Novel Characteristics of a Myosin Isolated from Mammalian Retinal Pigment Epithelial and Endothelial Cells*

(Received for publication, August 22, 1996, and in revised form, November 22, 1996)

Mark C. Alliegro† and Laura A. Linz
From the Department of Anatomy, Louisiana State University Medical Center, New Orleans, Louisiana 70112

The retinal pigment epithelium (RPE)† is composed of a population of cuboidal to low columnar cells sandwiched between the neural retina and the highly vascularized choroid layer of the eye. Although RPE cells are normally nonproliferative and nonmigratory, they are highly active in other respects. They are indispensable for the function and survival of their immediate neighbors, the rods and cones, and malfunction in the RPE form the basis for a variety of blinding disorders.

The RPE cell feature that is perhaps most directly related to vision is the presence of dense pigment granules in their cytoplasm. These granules absorb light that has passed through the neural layers of the retina, preventing backscatter and unwanted reflection. The RPE also performs a variety of metabolic functions in support of the neural retina. By means of tight junctions, they create the blood-ocular barrier that regulates the passage of molecules between the retina and choroidal capillaries (1). They are, therefore, primary determinants of the photoreceptor microenvironment. The RPE is also responsible for phagocytosis and disposal of shed photoreceptor outer segments (2). In the absence of this function, degeneration of the neural retina ensues. Moreover, RPE are essential for production of the visual pigment rhodopsin (3). During the visual cycle, the 11-cis-retinaldehyde chromophore of rhodopsin present in photoreceptor cells is reduced to all-trans-retinol.

To regenerate rhodopsin, the spent product is transferred to the RPE where it is isomerized to the 11-cis form once again. Directly and indirectly, therefore, the RPE is essential for vision.

As in all cellular systems, carbohydrate recognition molecules are thought to figure prominently in RPE physiology (and pathophysiology). For example, a mannose receptor present in the RPE plasma membrane has been shown to mediate phagocytosis of rod outer segments (4). Also, changes in the expression of specific carbohydrate moieties accompanying retinal pathology have been demonstrated in lectin binding studies (5, 6). However, identification of endogenous retinal lectins and delineation of the specific roles they play have been elusive. In the case of galactose-binding proteins, a useful method of isolation from cell homogenates is affinity chromatography on unconjugated Sepharose 4B (for examples, see Vasta and Marchalonsis (7) and Alliegro et al. (8)). Sepharose, being a trade name for agarose, is a linear polymer of D-galactose and 3,6-anhydrogalactose. We have used this approach to identify and isolate carbohydrate binding molecules from mammalian RPE, and this report describes the isolation and initial characterization of one such protein, called piglet (for pigment epithelial lectin). Piglet is a high $M_r$ oligomeric protein that binds avidly to Sepharose and displays several hallmark features of cytoplasmic myosin. Our data suggest that piglet is a new member of the growing myosin superfamily.

MATERIALS AND METHODS

Cell Culture—Reagents for cell culture were purchased from Life Technologies, Inc. and Sigma. Cells were cultured under routine conditions in minimal essential medium plus 15% fetal bovine serum and 100 units/ml penicillin + 100 µg/ml streptomycin (MEM-15). All cell types not purchased from American Type Culture Collection (Rockville, MD) were isolated using methods described previously (9), except D407 RPE cells (10), which were generously provided by Dr. Alberta Davis.

Protein Isolation and Characterization—Cells were harvested mechanically from Nunc (Milwaukee, WI) Delta-Square culture trays or T175 flasks after two or three rinses in ice-cold PBS. They were washed three additional times by centrifugation and resuspension in cold PBS, transferred to microcentrifuge tubes, and pelleted for 2 min at maximum speed, decanted, and frozen until approximately 8 ml of packed cells were accumulated. The thawed cells were pooled, homogenized, and sonicated in 50 mM Tris, pH 7.5, containing 1% Triton X-100, 7 mM EDTA, and 1% Me$_2$SO. The homogenate was centrifuged at 30,000 $\times$ g...
FIG. 1. RPE protein recovered from Sepharose 4B column with 8 M urea. A, elution profile; B, SDS-gel electrophoresis of the peak fraction in the presence (+) and absence (−) of dithiothreitol. Panel 1 in B, Coomassie blue-stained 7.5% polyacrylamide; panel 2, 3–10% polyacrylamide gradient. Migration of selected molecular mass markers are indicated by arrows.

for 45 min, and the supernatant was applied to a 1.5 × 2-cm column of Sepharose 4B at 4 °C. For convenience and maximum yield, the sample was usually cycled on the column overnight. To reduce the chances of nonspecific binding or precipitation within the column, the sample volume was doubled with homogenization buffer prior to affinity chromatography (halving overall protein concentration), and the column itself was preequilibrated with homogenization buffer. Following sample application the column was washed with at least 25 volumes of 50 mM Tris, 1% Triton, pH 7.5, then 50 volumes of 50 mM Tris, 1 M NaCl, and eluted with 8 M urea in 50 mM Tris.

For ATPase assays, fractions were pooled and dialyzed extensively in 50 mM Tris, pH 7.5, to remove urea. Samples were then concentrated by acetone or trichloroacetic acid precipitation, lyophilized, and resuspended in assay buffer. ATPase activity was determined in the presence of 4 mM MgCl₂ using methods described by Heinonen and Lahti (11). The phenol-sulfuric acid assay was used for estimation of carbohydrate (12). Deglycosylation experiments using neuraminidase, and O- and N-glycosidases alone and in combination were carried out as described by Matherly et al. (13). Glycosidases were purchased from Genzyme (Cambridge, MA).

Routine SDS-gel electrophoresis was performed according to the methods of Laemmli (14). Gradient gels were prepared using the manufacturer’s suggestions accompanying Hoeffer (San Francisco, CA) gradient makers. M₄ estimates under nonreducing conditions were made using the buffer system of Davies and Stark (15). Dimethyl suberimidate cross-linked standards for estimation of high M₄ proteins were purchased from Sigma.

Immunological Methods—For antibody production, purified piglet was resolved into α and β subunits on SDS-polyacrylamide gels. The α subunit was excised, periodate-treated to disrupt carbohydrate epitopes (16), and used to immunize female New Zealand White rabbits. Antibodies to platelet myosin and isoform-specific antibodies to non-muscle myosin II heavy chain (NMMHC) were gifts of Dr. Robert Adelstein. Antiserum was purchased from Sigma. Western blot techniques were performed as described by Towbin et al. (17). For immunocytochemistry, cells grown on sterile coverslips were fixed in 2.5% paraformaldehyde for 5 min, followed by 3 min in 0.5% Triton X-100 and 8 min in methanol at −20 °C. We found that antigenicity was greatly reduced using formaldehyde or glutaraldehyde fixatives. Primary antiserum was used at a dilution of 1/500 to 1/1000 in PBS plus 1.5% normal goat serum. Labeled specimens were viewed with a Leica (Heidelberg, Germany) confocal laser scanning microscope.

cDNA Analysis—A bovine endothelial cell (EC) cDNA library in λ-zap was obtained from Stratagene (La Jolla, CA) and screened with α-piglet antibodies according to standard methodologies (18). Nested, undirectional deletions were prepared from cesium chloride-purified plasmid DNA using the Promega (Madison, WI) Erase-a-Base sytem. Sequenza Version 2.0 (U. S. Biochemical Corp., Cleveland, OH) was used in cDNA sequencing. Sequences were assembled and analyzed using IBI (New Haven, CT) MacVector software. Sequence data base inquiries were made using the BLAST E-mail server (19).

RESULTS

Isolation of Sepharose-binding Polypeptides from RPE and EC—Following extensive detergent and high salt washes, RPE protein retained on the Sepharose column was eluted with 8 M urea in a single, symmetrical peak. Recovery approximated 1 mg/8 ml packed cells. This yield seems to decrease with increasing passage of cells. When peak fractions were analyzed on Coomassie blue-stained SDS-gels, two polypeptides were observed (Fig. 1) migrating with apparent molecular mass of 210 and 47 kDa (2). Both polypeptides aggregated at the stack/running gel interface in the absence of reducing agents. Gradient and low percentage acrylamide gel formats were used to resolve the aggregate as a single band with a molecular mass of 500–550 kDa, suggesting that the two polypeptides observed on reducing gels are subunits of one larger protein. Given the size of the individual subunits and their relative molar ratios estimated from gels, it is likely that the disulfide-bonded holoprotein is composed of two α (210 kDa) and two β (47 kDa) subunits.

Results of phenol-sulfuric acid assays yielded variable estimates for carbohydrate associated with the protein, ranging from 5 to 20%. However, we found that exhaustive glycosidase treatment did not alter migration in SDS-gels, so carbohydrate detected in the above assays may not be covalently bound. The protein has been tentatively named “piglet” for pigment epithelial lectin, but monosaccharide specificity has not yet been determined.

Piglet Distribution—Piglet was also isolated in similar quantities from bovine aortic EC. An additional polypeptide of 55 kDa was present in these preparations, which did not aggregate with oligomeric piglet in the absence of reducing agents. The two polyclonal antibodies generated against the α subunit of RPE piglet recognized EC piglet. The one antibody generated against EC piglet also recognized the RPE protein with equal avidity. These antibodies were used to survey a panel of other cell types for piglet expression (Fig. 2). Although present in RPE and EC from several species, immunoreactivity was not

2 A third polypeptide in these preparations, found in trace quantities and observable only on silver-stained gels, has been described in another report (38).
readily detected in any of the mesenchymal cell types examined (human and rabbit fibroblasts, human astrocytes, human glioma, and mouse myeloma). In a nonsystematic survey of available tissues, piglet was identified by Western blot in cat spleen, lung, and liver; bovine eye and kidney; mouse spleen, lung, eye, kidney, brain, and liver; and rat spleen, lung, eye, and placenta. Piglet was not detected in rat liver.

Piglet immunoreactivity was greatly affected by fixation and histological preparation. Consistent labeling of cultured cells was obtained with relatively mild paraformaldehyde fixation followed by Triton and methanol permeabilization, as described under “Materials and Methods.” Under these conditions, immunofluorescent confocal microscopy revealed a cytoplasmic, filamentous pattern of staining (Fig. 3). In human RPE the filaments appeared fine and highly woven. In EC the filaments were more prominent, resembling stress fibers. Filaments were most pronounced in the transformed RPE cell line, D407, and often exhibited a banded pattern (see Fig. 5). Regardless of cell type, method of detection, or precise dimensions, filaments were the common theme. Identical patterns were observed with all three anti-piglet antibodies in double label immunofluorescence.

Piglet Is a Myosin-like Molecule—Concurrent with these findings, we obtained a partial sequence of 1142 base pairs on piglet from a bovine cDNA clone (a-piglet 1.3) identified with our anti-piglet antibodies. Nucleotide and predicted amino acid sequences are shown in Fig. 4. The sequence shows 92% identity with the C terminus of human NMMHC. A five-amino acid (ARAAB) extension is present on the predicted C terminus of piglet. Interestingly, piglet exhibits a 67-nucleotide domain in the presumptive 3'-UTR with 65% identity to rat Munc-18, a mammalian homologue of the Caenorhabditis elegans unc-18 gene (20, 21). Unc-18 (for "uncoordinated") mutations lead to a paralytic phenotype, presumably due to a failure in acetylcholine release. The 3’-UTR for certain proteins, such as actin, are thought to be important for subcellular localization of message (as well as determining message stability). Functional implications for this similarity between the piglet and Munc-18 3’-UTRs would be purely speculative at this point.

The observed sequence identity with NMMHC was congruent with the subcellular localization just described, but other features of piglet were clearly at odds with characteristics thought typical of myosins. We therefore tested the notion that piglet is a myosin using several approaches. We first posed the question of whether piglet is co-distributed in cells with the actin cytoskeleton. Using the transformed human RPE cell line, D407, and polyclonal anti-mammalian actin antibodies, we found that this was indeed the case (Fig. 5). Actin was found, as expected, in prominent stress fibers, extending into filopodia, and in a diffuse cytoplasmic pool. Anti-piglet labeled RPE with an identical pattern, with the exception that a subpopulation of fine actin-containing filopodia seemed to be devoid of piglet immunoreactivity.

We next determined that purified piglet contains an actin-
activated ATPase activity, a hallmark of myosin. We used commercially available skeletal muscle myosin as a positive control in a colorimetric phosphomolybdate assay for inorganic orthophosphate (11). One unit of activity was defined as \( \Delta A_{495 \text{ nm}} \) of 0.001/min. Specific activity varied greatly between preparations, but our consistent finding was that piglet exhibited at least as much activity as the myosin control: 215 ± 80.5 units/mg piglet versus 103 ± 32.7 units/mg for the myosin control. This considerable activity relative to the myosin control hedges against the possibility that the ATPase activity in our piglet preparations was due to contaminating trace quantities of a conventional RPE myosin. Addition of actin (0.825 µg/µl) increased the piglet activity by 3.3-fold; myosin activity was increased by 2-fold. These figures are comparable to the levels of actin activation reported previously in the literature for a variety of myosin isoforms (for examples, see Refs. 22–24). The quantities of purified protein available did not permit a more extensive characterization of the piglet ATPase activity, particularly with regard to cation dependence.

NMMHC has been shown to exist in A and B isoforms (25–28). While often found in the same cell, these isoforms are transcribed by separate genes and may exhibit distinct subcellular localization patterns (29, 30). Isoform-specific antibodies have been generated by several laboratories and used in the analysis of NMMHC from a variety of species. In addition to its recognition by broad-spectrum antibodies generated against platelet-derived NMMHC (Fig. 6, lane 2), we have found that piglet reacts avidly with antibodies to NMMHC-A isoform. The antibody used in lane 3 of Fig. 6 is a polyclonal antibody generated against a 12-amino acid cassette found specifically in NMMHC-A. Piglet did not react with isotype B-specific antibodies, nor with antibodies to myosins V, VIIa, and IXb. The latter three are widely expressed “unconventional” myosins and comparable in molecular mass to piglet (190, 240, and 230 kDa, respectively) (31). It has been shown previously that myosin VIIa is expressed in mammalian RPE (32). Lastly, it should be noted that anti-piglet antibodies reacted poorly or not at all with NMMHC-A in rabbit (not shown) or human fibroblasts (Fig. 6B, lanes 1–4; also see Fig. 2).

**DISCUSSION**

The first molecular motor to be discovered was conventional, two-headed myosin II. Because of its quantity and easy recognition in muscle cells and its importance in cellular contraction, myosin II has been extensively studied and well characterized at the molecular and biophysical levels. Not long after the finding that myosins II were present in non-muscle cells, Acanthamoeba myosin-I was identified as the first “unconventional” myosin. Since then, and particularly in the last few years, the field has blossomed with the discovery of approximately two score of unconventional myosins and the realization that even “conventional” myosin II comes in a variety of flavors. It has also become apparent that multiple classes of myosin may be expressed in a single cell type. Bement et al. (33), for example, used a polymerase chain reaction approach to amplify 11 separate myosins in the human epithelial cell line Caco2.

A nomenclature system is just now beginning to take shape. The “myosin superfamily” is currently divided into 11 classes, some with several subclasses (31). Although no sequence data are available for at least two of the newly identified myosins (24, 34) and full sequence is available only for a limited number, several consensuses have emerged. All of these molecules include a relatively conserved N-terminal head domain within which lies the motor activity. There are possible exceptions, such as the Drosophila class III myosin, ninaC (31). The neck region of all known myosins contain a variable number of ~23-amino acid repeats known as the IQ motif. This domain is the putative site of light chain binding. Following the neck domain is the carboxyl-terminal tail region, which is generally the site of highest variability between myosin classes. In myosins II, the tail region consists primarily of coiled coil α-helix thought to permit dimer and filament formation. In other myosin classes the tail may be truncated and unsuitable for dimerization, or contain sites for membrane binding or signal transduction.

Our initial conception of RPE piglet was as a disulfide-bonded, oligomeric, carbohydrate-binding glycoprotein. We were mildly surprised to find the protein distributed intracellularly as a filamentous network, and sequence homology with

![Image](image-url)
myosin II was thoroughly unexpected. We therefore carefully reviewed and confirmed our existing data reflecting the distinctly “unmyosin-like” characteristics of piglet. First, we repeatedly found that piglet bound to Sepharose 4B and withstood extensive detergent and high salt washes. We subsequently learned that a significant portion of the bound protein withstood even 8 M urea. This was determined by binding radioiodinated piglet to Sepharose in small batch preparations, eluting with 8 M urea, and then boiling the Sepharose resin prior to SDS-gel electrophoresis. More than 50% of the bound protein was recovered in the last step. In contrast, conventional myosin is often sieved through large preparative Sepharose 4B columns as a final separation from actin (see Refs. 22, 23, and 35 for examples). Second, on SDS-gels in the absence of reducing agents, piglet seems to migrate as a heterotetramer with 210- and 47-kDa subunits.3 We were not aware of any myosins known to participate in disulfide bond formation. Third, piglet tested positive for carbohydrate in our phenol-sulfuric acid assays. Neither were we aware of any myosins known to participate in disulfide bond formation. However, as mentioned earlier, two observations serve to qualify the notion of covalently bound carbohydrate; the variable results we obtained suggesting that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin.

Like so many of the newly discovered myosins, piglet’s function in RPE cell behavior is not yet known. There is, in fact, much to be uncovered concerning the cytoskeletal organization of RPE in general. For example, an intermediate filament network composed of cytokeratins 8 and 18 is thought to be diagnostic of RPE among other retinal cells, but vimentin is much to be uncovered concerning the cytoskeletal organization of RPE in general. For example, an intermediate filament network composed of cytokeratins 8 and 18 is thought to be diagnostic of RPE among other retinal cells, but vimentin is known to be expressed in the RPE of at least two vertebrate species thus far (chicken and cow) (36). The desmosomal and zonulae adherens protein, plakoglobin, has been identified in RPE of all species examined. However, desmoplakin and desmoglein are not expressed in chicken or rat RPE (36). Whether desmoplakin is expressed in chicken filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin. Regardless of piglet’s status as a glycoprotein, the first two “unmyosin-like” features described above were enough to prompt further analyses along these lines. We therefore directed our subsequent studies at determining if piglet exhibited other features that were diagnostic of myosin. We found that piglet was co-distributed in cells with actin filaments, and that the molecule exhibited actin-enhanced ATPase activity and reacted avidly with antibodies to platelet myosin as well as the isoform-specific C-terminal domain of NMMHC-A. In the latter case, it should be noted that NMMHC-A is reportedly 15 kDa smaller than piglet and is expressed in fibroblasts (28), whereas piglet is not. These observations suggest to us that piglet is a new, unconventional myosin.