Lysosome-Associated Membrane Protein-1 (LAMP-1) Is the Melanocyte Vesicular Membrane Glycoprotein Band II

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Coated vesicles play a critical role in the process of melanogenesis. Antisera raised against a coated vesicle fraction from mouse melanoma cells recognize two major glycoprotein antigens, band I (47–55 kd) and band II (90–120 kd). We demonstrate that band II is lysosome-associated membrane protein 1 (LAMP-1) by the following criteria: 1) the molecular weight and abundance of LAMP-1 varies among tissues but is always identical to that of band II; 2) band II and LAMP-1 co-migrate in sucrose gradient sedimentation studies; 3) immunodepletion of cell extracts with antivesicle serum removes all LAMP-1; and 4) intact organelles immunoisolated with antivesicle serum contain band II and LAMP-1. Our results further confirm the long-suspected relationship between melanosomes and the lysosomal lineage of organelles. *J Invest Dermatol 100:110–114, 1993*

Coated vesicles were first implicated in the process of melanosomal biogenesis by studies employing the electron microscope [1–3]. From these studies, a model was developed in which vesicles containing tyrosinase arise from the Golgi apparatus and fuse with stage II melanosomes, thereby initiating the process of melanogenesis (see [4] for review). Subsequently, by adapting techniques developed for the isolation of coated vesicles from brain, highly purified coated vesicle fractions were prepared from murine and hamster melanoma cells and shown to contain high levels of tyrosinase, dopachrome tautomerase (formerly called dopachrome conversion factor), and gammaglutamyl transferase, three enzymes implicated in melanogenesis [5,6]. Dihydroxyindole and dihydroxyindole-2-carboxylic acid, two important melanogenic precursors, are also present in the vesicular fraction [7].

We have recently shown [8] that rabbit antisera generated to a highly purified vesicle fraction from Cloudman S91 mouse melanoma cells recognize two major glycoprotein antigens: Band I (47–55 kd) and band II (90–120 kd). Although both of these integral membrane proteins are present in the vesicular fraction of mouse melanoma cells and normal mouse melanocytes, only band I is specific to melanocytes [8]. In contrast, band II is present in all tissues examined, including brain, kidney, liver, and lung.

LAMP-1 (lysosome-associated membrane protein 1) is one of three major glycoproteins of the lysosomal membrane [9]. Like band II, it migrates broadly on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent Mr of 90–120 kd [9]. Due to the similarity in their molecular weights and the fact that a number of mutations, such as the *beige* mutation in mice [10] and Chediak-Higashi syndrome in humans [11], affect both melanosomes as well as lysosomes and lysosomally derived organelles [10], we sought to determine whether band II and LAMP-1 were related.

Our results, using a combined immunologic, biochemical, and cellular approach, demonstrate that LAMP-1 is the melanocyte vesicular membrane glycoprotein band II, and thereby confirm a long-suspected relationship between melanosomes and the lysosomal lineage of organelles.

METHODS

Cell Culture Cloudman S91 cells were cultured as previously reported [12]; Melan-a cells were obtained from D. Bennett via V. Hearing (NCI) and cultured as described [13].

Immunologic Reagents and Techniques The preparation and characterization of rabbit antiserum to a purified vesicular fraction from Cloudman S91 cells was described previously [8]. Rat monoclonal antibodies directed against murine LAMP-1 (1D4B) and another directed against murine LAMP-2 (ABL-93) [14] were purchased from the Developmental Studies Hybridoma Bank (University of Iowa and Johns Hopkins University). Rabbit anti-rat immunoglobulin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).

Metabolic labeling with [^{35}S] methionine, immunoprecipitation, SDS-PAGE and immunoblotting were all performed as described previously [8,15]. Brain, kidney, and liver from adult C57BL/6J mice and Cloudman S91 cells were homogenized and extracted as described [8].

Immunoisolation using protein A–coated magnetic beads was an adaptation [8,16] of a method developed for the immunoisolation of secretory vesicles from neuronal and endocrine cells [17].

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Abbreviations:

LAMP: lysosome-associated membrane protein
PBS: phosphate-buffered saline
SDS-PAGE: Sodium dodecylsulfate–polyacrylamide gel electrophoresis
TBS: Tris-buffered saline

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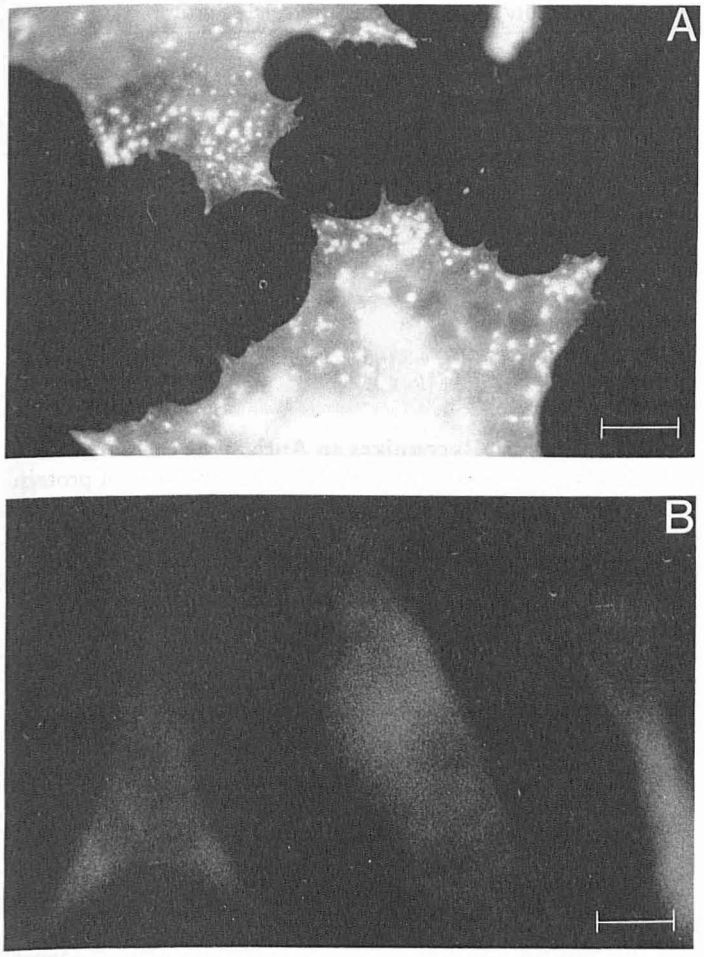

Figure 1. Immunolocalization of antigens recognized by antivesicle serum. Cloudman S91 cells were permeabilized and immunofluorescence performed with antivesicle serum (*A*) or pre-immune serum (*B*) as described in *Materials and Methods*. Note the intense granular pattern evident in *A*. *Bar*, 4 microns.

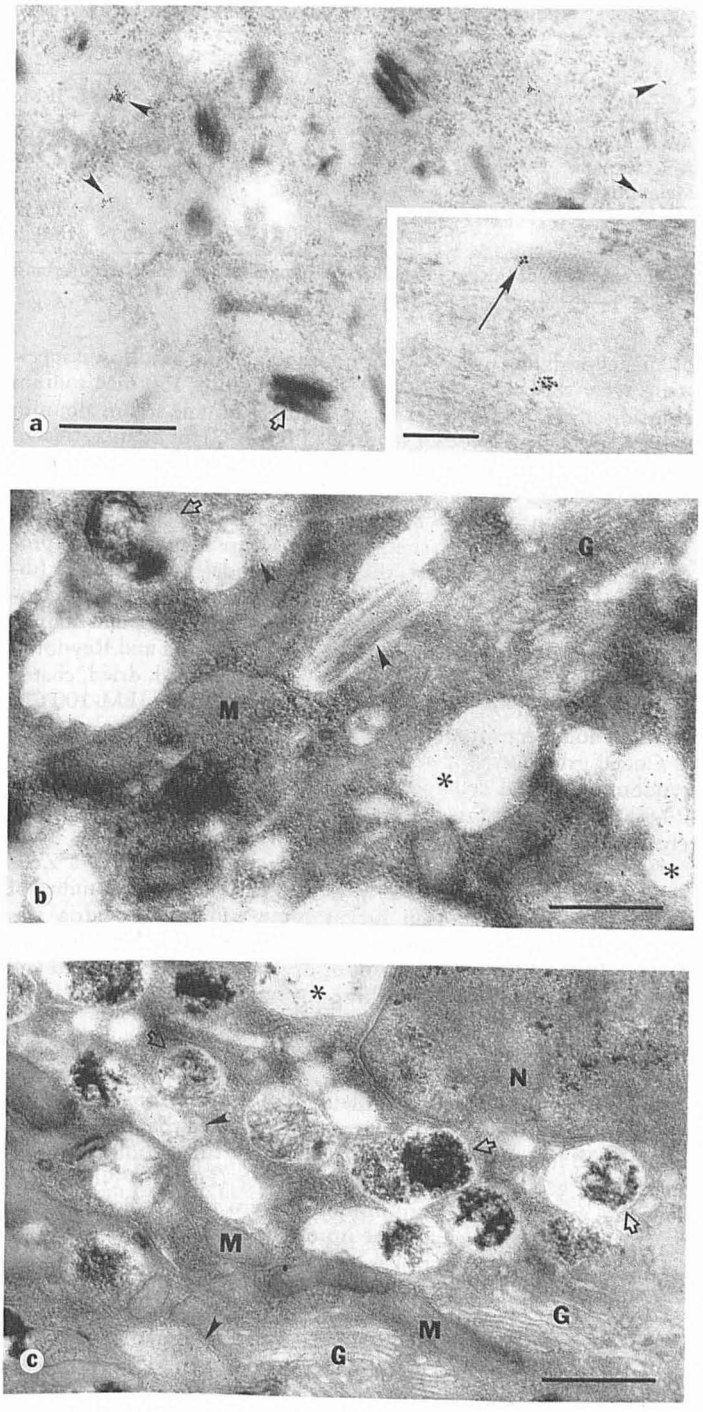

Figure 2. Immunoelectronmicroscopy using antivesicle serum. Cloudman S91 melanoma cells (*a*) untreated or (*b* and *c*) treated with MSH and isobutylmethylxanthine and immunostained with antivesicle serum at (*a*) 1:10,000 to dramatically reduce nonspecific background staining or (*b* and *c*) 1:100. Gold particles predominate on stage I–II melanosomes (*arrowheads* and *arrow* in *inset* to *a*), and on large, electron lucent vacuoles *(asterisks)* that resemble late endosomes. We have previously demonstrated that these latter organelles are LAMP-1 positive and represent putative melanosome precursors [16]. Stage III–IV melanosomes *(open arrows)* have far fewer gold particles than do the other organelles. (G, Golgi apparatus; M, mitochondria; N, nucleus.) *Bars*, *a*, 0.54 microns; *inset* to *a*, 0.16 microns; *b* and *c*, 0.60 microns.

For immunofluorescence microscopy, Cloudman S91 cells were cultured on cover slips for 48 h, rinsed in phosphate-buffered saline (PBS), and fixed for 30 min in PBS containing 3% paraformaldehyde and 0.02% glutaraldehyde, followed by three rinses in PBS. Cells were permeabilized in acetone at −20°C for 15 seconds, rinsed in PBS, exposed to antivesicle serum for 1 h at 37°C, and rinsed five times in PBS prior to exposure to Texas-red–labeled anti-rabbit immunoglobulin antibody (Amersham, Arlington Heights, IL) for 1 h at 37°C. Unbound antibody was removed by washing the cells five times in PBS. Specificity and background were checked by labeling parallel cultures with only Texas Red–labeled immunoglobulin.

For immunoelectron microscopy, Cloudman S91 cells were seeded into Lab Tek chamber slides (Nunc, Inc., Naperville, IL), and subsequently embedded as follows [18]: cells were washed twice in PBS, fixed with 4% paraformaldehyde, 1% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer containing 0.5 mM calcium chloride (pH 7.4) for 20 min at room temperature, washed three times, and incubated in 0.25% tannic acid for 10 min at room temperature. Cells were washed three times, quenched in 50 mM ammonium chloride for 20 min at 0°C, washed four times in 0.1 M maleate buffer (pH 6.5), treated with 2% uranyl acetate (pH 4.0) for 30 min at 0°C, dehydrated with acetone, and infiltrated with Lowicryl K4M at −20°C and the embedding medium polymerized under ultraviolet irradiation at −20°C for 2 d.

Embedded cells were mounted and sectioned, and sections placed

Table I. Density of Gold Labeling on Organelles Immunocytochemically Stained with Antivesicle Serum[a]

Cells	Antiserum (dilution)	Number of Gold Particles/Micron²		
		Stage I–II Melanosomes	State III–IV Melanosomes	Mitochondria
Amelanotic	αv (1:100)	153.9 ± 14.1	—	10.4 ± 1.1
Amelanotic	αv (1:200)	75.7 ± 3.9	—	8.3 ± 1.0
Melanotic	αv (1:100)	100.8 ± 5.7	61.5 ± 5.4	14.4 ± 1.9
Melanotic	αv (1:200)	84.9 ± 4.2	59.8 ± 4.5	15.1 ± 2.2
Melanotic	Preimmune (1:200)	10.0 ± 2.9	10.0 ± 2.0	9.0 ± 2.2

[a] The subcellular distribution of the antigens recognized by the antivesicle serum in Cloudman S91 cells was analyzed by immunoelectronmicroscopy and the density of gold labeling quantified as detailed in *Materials and Methods.*

on nickel grids and processed for immunolabeling as follows: specimens were washed with 50 mM Tris containing 150 mM sodium chloride at pH 7.4 (TBS), blocked with 1% bovine serum albumin in TBS containing 0.02% Tween 20 (TBS-ICC) for 10 min, treated with the primary antisera diluted in TBS-ICC for 2 h at room temperature and subsequently washed extensively. Primary antisera consisted of antivesicle or pre-immune rabbit serum (at 1:100, 1:200, or 1:10,000). Washed specimens were incubated with protein A conjugated to 5-nm-diameter colloidal gold particles (diluted 1:10 in TBS) for 1 h at room temperature, washed extensively, stabilized with 2% glutaraldehyde for 5 min, and stained sequentially with 2% osmium tetroxide for 15 min and Reynold's lead citrate for 3 min. Specimens were then washed, dried, coated with Formvar, viewed, and photographed in a JEOL JEM-100 CX transmission electron microscope.

Coded grids were viewed and the perinuclear area from 20–30 randomly selected cells per sample group were photographed at 20,000 times and subsequently printed to 50,000 times. Each micrograph contained on average 15 melanosomes. Prints were mounted on a 45-position menufield digitizing tablet of a Zeiss interactive digital analysis system. The perimeter and number of gold particles within each melanosome and mitochondria was traced and counted using a pen cursor. By computer, the number of gold particles per square micron of organelle was determined and means calculated prior to breaking the code.

RESULTS

Antivesicle Serum Recognizes Stage I and II Melanosomes Immunofluorescent analysis utilizing antivesicle serum and permeabilized Cloudman S91 melanoma cells revealed that the antibody recognized intracellular granules distributed throughout the cytoplasm and grouped in the perinuclear area (Fig 1*A*). No specific labeling was seen with pre-immune serum (Fig 1*B*). When the antivesicle serum was used for immunoelectronmicroscopy, gold particles were localized to spherical or "gourd" shaped vacuoles, many of which contained poorly organized matrix material (stage I melanosomes) or well-arranged matrix filaments (stage II melanosomes) (Fig 2). The normally amelanotic Cloudman cells can be induced to melanize by combined treatment with MSH and isobutylmethylxanthine, which we have previously shown to result in the induction of stage III and IV melanosomes [19]. When cells treated in this fashion for 48 h were analyzed with the antivesicle serum, some labeling of stage III–IV melanosomes could be noted, but the predominant structures labeled were still stage I and II melanosomes (Fig 2*B,C*). These observations were further confirmed by quantification of the number of gold particles per square micron (Table I).

We have recently shown [16] that in highly melanized cells, stage I and, to a lesser extent, stage II melanosomes, contain both tyrosinase-related protein-1 and LAMP-1, a major lysosomal membrane glycoprotein, whereas these proteins are absent or present in very low quantities in the tyrosinase-rich stage III and stage IV melanosomes. Because the antigen(s) recognized by the antivesicle serum are also present for the most part in stage I–II melanosomes, and because band II, one of the two antigens recognized by the antivesicle serum, has a molecular weight of 90–120 k [8], similar to that reported for LAMP-1 [9], we sought to determine whether band II could be identical to LAMP-1.

Antivesicle Serum Recognizes an Antigen of the Same Molecular Weight as LAMP-1 When equal quantities of protein from extracts of Cloudman S91 melanoma cells as well as from brain, kidney, and liver from C57BL6/J mice were subjected to SDS-PAGE and analyzed by Western immunoblotting, both the antivesicle serum and a monoclonal antibody to mouse LAMP-1 recognized a broadly migrating band of Mr 90–120 kd (Fig 3). The Mr and abundance of this protein varied from tissue to tissue, but the results obtained with both antibodies were always similar (Fig 3*A,B*). In contrast, a monoclonal antibody to mouse LAMP-2 recognized a band whose abundance and molecular weight could be readily differentiated from that of band II (Fig 3); unlike band II and LAMP-1, for example, LAMP-2 is present in very low quantities in brain (Fig 3).

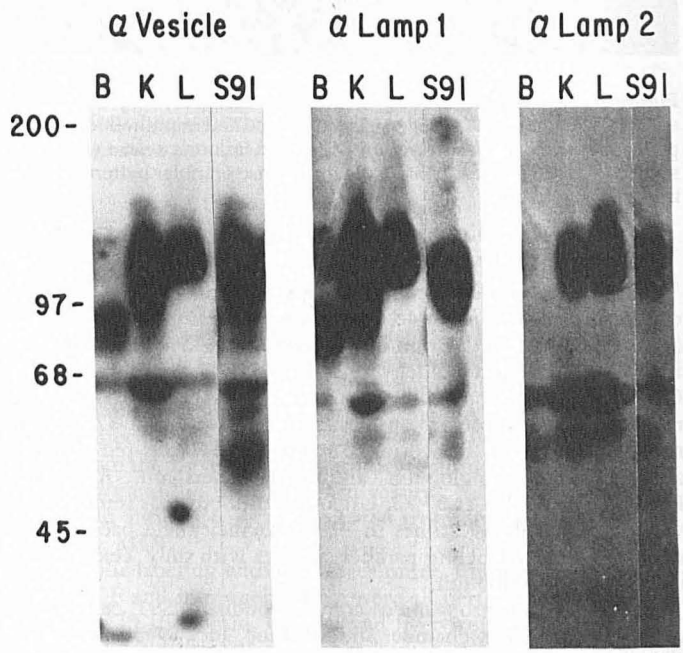

Figure 3. Western blotting analysis. Twenty micrograms of extracts of brain (B), kidney (K), and liver (L) from C57BL/6J mice, and of cultured Cloudman S91 melanoma cells (S91), were subjected to SDS-PAGE analysis and then immunoblotted with rabbit antivesicle serum (α vesicle), a rat monoclonal against murine LAMP-1 (αLAMP-1) and against murine LAMP-2 (αLAMP-2). The migration of molecular weight markers is shown at *left.* Note that the Mr of the 90–120-kd band recognized by the antivesicle serum matches that of LAMP-1, but is different from that of LAMP-2 (best seen in *B* lanes). The antivesicle serum also recognized a broadly migrating band of Mr 45–60 kd (band I) that is not recognized by the two anti-LAMP antibodies.

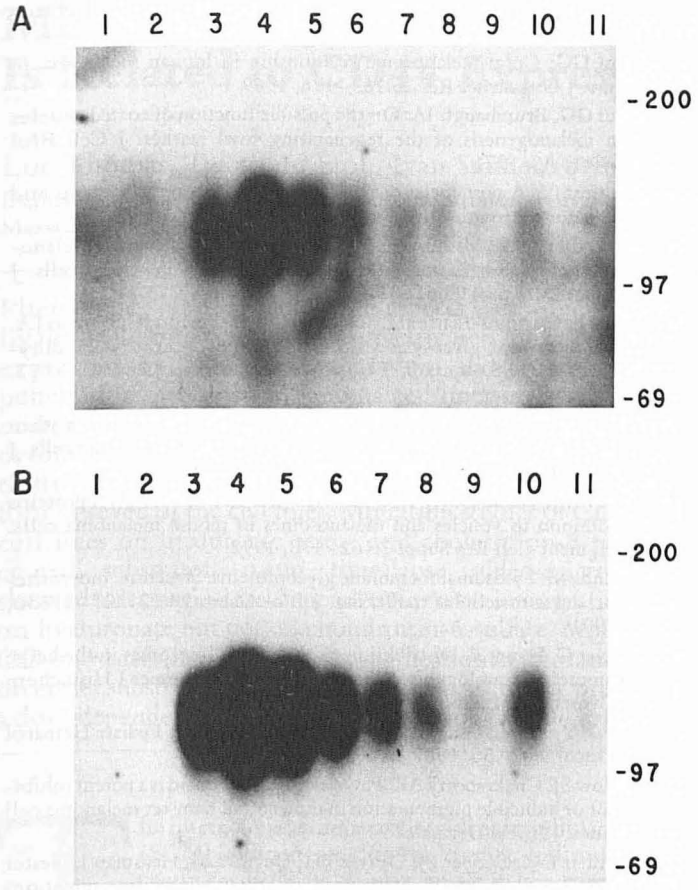

Figure 4. Sucrose density sedimentation analysis of band II/LAMP-1. A detergent-solubilized extract of melanoma cells was subjected to sedimentation in a 10–40% sucrose gradient as described in *Methods.* Fractions were collected from the top of the gradient, and equal quantities of each fraction were subjected to SDS-PAGE and immunoblotting with antivesicle serum (*A*) and anti LAMP-1 (*B*). Note that the Mr 90–120-kd antigen recognized by both antibodies migrates with a peak at fraction 3–5, and a smaller peak in fraction 10.

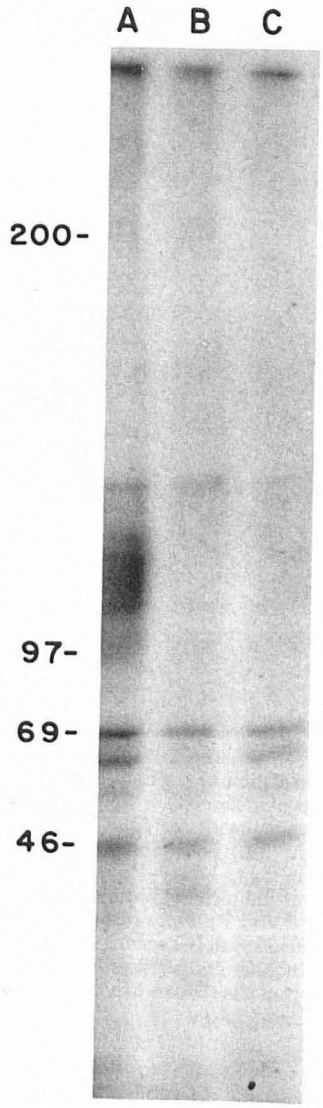

Figure 5. Immunoprecipitation analysis. Extracts of Cloudman S91 cells metabolically labeled with [^{35}S] methionine were pre-cleared with normal rabbit serum *(lane A)*, antivesicle serum *(lane B)*, or a monoclonal antibody to LAMP-1 *(lane C)*, followed by immunoprecipitation with antibody to LAMP-1. In *lane A*, a Mr 90–120-kd band is specifically immunoprecipitated, but pre-clearing with the antibodies to vesicles or LAMP-1 quantitatively removes all immunoreactive material from the extract.

LAMP-1 and Band II Co-Sediment on Sucrose Gradients To determine if the native size/molecular weight of LAMP-1 and band II were similar, detergent-solubized extracts of immortalized cultured mouse melanocytes (melan-a cells [13]) were centrifuged through sucrose density gradients and the collected fractions were subjected to Western immunoblotting analysis with antivesicle serum and αLAMP-1. The results in Fig 4 demonstrate that under these non-denaturing conditions, the sedimentation of band II and LAMP-1 are identical.

Antivesicle Serum Can Immunodeplete LAMP-1 αLAMP-1 precipitates a Mr 90–120 kd antigen from extracts of Cloudman S91 cells metabolically labeled with ^{35}S methionine (Fig 5). We have previously shown [8] that, from these same cells, antivesicle serum immunoprecipitates two antigens of Mr 47–55 kd (band I) and 90–120 kd (band II). When extracts of metabolically labeled cells were first subjected to immunoprecipitation with antivesicle serum and subsequently with αLAMP-1, antivesicle serum quantitatively removed all LAMP-1 reactivity from the extracts (Fig 5). Preprecipitation with control rabbit serum had no effect on the amount of LAMP-1 subsequently precipitated. As a positive control, preprecipitation with αLAMP-1 was performed. As expected, it removed all LAMP-1 from the extracts.

Antivesicle Serum Immunoisolates Organelles Containing LAMP-1 When magnetic beads coated with antivesicle serum were used to immunoisolate intact organelles from postnuclear supernatants of cultured melan-a cells, and the immunoisolated organelles subjected to Western immunoblotting, the isolated organelles were rich in LAMP-1 (Fig 6). In contrast, if beads coated with control serum were used, no LAMP-1 was isolated. (The reverse experiment could not be performed because the epitope recognized by αLAMP-1 is intraluminal, and thus inaccessible in intact organelles.)

DISCUSSION

A relationship between lysosomes and melanosomes has long been suspected. Lysosomal enzymes are transported from the trans-Golgi network to lysosomal precursors via a subset of coated vesicles [20]. Acid phosphatase is a well-described marker for lysosomes and certain other trans Golgi network-derived secretory organelles; its presence has been demonstrated in melanosomes by both biochemical and histochemical means [21–23]. At least 10 different recessive mutations in the laboratory mouse affect coat color, ocular pigmentation, and the secretion of lysosomal hydrolases or lysosomal struc-

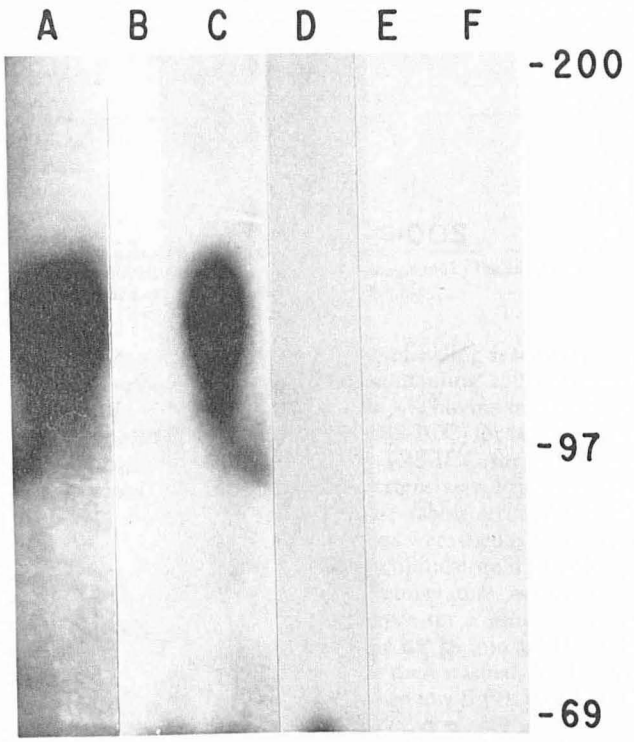

Figure 6. Immunoisolation of organelles with antivesicle serum. Postnuclear supernatants of melan-a cells were subjected to immunoisolation as described [8,16] using beads coated with antivesicle serum *(A,C,D,F)* or pre-immune serum *(B,E)*. Following SDS-PAGE, immunoblotting was performed with antibody to LAMP-1 *(lane A)*, antivesicle serum (*lanes B* and *C*), a control rat monoclonal antibody *(lane D)*, and pre-immune rabbit serum (*lanes E* and *F*). Note that beads coated with antivesicle serum immunoisolated organelles coating both LAMP-1 and band II (*lanes A* and *C*), whereas neither antigen was brought down by beads coated with pre-immune serum *(lane B)* or when immunoblotting was performed with control antibodies *(D–F)*.

ture [10,24]. Furthermore, histochemical evidence suggests that the interior of melanosomes, like that of endosomes and lysosomes, becomes acidified [25].

Our results demonstrate that one of the major lysosomal membrane glycoproteins, LAMP-1, is band II, present in subcellular organelles implicated in melanosomal biogenesis. We have recently reported that there is an early melanosomal compartment, perinuclear in distribution, that is rich in both LAMP-1 and tyrosinase-related protein-1, the product of the murine *brown* locus. These data show that there may be proteins shared by both organelles of the lysosomal lineage and melanosomes. Whether this is because melanosomes and lysosomes share a common biogenetic pathway up to a point and then diverge, or because the melanosome represents a "specialized" lysosome unique to the melanocyte, remains to be determined.

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