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Identification and characterization of the nano-sized vesicles released by muscle cells

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ABSTRACT

Several cell types secrete small membranous vesicles that contain cell-specific collections of proteins, lipids, and genetic material. The function of these vesicles is to allow cell-to-cell signaling and the horizontal transfer of their cargo molecules. Here, we demonstrate that muscle cells secrete nano-sized vesicles and that their release increases during muscle differentiation. Analysis of these nanovesicles allowed us to characterize them as exosome-like particles and to define the potential role of the multifunctional protein Alix in their biogenesis.

1. Introduction

Animal cells release nano-sized single membrane vesicles that function in signaling and the horizontal transfer of their cargo molecules (proteins, mRNAs, and microRNAs) to other cells [1–3]. Research on extracellular nanovesicles has focused primarily on the immune system and tumor cells. Recently, it has also been reported that a skeletal muscle cell line (C2C12) can release vesicles [4]; however, how muscle cells generate these vesicles and what their effectors are remain largely unknown.

Alix is an evolutionarily conserved adaptor protein that has been implicated in cytoskeleton and membrane remodeling [5–7]. In line with these reports, we have recently shown that Alix also plays a part in actin cytoskeleton remodeling in muscle cells, and that the Ozz-E3 ubiquitin ligase is crucial for the regulation of this function of Alix [8]. In addition, it has been suggested that, because Alix can interact with both a lipid and proteins and retains a characteristic "banana-shaped" structure, it can generate or scaffold a negative curvature within the membrane as part of the inward budding process within the endocytic pathway [9,10]. An interplay between F-actin and membrane-bending proteins like Alix is thought to occur, in order to promote a negative curvature of the membrane during processes such as filopodia formation, vesicle budding and midbody abscission. In fact, we and others have demonstrated that Alix downregulation leads to a decrease in the number of late endosomes and muscle podia, and to alterations in their composition [7,8]; thus, Alix loss of function may interfere with mechanisms that regulate membrane dynamics. Here, we demonstrate that differentiated muscle cells release nanovesicles extracellularly and that the loss of Alix alters the budding and composition of these vesicles.

2. Materials and methods

2.1. Antibodies and reagents

Commercial antibodies included mouse anti-AIP1/Alix for immunoblotting (BD Transduction Labs) and anti-Alix (Santa Cruz Biotechnology) for immunogold electron microscopy, alpha-enolase (Santa Cruz Biotechnology), anti-CD63 (Santa Cruz Biotechnology), anti-Hsp70/Hsc70 (Novus Biologicals), anti-Elongin C (BD Biosciences), anti-MyHc (MF20, Developmental Studies Hybridoma

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Bank), anti-Myogenin (Santa Cruz Biotechnology), anti-MyoD (Santa Cruz Biotechnology), anti-Bcl-2 (Calbiochem), anti-Bax (Calbiochem), anti-PARP (Cell Signaling Technology). Rabbit anti-Ozz antibody was prepared as described [11].

siRNAs targeting Alix and standard negative controls, and the transfection reagent were purchased from Dharmacon, as reported [8].

2.2. Cell culture methods

For three-dimensional cultures, C2C12 were cultured as reported [8]. Primary myoblast cultures were established as described previously [11].

2.3. Purification of nanovesicles by differential ultracentrifugation

Nanovesicles were isolated and quantified according to a previously published method [12]. This isolation method included a penultimate centrifugation step (10,000×*g* for 30 min) that allowed the removal of small cell debris and larger microvesicles for the subsequent pelleting of nanovesicles, comprised mainly of exosomes [13]. After washing, the pellet is resuspended either in RIPA buffer or 4% PFA, for further immunoblot or electron microscope analyses, respectively. To have an estimation of the amount of secreted nanovesicles, we quantified and compared the total protein content of the vesicle lysates using the Bradford assay [12].

2.4. Electron microscope analysis and immunogold labeling of muscle-derived nanovesicles

Nanovesicle pellets fixed in 4% PFA were mounted on Formvar–carbon coated EM gold grids by layering 10-μl drops of vesicle preparations, and letting grids air dry. Grid-mounted preparations were stained with uranyl acetate and lead citrate, and subsequently observed under the JEM-1220 (Jeol) electron microscope at 120 kV.

Muscle cells were 3D cultured [8] and inserts were fixed in PFA 4%. Inserts were then dehydrated in alcohol and embedded in liquid LR White Medium Grade Resin before inclusion in gelatine capsules (EMS). Samples were then cut into 70 nm-thick ultrathin sections and layered onto Formvar coated gold grids (EMS). For immunogold staining, the grids were rinsed in water, incubated in citrate buffer, and blocked in 3% BSA-c in T-PBS. Grids were then incubated with anti-Alix, washed in T-PBS, incubated with AuroProbe EM secondary antibody. The grids were post-fixed with 2% glutaraldhyde in PBS, and contrasted using conventional techniques.

3. Results and discussion

3.1. Budding of nanovesicles from the plasma membrane of differentiated muscle cells

Exosomes are nano-sized (50–100 nm in diameter) membrane-bound vesicles released by a variety of cell types and specified by a plethora of protein markers, including Alix [1–3]. In contrast, microvesicles tend to constitute a larger and more heterogeneous population of extracellular vesicles, ranging from 50 to 1000 nm in diameter [14]. Transmission electron microscopy analyses of 3D cultures of C2C12 myotubes revealed that nanovesicles, ranging from 50–100 nm and with an average diameter of 80 nm, were released by direct budding from the plasma membrane (Fig. 1A). 3D cell cultures exhibit more in vivo-like responses allowing us to visualize, by immunogold labeling, the distribution of Alix in close proximity to the surface of budding nanovesicles and decorating released nanovesicles (Fig. 1B). The size of these muscle-derived

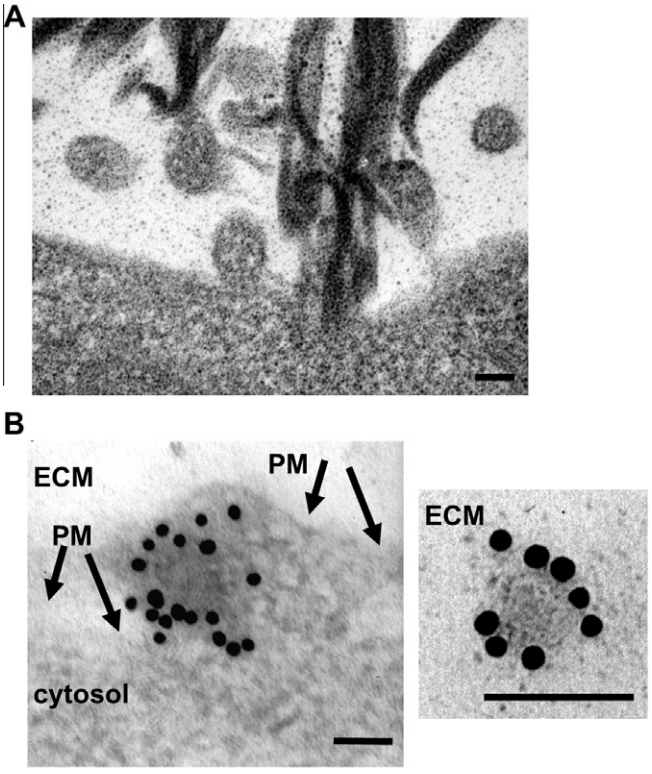

Fig. 1. Muscle cells release nanovesicles. (A) Electron microscope analyses of 3D cultures of differentiating C2C12 cells show the presence of outward budding intermediates (nascent vesicles) at the plasma membrane with an average diameter of 80 nm (scale bar = 100 nm). (B) Transmission electron microscopy shows immunogold labeling of Alix in the nanovesicles from 3D cultures of differentiated C2C12 myotubes (10 nm gold particles). Left panel shows a nascent nanovesicle, right panel shows a released vesicle (PM = plasma membrane, ECM = extra-cellular matrix) (scale bar = 100 nm).

vesicles and the presence of Alix are compatible with what has been described for exosomes from other cells [12–15].

Together, these observations indicate that skeletal muscle cells can produce and release nanovesicles directly from the plasma membrane. This modality of vesicle secretion is consistent with that previously reported for both microvesicle [16] and nanovesicle biogenesis; in the latter, human CD4+ T cells and certain T cell lines release nanovesicles—defined as exosomes—directly from subdomains of their plasma membrane [15,17,18]. However, it contrasts with the canonical view of exosome release as a delayed process in which vesicles bud first into an intermediate structure, the multivesicular bodies (MVB), and are subsequently released upon endosome–plasma membrane fusion [1,3,13]. Nevertheless, both modes of vesicle secretion involve outward nanovesicle budding and a negative curvature of specialized endosome-like membranes, either within the MVB or at the plasma membrane [14]. Whether the nanovesicles originating from these two mechanisms carry the same cargo molecules from the cell and are associated with the same protein sorting machinery is currently unknown. In this respect, it is conceivable that Alix, in addition to its canonical involvement in the biogenesis of MVBs and in viral budding, processes that involve a negative curvature of the membrane, may also participate in the direct budding of nanovesicles from the plasma membrane of muscle cells. Supporting this idea, Alix, which is mainly cytoplasmic, was found associated not only with actin and the membrane of internal organelles but also with sites of exosome biogenesis at the plasma membrane of T-cells, together with other exosomal proteins and the lipid lysobisphosphatidic acid (LBPA) [6–8,15].