Isolation of highly purified, functional endosomes from toad urinary bladder

Timothy G. HAMMOND,*|| D. James MORRÉ,† H. William HARRIS Jr.‡ and Mark L. ZEIDEL‡§

*University of Wisconsin Hospitals and Clinics, and William S. Middleton Memorial V.A. Hospital, Madison, WI, U.S.A.,

†Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, IN, U.S.A., ‡Renal Division, Children's Hospital Medical Center, Boston, MA, U.S.A., and §Renal Section, Medical Service, Brockton-West Roxbury Department of Veteran's Affairs Medical Center, West Roxbury, MA, U.S.A.

Endosomes are difficult to isolate as they share size and density properties with much more abundant cellular organelles such as mitochondria. In cultured cell lines the tandem use of charge-dependent isolation techniques and differential centrifugation is necessary to isolate endosomes. Endosomal populations of the toad urinary bladder are of special interest because they are thought to contain a water channel. Understanding of the molecular structure of the water channel has been constrained, as there is currently no practical method to isolate functional water-channel-containing vesicles. This study reports the tandem use of charge-dependent techniques and centrifugation to isolate populations of endosomes from the toad urinary bladder. To purify water-channel-containing vesicles aqueous two-phase partition was utilized to fractionate a preparation partially purified by differential centrifugation. Populations of endosomes were analysed by small-particle flow cytometry techniques. A 5-fold enrichment in endosomes, achieved with aqueous two-phase

partition, allowed us to identify two populations of endosomes of diverse size in a toad bladder endosomal fraction. Pre-enrichment also improved the efficiency of flow cytometry sorting, allowing isolation of the two endosomal populations in sufficient quantities for secondary analysis. A population of larger endosomes had very high water permeability, indicating the presence of water channels. The two populations had different SDS/PAGE fingerprints. Electron micrographs of the flow-sorted material shows a uniform population of membrane vesicles devoid of mitochondria and other identifiable cellular organelles. Hence, aqueous two-phase partition and flow cytometry allow identification of two populations of endosomes in the toad urinary bladder which have diverse structural and functional properties. Isolation of functional water-channel-containing vesicles allows co-localization of water-channel function with candidate water-channel proteins.

INTRODUCTION

Endosomal populations in toad urinary bladder epithelial cells are the subject of intense study because the vesicles are thought to contain water channels (Harris et al., 1991a). Isolation of these vesicles remains perplexing as they have a buoyant density very similar to other much more abundant cellular organelles. Separative techniques which rely on differences in size and density yield an intermediate pellet enriched in the endosomes of interest, but still largely composed of mitochondria and endoplasmic reticulum (Shi et al., 1990; Harris et al., 1991b). Density shifting is currently the only procedure which isolates to great purity the endosomal population from this tissue; however, it is of limited value as it renders the water channels non-functional. Analysis of the molecular machinery of endocytosis is constrained by difficulties in the isolation of endosomal elements.

Flow cytometry provides both an assay for endosomal elements, and the potential to isolate homogeneous populations of endosomes by flow sorting (Wilson and Murphy, 1988; Hammond et al., 1993). Pioneering attempts to identify and isolate endosomes by flow cytometry were limited by the small proportion of particles of interest in the fractions examined, and the modest noise-to-signal ratio of entrapped fluorescent markers (Wilson and Murphy, 1988; Murphy et al., 1989). Progress in the detection of fluorescence signals has been dramatic, as special adaptations of flow cytometry electronics and fluidics which optimize small-particle techniques (Hammond et al., 1993) have accompanied detection of single fluorescent molecules (Jett et al., 1989). Flow cytometry analysis of endosomal populations now resolves multiple endosomal populations in a variety of tissues (Hammond et al., 1993).

The successful isolation of endosomal populations from cultured cell lines gives us some important clues for how to approach isolation of endosomal populations from toad bladder epithelial cells (Marsh et al., 1987). The key to isolation of endosomes in cultured cells is the combination of freeflow electrophoresis, a separative method relying on charge, with separative methods relying on size and density. Although freeflow electrophoresis requires quantities of material which are unrealistic to obtain from toad bladders, aqueous two-phase partition is a technique which separates according to charge and hydrophobicity, and has been adapted to minute quantities of tissue (Mircheff et al., 1984; Morré et al., 1989). Our strategy was to fractionate by aqueous two-phase partition an intermediate pellet of endosomes prepared from toad bladder epithelial cells by differential centrifugation. Flow cytometry will then identify populations and isolate them for secondary analysis.

MATERIALS AND METHODS

Preparation of intermediate-pellet vesicles: partial purification by differential centrifugation

Hemibladders were isolated from pithed Dominican toads (*Bufo marinus*, National Reagents, Bridgeport, CN, U.S.A.). Endosomes were loaded with carboxyfluorescein with minimal labelling of apical membranes by following a protocol identical to several published studies (Harris et al., 1990a,b; Zeidel et al., 1992). Bladders are initially stimulated with anti-diuretic hormone (ADH) with isotonic luminal buffer (i.e. an absence of transepithelial osmotic gradient). Substitution of a hypotonic luminal buffer and withdrawal of ADH is associated with a wave of luminal endocytosis. Carboxyfluorescein in the hypotonic

buffer is taken up into the endocytotic pathway. Endocytosis is arrested by placing the bladder in ice-cold buffer, washing the lumen repeatedly, and scraping off the epithelium. The epithelial cells are homogenized and endosomes enriched by differential centrifugation. The resultant intermediate pellet is largely made of endosomes, mitochondria and endoplasmic reticulum (Harris et al., 1990a,b).

Enrichment of endosomes before isolation by flow cytometry: aqueous two-phase partition

Aqueous two-phase partition of the intermediate-pellet fraction was performed using the method of Morré and Morré (1989) with several modifications as detailed below. Stock solutions of 20% (w/v) dextran T-500 (Pharmacia) and 40% (v/v) poly(ethylene glycol) 3350 (PEG, Union Carbide) were prepared in double-distilled water on a weight for weight basis (Morré and Morré, 1989). A 6.0% weight-for-weight two-phase system was prepared by adding 4.8 g of 20% dextran stock to 2.4 g of 40% PEG stock solution in a preweighed centrifuge tube on ice. After adding 320 μ l of 0.2 M potassium phosphate buffer (pH 7.2) the total mass of the contents of the centrifuge tube was made up to 14 g with distilled water. The membrane fractions to be separated were resuspended in a minimum volume of homogenization solution and added to the two-phase system. The total mass of the contents of the centrifuge tube was brought up to 16 g with buffer. The tube containing the suspension was covered with parafilm and inverted exactly 40 times at 4 °C. The resulting suspension was centrifuged (100 g, 5 min) to separate the phases (Morré and Morré, 1989). The less dense upper phase was collected with a Pasteur pipette.

Flow cytometry analysis and sorting of endosomal populations

Flow cytometry analysis and sorting were performed on Becton Dickinson FACStar and FACStar Plus flow cytometers utilizing small-particle techniques (Jett et al., 1989; Wilson and Murphy, 1988; Hammond, 1990; Hammond et al., 1993).

Electron microscopy of endosomal preparations

Samples were fixed for electron microscopy using 2% (w/v) glutaraldehyde in PBS. The samples were then transferred to 1% (w/v) osmium tetroxide in 0.05 M sodium phosphate (pH 7.2) for several hours, then the samples were dehydrated in an acetone series, followed by embedding in Epon (Morré and Morré, 1989). Lead-stained thin sections were examined and photographed using a Phillips EM/200 electron microscope.

SDS/PAGE quantification of vesicle proteins

Proteins of the vesicle populations sorted by flow cytometry were fractionated by SDS/PAGE, transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, U.S.A.), and stained with 0.1% Amido Black. After destaining with methanol/water/acetic acid (10:9:1, by vol.) we quantified the blot proteins using a Zeineh soft-laser densitometer (Biomed Instrument Co., Fullerton, CA, U.S.A.) (Harris et al., 1991b).

Measurement of osmotic water permeability

Stopped-flow measurements of osmotic water permeability were performed on an Applied Photophysics SF.17MV (Applied Photophysics, Leatherhead, U.K.). An abrupt increase in extra-

vesicular osmolality led to efflux of water from the vesicles, increasing the intravesicular concentration of carboxyfluorescein, which in turn resulted in a concentration-dependent self-quenching of fluorescence (Chen et al., 1988; Harris et al., 1990a; Zeidel et al., 1992). Previous data (Zeidel et al., 1992) has demonstrated that the vesicles act as perfect osmometers over the range of osmolalities in the experiments.

Statistics

Results were analysed statistically by one-way analysis of variance, and Scheffe's post-hoc comparison. Results are expressed as means $\pm$ S.E.M.

Figure 1 Flow cytometry analysis of serial aqueous two-phase partition fractions of a toad bladder intermediate pellet prepared by differential centrifugation

Panels a-h show log[fluorescein fluorescence] versus number of events per channel. Autofluorescence from a bladder without fluorescein-dextran (a) contrasts with the fluorescence of fluorescein-loaded endosomes in the intermediate pellet before fractionation (b). Serial aqueous two-phase fractions are 6.4% upper phase (c), 6.2% upper phase (d), 6.0% upper phase (e), 5.8% upper phase (f), 5.6% upper phase (g), and 5.4% upper phase (h). Data is representative of four similar experiments. Projection plots of toad bladder endosomes loaded with non-fluorescent (i) or fluorescein-dextran (j) allows identification of populations for flow sorting.

Figure 2 Electron micrographs of endosomal fractions

(a) Intermediate pellet of partially purified endosomes prepared by differential centrifugation. Abbreviation: m, mitochondria. Arrows indicate vesicles. (b) Aqueous two-phase partition fractionation of the intermediate pellet. The 6.4% upper phase consists of large (large arrows) and small (small arrows) vesicles. (c) A 5.4% upper phase consists mainly of mitochondria and other contaminants. Scale bar = 0.5 μm .

RESULTS

Aqueous two-phase partition of toad urinary bladder endosomes

To isolate toad urinary bladder water-channel-containing vesicles aqueous two-phase partition was used to fractionate an intermediate pellet prepared by differential centrifugation (Figure 1). Each panel of this figure shows a histogram of log[fluorescein fluorescence] versus the number of events per channel. Figure 1(a), a control without fluorescent dextran, defines the level of particle autofluorescence. Figure 1(b), an intermediate pellet before aqueous two-phase partition, shows biphasic peaks of fluorescent particles and a small population of very bright particles. In Figure 1(c), the 6.4% upper phase from aqueous two-phase partition shows heavy enrichment in a very bright population. Figures 1(d)–1(g), representing 6.2%, 6.0%, 5.8%

and 5.6% upper phases, show many fluorescent particles, but few bright particles. The 5.4% upper phase has few, if any, fluorescein-positive particles.

In subsequent studies aiming to isolate populations by flow cytometry sorting we prepared a single 6.0% upper-phase fraction, which contained all the particles shown as 6.4%, 6.2% and 6.0% upper phases (Figures 1i and 1j). A 6.4% upper phase has the largest concentration of bright particles, but the final yield was very modest (< 20% of the total large vesicles). A 6.0% upper phase was the most practical compromise of purity and yield. By flow cytometry the percentage of large fluorescein-containing particles (putative water-channel-containing vesicles) increased from $3 \pm 1\%$ to $15 \pm 3\%$ ($n = \text{five preparations}$; $P < 0.005$) when two-phase-purified particles were analysed as opposed to particles from unpurified intermediate pellets.

Figure 3 Electron micrographs of water-channel-containing vesicles isolated by flow cytometry sorting

Water-channel-containing vesicles were purified from intermediate pellet using aqueous two-phase partition and flow cytometry. (a) A survey micrograph shows the homogeneity of the preparation and the absence of identifiable organelles. Vesicles have a corrugated membrane contour, with an electron-dense amorphous content in several endosomes. (b) A higher magnification of the flow-cytometry-sorted water-channel-containing vesicles shows more detail. These data are representative of multiple electron micrographs obtained from two flow-purified preparations. Scale bar = 1 μm .

Electron microscopy of endosomal preparations

When examined by electron microscopy, the unfractionated intermediate pellet is seen to be a mixture of membrane elements, including mitochondria, endoplasmic reticulum and vesicles of diverse sizes (Figure 2a). The dominant membrane element in the 6.4% upper-phase fraction, determined by electron microscopy, is the membrane vesicle (Figure 2b). Larger thin-walled membrane vesicles (large arrows) contrast with smaller thick-walled membrane vesicles (small arrows). The possibility that these apparently smaller thick-walled vesicles are grazed profiles of larger structures cannot be excluded. In the 5.4% upper phase, the dominant membrane types are mitochondrial and endoplasmic reticular (Figure 2c).

Isolation by flow cytometry of water-channel-containing vesicles from intermediate-pellet fractions

To determine whether the population of highly fluorescent larger endosomes contains water channels, this population was isolated by flow cytometry sorting and its water permeability studied. The homogeneity of the putative water-channel-containing vesicles

isolated by flow cytometry sorting is shown on a survey electron micrograph (Figure 3a), while the details of the membranes are seen better at higher magnification (Figure 3b). Flow cytometry sorting of this population results in a 73–150-fold purification (range for $n = 4$) of fluorescein over intermediate pellet, or a 328–675-fold increase in purity over the initial bladder homogenate (Harris et al., 1986). Flow cytometry re-analysis of sorted endosomes demonstrated over 99% purity using fluorescein fluorescence as a marker.

On electron micrographs the purified vesicle population appears uniform, having very few contaminating organelles (Figure 3). Flow cytometry purifies $1\text{--}3 \times 10^6$ water-channel-containing vesicles from four toads, yielding 15–40 μg of protein after completing a protocol of 4 h duration.

Measurement of osmotic water permeability (P_f)

To determine whether water-channel function is present in the population of large endosomes, fractions isolated by flow cytometry sorting were studied by stopped-flow fluorimetry. Isolated vesicles exhibited a high P_f value, indicative of the presence of functional water channels (Figure 4). The maximal P_f

Figure 4 Water flux in intermediate-pellet- and flow-cytometry-purified water-channel-containing vesicles

Water flux was measured in water-channel-containing vesicles loaded with carboxyfluorescein and partially purified as an intermediate pellet by differential centrifugation (a) (representative of $n = 24$ experiments). Water flux was also measured in water-channel-containing vesicles further purified by aqueous two-phase partition and flow cytometry (b) (representative of $n = 4$ experiments).

for four such preparations (using the upper 25 % of diameters for flow-purified vesicles) averaged 0.47 ± 0.09 cm/s; the P_t value obtained using the average diameter for flow-purified vesicles was 0.35 ± 0.07 cm/s. These values are similar to, or greater than, those obtained in the intermediate-pellet fraction in previous studies (0.15 ± 0.03 cm/s, $n = 24$, Harris et al., 1991a). These results provide strong evidence that the endosomes purified by flow cytometry contain functional water channels. Fluorescence in the population of small endosomal vesicles was nearly undetectable in the stopped-flow fluorimeter, precluding direct measurement of P_t in this population.

Figure 5 SDS/PAGE analysis of populations of endosomes in the intermediate-pellet fraction purified by flow cytometry sorting

Intermediate-pellet fractions containing endosomes loaded with F-dextran were isolated by flow cytometry sorting, subjected to SDS/PAGE and the resulting proteins stained with Coomassie Blue. Left panel: Population of large endosomes. The large arrowhead points to bands of 55 and 53 kDa that are candidate ADH water-channel proteins. The smaller arrowhead at the bottom of the gel lanes denotes the position of the dye front of the gel. There was essentially nothing detectable larger than the bands at the top of the gel as shown. Right panel: Population of small endosomes. SDS/PAGE protein bands from the population of small endosomes purified by flow sorting are distinct from those of the larger population. Larger arrows denote the absence of either the 55/53 or 110–90 kDa bands that are prominent in the large endosomes. Smaller arrowheads denote prominent protein bands of 80 kDa and 46 kDa. The results are representative of $n = 3$ experiments.

SDS/PAGE quantification of vesicle proteins

The protein compositions of populations of small and large endosomes isolated by flow cytometry sorting was examined by SDS/PAGE. The large population of endosomes contains prominent bands at 55, 53, 46 and 17 kDa (Figure 5), with no manifest bands above 80 kDa on the gel. These bands are consistent with putative water-channel components isolated in endosomes prepared by the density-shift technique. In contrast, the population of small vesicles has a disparate protein composition with several relatively high-molecular-mass bands.

DISCUSSION

Attempts to understand the molecular mechanisms which govern changes in water permeability of the toad urinary bladder have been hampered by our inability to isolate water-channel-containing vesicles in a functional form. Isolation of endosomes by established separative techniques has been limited by the nearly homogeneous size and density of water-channel-containing vesicles and other subcellular organelles. Similarly, at present we lack a high-affinity specific probe for affinity purification or immunoprecipitation of pure vesicles (Harris et al., 1991a). Water-channel-containing vesicles can be isolated by a density-shift procedure, but they are no longer functional (Harris et al., 1987). Thus, the techniques described in this report provide methods that for the first time allow isolation of water-channel-containing vesicles which are functional.

Two-phase aqueous partition has been used extensively to separate cellular organelles in plants (Albertsson et al., 1982; Sandelius and Morré, 1987). Aqueous two-phase partition was only recently applied to animal tissues, and there is scant functional data on fractionated membranes (Mircheff et al., 1984; Morré and Morré, 1989). We applied aqueous two-phase partition to the fractionation of a crude toad bladder endosomal preparation (intermediate pellet). Endosomes contained within the intermediate pellet are enriched more than five times by this technique with bulk removal of the contaminating mitochondria.

The enrichment in endosomes by aqueous two-phase partition allowed us to construct flow cytometry diagrams in which populations of vesicles are clearly delineated, and greatly improved the efficiency of isolation by flow cytometry sorting. Furthermore, the isolated vesicles remain fully functional, with high water permeability demonstrable in the population of larger vesicles, and brisk ATP acidification in the population of smaller vesicles (Zeidel et al., 1992).

At least two populations of endosomes are identifiable in toad bladder epithelial cells. Two endosomal populations of disparate size are demonstrable in an intermediate pellet on flow cytometry analysis (Zeidel et al., 1992). The more abundant population of smaller vesicles has brisk H^+ -ATPase activity, while the less abundant population of larger vesicles has no detectable H^+ -ATPase activity. The current findings that these populations have disparate protein contents demonstrates that the populations are distinct and not homogenization products of each other with different buffering capacities.

Isolating the population of large vesicles by flow cytometry sorting provides evidence that the preparation contains highly purified water-channel-containing vesicles. First, electron micrographs demonstrate that flow-purified vesicles were a homogeneous population of endosomes, with no detectable contamination with mitochondria or other organelles. Secondly, flow-purified endosomes exhibit a 73–150-fold increase in the specific activity of entrapped fluorescein over that in the intermediate pellet. Thirdly, purified endosomes exhibit a high P_i value, indicating that they contain functional water channels. Thus, aqueous two-phase partition coupled to flow cytometry results in a highly purified fraction of functional water-channel-containing vesicles.

This study co-localizes water-channel function, high P_i , with candidate water-channel protein elements. The 12 or so protein bands characteristic of water-channel-containing vesicles isolated by the density-shift procedure (Harris et al., 1987) were observed in our flow-cytometry-sorted population of large vesicles. Using flow cytometry isolation, the vesicles remained functional, and water-channel function co-localized with the putative water-channel proteins. Further studies using flow-purified vesicles can now delineate the function of each of these constituent vesicle proteins.

The intermediate-pellet fraction also contains another population of fluorescent vesicles. These are smaller than water-

channel-containing vesicles and therefore contain much less total fluorescence. Unfortunately, the amount of these smaller vesicles obtained by flow cytometry was too small to permit characterization of permeability properties. However, pooling of several preparations permitted analysis of protein structure, and demonstrated it was distinct from that of the larger intermediate-pellet endosomes. The distinct physiological function (acidification in the presence of ATP) (Zeidel et al., 1992) and the different protein structure demonstrates that this smaller fraction represents a distinct subpopulation of toad bladder endosomes.

Portions of this study were presented at the American Society of Nephrology Meeting (December 1990), the Society for Analytical Cytology Meetings (October 1991), and the Eighth International Conference on Two-Phase Aqueous Partition (August 1991). The work was supported by grants from the National Kidney Foundation of Wisconsin (T.G.H.), the Wisconsin Affiliate of the American Heart Association (T.G.H.), NIH RO-1 DK43955 (M.L.Z.), DK46117 (T.G.H.) and DK38744 (H.W.H.), Career Development and Merit Review Awards from the Medical Research Service of the Department of Veterans Affairs (M.L.Z.), an Established Investigator Award of the American Heart Association (H.W.H.), and an American College of Physicians Teaching and Research Scholar Award (T.G.H.).

REFERENCES

- Albertsson, P. A., Andersson, B., Larsson, C. K. and Akerlund, H. E. (1982) *Methods Biochem. Anal.* **28**, 115–150
- Chen, P. Y., Pearce, D. and Verkman, A. S. (1988) *Biochemistry* **27**, 5713–5718
- Hammond, T. G. (1990) *Kidney Int.* **38**, 5–11
- Hammond, T. G., Majewski, R. R., Morré, D. J., Schell, K. and Morrissey, K. W. (1993) *Cytometry* **14**, 411–418
- Harris, H. W., Jr., Handler, J. S. and Blumenthal, R. (1990a) *Am. J. Physiol.* **258**, F237–F244
- Harris, H. W., Jr., Kikeri, D., Janoshazi, A., Solomon, A. K. and Zeidel, M. L. (1990b) *Am. J. Physiol.* **259**, F366–F371
- Harris, H. W., Jr., Murphy, H. R., Willingham, M. C. and Handler, J. S. (1987) *J. Membr. Biol.* **96**, 175–186
- Harris, H. W., Jr., Wade, J. B. and Handler, J. S. (1986) *Am. J. Physiol.* **251**, C274–C284
- Harris, H. W., Jr., Strange, K. and Zeidel, M. L. (1991a) *J. Clin. Invest.* **88**, 1–8
- Harris, H. W., Jr., Zeidel, M. L. and Hosselet, C. (1991b) *Am. J. Physiol.* **261**, C143–C153
- Jett, J. H., Keller, R. A., Martin, J. C., Marrone, B. L., Moyzis, R. K., Ratliff, R. L., Seitzinger, N. K., Sheba, E. B. and Stewart, C. C. (1989) *J. Biomol. Struct. Dynamics* **7**, 301–309
- Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I. and Helenius, A. (1987) *J. Cell Biol.* **104**, 875–886
- Mircheff, A. K., Ives, H. E., Yee, V. J. and Warnock, D. G. (1984) *Am. J. Physiol.* **246**, F853–F858
- Morré, D. J. and Morré, D. M. (1989) *BioTechniques* **7**, 946–958
- Morré, D. M., Morré, D. J., Snyder, T., Li, W., Erb, A. and Sammons, D. W. (1989) in *Separation Using Aqueous Phase Systems in Cell Biology and Biotechnology* (Fisher, D. and Sutherland, I., eds.), p. 115. Plenum Press, New York
- Murphy, R. F., Roederer, M., Sipe, D. M., Cain, C. C. and Bowser, R. (1989) in *Flow Cytometry: Advanced Research and Clinical Applications* (Yen, A., ed.), pp. 221–254. CRC Press, Boca Raton
- Sandelius, A. S. and Morré, D. J. (1987) *Plant Physiol.* **84**, 1022–1027
- Shi, L.-B., Brown, D. and Verkman, A. S. (1990) *J. Gen. Physiol.* **95**, 941–960
- Wilson, R. B. and Murphy, R. F. (1988) in *Cell-Free Analysis of Membrane Traffic* (Morré, D. J., Howell, K. E., Cook, G. M. W. and Evans, W. H., eds.), pp. 153–168. Alan R. Liss, New York
- Zeidel, M. L., Hammond, T. G. and Harris, H. W., Jr. (1992) *Am. J. Physiol.* **263**, F62–F76