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Analytical characterization of plasma membrane-derived vesicles produced via osmotic and chemical vesiculation

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ABSTRACT

Plasma membrane-derived vesicles are being used in biophysical and biochemical research as a simple, yet native-like model of the cellular membrane. Here we report on the characterization of vesicles produced via two different vesiculation methods from CHO and A431 cell lines. The first method is a recently developed method which utilizes chloride salts to induce osmotic vesiculation. The second is a well established chemical vesiculation method which uses DTT and formaldehyde. We show that both vesiculation methods produce vesicles which contain the lipid species previously reported in the plasma membrane of these cell lines. The two methods lead to small but statistically significant differences in two lipid species only; phosphatidylcholine (PC) and plasmalogen phosphatidylethanolamine (PEp). However, highly significant differences were observed in the degree of incorporation of a membrane receptor and in the degree of retention of soluble cytosolic proteins within the vesicles.

1. Introduction

The cellular plasma membrane, a complex assembly of lipids and proteins, plays a critical role in cell physiology [1–4]. The membrane provides the barrier, and mediates the communication, between the cell and its environment. The processes that occur in the plasma membrane, such as ion conduction, nutrients uptake, and signal transduction, are critical for cell function [5–8]. It is often difficult, however, to study these processes in the plasma membrane of living cells, and thus biophysicists and biochemists often rely on model membrane systems. One such model system is plasma membrane-derived vesicles, which bud off cells in response to external stress [9,10]. These vesicles are derived from the native cellular membrane, and are thus more native-like than vesicles made of synthetic lipids. They are increasingly used in studies of lipid–lipid, lipid–protein and protein–protein interactions, and have already yielded new knowledge about lipid domains and receptor interactions in the membrane [11–15]. Often, however, they are not well characterized in terms of their lipid and protein content.

The most widely used vesiculation method, developed in the 1960s, utilizes the chemicals formaldehyde and dithiothreitol (DTT) to stress the cells and to induce an apoptosis-like response [9,16,17]. Vesicles can be produced with this method from a variety of mammalian cells including human embryonic kidney (HEK) 293 T cells, Chinese hamster ovary (CHO) cells, A431 human epidermoid carcinoma cells, 3 T3 fibroblasts, endothelial cells, a variety of other cancer cells, and macrophages [9,12,16–21]. These vesicles have been used widely in the literature to study lipid domains, but concerns may arise in some cases due to the presence of DTT, a reducing agent, as well as formaldehyde, a molecular cross-linker. Thus, an alternative vesiculation method, which utilizes osmotic stress rather than chemicals, was recently developed [10]. In this method, vesiculation is induced by incubating cells with a buffer which contains high concentration of chloride salts. This osmotic method has been used to produce vesicles from CHO and A431 cells, in the absence of DTT and formaldehyde. The overall appearance of the vesicles produced with the osmotic stress method and the DTT/formaldehyde method is very similar [10], and both vesicle preparations have been used successfully in studies of protein interactions in membranes [18].

Here we sought to characterize and compare the vesicles produced by chemical and osmotic vesiculation and to identify differences that might exist between the two types of vesicles. In particular, we characterized and compared A431 chloride salt vesicles and A431 DTT/formaldehyde vesicles. As a control, we also characterized CHO DTT/

Abbreviations: FC, free cholesterol; CE, cholesteryl ester; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; Cer, ceramide; SM, sphingomyelin; dhSM, dihydrosphingomyelin GalCer, galactosylceramide; Sulf, sulfatide; GlcCer, glucosylceramide; LacCer, lactosylceramide; GM3, monosialodihexosylganglioside 3; PA, phosphatidic acid; PC, phosphatidylcholine; PCe, etherphosphatidylcholine; PE, phosphatidylethanolamine; PEp, plasmalogen phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, diacylglycerol; LPC, lysophosphatidylserine; LPCe, lysoetherphosphatidylcholine; LPE, lysophosphatidylethanolamine; LPEp, lysoplasmalogen phosphatidylethanolamine; LPI, lysophosphatidylinositol; BMP, bismonoacylglycerol; APG, acyl phosphatidylglycerol; NAPE, N-Acyl phosphatidylethanolamine; NAPEp, N-Acyl plasmalogen phosphatidylethanolamine; NAPS, N-Acyl phosphatidylserine

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formaldehyde vesicles, with the goal of comparing differences due to vesiculation method and differences due to cell type.

2. Materials and methods

2.1. Cell culture and vesiculation

Chinese hamster ovary (CHO) and A431 cells were cultured in T75 flasks. These cells were vesiculated at 70% confluency using a DTT/formaldehyde buffer [9] or a chloride salt osmotic buffer [10].

2.2. Vesicle lipid pelleting

Centrifugation was performed at 125 ×*g*, 4 °C to pellet the cell debris. A second centrifugation was performed at 25,000 ×*g* for 45 min at 4 °C to pellet the vesicles. The supernatant was discarded.

2.3. Liquid chromatography mass spectrometric (LC–MS) analysis of lipids and cholesterol

Three independent samples were prepared for each vesicle type. After pelleting, lipids and cholesterol were extracted as described previously using a modified Bligh/Dyer procedure, spiked with appropriate internal standards [22], and analyzed using a 6490 Triple Quadrupole LC–MS system (Agilent Technologies, Santa Clara, CA). Glycerophospholipids and sphingolipids were separated with normal-phase HPLC as described before [22], with a few changes. An Agilent Zorbax Rx-Sil column (inner diameter 2.1 × 100 mm) was used under the following conditions: mobile phase A (chloroform:methanol:1 M ammonium hydroxide, 89.9:10:0.1, v/v) and mobile phase B (chloroform:methanol:water:ammonium hydroxide, 55:39.9:5:0.1, v/v); 95% A for 2 min, linear gradient to 30% A over 18 min and held for 3 min, and linear gradient to 95% A over 2 min and held for 6 min. Sterols and glycerolipids were separated with reverse-phase HPLC using an isocratic mobile phase as before [22] except with an Agilent Zorbax Eclipse XDB-C18 column (4.6 × 100 mm).

Quantification of lipid species was accomplished using multiple reaction monitoring (MRM) transitions [22] in conjunction with referencing of appropriate internal standards: PA 14:0/14:0, PC 14:0/14:0, PE 14:0/14:0, PG 15:0/15:0, PI 16:0/16:0, PS 14:0/14:0, BMP 14:0/14:0, APG 14:0/14:0, LPC 17:0, LPE 14:0, LPI 13:0, Cer d18:1/17:0, SM d18:1/12:0, dhSM d18:0/12:0, GalCer d18:1/12:0, GluCer d18:1/12:0, LacCer d18:1/12:0, D_7-cholesterol, CE 17:0, MG 17:0, 4ME 16:0 diether DG, D_5-TG 16:0/18:0/16:0 (Avanti Polar Lipids, Alabaster, AL).

2.4. Thin layer chromatography

The pelleted plasma membrane vesicles were resuspended in distilled water. The solution was dried in a rotory evaporator and the lipids were extracted using the Folch method with chloroform:methanol:distilled water (2:1:1 (v/v)) at room temperature [23]. The extracts were dried under a stream of N_2 gas and resuspended in a chloroform:methanol mixture (2:1 (v/v)). One dimensional thin layer chromatography (TLC) was used to analyze the lipid content. Whatman flexible silica gel G plates were spotted with solutions containing the extracted lipids, as well as the following lipid standards: 1-palmitoyl-2-deoyl-sn-glycerol-3-phosphocholine (POPC), 1-palmitoyl-2-deoyl-sn-glycerol-3-phospho-L-Serine (POPS), 1-palmitoyl-2-deoyl-sn-glycerol-3-phosphoethanolamine (POPE), Sphingomyelin (from Porcine brain), and cholesterol (Avanti Polar Lipids Inc). The plates were then placed in a chamber with a chloroform:methanol:7-N NH_4OH (65:27:5 (v/v)) solution to separate the different lipid components. After separation, the lipid components were visualized in iodine vapor.

2.5. ^{31}P NMR phospholipid analysis

Vesicle pellets were resuspended in distilled water. The solution was dried in a rotory evaporator and the lipids were extracted using the Folch method with chloroform:methanol:distilled water (2:1:1 (v/v)) at room temperature [23]. The extracts were dried under a stream of N_2 gas and resuspended in a chloroform:methanol mixture (2:1 (v/v)). 20–30 mg of lipid extracts from each vesicle type were sent to Avanti Polar Lipids Analytical Services for ^{31}P NMR analysis. A Bruker Avance™ III 400 mHz with a 5 mm BBFO Probe NMR spectrometer was used to characterize the phospholipid composition of the samples dissolved in 1 mL of detergent.

2.6. Annexin V binding to plasma membrane derived vesicles

Vesicles were incubated for 1 h with fluorescein-conjugated Annexin V using the LI2004 Annexin V detection kit (Molecular Probes). Images were recorded in a Nikon confocal microscope.

2.7. Plasmids for vesicle content leakage assays

The wild type human fibroblast growth factor receptor 2 (FGFR2) plasmid was a gift from Dr. Moosa Mohammadi (NYU). The FGFR2-mCherry plasmid was constructed by fusing mCherry to the C-terminus of full length FGFR2. The plasmid encoding 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1-GFP (Plcδ1-PH-GFP) was obtained from Dr. Tamas Balla (NIH). The plasmids encoding Intersectin II-GFP, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1-GFP (Plcγ-GFP) and Protein kinase C theta-GFP (PKCΘ-GFP) were a kind gift from Dr. Christoph Wuelfing (University of Bristol). The Growth factor receptor-bound protein 2-Venus (Grb2-Venus) plasmid was a gift from Dr. Jin Zhang (Johns Hopkins). The VVVVVV (Venus x 6) plasmid was purchased from Addgene (courtesy of Dr. Steven Vogel, NIH).

2.8. Western blot analysis of EGFR in A431 vesicles

The vesicle pellets were lysed with lysis buffer (25 mM Tris–HCl, 0.5% Triton X-100, 20 mM NaCl, 2 mM EDTA, phosphatase inhibitor and protease inhibitor, Roche Applied Science). The lysates were loaded onto 3–8% NuPAGE®Novex®Tris–Acetatemini gels (Invitrogen, CA). The proteins were transferred onto a nitrocellulose membrane, and blocked using 5% milk in TBS. EGFR was detected with anti-EGFR receptor antibodies (2232 s, Cell Signaling Technology USA), followed by anti-rabbit HRP conjugated antibodies (W4011, Promega). The proteins were visualized with the Amersham ECL detection system (GE Healthcare) as described previously [24,25].

2.9. EGF-Rhodamine binding to EGFR in A431 vesicles

Vesicles were incubated with 1ug/mL of Epidermal Growth Factor–Tetramethylrhodamine Conjugate (E3481, Molecular Probes) for 1 h, and were then imaged in the confocal microscope.

3. Results

3.1. Different methods of vesicle production lead to small, but statistically significant differences in cholesterol and lipid composition

We first compared the lipid and cholesterol content of the three types of vesicles used in this study: A431 chloride salt vesicles, A431 DTT/formaldehyde vesicles, and CHO DTT/formaldehyde vesicles using liquid chromatography–mass spectrometry (LC–MS). Three independent samples were prepared for each type of vesicle preparation. The vesicles were pelleted and the lipids were extracted as described in the Materials and methods section. The LC–MS results, shown in Fig. 1,