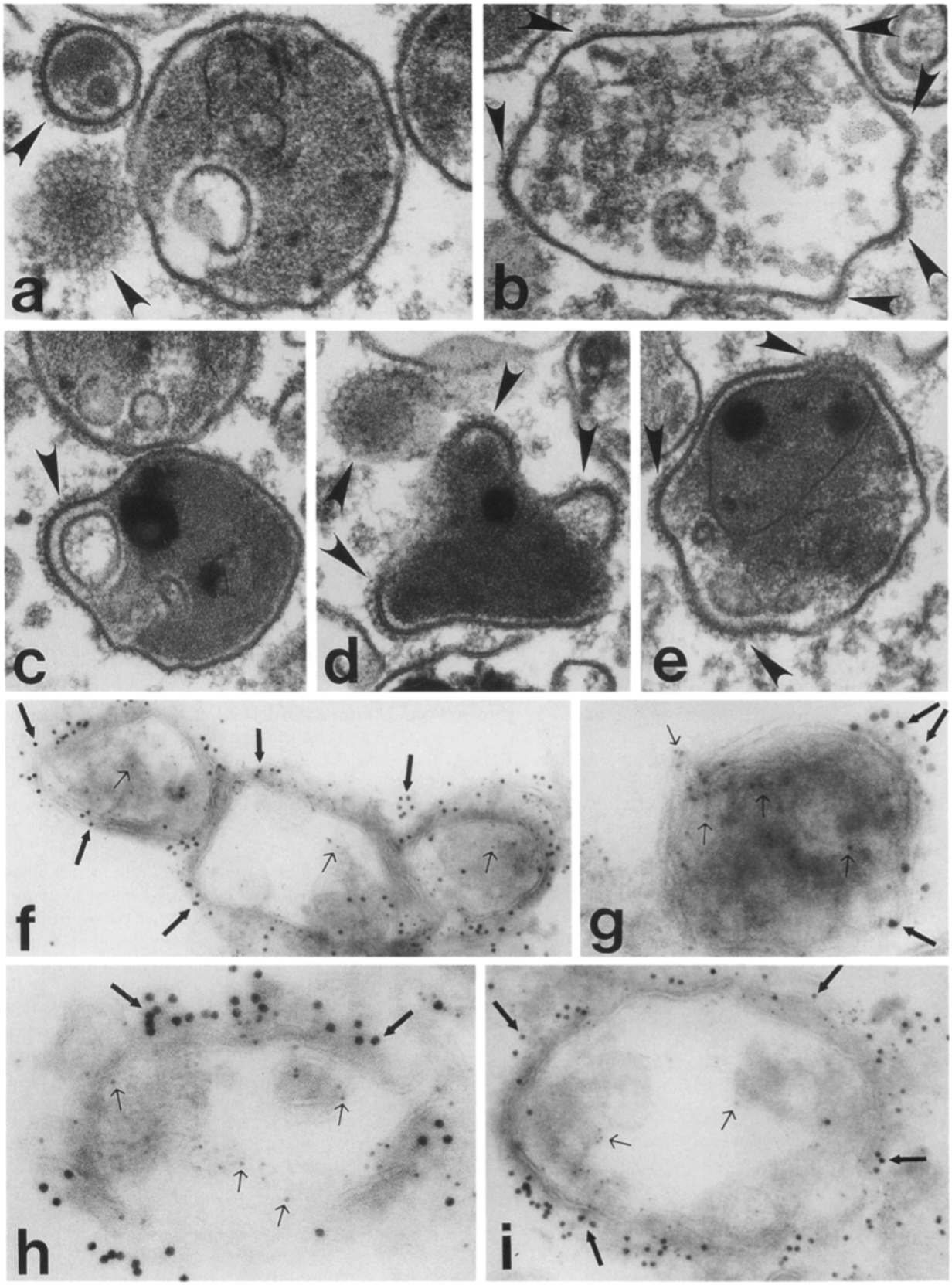

Figure 7. EM analysis of coat formation on lysosomes. After incubation with brain cytosol and ATP, lysosome pellets were prepared for EM. In conventional thin-sections, the purified lysosome preparation consists of a mixture of dense-core vesicles and multivesicular bodies. Membrane-associated clathrin coats (*arrows*) covering portions of the lysosome outer membrane and on emerging buds, as well as areas of assembled polyhedral clathrin lattice, are evident. Portions of several typical fields are shown in *a–e* to illustrate both the type of lysosomes that accumulate coats and differing extents of lattice curvature. (*f–i*) In immunolabeled cryosections of the purified lysosomes incubated together with brain cytosol and an ATP regeneration system, the α subunit of AP-2 (affinity-purified antibody C619-656, 12 nm gold; *bold arrows*) is localized along the outer membrane of the lysosome, in several instances clustered at what appear to be budlike structures. lgp120 (mAb Ly1C6, 6 nm gold; *fine arrows*) labeling is found near the peripheral membrane as well as in internal membrane elements.

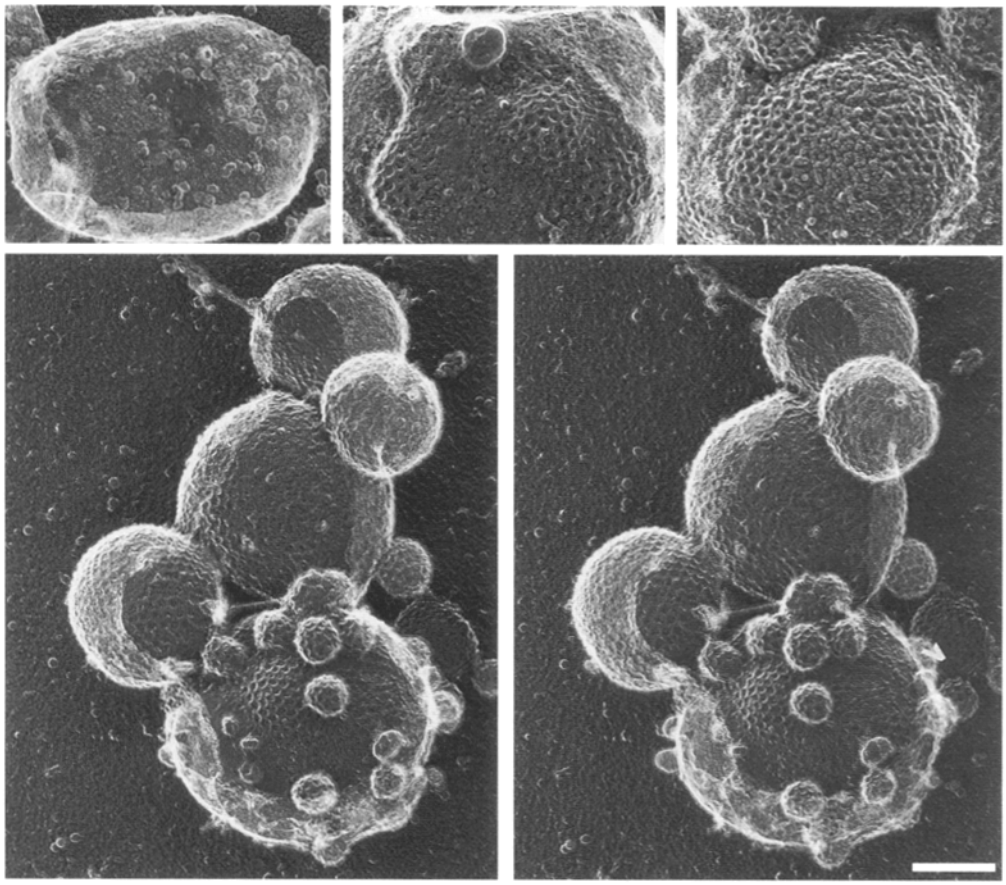

Figure 8. Rapid freeze–deep etch visualization of assembled clathrin coats. Purified lysosomes reveal a membranal substructure, with the peripheral sector of the vacuolar-type proton ATPase visible as peglike structures on the membrane (*upper left*). After incubation with cytosol and ATP at 37°C, discrete patches of assembled polyhedral clathrin lattice are now evident (*upper center* and *right*, and *lower panels*). In the lower panels, a stereo pair of a cluster of lysosomes showing differing degrees of lattice curvature and bud formation is shown. The images in this figure were kindly prepared by John Heuser. Bars: (*upper panels*) 150 nm; (*lower panels*) 200 nm.

are now seen on the lysosome exterior. The number and size of the assembling coats vary. In some instances, highly curved, ~100-nm buds are evident, giving the impression that the buds are nearing the fission step (Fig. 8, *lower panels*). More commonly, the latticework displayed a less pronounced degree of curvature, but each coat is clearly composed of normal polyhedral units (Fig. 8, *upper center* and *right*). On some lysosomes, quite expansive regions of assembled flat lattice are also seen. These experiments confirm that the clathrin coat proteins recruited onto lysosomes assemble into normal structures and initiate budding and, taken together with the biochemical data, provide compelling evidence of the assembly of AP-2–containing clathrin coats on lysosomes.

Relocation of Clathrin Coat Components onto Lysosomes in Intact Cells

A dramatic change in the subcellular distribution of both AP-2 and clathrin occurs when intact cells are treated with chlorpromazine, imipramine, or other cationic amphiphilic drugs (CADs) (68). Within 30 min of adding the drug, the normal steady-state pattern of fine punctate staining principally over the cell surface is replaced with AP-2 and clathrin labeling of larger organelles, scattered throughout the cytoplasm (68). The appearance of this compartment onto which AP-2 and clathrin redistribute is reminiscent of lysosomes, and EM identification of the coated structures as mature multivesicular bodies (68) is consistent with an assignment as lysosomes. To ascertain whether these structures are in fact lysosomes, sucrose-loaded NRK cells were treated with chlorpromazine, and then the intracellular location of AP-2 and clathrin was determined. Although visualization of AP-2 is more difficult after chlorpromazine treatment because the cells tend to round up and begin to detach slowly from the coverslip, the periphery of many of the sucrose-swollen lysosomes are labeled by antibodies directed against the α subunit of AP-2 (not shown). The drug also causes a significant redistribution of intracellular AP-2 into the cytosol, with a consequent increase in immunofluorescence. A clearer picture of the effect of the drug on clathrin and AP-2 is therefore obtained upon confocal analysis. From serial optical sections through the cell, it is obvious that many of the lgp120-positive swollen lysosomes contain both AP-2 and clathrin on the limiting membrane. A single optical section is shown to illustrate this in Fig. 9 (*a* and *b*). Several lgp120-positive structures can also be seen that do not appear to contain AP-2 or clathrin. Imipramine induces a similar relocation of clathrin and AP-2 in intact NRK cells, but it is better tolerated by the cells. A striking expansion of the lysosome compartment accompanies the accumulation of AP-2 (compare Fig. 9 *e* and 9 *f* with the control panels *c* and *d*) and clathrin (not shown) on these structures in the presence of the drug. Dilation of the lysosomal lumen could suggest that the clathrin coat assembly in the presence of CADs might be nonproductive. Both of these drugs appear to shift the equilibrium of AP-2 and clathrin binding from the plasma membrane onto lysosomal elements, and, although the biochemical basis for this perturbation is not yet clear, these results clearly show extensive assembly of clathrin coats specifically on lysosomes within intact cells.

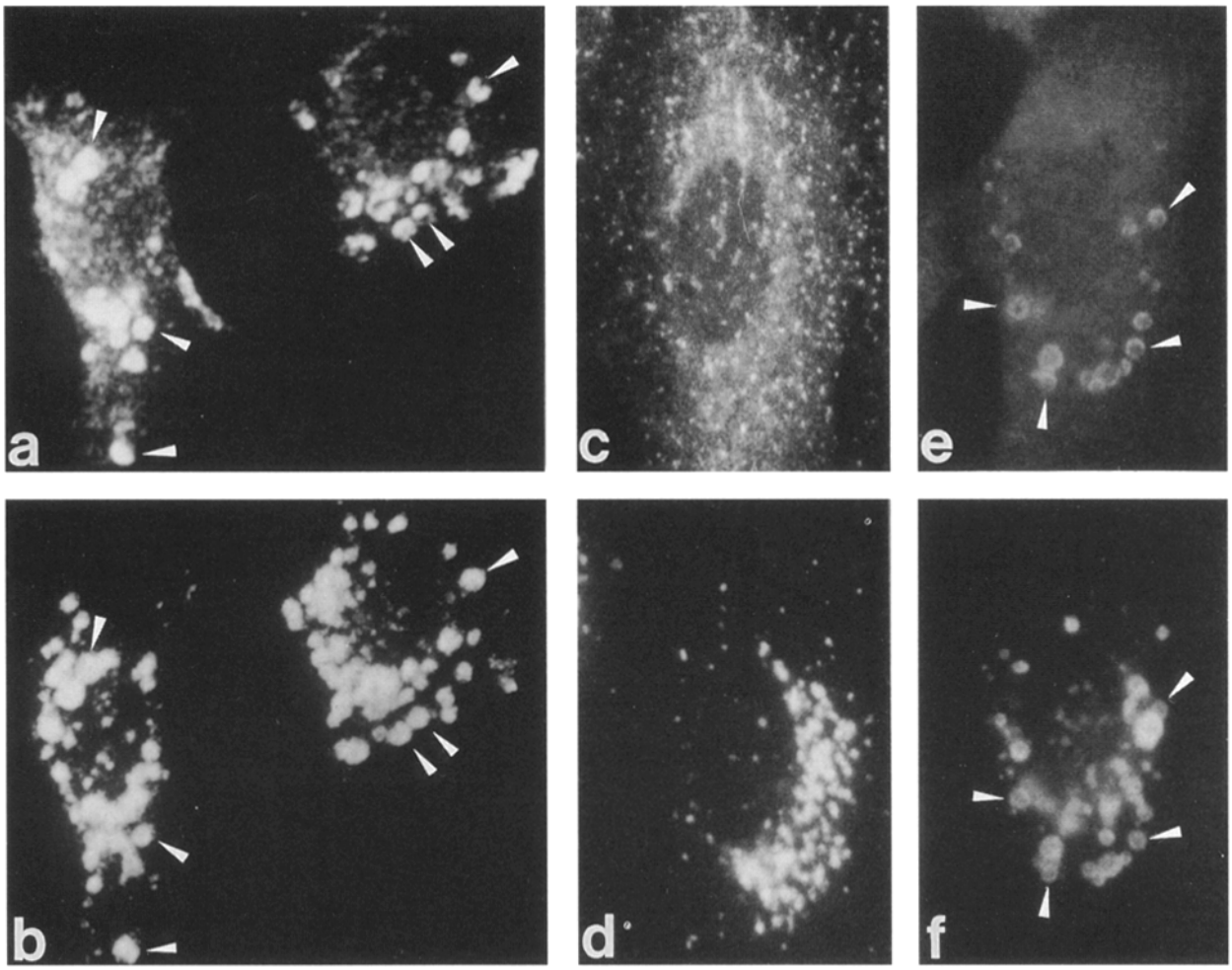

Figure 9. Redistribution of AP-2 onto lysosomes in the presence of chlorpromazine or imipramine. In *a* and *b*, sucrose-loaded NRK cells were incubated with 40 μM chlorpromazine at 37°C for 30 min before fixation and subsequent confocal microscopic analysis using affinity-purified anti–AP-2 α_c subunit antibodies C619-656 (*a*) and the anti-lgp120 mAb Ly1C6 (*b*). In *c–f*, NRK cells were incubated either with (*e* and *f*) or without (*c* and *d*) 50 μM imipramine at 37°C for 60 min before fixation and subsequent analysis by conventional indirect immunofluorescence using an anti–AP-2 α subunit mAb AP.6 (*c* and *e*) and affinity-purified anti–rat lgp120 antibodies (*d* and *f*). Notice that the imipramine-induced swelling of lgp120-positive lysosomes is accompanied by AP-2 (and clathrin) relocation onto these expanded structures.

Discussion

Several independent studies have provided convincing evidence that lysosomal membrane glycoproteins continually cycle from lysosomes to endosomal compartments and the cell surface (3, 4, 21, 22, 39, 40, 52). However, the mechanistic basis for this protein movement remains to be determined. Here we demonstrate that AP-2–containing clathrin coats and vesicles form on lysosomes, representing a plausible vesicular intermediate for protein trafficking out of this compartment. This process is quite specific. When cytosol and ATP are added to permeabilized cells, AP-2 and clathrin are recruited onto lysosomes, but no binding of AP-1 to this organelle is observed. The same results are obtained when purified lysosomes are incubated with cytosol and ATP, ruling out that coat assembly is a consequence of the digitonin treatment we have used to permeabilize the cells. Recruitment is temperature dependent and requires a source of hydrolyzable ATP. Interestingly, this coat assembly does not depend on GTP or the small GTP-binding protein ARF1, both of which are essential for the recruitment of AP-1 and clathrin onto Golgi membranes (37, 55, 63, 66, 72). In this respect, AP-2–dependent coat formation on lysosomes resembles the recruitment of this adaptor complex onto the plasma membrane, where it is known that AP-2 binding is not blocked by BFA (55, 72), an inhibitor of ARF1 recruitment onto membranes (17, 31).

If lysosome-derived clathrin-coated vesicles are authentic vesicular intermediates, why have they not been detected previously? The answer, we believe, relates to the steady-state distribution of AP-2 and clathrin in intact cells. The majority of AP-2 is found in clathrin-coated structures at or near the plasma membrane. In immunofluorescence images, these appear in a random punctate distribution close to the cell surface. The diffuse fluorescent haze, out of the focal plane, is assumed to represent clathrin coats forming on the region of the plasma membrane enveloping the bulging nucleus. However, this must also

contain lysosomal-coated vesicles, deeper within the cytosol. Another factor to consider is our unexpected finding that the clathrin coats on lysosomes use the AP-2 adaptor complex, as do those at the cell surface. This makes it unlikely that lysosome-derived AP-2–containing coated vesicles could be distinguished by immunofluorescence from those budding from the plasma membrane near the perinuclear region of intact cells, and they would have been assumed to be part of the plasma membrane–derived clathrin-coated vesicle population. Similar reasoning might explain why the clathrin-coated vesicles that bud from endosomes have eluded detection until only recently (65). In fact, when other techniques have been used, clathrin coats have occasionally been observed on lysosomes. Clathrin-like plaques in conventional thin-sections of multivesicular bodies and lysosomes were noticed many years ago (19, 33, 45) and were confirmed to contain clathrin by immunocytochemistry (41). Therefore, we believe our in vitro assays have revealed a physiologic process that appears to be difficult to detect under most circumstances. The estimated rate constant for shuttling of lgp120 out of lysosomes is ~100-fold lower than that for internalization from the surface (40). Only perturbations of the normal steady state allow easy visualization of clathrin coats on lysosomes. Apparently in the permeabilized cell assays used here, the assembly of clathrin coats on lysosomes proceeds in an efficient manner, but the final fission reaction seems to be rate limiting, resulting in the accumulation of clathrin-coated structures that are readily detected. These predominate because we have used digitonin to permeabilize the cells, which fragments the plasma membrane and diminishes clathrin-coated pit assembly at the surface.

We have considered other possible interpretations for our data. It is widely accepted that AP-2 is exclusively associated with cell surface–derived clathrin-coated vesicles, while AP-1 drives the assembly of clathrin coats on the TGN (50, 59). In the few instances where AP-2 has been found on intracellular organelles other than the plasma membrane, the authors concluded that this represented a mistargeting phenomenon, provoked by the experimental conditions (60, 68). These groups propose that the membrane association of AP-2 depends on a membrane-bound docking protein that recycles through the endosomal system, returning to the plasma membrane where it again initiates the assembly of coated pits. Under normal circumstances when recycling, the AP-2–specific docking protein would be inactivated and unable to associate with soluble AP-2. Certain manipulations—CAD treatment of intact cells or, when using permeabilized cells, elevated concentrations of free calcium or GTPγS in the donor cytosol—were suggested to alter the on/off status of this docking protein. Thus, these investigators concluded that the binding of AP-2 and clathrin, to a compartment they both identify as multivesicular late endosomes, was an abnormal event (60, 68).

Several facts lead us to believe it unlikely that we are observing this same so-called mistargeting phenomenon. First, in our studies we have been unable to detect any effect of calcium on AP-2 recruitment onto lysosomes. Addition of calcium chelators to the binding assays was unable to abolish AP-2 binding, and treating NRK cells with the calcium ionophore A23187 has no effect on the intracellular distribution of AP-2 within the cell (data not shown). The best argument for a strict ATP dependence and against a specific requirement for calcium is the experiment presented in Fig. 2. Under identical assay conditions, AP-1 and AP-2 recruitment can be selectively induced by the addition of either GTPγS or ATP, respectively. The clathrin coat assembly we see on lysosomes also differs from mistargeting in that it is BFA insensitive and occurs on purified lysosomes without recruitment of ARF, even in the presence of GTPγS. In fact, using our biochemical recruitment assays with Golgi-enriched membrane preparations, we appear to be able to follow both types of AP-2 recruitment, the ATP-dependent type and the GTPγS-dependent type (Fig. 5).

The apparent similarity in the effects of calcium, chlorpromazine, and other calmodulin antagonists on AP-2 and clathrin distribution prompted Seaman et al. (60) and Wang et al. (64) to conclude that they were each following an analogous phenomenon, initiated by different pharmacological manipulations. What we observed in chlorpromazine- or imipramine-treated cells was that the membrane elements binding AP-2 are strongly lgp120 positive. This is in contrast with the findings of Seaman et al., showing that the intracellular membrane compartment onto which clathrin coats assemble in the presence of GTPγS is not particularly enriched in lgp120 (60). We therefore favor an alternate interpretation that, in intact cells, CADs stabilize clathrin coats formed on lysosomes. We suspect that chlorpromazine and imipramine also exert an effect on nucleation of clathrin-coated pits at the cell surface, rather than inappropriately activating the putative AP-2 docking protein on endosomes.

Another possible interpretation of our data is that the clathrin coat assembly we see on lysosomes is due to the direct association of AP-2 with the cytosolic domains of recycling receptors that have been transported to the lysosome for catabolism. This interpretation is unlikely for a number of reasons. First, the steady-state level of these receptors in lysosomes is very low and, in fact, not all receptors are metabolized in lysosomes. The lysosomal-protease inhibitors leupeptin and pepstatin A do not alter the half-lives of several receptors when added to cells in culture. In the case of the man-6-P/insulin-like growth factor II receptor, proteolysis at the plasma membrane appears to be the major site of turnover (12). Other receptors, like the EGF receptor, which do enter the lysosome (8), appear to be largely sequestered within intraluminal vesicular structures (23, 53), which would seem to preclude them from interacting with the AP-2 complex.

Regardless of the actual concentration of plasma membrane proteins containing functional internalization signals in the limiting membrane of lysosomes, their effect would be minor given the density of the lamps on the membrane. The lamps constitute between 10 and 50% of the membrane protein in lysosomes (2, 43). All members of the lamp family display short cytosolic domains with either a tetrapeptide YXX∅ (∅ representing a bulky hydrophobic residue) sequence or dileucine motif that direct the proteins into clathrin-coated vesicles at both the TGN and at the cell surface (20, 30, 57). Consequently, these sorting motifs would also be expected to interact with AP-2 adaptors recruited onto lysosomal membranes. The ability of

the lamps to be incorporated into clathrin-coated vesicles that assemble at multiple locations would effectively ensure a perpetual cycling through the exocytic/endocytic system, entirely consistent with the kinetic and morphologic data. Because rather extensive coat formation is seen on lysosomes, we cannot exclude the possibility that our experimental procedures have unmasked a population of proteins normally prevented from initiating clathrin coat assembly. If this is the case, then our experiments have revealed a novel, ATP-dependent regulatory step, an inhibitory control point that is distinct from that seen on endosomes, which can be overridden by GTPγS.

In view of the findings that AP-2 can assemble clathrin coats on lysosomes and on some endosomes as well (60; our unpublished data), we believe that the definition of AP-2 as the adaptor involved exclusively in the formation of plasma membrane clathrin-coated vesicles needs to be modified. In being able to bind to different membrane locations, AP-2 resembles the COPI coat that, in addition to playing an essential role in vesicular traffic between the Golgi and ER (6, 58), also binds to endosomes (70). Subtle differences in the subunit composition of the COPI promoter that associates with endosomes were observed (70), raising the possibility that different forms of AP-2 might bind to the plasma membrane and lysosomes. A number of different α subunit isoforms do exist (7), but, using several different antibodies against the α subunits, we have not found evidence to suggest selective recruitment of a particular isoform of AP-2 onto lysosomes.

Finally, our findings also raise the following issue: what really is a terminal compartment? The classical definition of de Duve (14) referred principally to the site where endocytosed material would be transported for digestion and reutilization as constituents. We now understand that trafficking to the lysosome is a complex process, regulated by an increasing number of intricate molecular interactions. Without any possibility of recycling, incoming proteins that play either a structural or regulatory role in lysosome fusion must be degraded, performing their cellular function only once. At other stations of both the biosynthetic and endocytic compartments, recycling of protein components is a common theme. A retrograde trafficking route out of the lysosomes could serve a variety of functions, including the recycling of regulatory elements (e.g., v-SNARES) to sustain traffic, the dynamic redistribution of membrane lipids, the delivery of lysosomal constituents, including hydrolases, lamps, and proton pumps, to upstream endocytic compartments, and, in certain cells, transport of antigenic peptides to a suitable loading compartment (29). The selective nature of coat-mediated vesicular traffic would ensure that the bulk of the luminal content remains within the terminal lysosomal compartment.

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