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Matrix vesicles isolated from mineralization-competent Saos-2 cells are selectively enriched with annexins and S100 proteins

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ABSTRACT

Matrix vesicles (MVs) are cell-derived membranous entities crucial for mineral formation in the extracellular matrix. One of the dominant groups of constitutive proteins present in MVs, recognised as regulators of mineralization in norm and pathology, are annexins. In this report, besides the annexins already described (AnxA2 and AnxA6), we identified AnxA1 and AnxA7, but not AnxA4, to become selectively enriched in MVs of Saos-2 cells upon stimulation for mineralization. Among them, AnxA6 was found to be almost EGTA-non extractable from matrix vesicles. Moreover, our report provides the first evidence of annexin-binding S100 proteins to be present in MVs of mineralizing cells. We observed that S100A10 and S100A6, but not S100A11, were selectively translocated to the MVs of Saos-2 cells upon mineralization. This observation provides the rationale for more detailed studies on the role of annexin-S100 interactions in MV-mediated mineralization.

1. Introduction

Physiological and pathological mineralization is initiated by matrix vesicles (MVs). MVs are cell-derived vesicular structures, nucleation sites for mineral formation, located within the extracellular matrix (ECM). Annexins are a dominant group of proteins present in MVs [1,2]. These calcium- and membrane-binding proteins appear to play a significant role in the functioning of MVs. As membrane proteins, annexins exhibit ion channel activities to sustain the influx of Ca^{2+} into MVs [3–5]. Inside MVs, soluble annexins may provide nucleation sites for minerals to grow [6]. Extravesicularly, annexins form anchors for ECM components [7]. Up to now, many reports have evidenced the involvement of AnxA5 and AnxA6 in MV-mediated mineralization. Unfortunately, this was not supported by the results obtained by knocking out annexin genes in animal models [8,9]. These reports did not consider the potential functional overlap by members of this large gene family or the possibility that pathology may become evident only if a defective mutant form of annexin is expressed [10].

Human osteosarcoma Saos-2 cells express the entire osteoblastic differentiation sequence from proliferation to mineralization and spontaneously release MVs [11]. Recently, several annexins (AnxA1, AnxA2, AnxA4-A7 and AnxA11) were identified in MVs of Saos-2 cells [12]. Therefore, not only AnxA5 or AnxA6 of MVs may be considered as mineralization regulators. It was evidenced that AnxA2 and AnxA6 content were significantly increased in mineralizing MVs in comparison to control MVs (both isolated from vascular smooth muscle cells) [13]. In this study, we investigated the higher content of several annexins in MVs isolated from mineralizing Saos-2 cells.

S100 proteins represent a large family of small EF-hand Ca^{2+}-binding proteins that exert both intracellular and extracellular functions. Previously, S100A4 and S100A8/A9 were found in differentiating osteoblasts and were shown to regulate matrix mineralization [14–16]. A proteomic study revealed S100A4 as the one and only representative of the S100s in MVs of osteoblasts [17]. However, the presence of S100s in MVs was not supported by other proteomic research [12,18]. This discrepancy is questionable as several S100s and annexins are known to interact with each other (AnxA1 binds S100A6 or S100A11, AnxA2 forms complexes with S100A4, S100A6, S100A10 or S100A11 and AnxA6 interacts with S100B, S100A6, S100A11, S100A1) to form complexes that exhibit biological activities [19–21]. Thus, we decided to test MVs isolated from Saos-2 cells for the presence of S100s described earlier for their interactions with annexins. Our findings greatly complement previous proteomic studies and create a new topic for further mineralization studies.

Since the involvement of MVs in pathological calcification (vascular smooth muscles or renal calcification) gathers more and more attention, the detailed investigation of annexins and S100s actions in these structures offers a way to uncover the mechanisms involved in these processes.

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2. Materials and methods

2.1. Cell culture and treatment

Human osteosarcoma Saos-2 cells (ATCC HTB-85) were cultured in McCoy's 5A media (ATCC) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma) and 15% FBS (v/v, Gibco). Mineralization was induced by treatment of Saos-2 cells for 7 days with 50 μg/ml ascorbic acid (AA, Sigma) and 7.5 mM β-glycerophosphate (β-GP, Sigma).

2.2. Preparation of matrix vesicles

MVs were harvested by different ultracentrifugation steps at 4 °C simultaneously from the cell cultures grown for 7 days under resting or stimulated conditions. After washing with PBS, cells were digested with 100 U/ml collagenase type IA in HBSS (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose, pH 7.6) at 37 °C for 3 h. The cells were released by gentle scraping and were centrifuged at 800 × g for 30 min (MPW-350R) to remove debris. The obtained supernatant was further subjected to centrifugation at 30,000 g for 30 min (Beckmann, Ti 60 rotor) to pellet basolateral membranes and microsomes. The supernatant was ultracentrifuged at 100,000 g for 30 min (Beckmann, Ti 60 rotor) to collect MVs. The MV pellet was dissolved in HBSS buffer and stored at −20 °C.

2.3. Alkaline phosphatase (ALP) activity assay

The ALP activity of MVs was determined using freshly prepared 10 mM p-NPP (4-nitrophenyl phosphate disodium salt hexahydrate) in reaction buffer (25 mM glycine, 25 mM piperazine, pH 10.4). The reaction was started by the addition of vesicles to the reaction buffer and incubated at 37 °C. The absorbance was measured in 30 s intervals at 420 nm in a BioMate3 spectrophotometer (Thermo Electron Co.). The ALP activity was quantified using a molar absorption coefficient of 17.8 cm^{-1} mM^{-1}. The protein content in particular fractions was previously measured by Bradford assay (BioRad Laboratories). The results are normalized as enzyme units in mol of p-NPP hydrolyzed per minute per milligram of total protein.

2.4. Visualization of alkaline phosphatase activity

Electrophoresis was performed in 7.5% (w/v) SDS–PAGE gels under mild-denaturing conditions. Protein samples (15 μg/well) were loaded in Tris buffer containing 2% SDS. After migration, gels were incubated in a buffer containing 0.1 M Tris–HCl, pH 9.6, 100 mM NaCl, 5 mM $MgCl_2$, 0.25 mM nitroblue tetrazolium (NBT) and 0.24 mM bromo-chloro-indolyl-phosphate (BCIP) until bands of sky blue colour were clearly visible.

2.5. Mineralization assay

MVs were suspended in SCL buffer (100 mM NaCl, 12.7 mM KCl, 0.57 mM $MgCl_2$, 1.83 mM $NaHCO_3$, 0.57 mM Na_2SO_4, 3.42 mM NaH_2PO_4, 5.55 mM D-glucose, 63.5 mM sucrose, 16.5 mM TES, pH 7.6) with or without 2 mM $CaCl_2$ to a final concentration of 50 μg of MV protein /ml. The samples were incubated at 37 °C in a 96-well microtitre plate without mixing and light scattering was read at 340 nm at 15-min intervals (SpectraMax M5e multi-detection reader, Molecular Devices). As a positive control of mineral formation, 4% DMSO in SCL buffer supplemented with 3.42 mM P_i and 2 mM $CaCl_2$ was used. As a negative control, vesicles incubated in SCL buffer containing 3.42 mM P_i but without 2 mM $CaCl_2$ were prepared.

2.6. Transmission electron microscopy (TEM)

A 10 μl aliquot of the MV suspension (30 μg proteins/mL) was dropped on formvar-coated carbon-labelled nickel grids (Agar Scientific Ltd). Prior to the complete drying of the samples, the grids were counterstained with 2% (w/v) uranyl acetate according to the negative staining method. The specimens were viewed with an electron microscope JEM-1400EX (JEOL Co) at 80 kV accelerating voltage.

2.7. EGTA treatment of MVs

Samples containing 0.33 mg protein/ml of MVs were incubated in SCL buffer pH 7.4 with either 2 mM $CaCl_2$ or with 10 mM EGTA at room temperature for 30 min. They were subjected to five cycles of freeze/thaw procedure (freezing in liquid nitrogen and thawing in a water bath at 37 °C). Then, both samples were centrifuged at 170,000 g for 20 min at 4 °C. The extracted proteins (supernatants) and MV membranes (pellets) were analyzed for the presence of annexins by 10% SDS–PAGE and immunoblotting.

2.8. SDS–PAGE and immunoblotting

The protein concentration of the vesicles fractions were determined using the Bradford method (BioRad Laboratories). Samples (25 μg) were suspended in Laemmli loading buffer and incubated at 95 for 5 min. Proteins were separated by SDS–PAGE gels and then electrotransferred onto nitrocellulose membranes (Mini-PROTEAN III, BioRad Laboratories). After 1 h blocking with 5% low fat milk in TBS (10 mM Tris, 100 mM NaCl), proteins were immunostained overnight with the following primary antibodies (from BD Transduction Laboratories: mouse monoclonal anti-AnxA2 IgG1 (1:2500), mouse monoclonal anti-AnxA4 IgG1 (1:2500), mouse monoclonal anti-AnxA6 IgG1 (1:2500); from Abcam: mouse polyclonal anti-AnxA1 (1:500), mouse monoclonal anti-AnxA5 IgG1 (1:1000), mouse monoclonal anti-AnxA7 (1:1000), mouse monoclonal anti-S100A6 (CACY-100) (1:1000), rabbit monoclonal anti-S100A10 (EPR3317) (1:5000), rabbit polyclonal anti-S100A11 (1:1000), rabbit monoclonal (EP1531Y) anti-Hsc70 (1:1000), rabbit polyclonal anti-gelsolin (1:1000), mouse monoclonal anti-bone alkaline phosphatase (0.G.2) (1:1000); from Sigma: rabbit polyclonal anti-myosin IIB (non-muscle) (1:500), mouse monoclonal anti-vinculin 1 IgG1 (1:500); from Calbiochem: mouse monoclonal anti-actin 1 (1:5000), in 2.5% low fat milk in TBST (TBS with 0.05% Tween-20). This was followed by incubation with HRP-conjugated secondary antibodies (ECL-Anti-mouse IgG-HRP, ECL-Anti-rabbit IgG-HRP, both from Amersham Biosciences). The blots were visualized by ELC kit (Amersham Pharmacia) according to the manufacturer's instructions.

2.9. Statistical analysis

Data were expressed as the mean ± SE (t student) of n observations.

3. Results

3.1. Ascorbic acid and β-glycerophosphate treated Saos-2 cells produce functional MVs with high ALP-activity

In the present work, we used AA and β-GP simultaneously to intensify Saos-2 cell mineralization. The comparison of MVs isolated from resting and isolated from 7 days mineralizing Saos-2 cells served as a model for the investigation of protein dynamics in different stages of mineralization. We conducted functional