Cathepsin D Precursors in Clathrin-coated Organelles from Human Fibroblasts

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ABSTRACT Coated vesicles were isolated from metabolically labeled human fibroblasts with the aid of affinity-purified antibodies against human brain clathrin and *Staphylococcus aureus* cells. The material adsorbed to the *S. aureus* cells was enriched in clathrin. When the *S. aureus* cells bearing the immunoadsorbed material were treated with 0.5% saponin, extracts containing the precursor form of cathepsin D were obtained. The extraction of the precursor was promoted in the presence of mannose 6-phosphate. Material adsorbed to *S. aureus* cells coated with control immunoglobulins was nearly free of clathrin and contained a small amount of the cathepsin D precursor (<20% of that adsorbed with anti-clathrin antibodies). The extraction of this cathepsin D precursor was independent of mannose 6-phosphate and was complete after a brief exposure to saponin. The amount of cathepsin D precursor in coated membranes varied between 0.4 and 2.5% of total precursor. Analysis of pulse chase-labeled fibroblasts revealed that cathepsin D was only transiently associated with coated membranes. The mean residence time of cathepsin D precursor in coated membranes was estimated to be 2 min. These observations support the view that coated membranes participate in the transfer of precursor forms of endogenous lysosomal enzymes to lysosomes.

Lysosomal enzymes are synthesized as precursors of larger molecular weight in the rough endoplasmic reticulum. The precursors are transported to lysosomes via the Golgi complex (reviewed in references 1 and 2). In the Golgi complex the lysosomal enzyme precursors become phosphorylated as a prerequisite of their segregation from the secretory products. The segregation depends on binding of the precursors to receptors specific for mannose 6-phosphate residues. It is a matter of debate whether the segregation and packaging of lysosomal precursors takes place in the *cis* or *trans* part of the Golgi complex (3, 4) and little is known about the subsequent pathway either. Friend and Farquhar (5) observed acid phosphatase-positive coated vesicles in the vicinity of the Golgi complex and suggested them to be the vehicle for lysosomal enzymes leaving the Golgi complex. This idea obtained support from recent observations on the presence of lysosomal enzyme precursors (6–8) and of the mannose 6-phosphate-specific receptors (3, 4, 9) in coated membranes prepared from various tissues.

The coated areas of membranes are implicated in membrane recycling (10). The major constituent of the coat is clathrin, which forms a three-dimensional meshwork at the cytosolic aspect of membranes. We have applied an immunological approach similar to that of Merisko et al. (11) to the isolation of clathrin-coated membranes. We report that in cultured human fibroblasts (*a*) radioactively labeled cathepsin D is transiently associated with clathrin-coated organelles; (*b*) the association is selective for the precursor of cathepsin D; and (*c*) the dissociation of the precursor from the organelles is stimulated by mannose 6-phosphate.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (specific activity, 1.2 Ci/μmol) and ^{14}C-methylated standards were from New England Nuclear (Dreieich, Federal Republic of Germany); and Immuno Precipitin (formaldehyde-fixed protein A-bearing *S. aureus* cells) was from Bethesda Research Laboratories (Neu Isenburg, Federal Republic of Germany). Affinity-purified rabbit antibodies against human placental cathepsin D have been described previously (12).

Purification of Human Clathrin

Human brain tissue stored not longer than 3 mo at −20°C was used as the starting material and the whole purification was performed at 4°C. The grey matter, 300 g, was homogenized in 300 ml of 0.1 M Tris-HCl, 1 mM EGTA, 0.5 mM $MgCl_2$, 3 mM NaN_3, pH 6.8, using ultraturrax (Janke & Kunkel, Staufen, Federal Republic of Germany). The homogenate was centrifuged 30

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min at 20,000 *g* and the pellet was re-extracted twice with 225 ml of buffer. Coated vesicles were collected by centrifugation of the combined extracts for 1 h at 85,000 *g*. They were resuspended and stirred in 100 ml of 0.5 M Tris-HCl, 1 mM EDTA, 1 mM mercaptoethanol, and 3 mM NaN_3, pH 8.0, overnight. After a centrifugation at 125,000 *g* for 1 h, an "alkaline extract" of crude coated vesicles was obtained. Clathrin was precipitated from this extract by adding $(NH_4)_2SO_4$, 0.17 g/ml, to the extract. The precipitate was resuspended in 5 ml of 10% glycerol, 1% SDS, 10 mM dithiothreithol, 125 mM Tris-HCl, pH 6.8 (buffer A). The suspension was dialysed against this buffer overnight, incubated at 95°C for 6 min, and applied to a 6% acrylamide slab gel. The electrophoresis was performed according to Laemmli (13) and clathrin was extracted from the gel by electroelution. About 3 mg of clathrin was obtained. Coated vesicles from placenta were prepared according to Pearse (14) in the presence of 1% Triton X-100 as described (8).

Anti-Clathrin Antibodies

Rabbits were immunized with 60–70 μg purified clathrin suspended in Freund's complete adjuvant. A total of 5 to 7 booster injections in Freund's incomplete adjuvant were given. Blood samples were collected following the second booster injection. The titer of anti-clathrin antibodies was monitored by an enzyme-linked immunosorbent assay (15). The titer remained constant after the second or third booster injection. Anti-clathrin antibodies were isolated by affinity chromatography on a clathrin-Sepharose 4B column (16).

Metabolic Labeling

Normal human skin fibroblasts were cultured as previously described (12). Cells grown to confluency in 75-cm^2 flasks were labeled for 3 h with 0.8 mCi [^{35}S]methionine as described (17). Labeling was terminated by harvesting or feeding with 2 ml of Eagle's minimum essential medium containing 7.5% fetal calf serum. Cells were harvested by scraping, washed twice in 0.15 M NaCl, and homogenized in 2.5 ml of 0.1 M 2[*N*-morpholino]ethane sulfonic acid, pH 6.5, 50 mM NaCl in a tight-fitting Dounce homogenizer by 50 strokes. The homogenate was centrifuged at 1,500 *g* for 10 min and the resulting pellet was re-extracted two more times. The three supernatants, containing 75 to 85% of radioactivity and of a lysosomal marker enzyme (β-hexosaminidase) were pooled, adjusted to 0.5 mM EGTA and 0.5 mM $MgCl_2$ using 20-fold concentrated stock solutions and centrifuged for 1 h at 100,000 *g*. The resulting pellet, containing 10–30% of the lysosomal marker was suspended in 1 ml of 0.1 M 2[*N*-morpholino]ethane sulfonic acid, pH 6.8 containing 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mg/ml bovine serum albumin, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin (buffer B) by 2 strokes in a Dounce homogenizer. The suspension was incubated for 1 h in Eppendorf microtubes with 20 mg of *S. aureus* cells followed by centrifugation for 40 s at 12,000 *g* in an Eppendorf microfuge. This treatment was repeated twice.

Immunoprecipitation of Coated Organelles and Extractions

The preabsorbed extracts were divided into two aliquots. The aliquots were mixed with 30 μg of either anti-clathrin or control rabbit immunoglobulins in 0.15 M NaCl in 20 mM 2[*N*-morpholino]ethane sulfonic acid, 50 mM Tris-HCl, pH 6.8. After incubation for 6 h on a shaker, 30 μl of a 10% suspension of *S. aureus* cells pretreated as described (18) was added. After shaking for 30 min, the *S. aureus* cells were pelleted by centrifugation for 40 s at 12,000 *g* in an Eppendorf microfuge. The pelleted *S. aureus* cells were washed five times with 1 ml buffer B. The first extraction was for 15 min in 1 ml of buffer B containing 0.5% saponin. Unless otherwise stated, the second extraction was in 1 ml of 50 mM Tris-HCl, pH 7.0, containing 0.5% saponin and 5 mM mannose 6-phosphate for 5 to 7 h. The final extract was obtained by heating the *S. aureus* cells for 6 min at 95°C in 0.1 ml of buffer A without dithiothreitol.

Immunoprecipitation of Cathepsin D

The extracts were adjusted to 1 ml with water and mixed with 1 ml 20 mM sodium phosphate, pH 7.4 containing 0.3 M NaCl, 1% sodium deoxycholate, 2% Triton X-100, 0.2% SDS, 4% bovine serum albumin, 2 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide, and 2 mM EDTA. Aliquots corresponding to 5% each of the fractions sedimenting at 1,500 *g*, sedimenting at 100,000 *g* or non-sedimenting, were diluted likewise. After incubation for 30 min with 20 mg *S. aureus* cells, the extracts were centrifuged for 1 h at 50,000 *g*. The supernatants were incubated overnight with anti-cathepsin D antibodies (3–6 μg, immunoprecipitating ≥90% of the cathepsin D polypeptides in the extracts). The immune complexes were collected with *S. aureus* cells, washed, solubilized, and separated by SDS PAGE (12.5% gels) as described (12). Labeled material was visualized by fluorography (19) and quantified by densitometry using UltroScan laser densitometer (LKB Instruments, Bromma, Sweden).

Other Procedures

ELECTRON MICROSCOPY: Electron microscopy of ultrathin sections of Immuno Precipitin sediments was performed as described (8).

WESTERN BLOTTING: For characterization of the affinity-purified anti-clathrin antibodies, the alkaline extract of a crude coated vesicle preparation from human brain (20 μg of protein) was applied to SDS PAGE (6% acrylamide). Parts of the gel were either stained with Coomassie Blue or subjected to electrotransfer to nitrocellulose. Conditions for blotting and detection of the antigen were as described (20).

RESULTS

Immunoadsorption of Clathrin and Coated Membranes

Throughout this study, affinity-purified antibodies raised in rabbits against human brain clathrin were used. These antibodies reacted in Western blots with an M_r 180,000 polypeptide corresponding to clathrin (Fig. 1). In combination with *S. aureus* cells the antibodies could be used as a specific immunoadsorbent for coated membranes as described by Merisko et al. (11). When *S. aureus* cells were opsonized with anti-clathrin antibody, and then incubated with Triton X-100–extracted coated vesicles from human placenta, numerous coated vesicles decorated the immunoadsorbent. Coated vesicles were not bound to *S. aureus* cells opsonized with control rabbit immunoglobulin (results not shown).

The suitability of the anti-clathrin antibody–*S. aureus* immunoadsorbent for isolation of clathrin and coated membranes from metabolically labeled human fibroblasts was tested as follows. Cells that had been labeled for 24 h with [^{35}S]methionine were extracted with detergent. The extract

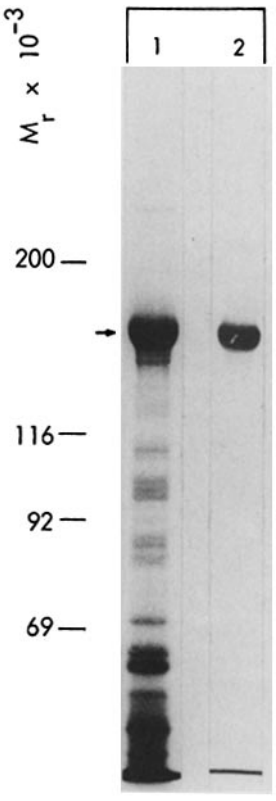

FIGURE 1 Specificity of anti-clathrin antibodies in Western blots. An alkaline extract of crude coated vesicles from human brain (20 μg protein) was separated in SDS PAGE (6% gel). The polypeptides were either stained with Coomassie Brilliant Blue (lane *1*) or transferred to nitrocellulose and analyzed for reactivity with the anti-clathrin antibody, 5 μg/ml (lane *2*). The positions of molecular weight standards are indicated.

was preadsorbed with *S. aureus* cells, clarified by high speed centrifugation and reacted with the immunoadsorbents. In the sample treated with anti-clathrin antibody, a major portion of the adsorbed radioactivity was present in an M_r 180,000 polypeptide as expected for clathrin (Fig. 2, lane *1*). In control cells treated with non-immune antibody, only a low molecular weight polypeptide (M_r < 40,000) which migrated at the dye front was found (Fig. 2, lane *2*). The pattern of the minor protein bands recovered along with clathrin from the labeled cells was similar to that of protein present in detergent-extracted coated vesicles that were isolated from human placenta and stained with Coomassie Blue (Fig. 2, lane *3*).

For isolation of coated organelles from labeled cells in the absence of detergent, conditions were chosen that minimize dissociation of clathrin from membranes. The cells were disrupted in 0.1 M 2[*N*-morpholino]ethane sulfonic acid, pH 6.5, 50 mM NaCl. The postnuclear supernatant was centrifuged for 1 h at 100,000 *g*. The resulting pellet was resuspended and used as source for isolation of coated membranes by immunoadsorption. Again, the M_r 180,000 band (clathrin) was the major constituent of the immunoadsorbed material (Fig. 2, lane *4*) and was not detected in the control (lane 5). In the absence of the detergent, the relative amount of smaller polypeptides accompanying clathrin was higher (Fig. 2*B*) than in the presence of the detergent (Fig. 2*A*). We have presumed that a portion of the smaller polypeptides accompanying clathrin represent the contents of the coated organelles.

Cathepsin D in Coated Organelles

In previous studies using coated vesicles prepared on a large scale, it has been shown that small amounts of lysosomal enzymes are associated with coated vesicles and that these fractions are enriched in the precursor forms of lysosomal

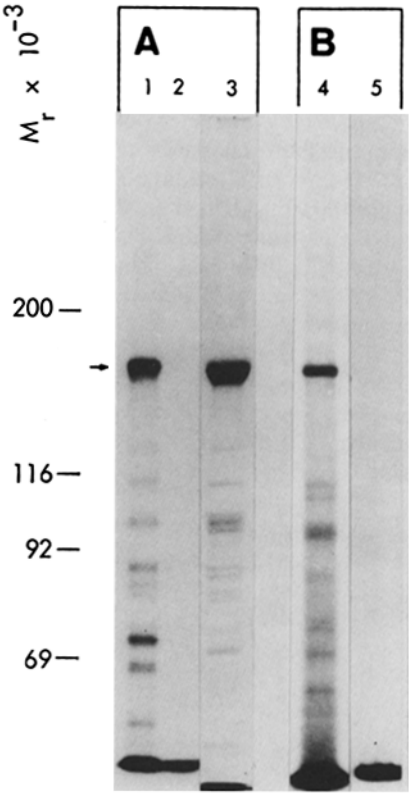

FIGURE 2 Immunoprecipitation of clathrin (*A*) and coated organelles (*B*). (*A*) Human skin fibroblasts were incubated for 24 h with 0.05 mCi of [^{35}S]methionine. After labeling, the cell layer was extracted with 50 mM Tris-HCl, pH 7.45, containing 1% Nonidet P-40 and 1% sodium deoxycholate. After centrifugation for 1 h at 100,000 *g*, the supernate was preadsorbed with *S. aureus* cells and reacted with 20 μg of anti-clathrin antibodies (lane *1*) or 20 μg of control rabbit immunoglobulins (lane *2*). The immune complexes were collected with the aid of pre-treated *S. aureus* cells, solubilized, and separated in SDS PAGE (6% gel) as described in Materials and Methods. Lane *3* shows, for comparison, the pattern of protein bands as stained with Coomassie Blue in coated vesicles prepared in the presence of detergent from human placenta. (*B*) A fraction containing coated vesicles was prepared from labeled fibroblasts and reacted with either anti-clathrin antibodies (lane *4*) or control rabbit immunoglobulins (lane *5*) as described in Materials and Methods. The opsonized vesicles were collected with the aid of pretreated *S. aureus* cells, solubilized, and separated by SDS PAGE (6% gel). The positions of molecular weight standards are indicated.

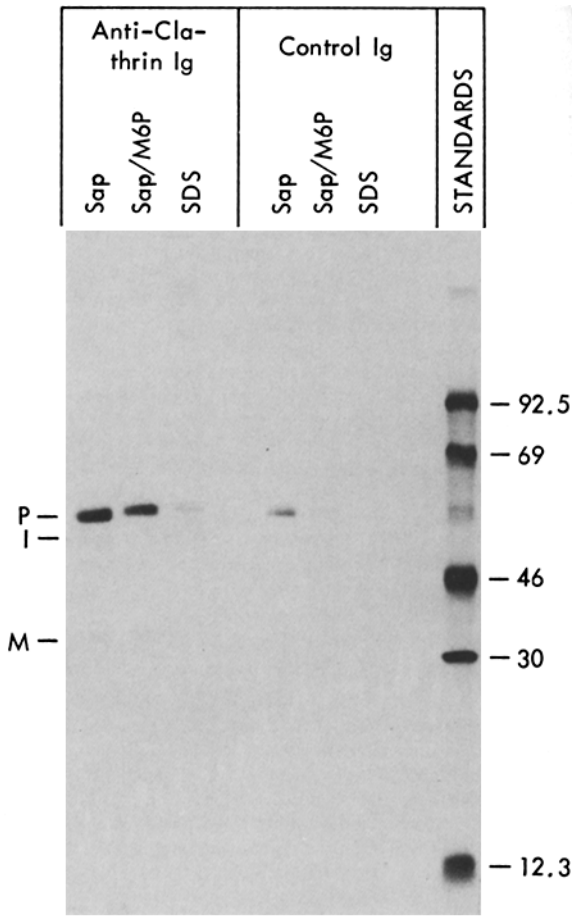

FIGURE 3 Association of cathepsin D with immunoadsorbed coated organelles. Fibroblasts were incubated for 3 h with [^{35}S]-methionine. A fraction containing coated vesicles was prepared and reacted with anti-clathrin antibody or control rabbit immunoglobulin. The material adsorbed to pretreated *S. aureus* cells was sequentially extracted with buffers containing 0.5% saponin, 0.5% saponin with 5 mM mannose 6-phosphate, and 1% SDS. Cathepsin D was isolated from the extracts by immunoprecipitation and separated in SDS PAGE (12.5% gel). The positions of precursor (*P*, 53,000 daltons), intermediate (*I*, 47,000 daltons), and mature (*M*, 31,000 daltons) forms of cathepsin D are indicated. The ^{14}C-methylated standards were phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome c (12,300).

enzymes. The immune technique described above should allow us to critically examine the presence of the precursor form of a lysosomal enzyme in the minute amounts of material that are available from cultured cells. Cathepsin D was chosen, for it is the most sensitive marker for lysosomal enzymes available to us.

Fibroblasts were labeled with [^{35}S]methionine for 3 h. Association of the labeled cathepsin D with coated membranes present in the material sedimenting at 100,000 *g* was examined by (*a*) opsonizing the membranes with antibody, (*b*) binding the opsonized membranes to *S. aureus* cells, (*c*) extracting the contents of the bound coated organelles in the presence of saponin, and (*d*) immunoprecipitating cathepsin D from the extracts. In the extracts, the M_r 53,000 precursor form represented >90% of the cathepsin D polypeptides (Fig. 3). The amount of the precursor recovered from membranes bound in the presence of the anti-clathrin antibody was about seven times that in the presence of control immunoglobulin. The yield of the precursor was not changed when the amount of the anti-clathrin antibody was halved or doubled (not shown). The same result was obtained when protein A–Sepharose 4B was substituted for *S. aureus* cells. In several experiments it was observed that ⅔ to ¾ of the precursor were extracted by exposing the adsorbed material briefly (15 min) to a 0.5% solution of saponin. The remainder was nearly completely released during a prolonged (5–7 h) extraction in the presence of saponin and mannose 6-phosphate. In the final extracts with 1% SDS, we obtained merely a trace of the precursor.

Previously, biochemical (9) as well as cytological (21, 22) data have been presented showing that coated membranes from various sources contain mannose 6-phosphate receptors. It came as a surprise, therefore, that the precursor was readily eluted by saponin from the material immunoadsorbed in the presence of the anti-clathrin antibody. Either only a small portion of the precursor in the coated organelles was associated with the receptor or the precursor readily dissociated from the receptor upon the dilution in the presence of saponin. To see if there is any dependence of the elution on mannose 6-phosphate, the conditions were varied as shown in Fig. 4*A*. The elution with saponin was followed by elution for 7 h with either saponin and mannose 6-phosphate or saponin alone. In the former scheme, essentially all precursor was eluted in the two steps, whereas in the latter at least 10% of the precursor remained in the sedimenting material and was eluted in a subsequent extraction in the presence of mannose 6-phosphate.

In Fig. 4*A*, it is shown that after a 3-h pulse labeling, comparable amounts of radioactivity were present in the precursor, intermediate, and mature forms of cathepsin D in the subcellular fractions. In contrast, in the coated organelles isolated by the immunoadsorption, the precursor was ~10

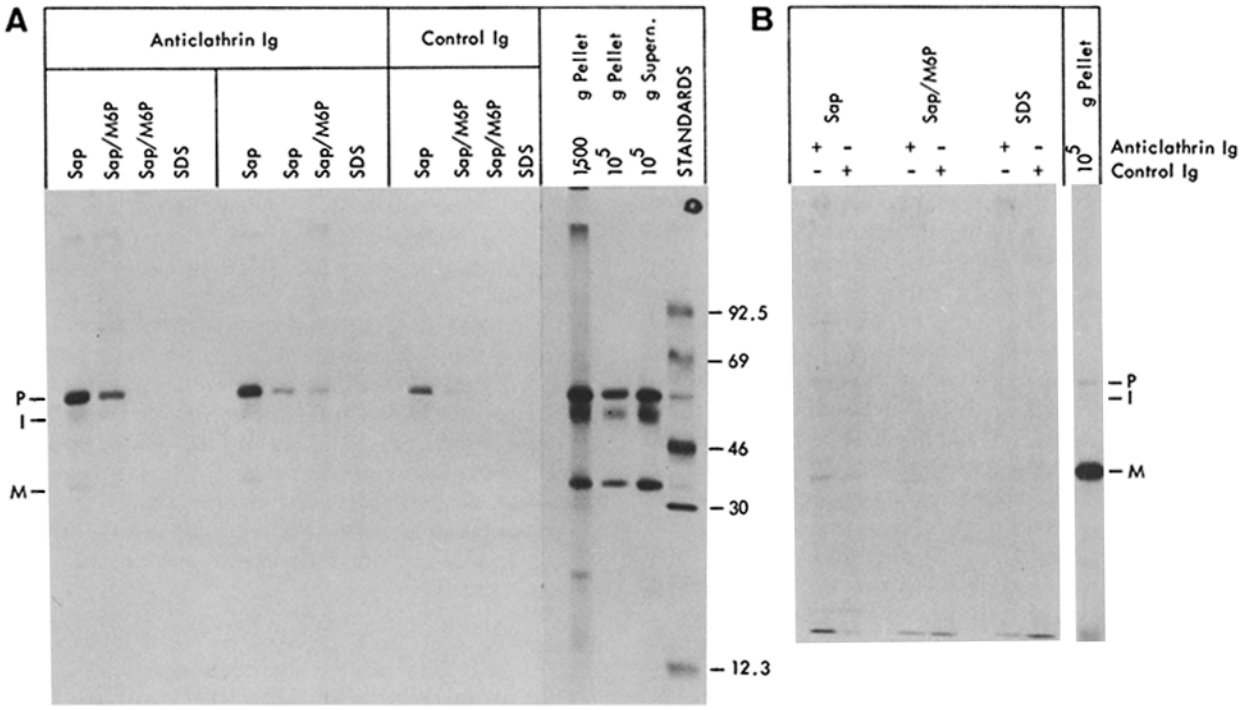

FIGURE 4 Selective presence of the precursor form of cathepsin D in the immunoadsorbed coated organelle and enhancement of the extraction of the precursor with mannose 6-phosphate. (*A*) Fibroblasts were incubated for 3 h with [^{35}S]methionine. The material adsorbed (*S. aureus* cells in the presence of anti-clathrin antibodies or control rabbit immunoglobulins) was sequentially extracted with buffers containing 0.5% saponin, 0.5% saponin and 5 mM mannose 6-phosphate, or 1% SDS in the sequence indicated above the lanes. The three sequential extractions with the saponin-containing buffers were for 15 min, 7 h, and 5 h, respectively. Cathepsin D was immunoprecipitated from the extracts as well as from aliquots corresponding to 5% of the cell fractions pelleting at 1,500 *g* or 100,000 *g*, or remaining soluble after centrifugation at 100,000 *g* (for details see section Materials and Methods). The relative amount of cathepsin D precursors extracted from the immunoadsorbed fractions were 1 (lanes *1–4*), 0.78 (lanes *5–8*), and 0.13 (lanes *9–12*). (*B*) Fibroblasts were labeled for 3 h with [^{35}S]methionine followed by a chase for 10 h in medium supplemented with 0.25 mg/ml methionine. The material obtained after immunoadsorption with anti-clathrin or control antibodies was extracted as indicated above the lanes and the extracts were analyzed for cathepsin D polypeptides. The positions of cathepsin D polypeptides (*P, I, M*) are indicated. For symbols, standards, and gel concentration see legend to Fig. 3.

times more strongly labeled than the other forms of cathepsin D. After a 10-h chase, >90% of cathepsin D was processed to the mature form (Fig. 4 *B* shown for the subcellular fraction, from which the coated membranes were isolated). In the immunoadsorbed membranes, the precursor could not be detected any more, whereas the amount of the mature enzyme was hardly increased. After the pulse—and after the chase as well—similar amounts of mature cathepsin D were immunoadsorbed with either the anti-clathrin or the control antibody. In all these samples the extraction of the mature polypeptide seemed to be complete with a single brief saponin wash.

DISCUSSION

Specific anti-clathrin antibody can be exploited in immunoadsorption of coated vesicles (11). We prepared and characterized an antibody against human clathrin. The antibody mediated adsorption of human placental-coated vesicles to *S. aureus* cells and could be used to isolate clathrin and associated material from human fibroblasts. Previously, in coated membranes purified from rat liver and human placenta by conventional procedures, precursor and processed forms of lysosomal enzymes were detected in about equal amounts (7, 8). This suggested that coated membranes may participate in the transport of newly synthesized lysosomal enzymes and also in the exchange of mature lysosomal enzymes between the lysosomes or in the processing of the precursors. The present results indicate that, depending on isolation procedure, coated organelles may be obtained more or less free of the mature form of lysosomal enzymes. The specific nature of the association of the precursor of cathepsin D with the coated organelles was indicated by a decrease in the total amount of the immunoadsorbed radioactive precursor upon the chase during which the precursor was converted to the mature enzyme, and by differential immunoadsorption of the precursor in the presence of anti-clathrin and control antibody. Usually a small amount of the mature cathepsin D also was found in the adsorbed material. However, unlike with the precursor, there was little difference in the binding of the mature cathepsin D when either anti-clathrin or control antibody was used, or when the precipitation was performed after either a 3-h pulse (preferential labeling of the precursor form) or a 3-h pulse/10-h chase (preferential labeling of the mature form) labeling. In addition, the mature form seemed to be more readily extracted from the immunoadsorbed material than the precursor. Lastly, there is strong evidence that the mature cathepsin D (M_r 31,000) is formed from the M_r 47,000 intermediate within the dense lysosomes (12, 23). We think that the mature forms of lysosomal enzymes in various preparations of coated organelles are due to contamination that may be difficult to avoid simply because the cells contain an overwhelming (100-fold or higher) excess of the mature over the precursor forms of lysosomal enzymes. We conclude that the transient association of the precursor form of cathepsin D in the coated organelles supports the idea on the involvement of coated organelles in the intracellular transport of the newly synthesized lysosomal enzymes (5).

The newly synthesized cathepsin D persists in the cell as precursor for ~2 h (12). A metabolic labeling for 3 h, therefore, is expected to ensure a constant specific radioactivity in the precursor in all compartments of the cell it is passing through. To estimate the mean residence time of cathepsin D precursors in coated membranes, we compared the amount of cathepsin D in the postnuclear supernatant with that in coated membranes. On average, ‰ of the total precursor is associated with coated vesicles (range = 0.4–2.5%, n = 5; see an example in Fig. 4). It appears, therefore, that the mean residence time of the precursor in the coated vesicles is ~2 min. This estimation is based on the assumption that coated membranes in the postnuclear supernatant are recovered quantitatively by our procedure, that the nuclear fraction is not selectively enriched in or depleted from coated membrane-associated cathepsin D, and that the immunoprecipitation of cathepsin D is quantitative. While the immunoprecipitation of coated membranes and cathepsin D was found to be essentially quantitative, the other assumption could not be tested.

The cellular compartments donating and receiving the lysosomal precursors to and from the coated membranes were not identified in the present contribution. The observation that elution of the precursor of cathepsin D from coated membranes is facilitated in the presence of mannose 6-phosphate (reference 7 and Fig. 4) suggests that the coated organelles bearing lysosomal enzyme precursors mediate the transport of lysosomal enzymes from the Golgi apparatus and/or the plasma membrane to prelysosomal organelles where receptors and lysosomal enzymes are thought to be separated (21, 24, 25). In our experiments as well as in those of Campbell and Rome (7), most of the lysosomal enzymes could be eluted even in the absence of mannose 6-phosphate. Either a portion of lysosomal enzymes in the coated organelles is not bound to the receptors, or they dissociate from the receptors upon the dilution in the presence of a detergent. The reports of Brown et al. (22, 26) on redistribution of mannose 6-phosphate receptors in coated vesicles and Golgi apparatus in conditions with abnormal transport of lysosomal enzymes lend further support to the idea that mannose 6-phosphate receptors in coated vesicles participate in the transport of lysosomal enzymes from the Golgi apparatus. There is evidence from cytological studies that coated vesicles participate also in receptor-mediated endocytosis of lysosomal enzymes (6). (For detailed discussion on the role of mannose 6-phosphate receptors in transport of lysosomal enzymes to prelysosomal organelles, see recent reviews in references 1 and 2.)

Studies on the kinetics of labeling and on the structure of carbohydrates in lysosomal enzymes in coated organelles, and studies on the effect of inhibitors affecting the transport of lysosomal enzymes at selective sites may provide new information on transport of lysosomal enzymes and the functions of coated organelles.

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