Published in final edited form as:

Cell Motil Cytoskeleton. 2005 September ; 62(1): 13–26.

The unconventional myosin-VIIa associates with lysosomes

Lily E. Soni, Carmen M. Warren, Cecilia Bucci¹, Dana J. Orten², and Tama Hasson

Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093

1Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Universita degli Studi di Lecce, 73100 Lecce

2Center for Hereditary Communication Disorders, Boys Town National Research Hospital, 555 N. 30th Street, Omaha, NE 68131-9909

Abstract

Mutations in the myosin-VIIa (*MYO7a*) gene cause human Usher disease, characterized by hearing impairment and progressive retinal degeneration. In the retina, myosin-VIIa is highly expressed in the retinal pigment epithelium, where it plays a role in the positioning of melanosomes and other digestion organelles. Using a human cultured retinal pigmented epithelia cell line, ARPE-19, as a model system, we have found that a population of myosin-VIIa is associated with cathepsin D- and Rab7-positive lysosomes. Association of myosin-VIIa with lysosomes was Rab7 independent, as dominant negative and dominant active versions of Rab7 did not disrupt myosin-VIIa recruitment to lysosomes. Association of myosin-VIIa with lysosomes was also independent of the actin and microtubule cytoskeleton. Myosin-VIIa copurified with lysosomes on density gradients, and fractionation and extraction experiments suggested that it was tightly associated with the lysosome surface. These studies suggest that myosin-VIIa is a lysosome motor.

Keywords

unconventional myosin-VIIa; actin; pigmented epithelial cell; Usher disease; Blindness; Deafness

Abbreviations

Myosin-VIIa: myo7a; Retinal pigmented epithelia: RPE; Rod outer segment: ROS

INTRODUCTION

Myosin-VIIa (myo7a) is an unconventional myosin required for sensory cell development and maintenance. Mutations in the myosin-VIIa gene cause human Usher disease, characterized by hearing impairment, balance dysfunction and progressive retinal degeneration (Ahmed et al. 2003; Weil et al. 1995). In the retina, myosin-VIIa is expressed in two adjacent cell types, the photoreceptors and the retinal pigment epithelia (RPE), and of these two cell types, myosin-VIIa is most highly expressed in the RPE (Hasson et al. 1995; Wolfrum et al. 1998).

The RPE is dedicated to the daily phagocytosis of rod outer segments (ROS) shed by the photoreceptor cells as part of the daily light/dark cycle of photoreceptor renewal (Zinn and Marmor 1979). Each day with light onset the shedding of distal disks from the outer segments

Address correspondence to: Tama Hasson, Ph.D., University of California at San Diego, Division of Biological Sciences, Section of Cell and Developmental Biology, 2129 Bonner Hall, MC 0368, 9500 Gilman Drive, La Jolla, CA 92093-0368, Phone: 858-822-3033; Fax: 858-822-3034, Email: tama@ucsd.edu.

is coordinated with their phagocytosis into the RPE. This phagocytosis is a multistep process that can be divided into three major stages: binding, ingestion and digestion.

The apical surface of the RPE is comprised of specialized actin-rich microvilli that are tightly associated with the photoreceptors and are involved in ROS binding. Mutations that block the binding or ensuing ingestion step lead to an accumulation of older disks at the tips of the photoreceptors which eventually becomes toxic to the photoreceptors and leads to retinal degeneration (Chaitin and Hall 1983). Digestion of the ROS within the RPE is also essential. Accumulation of undigested ROS material within the RPE, whether due to mutations in lysosomal proteases (Rakoczy et al. 2002) or due to accumulation of natural non-degradative substrates (e.g. lipofuscin, Finnemann et al. 2002), similarly causes photoreceptor cell death. Therefore, a tight coupling between photoreceptor outer segment shedding and RPE phagocytosis is necessary for retinal health.

To determine myosin-VIIa's function in retina, attention has turned to the phenotypes observed in *shaker-1* myosin-VIIa knockout mice. Although *shaker-1* mice exhibit the inner ear defects seen in Usher patients, no retinal degeneration is evident (Hasson et al. 1997; Liu et al. 1999). A slight slowing of opsin transport along the length of the photoreceptor outer segment was noted, suggesting a potential role for myosin-VIIa in the transport of opsin through the connecting cilium of photoreceptor cells (Liu et al. 1999). However, given the tight coupling between opsin delivery to the outer segment and the process of outer segment renewal and RPE phagocytosis, this slowing could also reflect an alteration in the kinetics of RPE phagocytosis of the spent ROS.

In vivo, RPE phagocytosis is a highly regulated process that follows a circadian rhythm. Following its daily burst of ROS phagocytosis, a rapid digestion of the internalized ROS takes place. This process is initiated by the sequential fusion of endosomes, lysosomes, and melanosomes with the ROS containing phagosomes, creating the so-called phagolysosome (reviewed in Vieira et al. 2002). Actin may facilitate fusion between endocytic compartments and existing phagosomes, as judged by studies that depolymerize actin (Jahraus et al. 2001). A class I myosin has been implicated in membrane trafficking occurring between endosomes and lysosomes (Raposo et al. 1999), so it was proposed that myosin-VIIa may play a similar role in organelle function in RPE.

Recent analysis of *shaker-1* phenotypes has focused on the rates of phagocytosis of ROS by RPE isolated from the myosin-VIIa knockout mice. Of note, both in vivo and in primary culture, the RPE lacking myosin-VIIa exhibited normal adhesion and ingestion of ROS (Gibbs et al. 2003). Therefore myosin-VIIa does not play a role in either ROS binding or initial uptake. Myosin-VIIa may play a later role in ROS phagocytosis, however. The transport of the ingested ROS out of the apical region of the cell was inhibited in *shaker-1* mice (Gibbs et al. 2003). There was evidence for a diminished digestion of the ingested ROS possibly due to the delay in basal transport of phagosomes to lysosomes (Gibbs et al. 2003). Based on this data they hypothesized that myosin-VIIa was acting late during phagocytosis to facilitate positioning of the phagosome for phagosome-lysosome fusion.

shaker-1 RPE also exhibited mispositioned melanosomes, pigment containing organelles that derive from lysosomes (Liu et al. 1998). Instead of being transported to the apical projections, in *shaker-1* RPE the melanosomes were retained in a perinuclear region (Liu et al. 1998). Electron microscopy data suggested that myosin-VIIa was on a subset of melanosomes (El-Amraoui et al. 2002; Gibbs et al. 2004), but the association of myosin-VIIa with a definitive digestion organelle has not been rigorously shown.

The differentiation of the RPE in vivo is influenced by diffusible factors secreted from neighboring retina layers. Because many of these differentiation factors are still unknown, a

cultured RPE line that reproduces all the unique properties of the RPE does not exist. In this study, we utilized the RPE-derived model cell line, ARPE-19. ARPE-19 is a nonimmortalized cell line that forms a polarized monolayer and differentiates under specific culture conditions (Dunn et al. 1996). After differentiation in culture, the ARPE-19 cells assemble apical microvilli and exhibit tight junctions (Dunn et al. 1996). The cells express many RPE specific gene products (e.g. cellular retinaldehyde binding protein CRALBP). They also phagocytose apically presented ROS, albeit with slower kinetics and without circadian regulation (Campochiaro et al. 1991; Dunn et al. 1996; Tian et al. 2004; Turowski et al. 2004). In addition, subretinal transplantation of ARPE-19 cells into developing dystrophic rat retinas, which are genetically impaired in RPE function, has been shown to rescue RPE function (Lund et al. 2001). Therefore, ARPE-19 cells retain the capacity to become fully functioning RPE and are a good model for the study of the certain aspects of RPE biology. Using this cell line and a series of monoclonal antibodies, we have found that myosin-VIIa is a lysosome-associated molecular motor.

MATERIALS AND METHODS

Cell Culture

The retinal pigmented epithelial cell line ARPE-19 (Dunn et al. 1996) was grown at 37°C with 5% CO₂ in DMEM-F12 with 10% FBS, fungizone (Gibco-BRL) and glutamine (Gibco-BRL). ARPE-19 cells were differentiated by switching cells to low serum media (DMEM-F12 with 1% FBS and 10nM retinoic acid) once cells reached confluency and maintained for 8 weeks at 37°C with 5% CO₂. HepG2 and HeLa (ATCC) were maintained at 37°C with 5% CO₂ in DMEM supplemented as above.

Antibody production

Monoclonal antibodies were generated to amino acids 927-1203 of human myosin-VIIa transcript 2 (accession #U55209; Chen et al. 1996). A SacI-XhoI cDNA fragment encoding this region was cloned into pQE30 (Qiagen) to create a HIS-tagged-Myosin-VIIa fusion protein for injection and also cloned into pGEX-2T (Pharmacia) to produce a GST-tagged-Myosin-VIIa fusion for use in ELISA assays to test antigen specificity.

For injection, HIS-tagged-Myosin-VIIa fusion protein inclusion bodies were solubilized in 8 M Urea, 0.5% CHAPS, 20 mM Tris-HCl, pH 8. HIS-tagged-Myosin-VIIa was bound to and renatured on a Chelating Sepharose Fast Flow Column (Amersham Pharmacia Biosciences) using Ni²⁺ as the transition metal in 20 mM Tris, pH 8, 0.5 M NaCl before elution with 250 mM Imidazole, 20 mM Tris-HCl, pH 8, 0.05% CHAPS + protease inhibitor cocktail (1 mM PMSF and 20 µg/ml each leupeptin, antipain, chymostatin, and pepstatin). Pooled fractions were dialyzed against PBS with 0.1 mM EDTA, 0.5% CHAPS, 0.3 M Urea and the protease inhibitor cocktail. This buffer alone caused no immune reaction when tested with the RIBI adjuvant system in mice.

To develop an antigen-specific ELISA, DH5α bacteria transformed with the pGEX-Myosin-VIIa construct or mock transformed bacteria were grown in LB/glucose medium supplemented with 50µg/ml carbenicillin, and induced with IPTG. Inclusion bodies were resuspended in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1 mM PMSF and 20 µg/ml each of leupeptin, bestatin, and trans-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (Sigma). Proteins were solubilized as described (Frangioni and Neel 1993). Lysates from fusion-protein expressing or control cultures were diluted in PBS in a range from 1:100 to 1:2500, and incubated on 96-well PVC microtiter plates at 4°C overnight. Wells were reacted with pre-immune and immune sera (see below) and also used to test hybridoma supernatants.

Two adult female Balb/c mice were immunized intraperitoneally with 250 µg purified HIS-tagged-Myosin-VIIa fusion protein antigen in the RIBI adjuvant system (RIBI Immunochemical Research). The animals were subsequently injected at 3-week intervals with 100 µg antigen. After the third injection, animal serum was assayed for immune response by the antigen-specific ELISA described above. One immune reactive animal was boosted with an injection of 87 µg antigen one month later. After ten days, a final 100 µg boost was administered and five days later the splenocytes were fused (4:1 ratio) with MOPC-21 NS-1 murine myeloma cells and grown in selective HAT medium. Cells from wells showing specific activity against the GST-tagged Myosin-VIIa fusion protein (8/864) were expanded and frozen. Four high titered wells (138-1; 138-2; 138-3; 138-4) were cloned by limiting dilution in growth factor supplemented media (IGEN Inc.) and re-screened by ELISA. Monoclonal 138-1 (IgG₁, kappa isotype) was chosen for further study and used at 1:2500 for immunoblot and 1:250 for immunocytochemistry.

Other Antibodies

Antibodies used in this study were from the following sources: Sigma: Tubulin (tub2.1), Actin (AC-74) and Hsp-60. The Developmental Studies Hybridoma Bank at the University of Iowa: Lamp1 (H4A3), Lamp2 (H4B4). Calbiochem: Cathepsin D. Research Diagnostics: GAPDH. BD Transduction Labs: EEA-1. Jackson Immunochemicals: rabbit non-immune IgG, and FITC-, Rhodamine- donkey anti-rabbit and donkey anti-mouse secondary antibodies. All purchased antibodies were used within the concentration range recommended by the manufacturer. Rabbit affinity purified antibodies directed to the tail domain of human myosin-VIIa (Hasson et al. 1995) were used as previously described.

Immunoblot analysis

Proteins isolated from cultured cells and tissues were prepared for gel analysis as described (Hasson and Mooseker 1994). Human retina was generously provided by Dr. Paul Wong, University of Alberta, Canada. Separated proteins were transferred to nitrocellulose, and the filter blocked with 5% non-fat dry milk in TBS before incubation with antibodies diluted in TBS + 0.05% Tween-20 for 2 hours at room temperature. Filters were washed extensively with TBS-Tween-20 before incubation for 30 min with 40 mU/ml horseradish peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG or anti-goat IgG (Jackson Immunochemicals). The signal was visualized using a chemiluminescence detection kit (Pierce) and X-OMAT RP film (Kodak).

Immunoprecipitations

ARPE-19 cells from a confluent T75 flask was rinsed twice with cold PBS. 1ml of ice-cold homogenization buffer (PBS, 1% Tx-100, 10mM ATP, 0.2 mM DTT, 1mM K-EGTA, 10mM MgCl₂) plus protease inhibitors (0.2mM Pefabloc, 50 µg/ml PMSF, 20 µg/ml chymostatin and 2 µg/ml each: aprotinin, leupeptin, pepstatin A), was added to the flask and rocked at 4°C for 45 mins. Cells were scraped and homogenized with a 25-gauge needle on ice. Cell debris was removed by centrifugation at 20,000xg for 10mins at 4°C. 0.18 mg protein A-Sepharose beads (Pharmacia) was added per ml of supernatant and incubated for 30–45 mins at 4°C to preclear the extract. The cleared supernatant was divided into two 0.5ml fractions, and 6 µg of affinity purified rabbit anti-myosin-VIIa antibody (αmyo7a) or rabbit nonimmune IgG (Jackson) was added to each aliquot and rotated at 4°C for 3 hrs. 0.09 mg protein A-Sepharose beads were added and the sample further rotated for 1 hr at 4°C. The immunocomplexes were collected by centrifugation at 3,800xg for 5 mins at 4°C. The supernatants were saved for gel analysis. The pellets were washed 4 times with homogenization buffer, and gel samples were prepared by adding boiling sample buffer. Samples were separated on a 5–20% SDS-PAGE gel and immunoblotted as described above or Coomassie stained.

Immunofluorescence Microscopy

Frozen sections of rat retina were prepared and stained as described in Hasson & Mooseker (1994). Coverslip grown cells were processed for immunofluorescence in six-well plates as follows: Cells were washed in PBS with 100 μ M CaCl_2 and 1 mM MgCl_2 (PBS-CM) before fixation at room temperature for 60 mins in 4% electron microscopy grade paraformaldehyde (PFA; EM Sciences) in PBS-CM. Fixed cells were permeabilized by treatment for 5 mins at -20°C in methanol. Coverslips were blocked for 30 mins at room temperature with 3% Bovine Serum Albumin (BSA) and 1% Normal Donkey Serum (NDS) in PBS. Primary antibodies were diluted in PBS+ 3% BSA + 1% NDS and incubated with coverslips for 2 hours at 37°C . Coverslips were washed three times for 15 mins in 0.5% BSA-PBS before incubation with secondary antibodies (diluted 1:100 in PBS+ 3% BSA + 1% NDS) for 2 hours at 37°C . Coverslips were washed 3 times for 15 mins in 0.5% BSA-PBS then mounted in Vectashield immunofluorescence mounting media (Vector Labs) and sealed with nail polish to minimize photobleaching.

Samples were observed with a filter free laser scanning confocal microscope (Leica SP2-AOBS) equipped with a 100x/1.32 NA Plan Apo oil objective or with a Leica DMR light microscope fitted with an ORCA Digital Camera. Bleed-through during conventional light microscopy was minimized with the use of both excitation and emission filters and confirmed in control stainings lacking primary antibodies. Images were captured using either Leica TCS software for confocal or OpenLab for conventional light microscopy. All digital images were analyzed using Adobe Photoshop and percent overlap was quantified by scoring individual cathepsin-D positive lysosomes for the presence or absence of myosin-VIIa stain.

Drug Treatments

Cytochalasin D (CD; ICN) was stored at a stock concentration of 500mM in DMSO at -20°C and diluted to 5 μ M in cell culture media. ARPE-19 CELLS on coverslips were incubated with CD or equivalent amount of DMSO for mock treatment at 37°C for 15 mins. Coverslips were then rinsed twice in PBS-CM and processed for immunofluorescence as described previously. Nocodazole (ICN) was stored at a stock concentration of 5 mg/ml in DMSO at -20°C and diluted to 33 μ M in cell culture media. ARPE-19 cells were incubated with nocodazole or equivalent amount of DMSO for mock treatment at 37°C for 2h. Coverslips were processed for immunofluorescence as described above.

Isolation of lysosomes from ARPE-19 cells: Metrizamide discontinuous gradient

A procedure was modified from (Schutt et al. 2002). Confluent differentiated ARPE-19 cultures from ten T-75 flasks were rinsed twice with room temperature PBS, then twice with room temperature HK buffer (10mM HEPES, 250mM sucrose, 10mM KCl, pH 7.4). Cells were harvested by scraping into ice-cold HEK buffer (10mM HEPES, 10mM EDTA, 10mM KCl, pH 7.4) plus 250mM sucrose and centrifugation at 2800xg for 15 mins. The cell pellets were resuspended in 2mls of HEK buffer with protease inhibitors. Cells were homogenized with 35 strokes of a tight-fitting dounce. A postnuclear supernatant (PNS) was prepared by low-speed centrifugation at 1300g for 5 mins at 4°C . To maximize the yield of cytoplasmic organelles, nuclear pellets (NP) were resuspended in HEK buffer plus 250mM sucrose, dounced again and a second PNS prepared by centrifugation at 1,300g for 5 mins at 4°C . The two PNS fractions were combined and layered on a discontinuous density gradient consisting of 1.8ml 40% metrizamide, 1.8ml 20% metrizamide, 4.7ml 6% Percoll in HEK buffer and centrifuged in a Beckman SW41 Ti rotor for 15 min at 50,000g. The lysosomes are enriched at the 20/40% metrizamide interface in a milky, white band. 1ml fractions were collected from the bottom of the gradient and analyzed by SDS-PAGE and immunoblot.

Isolation of lysosomes from ARPE-19 cells: Self-generated Optiprep gradient

Postnuclear supernatants were prepared as described above except the homogenization buffer was HEMK (10mM HEPES, 1mM K-EGTA, 10mM MgCl₂, 10mM KCl, 5mM ATP, 100µg/ml Bovine Serum Albumin, pH 7.4) plus 250mM sucrose and protease inhibitors as mentioned above. The PNS was adjusted to 20% Optiprep (60% iodixanol solution; Axis Shield) and loaded into 5 ml Optiseal tubes for the Beckman VTi65.2 rotor. Gradients were centrifuged at 350,000g for 3h at 4° C. 0.25ml fractions were collected with a micropipette from the bottom of the gradient and analyzed by SDS-PAGE and immunoblot.

Extraction and proteolysis of lysosomal membranes

Fractions enriched for lysosomes from Optiprep gradients were pooled and 50µl samples were used for extraction or trypsin digestion experiments. To pellet the lysosomes, samples were diluted 1:1 with HEMK sucrose buffer and centrifuged at 20,000xg for 20 mins at 4° C. For extraction studies, organelle pellets were resuspended in HEMK sucrose buffer alone, or in HEMK sucrose containing 1% Tx-100, 1.6M Urea, or 1M NaCl. Samples were placed on ice for 30 mins then layered over a 0.5 ml 50% sucrose cushion and spun at 20,800xg for 20mins to separate proteins dissociated from the organelles. Supernatants and pellets were analyzed by SDS-PAGE and immunoblot.

For trypsinization studies, pelleted lysosomes were resuspended in HEMK sucrose buffer alone or HEMK sucrose buffer containing 100 µg/ml trypsin (TPCK-treated in 50mM Tris, pH 8; Sigma) or 100 µg/ml trypsin pretreated with 2 mg/ml soybean trypsin inhibitor (Sigma). Samples were incubated at room temperature for 30 mins. Trypsinization was stopped by addition of soybean trypsin inhibitor to 2 mg/ml. Digested proteins were separated from intact lysosomes by centrifugation through a 50% sucrose cushion and analyzed as described above.

RESULTS

Expression of Myosin-VIIa in retina and cultured RPE

Myosin-VIIa is only expressed in epithelial cell types in a subset of tissues (Hasson et al. 1995; Sahly et al. 1997; Wolfrum et al. 1998). We evaluated a series of RPE sources for myosin-VIIa expression and identified ARPE-19 cells as a human cultured cell line that expressed this protein. Both an affinity purified rabbit polyclonal antibody (Hasson et al. 1995) and newly created mouse monoclonal antibodies (see materials and methods) detected a 240kDa band in human retina (Fig. 1B) and ARPE-19 cells (Fig. 1C; and data not shown) in immunoblot analysis. This band was confirmed as myosin-VIIa in immunoprecipitation studies (Fig. 1D); the 138-1 mouse monoclonal antibody to myosin-VIIa (mmyo7a) detected myosin-VIIa immunoprecipitated with the affinity purified rabbit antibody (rmyo7a). In addition, the immunoprecipitated band was positively identified as myosin-VIIa by tandem mass spectrometry analysis (data not shown).

Myosin-VIIa expression has been reported in liver (Hasson et al., 1995), and immunoblot evaluation of HepG2 cells, a human epithelial hepatocellular carcinoma cell line, revealed that this cell line also expressed myosin-VIIa (Fig. 1C). Scanning densitometry suggested HepG2 cells expressed 20% the level of full-length myosin-VIIa as compared to ARPE-19 cells. HeLa cells, which are fibroblastic and are not predicted to express myosin-VIIa based on tissue analyses, did not express detectable myosin-VIIa protein (Fig. 1C).

Myosin-VIIa is highly susceptible to proteolytic enzymes. In HepG2 and ARPE-19 cells, both the polyclonal and monoclonal myosin-VIIa antibodies recognized a lower molecular weight band at ~80 kD (Fig. 1C). The 80kDa protein was also immunoprecipitated with the polyclonal myosin-VIIa antibody, and this immunoprecipitated species was detected with the both the

polyclonal and monoclonal antibody (Fig. 1D). We conclude that the extra band commonly seen in immunoblots is likely a degradation product or an uncharacterized splice form of myosin-VIIa, since all the antibodies prepared recognize both species. Here we present a detailed characterization of the 138-1 monoclonal antibody (m α myo7a).

Localization of Myosin-VIIa to lysosomes in retina pigment epithelia

Several studies have found that myosin-VIIa is enriched in the apical region of the RPE (Hasson et al., 1995; El-Amraoui et al., 1996; Gibbs et al., 2004). Both traditional light microscopy and confocal microscopy of rat retina cryosections revealed that, like rabbit antibodies to myosin-VIIa, the 138-1 monoclonal anti-myosin-VIIa antibody (m α myo7a) specifically recognized myosin-VIIa in RPE (Fig. 2). Higher magnification revealed that myosin-VIIa was localized to apical punctate structures resembling a vesicular compartment directly above the nucleus. The apical region of the RPE contains abundant melanosomes, lysosomes and phagosomes (Bosch et al., 1993) and indeed myosin-VIIa has been shown to be associated with melanosomes in mouse RPE (Gibbs et al., 2004). However, myosin-VIIa has also been implicated in the late stages of ROS phagocytosis (Gibbs et al., 2003). Therefore, we examined in more detail the subcellular localization of myosin-VIIa utilizing both 8-week differentiated ARPE-19 cells and subconfluent ARPE-19 cells as our model system.

Immunofluorescence staining of differentiated (Fig. 3A) and subconfluent (Fig. 3B) ARPE-19 cells with the monoclonal anti-myosin-VIIa antibody revealed that myosin-VIIa is enriched on an array of organelles adjacent to the nucleus, with some vesicles distributed throughout the cytoplasm. To determine if these represented a known vesicular compartment, we double stained ARPE-19 cells with antibodies to myosin-VIIa and organellar markers, including markers for the early endosomes (EEA1, Rab5), and Golgi (TGN38, β -cop) (data not shown). Confocal microscopy revealed that none of these markers overlapped with the myosin-VIIa-associated compartment. However, we found that a majority of the myosin-VIIa-associated organelles overlapped with cathepsin D, a marker for lysosomes (Fig. 3A-B).

In the undifferentiated ARPE-19 cells, cathepsin D overlapped with myosin-VIIa on the vesicles just outside of the perinuclear area where the vesicles are less dense (Fig. 3B: d-f). Quantitation revealed that ~65% of the cathepsin D positive lysosomes also stained for myosin-VIIa. In differentiated ARPE-19 cells, however, essentially all lysosomes were decorated with the 138-1 monoclonal antibody (Fig. 3A). The diameter of these myosin-VIIa positive lysosomes was on average 0.5 μ m. Association of myosin-VIIa with lysosomes was not unique to RPE. In HepG2 cells and the Cos-7 monkey kidney cell line, myosin-VIIa also overlapped with lysosomal markers (Fig. 3C). Myosin-VIIa's association with lysosomes may therefore be a general phenomenon in all myosin-VIIa expressing cells.

Localization of Myosin-VIIa to lysosomes is Rab7 independent

In order to verify that the myosin-VIIa-decorated vesicles were indeed lysosomes, we compared their location to Rab7, which associates with both late endosomes and lysosomes (Fig. 4A). Rab7 is a small GTPase regulator of lysosome (Bucci et al. 2000; Meresse et al. 1995; Vitelli et al. 1997), late endosome (Feng et al. 1995) and melanosome trafficking (Gomez et al. 2001). ARPE-19 cells transfected with a wildtype version of Rab7 fused to the green fluorescent protein (GFP-Rab7wt) were stained for myosin-VIIa and, similar to the colocation seen with cathepsin D antibodies, the majority of the myosin-VIIa-positive vesicles were decorated with GFP-Rab7wt (Fig. 4A). Control experiments staining for Lamp1, Lamp2 and Cathepsin D confirmed the lysosomal localization of GFP-Rab7wt (data not shown). Not all the Rab7 associated organelles stained with antibodies to myosin-VIIa, however, perhaps reflecting the fraction of GFP-Rab7wt that is associated with late endosomes.

The colocalization of myosin-VIIa and Rab7 suggested that myosin-VIIa targeting to lysosomes could be Rab7 dependent, as myosin-VIIa has been shown to associate with Rab effectors in other systems (El-Amraoui et al. 2002; Fukuda and Kuroda 2002). To test this hypothesis, we evaluated whether mistargeting of myosin-VIIa occurred in the presence of a dominant active Rab7(Q67L, GTP-bound), or a dominant negative Rab7(T22N, GDP-bound). Cells expressing dominant active GFP-Rab7(Q67L) exhibit excessive fusion of lysosomes leading to enlarged lysosomal vesicles aggregated near the nucleus (Bucci et al. 2000; Vitelli et al. 1997). In GFP-Rab7(Q67L) expressing ARPE-19 cells, myosin-VIIa aggregated near the nucleus, where it overlapped with GFP-Rab7(Q67L) (Fig. 4B). This suggested that myosin-VIIa targeting to lysosomes was not perturbed by increased fusion and aggregation of these lysosomes, but did not rule out a role for Rab7 in the recruitment of myosin-VIIa to lysosomes.

We next evaluated the effect of dominant negative Rab7(T22N) expression on myosin-VIIa location (Fig. 4C). Locked in the GDP-bound form, Rab7(T22N) is not recruited to the lysosome surface and its overexpression leads to a defect in lysosome fusion (Bucci et al. 2000; Vitelli et al. 1997). As predicted, overexpression of GFP-Rab7(T22N) in ARPE-19 cells resulted in a fragmentation of lysosomes into smaller vesicles. Although smaller in size, these lysosomes are still functional (Bucci et al. 2000; Vitelli et al. 1997) and contain cathepsin D (Fig 4C). Staining for myosin-VIIa in cells overexpressing GFP-Rab7(T22N) revealed that myosin-VIIa was associated with the dispersed lysosomes. In untransfected cells, however, myosin-VIIa remained associated with lysosomes clustered near the nucleus (Fig. 4C). Quantitation of overlap between myosin-VIIa and cathepsin D in cells expressing GFP-Rab7(T22N), GFP-Rab7(Q67L) or GFP-Rab7(wt) revealed that under all conditions, approximately 65% of the distinct cathepsin D-positive lysosomes also stained for myosin-VIIa. Therefore, association of myosin-VIIa with lysosomes is independent of Rab7.

Myosin-VIIa association with lysosomes is independent of the cytoskeleton

To determine whether the actin and microtubule cytoskeleton were required for myosin-VIIa targeting to lysosomes, we treated ARPE-19 cells with both cytochalasin D, to depolymerize actin and nocodazole, to depolymerize microtubules (Fig. 5). Previous studies have shown that long range lysosomal motility occurs along the microtubule cytoskeleton (Matteoni and Kreis 1987). Treatment of ARPE-19 cells with 33 μ M nocodazole for 2h led to depolymerization of microtubules. There was only a diffuse staining with a tubulin-specific antibody in treated cells as compared to the filamentous microtubule staining seen in untreated cells (Fig. 5A). As expected, this perturbation of the microtubule network led to a repositioning of the lysosomes. Lysosomes in mock treated cells were highly concentrated in the perinuclear area, whereas in nocodazole treated cells lysosomes were dispersed in clusters throughout the cell body. Staining for myosin-VIIa in nocodazole treated cells showed that myosin-VIIa remained associated with the dispersed lysosome clusters, suggesting that targeting of myosin-VIIa to lysosomes is independent of microtubules.

Since myosin-VIIa is an actin-dependent motor, actin association may be necessary for its targeting to lysosomes. To test this, we treated ARPE-19 cells with 5 μ M cytochalasin D for 15mins to disrupt the actin cytoskeleton. This treatment led to an obvious loss of cortical actin and stress fibers without affecting the gross morphology of the cells (Fig. 5B). Staining with cathepsin D showed that actin depolymerization did not affect lysosomal organization nor myosin-VIIa's association with lysosomes. Myosin-VIIa is therefore not the primary motor for lysosome positioning in the RPE, supporting previous statements that the organization of lysosomes in *shaker-1* RPE is normal (Gibbs et al. 2003). Instead, myosin-VIIa may play a specialized role in directing lysosome motility or fusion.

Myosin-VIIa copurifies with lysosomes

To confirm that myosin-VIIa is a lysosome-associated motor, we purified lysosomes from differentiated ARPE-19 cells. Postnuclear extracts derived from confluent cultures of ARPE-19 cells were sedimented at high speed through a metrizamide/percoll density gradient (Fig. 6A). Purification of the lysosomes was determined by immunoblot analysis using antibodies against cathepsin D and myosin-VIIa (Fig. 6B) and quantified by scanning densitometry (Fig. 6C). As judged by cathepsin D content, lysosomes were enriched at the 20/40% metrizamide interface and in the 20% metrizamide fractions, fractions 2–4 (Fig. 6B). These fractions were relatively free of contamination by soluble proteins as demonstrated by the lack of GAPDH, a soluble protein marker (Fig. 6B). Myosin-VIIa cofractionated with the lysosomes in fractions 2 and 4 and densitometry (Fig. 6C) revealed that ~11% of the soluble myosin-VIIa was found in these lysosome-enriched fractions.

Although the metrizamide gradient gave highly purified lysosomes suitable for analytical purposes, the total recovery of lysosomes in these purified peak samples was very low making this procedure impractical for use in further biochemical analyses. In order to improve the total yield of lysosomes, we used a self-generated 20% optiprep gradient (Fig. 6D). Separation in this type of gradient is based on buoyant density of organelles with the densest material found at the bottom of the gradient. Soluble proteins and dense organelles settle towards the bottom at 1.14–1.17 g/ml, whereas lysosomes and other buoyant membranes float near the top of the gradient between 1.08–1.1 g/ml.

In 20% gradients, myosin-VIIa is resolved into two populations. The majority is found near the top of the gradient with the buoyant vesicles in fractions 15–18 (Fig 6E). Lamp1, a marker for lysosomes, also peaked in fractions 15–18, confirming that these fractions contain lysosomes. A population of myosin-VIIa in fractions 1–5 represented the soluble pool (Fig. 6E). A minor subpopulation of denser Lamp1 positive membranes was found in fractions 6–9, which may reflect a population of late endosomes or denser lysosomes. Overall, this fractionation is consistent with our immunofluorescence data showing that myosin-VIIa is on a subset of lysosomes (Fig. 3).

The lysosomes were well separated from mitochondria on Optiprep gradients. Mitochondria were found primarily at the bottom of the gradient as Hsp60, a mitochondrial matrix protein, was enriched in fractions 4–7 with only a minor amount found at the top of the gradient (Fig. 6E). This light mitochondrial fraction may contain mitochondria fragmented during homogenization or may reflect a normal variation in the mitochondria of ARPE-19 cells.

Other vesicular compartments such as early endosomes (as judged by the presence of the endosome protein, EEA1) and Golgi-derived vesicles (as judged by β -cop; data not shown) also fractionated near the top of the gradient (Fig. 6E). However, myosin-VIIa does not colocalize with these markers by immunofluorescence suggesting that myosin-VIIa is not associated with these organelles. In sum, the cofractionation of myosin-VIIa and lysosomes through two different gradient steps suggests that myosin-VIIa is associated with lysosomes or a lysosome-like compartment.

Myosin-VIIa is a peripheral membrane protein

In order to verify that myosin-VIIa is associated with the surface of lysosomes, we tested its ability to remain associated with the organelles under various extraction and proteolytic conditions. We subjected Optiprep membrane fractions enriched for myosin-VIIa and lysosomal markers to 1% Triton X-100 detergent, to solubilize all membrane proteins, to 1.6M urea to denature all proteins not protected by membranes and to 1M salt to strip organelles of peripheral membrane proteins. As shown in Fig. 7A, myosin-VIIa's interaction with organelles

is sensitive to detergent, urea and salt, suggesting that it is a surface protein, whereas Lamp1, an integral membrane protein can only be removed from lysosomal membranes by detergent solubilization.

To further confirm that myosin-VIIa is peripherally associated with lysosomes, we tested its accessibility to proteases. If myosin-VIIa was associated with a receptor on the cytoplasmic surface of lysosomes, then it would be sensitive to trypsin digestion. However if myosin-VIIa resided in the lysosomal membrane or lumen, then it should remain protected in a trypsin assay. Partially purified lysosomes were concentrated and resuspended in either HEMK sucrose buffer alone, 100 µg/ml trypsin or 100 µg/ml trypsin pretreated with trypsin inhibitor. As seen in Fig. 7B, myosin-VIIa is indeed sensitive to trypsin, whereas Lamp1, an integral membrane protein, is protected from proteolysis. Pretreatment with trypsin inhibitor completely inhibited proteolysis of myosin-VIIa showing that loss of myosin-VIIa is due specifically to trypsin digestion. These data suggest that myosin-VIIa is associated with the surface of lysosomes where it may function as a lysosomal motor.

DISCUSSION

Outer segment phagocytosis is essential for retina function as defects result in photoreceptor cell death and progressive retinal degeneration much like that seen in Usher patients (Mullen and La Vail 1976). *Shaker-1* retinal epithelia which lack myosin-VIIa exhibit normal levels of outer segment binding and phagosome formation, however phagosomes containing ROSs accumulate in apical regions because their transport to basal regions, where digestion is thought to occur, is inhibited (Gibbs et al. 2003). It is hypothesized that this change in ROS digestion dynamics over time would lead to the retinal degeneration seen in USH1B patients.

A second defect seen in myosin-VIIa null RPE is that melanosomes, the lysosome related pigment granules, are mispositioned; they remain basal and do not enter apical actin-rich domains (Liu et al. 1998). Although melanosomes have not been shown definitively to have degradative properties, they are able to fuse with phagosomes and express many lysosomal hydrolases including β-glucuronidase, β-galactosidase, β-hexosaminidase and cathepsin B and L (Diment et al. 1995). Fusion of phagosomes with melanosomes is most prominently observed in cells overloaded with ROS or undigestible products (Orlow 1995; Schraermeyer et al. 1999). Recent studies show that a large fraction of myosin-VIIa in primary RPE cells is found on melanosomal membranes (Gibbs et al. 2004). Our studies with a new myosin-VIIa antibody have found that a population of myosin-VIIa also associates specifically with lysosomes, an organelle known to be required for ROS digestion.

We propose that myosin-VIIa has the capability to associate with organelles in the melanosome-lysosome pathway. In cultured cell lines such as ARPE-19 or COS-7, which produce very few or no melanosomes, myosin-VIIa associates predominantly with lysosomes; in retina tissue, however, myosin-VIIa is more evident on melanosomes. Lysosome organization is maintained by the microtubule network with its motors, as has been shown for other cell types (Matteoni and Kreis 1987). Gibbs et al. (2004) suggested that myosin-VIIa may play a role in short range movements and temporary tethering of melanosomes. Perhaps this is also true for myosin-VIIa found on lysosomes.

Overall, a complex series of phenotypes is seen in *shaker-1* RPE including mislocation of organelles and a delay in ROS digestion. We suggest that these phenotypes can be explained if myosin-VIIa is an auxiliary lysosome and melanosome motor that directs or regulates transport of digestion organelles out to the apical processes during periods of intense phagocytic activity. A microtubule dependent mechanism is responsible for the rapid relocation of phagosomes from the apical processes back to the base of the cell (Herman and Steinberg

1982). This microtubule dependent transport is not seen in *shaker-1* mice, however, as before transport can occur the ROS containing phagosomes must first sequentially fuse with apically located endosomes, lysosomes, and melanosomes (reviewed in Vieira et al. 2002; Bosch et al. 1993). Therefore, as a result of melanosome and lysosome mispositioning, there is also mispositioning of phagosomes resulting in an apparent delay in ROS digestion.

This role for myosin-VIIa is consistent with its characterized motor properties. At the cell cortex, actin filaments are anchored with their plus ends at the plasma membrane and their minus ends facing inwards. Microvilli, such as those evident on the pigment epithelia apical surface, are comprised of bundled actin filaments that are oriented in this fashion with minus ends inwards and plus ends out at the tips. Myosin-VIIa, like conventional myosin, travels toward the plus ends of the actin filament (Inoue and Ikebe 2003; Udovichenko et al. 2001). These in vitro motility studies are consistent with a role for myosin-VIIa in the outward movement of cargo such as lysosomes. It will be interesting to evaluate whether lysosome motility in *shaker-1* RPE is altered.

Acknowledgements

We thank Wen Hong Zhu for performing tandem mass spectrometry analysis. This work was supported by NIH core grant EY12598, and NIH grant (R01-EY12695) to T.H.

References

- Ahmed ZM, Riazuddin S, Riazuddin S, Wilcox ER. The molecular genetics of Usher syndrome. *Clin Genet* 2003;63:431–444. [PubMed: 12786748]
- Bosch E, Horwitz J, Bok D. Phagocytosis of outer segments by retinal pigment epithelium: phagosome-lysosome interaction. *J Histochem Cytochem* 1993;41:253–263. [PubMed: 8419462]
- Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B. Rab7: A key to lysosome biogenesis. *Mol Biol Cell* 2000;11:467–480. [PubMed: 10679007]
- Campochiaro PA, Hackett SF, Conway BP. Retinoic acid promotes density-dependent growth arrest in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 1991;32:65–72. [PubMed: 1846132]
- Chaitin MH, Hall MO. Defective Ingestion of Rod Outer Segments by Cultured Dystrophic Rat Pigment Epithelial Cells. *Invest Ophthalmol Vis Sci* 1983;24:812–820. [PubMed: 6345445]
- Chen ZY, Hasson T, Kelley PM, Schwender BJ, Schwartz MF, Ramakrishnan M, Kimberling WJ, Mooseker MS, Corey DP. Molecular cloning and domain structure of human myosin-VIIa, the gene product defective in Usher syndrome 1B. *Genomics* 1996;36:440–448. [PubMed: 8884267]
- Diment S, Eidelman M, Rodriguez GM, Orlow SJ. Lysosomal hydrolases are present in melanosomes and are elevated in melanizing cells. *J Biol Chem* 1995;270:4213–5. [PubMed: 7876179]
- Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res* 1996;62:155–169. [PubMed: 8698076]
- El-Amraoui A, Schonn J-S, Kussel-Andermann P, Blanchard S, Desnos C, Henry J-P, Wolfrum U, Darchen F, Petit C. MyRIP, a novel Rab effector, enables myosin VIIa recruitment to retinal melanosomes. *EMBO Rep* 2002;3:1–8.
- Feng Y, Press B, Wandinger-Ness A. Rab 7: an important regulator of late endocytic membrane traffic. *J Cell Biol* 1995;131:1435–1452. [PubMed: 8522602]
- Finnemann SC, Leung LW, Rodriguez-Boulan E. The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipids by the retinal pigment epithelium. *Proc Natl Acad Sci USA* 2002;99:3842–3847. [PubMed: 11904436]
- Frangioni JV, Neel BG. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. *Anal Biochem* 1993;210:179–187. [PubMed: 8489015]
- Fukuda M, Kuroda TS. Slac2-c (synaptotagmin-like protein homologue lacking C2 domains-c), a novel linker protein that interacts with Rab27, myosin Va/VIIa, and actin. *J Biol Chem* 2002;277:43096–43103. [PubMed: 12221080]

- Gibbs D, Azarian SM, Lillo C, Kitamoto J, Klomp AE, Steel KP, Libby RT, Williams DS. Role of myosin VIIa and Rab27a in the motility and localization of RPE melanosomes. *J Cell Sci* 2004;117:6473–83. [PubMed: 15572405]
- Gibbs DJK, Williams DS. Abnormal phagocytosis by retinal pigmented epithelium that lacks myosin VIIa, the Usher syndrome 1B protein. *Proc Natl Acad Sci U S A* 2003;100:6481–6486. [PubMed: 12743369]
- Gomez PF, Luo D, Hirosaki K, Shinoda K, Yamashita T, Suzuki J, Otsu K, Ishikawa K, Jimbow K. Identification of rab7 as a melanosome-associated protein involved in the intracellular transport of tyrosinase-related protein 1. *J Invest Dermatol* 2001;117:81–90. [PubMed: 11442753]
- Hasson T, Heintzelman MB, Santos-Sacchi J, Corey DP, Mooseker MS. Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. *Proc Natl Acad Sci USA* 1995;92:9815–9819. [PubMed: 7568224]
- Hasson T, Mooseker MS. Porcine myosin-VI: Characterization of a new mammalian unconventional myosin. *J Cell Biol* 1994;127:425–440. [PubMed: 7929586]
- Hasson T, Walsh J, Cable J, Mooseker MS, Brown SDM, Steel KP. Effects of shaker-1 mutations on myosin-VIIa protein and mRNA expression. *Cell Motil Cytoskel* 1997;37:127–138.
- Herman KG, Steinberg RH. Phagosome degradation in the tapetal retinal pigment epithelium of the opossum. *Invest Ophthalmol Vis Sci* 1982;23:291–304. [PubMed: 7107157]
- Inoue A, Ikebe M. Characterization of the motor activity of mammalian myosin VIIA. *J Biol Chem* 2003;278(7):5478–87. [PubMed: 12466270]
- Jahraus A, Egeberg M, Hinner B, Habermann A, Sackman E, Pralle A, Faulstich H, Rybin V, Defacque H, Griffiths G. ATP-dependent membrane assembly of F-actin facilitates membrane fusion. *Mol Biol Cell* 2001;12:155–770. [PubMed: 11160830]
- Liu X, Ondek B, Williams DS. Mutant myosin VIIa causes defective melanosome distribution in the RPE of shaker-1 mice. *Nature Genet* 1998;19:117–118. [PubMed: 9620764]
- Liu X, Udovichenko IP, Brown SDM, Steel KP, Williams DS. Myosin VIIa participates in opsin transport through the photoreceptor cilium. *J Neurosci* 1999;19:6267–6274. [PubMed: 10414956]
- Lund RD, Adamson P, Sauve Y, Keegan DJ, Girman SV, Wang S, Winton H, Kanuga N, Kwan AS, Beauchene L, et al. Subretinal transplantation of genetically modified human cell lines attenuates loss of visual function in dystrophic rats. *Proc Natl Acad Sci USA* 2001;98(17):9942–7. [PubMed: 11504951]
- Matteoni R, Kreis TE. Translocation and clustering of endosomes and lysosomes depends on microtubules. *J Cell Biol* 1987;105(3):1253–65. [PubMed: 3308906]
- Meresse S, Gorvel JP, Chavrier P. The rab7 GTPase resides on a vesicular compartment connected to lysosomes. *J Cell Sci* 1995;108:3349–3358. [PubMed: 8586647]
- Mullen RJ, La Vail MM. Inherited retinal dystrophy: Primary defect in pigment epithelium determined with experimental rat chimeras. *Science* 1976;192:799–801. [PubMed: 1265483]
- Orlow SJ. Melanosomes are specialized members of the lysosomal lineage of organelles. *J Invest Dermatol* 1995;105(1):3–7. [PubMed: 7615972]
- Rakoczy PE, Zhang D, Robertson T, Barnett NL, Papdimitriou J, Constable IJ, Lai CM. Progressive age-related changes similar to age-related macular degeneration in a transgenic mouse model. *Am J Pathol* 2002;161:1515–1524. [PubMed: 12368224]
- Raposo G, Cordonnier M-N, Tenza D, Menichi B, Durrbach A, Louvard D, Coudrier E. Association of myosin I alpha with endosomes and lysosomes in mammalian cells. *Mol Biol Cell* 1999;10:1477–1494. [PubMed: 10233157]
- Sahly I, El-Amraoui A, Abitbol M, Petit C, Dufier J-L. Expression of myosin VIIA during mouse embryogenesis. *Anat Embryol* 1997;196:159–170. [PubMed: 9278160]
- Schraermeyer U, Peters S, Thumann G, Kociok N, Heimann K. Melanin granules of retinal pigment epithelium are connected with the lysosomal degradation pathway. *Exp Eye Res* 1999;68(2):237–45. [PubMed: 10068489]
- Schutt F, Bergmann M, Holz FG, Kopitz J. Isolation of intact lysosomes from human RPE cells and effects of A2-E on the integrity of the lysosomal and other cellular membranes. *Graefes Arch Clin Exp Ophthalmol* 2002;240:983–988. [PubMed: 12483320]

- Thumann G, Bartz-Schmidt KU, Kociok N, Heimann K, Schraemeyer U. Ultimate fate of rod outer segments in the retinal pigment epithelium. *Pigment Cell Res* 1999;12(5):311–5. [PubMed: 10541040]
- Tian J, Ishibashi K, Handa JT. The expression of native and cultured RPE grown on different matrices. *Physiol Genomics* 2004;17(2):170–82. [PubMed: 14982971]
- Turowski P, Adamson P, Sathia J, Zhang JJ, Moss SE, Aylward GW, Hayes MJ, Kanuga N, Greenwood J. Basement membrane-dependent modification of phenotype and gene expression in human retinal pigment epithelial ARPE-19 cells. *Invest Ophthalmol Vis Sci* 2004;45(8):2786–94. [PubMed: 15277505]
- Udovichenko IP, Gibbs D, Williams DS. Actin-based motor properties of native myosin VIIa. *J Cell Sci* 2001;115:445–450. [PubMed: 11839794]
- Vieira OV, Botelho RJ, Grinstein S. Phagosome maturation: aging gracefully. *Biochem J* 2002;366:689–704. [PubMed: 12061891]
- Vitelli R, Santillo M, Lattero D, Chiariello M, Bifulco M, Bruni CB, Bucci C. Role of the small GTPase Rab7 in the late endocytic pathway. *J Biol Chem* 1997;272:4391–4397. [PubMed: 9020161]
- Weil D, Blanchard S, Kaplan J, Guilford P, Gibson F, Walsh J, Mburu P, Valera A, Levilliers J, Weston MD, et al. Defective myosin-VIIA gene responsible for Usher syndrome type 1B. *Nature* 1995;374:60–61. [PubMed: 7870171]
- Wolfrum U, Liu X, Schmitt A, Udovichenko IP, Williams DS. Myosin VIIa as a common component of cilia and microvilli. *Cell Motil Cytoskel* 1998;40:261–271.
- Zinn KM, Marmor MF, editors. 1979. *The retinal pigment epithelium*. Cambridge, Mass.: Harvard University Press.

Figure 1. Characterization of new myosin-VIIa-specific monoclonal antibodies

(A) Schematic of Myosin-VIIa showing region containing the epitopes recognized by our monoclonal and polyclonal myosin-VIIa antibodies. (B) 1 μg retina protein was separated on a 5–20% SDS-PAGE and probed with polyclonal rabbit (rαmyo7a; right lane) or monoclonal antibodies (138-1, -2, -3 and -4) to myosin-VIIa. A single 240 kD band was detected with all five antibodies. (C) 10 μg total protein from HepG2, ARPE-19, and HeLa cells was separated on a 5–20% SDS-PAGE and probed with both polyclonal rabbit (rαmyo7a) and monoclonal 138-1 (mαmyo7a) antibodies to myosin-VIIa. The 240kDa band is demarcated with an arrowhead. A lower ~80 kD band is most likely a degradation product of myosin-VIIa. (D) ARPE-19 cell extracts were incubated with rαmyo7a antibodies or rabbit non-immune (ni) IgG, and the immunocomplexes were analyzed by Western blot with rαmyo7a and mαmyo7a antibodies. Both antibodies recognize 240kD proteins (arrowhead) in the anti-myo7a immunoprecipitates. The position of the rabbit IgG is demarcated by an asterisk.

Figure 2. Localization of myosin-VIIa in rat retina

Adult pigmented rat retina cryosections were stained with m α myo7a antibody and Hoechst. Sections incubated with Hoechst and the secondary antibody alone served as the negative control to visualize tissue autofluorescence. Low magnification images (A) and high magnification images (C) were captured by optical sectioning with a laser scanning confocal microscope. The intermediate magnification images (B) were captured using conventional light microscopy. In all cases, the only specific staining was found in the RPE (R) layer. ONL (outer nuclear layer); IS (photoreceptor inner segments); OS (photoreceptor outer segments). In the overlaid images in (B) and (C), myosin-VIIa or the no primary control is shown red and nuclei are in blue. Bar=10 μ m.

Figure 3. A population of myosin-VIIa associates with lysosomes

Differentiated ARPE-19 cells (A), undifferentiated ARPE-19 cells (B), HepG2 cells (C: a-c) and Cos-7 cells (C: d-f) were paraformaldehyde-fixed and methanol permeabilized, then double stained with mαmyo7a antibody (red) and antibodies to the lysosomal enzyme, cathepsin D (green). For some samples, nuclei were stained with Hoescht (blue in A: c,f). A phase contrast image of HepG2 cells is shown in (C) panel c. The differentiated ARPE-19 cells were imaged using confocal microscopy whereas conventional light microscopy was used to image subconfluent ARPE-19, HepG2 and Cos-7 cells. Areas boxed in panels a-c of each figure are enlarged in the corresponding d-f panels. Filled arrows in A and B point to regions of

overlap between myosin-VIIa and cathepsin D (yellow). The hollow arrows in B point to cathepsin-D-positive lysosomes that do not have associated myosin-VIIa. Bar=10 μ m.

Figure 4. Myosin-VIIa association with Rab7 positive lysosomes is not dependent on the state of Rab7

ARPE-19 cells expressing (A) GFP-Rab7wt, (B) GFP-Rab7(Q67L) and (C) GFP-Rab7(T22N) were stained with mαmyo7a antibody (red in all panels). GFP fluorescence (green) is overlaid with the myosin stain in (A) panels c & f. In (B and C) cells were also stained with antibodies to Cathepsin D (green in overlay, panels f). The boxed areas in panels (a-c) are enlarged in the corresponding (d-f) panels. Small arrows in these enlargements indicate overlapping areas. Clustering of lysosomes in a GFP-Rab7(Q67L) transfected cells (B) is indicated by a large closed arrow in panels a-c. An untransfected cell, which shows a cluster of lysosome-associated myosin-VIIa near the nucleus, is pointed out with a large hollow arrow in (C). Bar=10μm.

Figure 5. Myosin-VIIa association with lysosomes is independent of the cytoskeleton
 (A) ARPE-19 cells untreated (a-b) or treated (c-h) with 33 μ M nocodazole for 2hrs to depolymerize microtubules were fixed in paraformaldehyde and double stained with antibodies to cathepsin D (b,d,f,h) and either tubulin antibody (a,c), or m α myo7a antibody (e,g). (B) ARPE-19 cells untreated (a-b) or treated (c-h) with 5 μ M cytochalasin D for 10 mins to depolymerize actin filaments, were fixed as described above and double stained with antibodies to cathepsin D (b,d,f,h) and either phalloidin-rhodamine (a,c), or m α myo7a antibody (e,g). The boxed areas in panels (e-f) are enlarged in the corresponding (g-h) panels. Bar=10 μ m.

Figure 6. Myosin-VIIa copurifies with lysosomes

(A) Postnuclear supernatants from ARPE-19 cells were fractionated on a discontinuous metrizamide/percoll gradient. A diagram of this gradient is shown. (see Materials and Methods). (B) Samples of the homogenate (Hom), first and second post nuclear supernatants (PNS1, PNS2) and fractions 1–13, ordered from highest to lowest density, were separated on a 5–20% SDS-PAGE and probed in a western blot analysis with antibodies to myosin-VIIa (myo7a), cathepsin D (cathD) or GAPDH, a marker for soluble proteins. (C) The immunoblots shown in panel (B) were analyzed by scanning densitometry. The total signal acquired for each fraction was plotted relative to the fraction with the highest value, arbitrarily set at 100%. (D) Preparative protocol using an Optiprep gradient to purify lysosomes from the postnuclear supernatants derived from ARPE-19 cells. (E) Samples of the homogenate (Input) and fractions 1–20, ordered from highest (1.17 g/ml) to lowest (1.06 g/ml) density were separated on a 5–20% SDS-PAGE and probed in a western blot analysis with antibodies to myosin-VIIa, the lysosomal protein Lamp-1, the mitochondrial component hsp-60, the early endosome marker EEA1, and actin.

Figure 7. Myosin-VIIa is peripherally associated with lysosomes

(A) Optiprep purified lysosomes were extracted for 30 mins on ice with HEMK sucrose buffer, or buffer containing 1% Tx-100, 1.6M Urea or 1M NaCl. (B) Optiprep purified lysosomes were incubated for 30 mins at room temperature with HEMK sucrose buffer containing no trypsin, 100 μ g/ml trypsin, or 100 μ g/ml trypsin pretreated with protease inhibitors. The extracted lysosomes were isolated by centrifugation (see materials and methods), separated on a 5–20% SDS-PAGE and probed in an immunoblot analysis with either antibodies to myosin-VIIa or Lamp1.