Inside-out: unpredicted Annexin A2 localisation on the surface of extracellular vesicles

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Abstract

Extracellular vesicles (EVs) contain many proteins, both cytosolic and surface bound. The current model for EV biogenesis dictates that cytosolic proteins remain in the lumen and cell surface proteins reside on the outside of vesicles. This is consistent with the traditional protein trafficking pathway, where proteins destined for the plasma membrane contain a signal sequence targeting them to the secretory pathway. According to this 'classical' pathway for membrane and secretory protein trafficking, proteins lacking a signal sequence should not reside at the cell surface. It has been shown that transmembrane proteins are retained in the membrane of EVs and RNAs reside in the lumen of EVs. However, there is little known about the packaging and location of other proteins enriched in EVs. Annexin A2 is a cytosolic protein abundant in EVs. We show for the first time that Annexin A2 is expressed not only in the lumen of EVs as predicted but also on the surface of EVs. This raises fundamental questions regarding Annexin A2 transport to the outer leaflet of the EV membrane as it lacks a signal peptide for secretion.

Figure

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Annexin A2 is located on the surface of extracellular vesicles. A) Workflow diagram for extracellular vesicle isolation by ultracentrifugation. B) Size distribution of PANC1 EVs isolated by ultracentrifugation. The diameter of EVs was measured by tunable resistance pulse sensing and data plotted as a percentage of the total count analysed in one experiment. The mean diameter is 153 nm and the mode diameter is 98 nm. C) PANC1 EVs are positive for the exosomal marker CD63 and negative for the Golgi protein GGA1. EVs and PANC1 cell lysate were resolved by western blot (under non-reducing conditions for CD63) and probed for CD63, GGA1 or actin. Representative western blot where n=3. D) PANC1 EVs contain acetylcholine esterase. EVs were lysed and acetylcholine esterase activity measured over time on a colorimetric substrate at an absorbance of 410 nm. Representative data n=2. E) Annexin A2 is on the surface of PANC1 EVs. Left: Diagram of the experiment showing the removal of surface bound Annexin A2 with EDTA treatment. Right: EVs treated with or without EDTA, EVs pellet and supernatant resolved by western blot. Annexin A2 is detected in both EVs pellet and only in the supernatant of EDTA treated EVs. The blot was also probed for actin as a control. PANC1 cell lysates were run on each blot as a positive control. Representative western blot where n>3. F) Mass spectrometry analysis of EDTA treatment as in E. Data plotted as the fold increase of protein in the supernatant with respect to untreated samples. Representative data from n=2. G) NSC EVs

treated with or without EDTA as in panel E (right). Representative western blot where n=2. H) Annexin A2 on the surface of EVs is digested by trypsin. Left: Diagram of the experimental concept, where trypsin cleaves surface bound Annexin A2. Right: EVs treated with trypsin in the presence or absence of triton (Tx-100) detergent resolved by western blot. Surface bound Annexin A2 is digested by trypsin and cleavage products detected. Two exposures shown for Annexin A2. Representative western blot where n>3.

Introduction

Practically all cells (under both physiological and pathological conditions) secrete small non-apoptotic vesicles (Colombo, Raposo et al. 2014). These include microvesicles and exosomes, collectively termed extracellular vesicles (EVs) [1]. Microvesicles are formed during plasma membrane shedding, whereas exosomes originate from the endosomal pathway [1](Lo Cicero_2015[2]). EVs contain a specific subset of lipids, RNAs and proteins that can be transferred between cells [3](Lo Cicero_2015[2])[4].

The current model for EV biogenesis dictates that cytosolic proteins are in the lumen and cell surface proteins are on the exterior of EVs. Annexin A2 is a cytosolic calcium dependent membrane binding protein lacking a signal sequence for secretion (leaderless protein). Annexin A2 is involved in many cellular processes including membrane trafficking events [5]. Through its membrane binding capacity, Annexin A2 has been shown to be involved in lipid organisation at sites of membrane-actin interactions, calcium-mediated endocytosis and may have a role in ion channel activity [6]. Along with several other proteins, Annexin A2 is consistently identified and enriched in EVs (Lo Cicero_2015[2]) (Exocarta/Vesiclepedia databases)[7]. Here we demonstrate that, despite being a cytosolic protein, Annexin A2 localises not only in the lumen but also on the surface of EVs.

Objective

To investigate the packaging and localisation of Annexin A2 in extracellular vesicles.

Results & Discussion

EVs were produced from the pancreatic cancer cell line, PANC1, and isolated by ultracentrifugation (Figure 1A) as described [3]. This cell line was chosen as it produces a large number of EVs and expresses a high level of Annexin A2. Isolated EVs were characterised according to size distribution and common exosomal markers.

Tunable resistance pulse sensing showed the purified EVs had a mean diameter of 153 nm and a mode of 98 nm (Figure 1B), consistent with the literature. EVs showed an enrichment in the exosomal marker CD63 and were negative for the Golgi-localized, ;2ÖV homology domain, ARF-binding protein (GGA1) (Figure 1C). EVs also contained acetylcholine esterase, as detected by a specific activity assay (Figure 1D). These results show that EVs had the expected size distribution and contained a large proportion of exosomes.Annexin A2 is a calcium dependent membrane binding protein. As such it is able to be removed from the membrane with calcium chelators, such as EDTA. To determine whether Annexin A2 is on EV surface, EVs were treated with EDTA for 30 min at 37 pC. The supernatant was then separated from the EVs by ultracentrifugation and Annexin A2 was detected by western blot. We found that Annexin A2 was present in the supernatant, which suggests its dissociation from the EVs membrane upon EDTA treatment (Figure 1E). This was unlikely to be due to EVs damage, as actin (present in the lumen of EVs) was not detectable in the EDTA supernatant (Figure 1E). Annexin A2 was also detectable in the EVs pellet, thus indicating that it is present both in the lumen as well as on the surface of EVs (Figure 1E). To confirm this result, samples were subjected to mass spectrometry analysis; we observed a two fold increase in the amount of Annexin A2 when EDTA was present (Figure 1F).

These experiments were repeated with EVs isolated from mouse neural stem cells (NSCs) and led to similar results, indicating that the presence of Annexin A2 on EVs surface is not restricted to one cell line (Figure 1G). A small amount of actin was also detected in the supernatant in both untreated and after EDTA, which could be due to some actin leaking from the EVs (Figure 1G). Nonetheless, we observed a clear-cut evidence with Annexin A2 that was found in EDTA supernatants only. In NSC experiments, we also used GAPDH as additional control, which was not present in supernatants so to confirm the integrity of EVs (Figure 1G).

To further confirm the presence of Annexin A2 on the surface of EVs, we used a protease protection assay. EVs were treated with trypsin with or without detergent for 30 min at 37°C; the EVs pellet was then separated from the supernatant by ultracentrifugation and Annexin A2 was detected by western blot (Figure 1H). Annexin A2 was liable to tryptic digest, further confirming its unpredicted presence on the surface of EVs. When detergent and trypsin were present, all the available Annexin A2 was digested to completion. This confirms that the Annexin A2 detected after tryptic digest, both the full length and smaller products, were protected from cleavage. As an internal control the blot was probed for actin, the vast majority of which remained intact after trypsin digestion, thus indicating the EVs were not damaged as a result.

Following the current model for EVs formation, cytosolic proteins should remain in the lumen of EVs. We report here the intriguing observation that the cytosolic protein Annexin A2 is present on the surface of EVs. How Annexin A2 is able to access the outer membrane of EVs

remains unclear and represents a major gap in our understanding of protein trafficking that will require further investigation.

It is known that a pool of Annexin A2 is found at the cell surface [8]. Therefore, it is possible that this surface pool of Annexin A2 is internalised during endocytosis upstream of EVs biogenesis and that could explain the localisation of Annexin A2 on the surface of EVs. Similarly, microvesicles are formed by budding from cell surface and thus may also contribute to the pool of Annexin A2 found on the surface of EVs. However, as described above, Annexin A2 lacks a signal sequence for secretion and therefore should not be able to access the cell surface [9]. In this scenario, Annexin A2 would have to cross the plasma membrane via an unconventional protein trafficking pathway, a hypothesis that remains to be tested [10][11][12]. It would also be interesting to test the presence of other leaderless proteins on the surface of EVs to understand whether this observation represents a common feature of such leaderless proteins or is restricted to Annexin A2.

Conclusions

In conclusion, we show for the first time that Annexin A2 has an unpredicted localisation on the surface of EVs.

Funding statement

This work was supported by Wellcome Trust Strategic Award [100574/Z/12/Z] and MRC Metabolic Diseases Unit [MRC_MC_UU_12012/5], from the Italian Multiple Sclerosis Association (AISM, grant 2010/R/31), the Italian Ministry of Health (GR08-7), the European Research Council (ERC) under the ERC-2010-StG Grant agreement n° 260511-SEM_SEM, the UK Regenerative Medicine Platform Acellular hub (Partnership award RG69889), and core support grant from the Wellcome Trust and MRC to the Wellcome Trust – Medical Research Council Cambridge Stem Cell Institute. FG is supported by a scholarship of the Gates Cambridge Trust.

Acknowledgements statement

We thank Frances
Richards (Cancer Research UK) for the PANC1 cell line and the CIMR/IMS
Proteomics Facility.

Ethics statement

Not applicable

Methods

Antibodies - Rabbit anti-human actin
(Sigma-Aldrich, A2066) used at 1:2000; mouse monoclonal anti-human Annexin A2
(BD Biosciences, 610069) used at 1:1000; mouse monoclonal anti-human CD63
(ThermoFisher Scientific, 10628D) used at 1:500. Q-27 mouse monoclonal anti-human GGA1 (Santa Cruz Biotechnology, sc-101257) used at 1:500. Secondary antibodies goat
anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2004) and goat anti-mouse IgG (Santa Cruz Biotechnology,
sc2005) were used at 1:2000. All antibodies were diluted in phosphate buffered
saline (PBS). Cell lines and cell culture – Human pancreatic cancer cell line
PANC1 [13] were maintained in DMEM
(Sigma-Aldrich, D6546) containing 10% (v/v) fetal bovine serum, 2 mM
L-glutamine, 1 mM penicillin and 1 mM streptomycin. Mouse neural stem cells
(NSCs) were cultured as previously described [14]. Extracellular vesicle isolation – PANC1 cells were grown to 70%
confluence in a T75 flask at which point the medium was changed to 10 ml serum
free DMEM and incubated for 24 hours. EVs were collected by ultracentrifugation
as previously described [3] . Briefly, the medium was collected
and sequentially centrifuged at 300 g for 15 min, 1,000 g for 15
min, 100,000 g for 90 min. The EVs pellet was washed in serum
free DMEM without phenol red (SF-DMEM; GIBCO, 21063) centrifuged at 100,000 g for 30-60
min. EVs were resuspended in SF-DMEM at 10 µl per 10 ml starting material. EVs
from NSCs were also produced as previously described [3]. Briefly, 12 million cells were
seeded per T75 flask and incubated overnight. The medium was collected and EVs
isolated as described above. Cell lysates - Cells were incubated in an
appropriated volume of ice cold lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1 mM
EDTA, 1% triton X-100, pH 6.8) at 4°C for 10 min. The
lysate was collected and insoluble material pelleted at 10,000 g for 10 min at 4°C. The supernatant was collected and sample buffer added (final: 50 mM
Tris-HCl, 2% (w/v) sodium dodecyl sulphate (SDS) 0.1% (w/v) bromophenol blue,
10% (v/v) glycerol, 100 mM DTT, pH 6.8). Samples were boiled for 10 min and
stored at -20°C. Tunable resistance pulse sensing (TRPS) – TRPS measurements were measured with
the qNano (Izon Science, UK). The polyurethane nanopore NP100 (Izon Science,
part A33255) was used for all measurements

and was axially stretched to 46.99
mm. EV samples were diluted in PBS as required and 40 µl loaded into the instrument.
Measurement time was up to 2 min depending on the instrument stability. The
system was calibrated with 200 nm polystyrene beads diluted in PBS. Data
analysis was carried out with Izon Control Suite software (Izon Science, UK). Acetylcholine esterase activity assay – 10 µl EVs were lysed in 0.5% (v/v)
triton X-100 in PBS and assayed for acetylcholine esterase activity with a
colorimetric assay kit from Abcam (ab138871) as per the manufactures
instructions. The absorbance was measured at 410 nm over time and plotted
against a buffer alone control. EDTA treatment – EVs (10-20 µl per treatment) were resuspended in
100 µl of either SF-DMEM or versene solution (GIBCO, 15040-066) containing 0.48 mM EDTA and
incubated at 37°C for 30 min. EVs were pelleted at
100,000 g for 30 min. The supernatant was collected and the pellet resuspended
in 100 µl SF-DMEM. All samples were mixed with sample buffer (as above) and
boiled for 10 min. Samples were analysed by western blotting. Western blotting
– All samples were resolved by 12% SDS-polyacrylamide gel electrophoresis
(PAGE) and transferred to polyvinylidene difluoride membranes for blotting.
Membranes were blocked with 0.05% (w/v) skim milk powder in PBS containing 0.1%
Tween-20 (PBS-Tween) for 30 min at room temperature. Membranes were then probed
with an appropriate dilution of primary antibody overnight at 4°C. Membranes were washed three times in PBS-Tween before incubation in
diluted secondary antibody for 1 hour at room temperature. Membranes were
washed as before and developed with ECL (Cyanagen, Westar XLS100) using a Bio
Rad Chemi Doc XRS system. Membranes were stripped with Restore plus
 (ThermoFisher Scientific, 46430) as per manufactures instructions. Mass spectrometry analysis – Samples prepared as described above
for EDTA treatment and submitted for mass spectrometry analysis using Thermo
Orbitrap Q Exactive with EASY-spray source and Dionex RSLC 3000 UPLC. For
graphical representation the following equation (ratio = (EDTA sup/EDTA pellet)+1 / (untreated sup/untreated pellet)+1) was used to obtain a ratio of
protein detected in the supernatant upon EDTA treatment with respect to the
untreated control.Trypsin treatment – EVs (10-20 µl per treatment) were resuspended in
100 µl of either SF-DMEM, trypsin solution (Sigma-Aldrich, 4674) diluted to 2.5
mg/ml SF-DMEM, 0.5% triton X-100 (Sigma-Aldrich, T8787) diluted in SF-DMEM or a
combination of both trypsin and triton X-100. EVs were incubated at 37°C for 30 min. EVs were then pelleted at 100,000 g
for 30 min. The supernatant was collected and pellet resuspended in 100 µl
SF-DMEM. Samples were mixed with sample buffer (as described above), boiled for
10 min and analysed by western blotting.

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