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Cooperative binding promotes demand-driven recruitment of AnxA8 to cholesterol-containing membranes

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ABSTRACT

The functionality of cellular membranes is critically determined by their lipid composition. Within the endolysosomal system, cholesterol is mainly found in more peripheral compartments. In contrast, cholesterol levels are low in late endosomes/lysosomes (LEL), and the occurrence of enlarged pools of this lipid is commonly linked to endolysosomal dysfunction. Here, we show that Annexin A8 (AnxA8), a member of the annexin family of Ca^{2+}-dependent membrane-binding proteins, participates in the endosomal regulation of cholesterol homeostasis. Depletion of AnxA8 caused accumulation of cholesterol in LEL, and pharmacological inhibition of the LEL cholesterol export recruited AnxA8 to the cholesterol-laden LEL. Biophysical analysis revealed that cholesterol enhanced the Ca^{2+}-dependent affinity of AnxA8 to lipid bilayers, and induced positive cooperativity of membrane binding. Our findings identify AnxA8 as a regulator of LEL cholesterol balance and point to altered membrane binding cooperativity induced by aberrant lipid composition in the target membrane as a means to control the demand-driven recruitment of this cytosolic regulatory protein.

1. Introduction

Lipids are key regulators in the maintenance and functionality of membrane systems within eukaryotic cells. Specific lipid signatures in cellular membranes substantially contribute to the biophysical properties such as curvature, stiffness, and rigidity [1,2]. Accordingly, proteins involved in membrane trafficking are often targeted to their destination compartments through their ability to recognize and bind specific lipids. In particular, local enrichment of lipids such as cholesterol, in membrane microdomains, through lipid-protein and protein-protein interactions, enables the formation of associated protein complexes at high density, thus forming spatially restricted functional units.

Members of the evolutionary conserved annexin multigene protein superfamily interact with negatively charged phospholipids, such as phosphatidylserine (PS), in a Ca^{2+} dependent manner [3,4]. Accordingly, most of their reported activities in the regulation of membrane dynamics have been attributed to this transient and reversible membrane binding behavior, e.g. in endocytosis, the pathway that ensures delivery of macromolecules and particles to their respective cellular destinations within eukaryotic cells. While peripheral early endosomes sort cargo for recycling to the plasma membrane or final degradation, the more perinuclear late endosomes and lysosomes not only function in the degradative pathway, but act as an additional sorting compartment from which cargo can be retrieved, and inter-compartmental trafficking, e.g. with the Golgi, and the endoplasmic reticulum, are known. Because of their more acidic nature, the occurrence of intraluminal vesicles, and their shared marker proteins such as Rab7 and CD63, the late endosomes and the lysosomes cannot be easily discriminated, hence we address these vesicles as LEL [1].

The considerable impact of lipids on endosomal membrane dynamics is drastically observed in lipid storage disorders, such as Niemann-Pick disease, type C1 (NPC1) [5]. This fatal autosomal recessive disease is caused by mutations in the late endosomal proteins NPC1 and 2, that act synergistically in the export of free (unesterified) cholesterol to other cellular destinations [6–8]. Accordingly, the expression of a defective NPC1 protein leads to a pathological

Abbreviations: Anx, annexin; AnxA8ins, siRNA-insensitive AnxA8; Ctrl, control; GFP, green fluorescent protein; IPTG, isopropyl-β-d-thiogalactose; LDL, low-density lipoprotein; LEL, late endosomes/lysosomes; NPC1, Niemann Pick disease type C1; PM, plasma membrane; PNS, post-nuclear supernatant; POPC, phosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; PS, phosphatidylserine; QCM-D, Quartz Crystal Microbalance with Dissipation; SEM, standard error of mean; shRNA, short hairpin RNA; SLBs, solid-supported lipid bilayers; WT, wild-type; YFP, yellow fluorescent protein

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accumulation of LEL cholesterol, with a concomitant cholesterol depletion at other cellular membranes, such as the plasma membrane (PM). This cellular imbalance of cholesterol distribution triggers major defects in membrane trafficking, highlighting the need to tightly control LEL cholesterol contents for proper cellular function. Under normal conditions, cholesterol is taken up from the surrounding media via endocytosis of low-density lipoprotein (LDL) particles that are hydrolyzed in LEL, where free cholesterol is then delivered to other cellular sites. The accumulation of this lipid in LEL, a compartment that under normal conditions contains only little cholesterol [9], is usually associated with a disturbed LEL functionality, notably impaired sorting and further trafficking of cargo.

We recently reported delayed trafficking through the degradative pathway and improper transport of CD63 from endolysosomes to the cell surface in the absence of the LEL-associated protein annexin A8 (AnxA8) [9,10], which prompted us to investigate a potential impact of this annexin on LEL cholesterol homeostasis. We found that depletion of AnxA8 caused LEL cholesterol storage, indicating that AnxA8 is involved in the export of cholesterol from LEL. Accordingly, we observed recruitment of AnxA8 onto LEL laden with cholesterol through pharmacological inhibition of NPC1. Analysis of the binding using solid-supported artificial lipid bilayers revealed that in the presence of cholesterol, AnxA8 binding to PS required less Ca^{2+}, yet remained strictly Ca^{2+}-dependent. Importantly, the AnxA8 binding behavior switched to positive cooperativity. Our observations suggest that AnxA8 is part of the LEL cholesterol balancing system, and that membrane binding cooperativity is used to dynamically control the demand-driven recruitment of such a cytosolic regulatory protein.

2. Material and methods

2.1. Plasmids, RNAi

The bacterial expression plasmid pET-23amod/anxA8, green fluorescent protein (GFP)-tagged wild-type AnxA8 (AnxA8-GFP) and siRNA-insensitive AnxA8ins-GFP carrying seven silent mutations in the siRNA-targeted region were described previously [10,11]. To generate the Ca^{2+}-insensitive AnxA8-CM-GFP mutant in which all type II Ca^{2+} binding sites are deleted, amino acid replacements E78A, D150A, E235A, and D313A were introduced by site-directed mutagenesis. Yellow fluorescent protein (YFP)-tagged murine wild-type NPC1 and the dominant negative loss-of-function NPC1 P692S mutant were described previously [12,13]. pEGFP-N3 (Clontech) served as a control. For AnxA8 depletion, AnxA8-specific siRNA [11] (5´-GGAGCGGAGAUUGACUUAAAdTdT-3) was used. The non-targeting Ctrl siRNA (AllStars negative control siRNA, Qiagen) served as negative control.

2.2. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) High Glucose (Sigma) supplemented with 10% fetal calf serum (Biochrom), 2 mM L-glutamine, 1% non-essential amino acid solution, 100 U/mL penicillin/100 µg/mL streptomycin at 7% CO_2 and 37 °C. Cells were transfected with Lipofectamine 2000 (ThermoFischer) according to the manufacturer's protocol.

2.3. Quantitative real-time reverse transcription-PCR (qPCR)

Total RNA was isolated using the RNAse Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was transcribed with High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific). qPCR was performed with the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) and primers obtained from Qiagen (ACTB, QT00095431), (B2M, QT00088935) or custom-made (AnxA8 5′-TGGGACCCTGATAAGAAACAT-3′, 5′-TCCTGGAGACTCTGGCTTCAT-3′; GAPDH 5′-ACCCACTCCTCCACCTTTG-3′, 5′-CTCTTGTGCTCTTGCTGGG-3′) using the LightCycler 480 II (Roche) according to the manufacturer's instructions. qPCR data were analyzed according to the $2^{-\Delta\Delta cp}$ method.

2.4. Isolation of murine lung primary cells

C57BL/6 mice and AnxA8 KO mice were C57BL/6 mice were kept in standard individually ventilated cages (IVC) under specific pathogen-free conditions and regular general health checks at the ZMBE animal housing facility. Animal work was not classified as “animal experimentation”, was carried out in compliance with German law of animal protection and the guidelines issued by the state of North Rhine-Westphalia, and was approved by the local authorities (LANUV, 84–02.04.50.15.041). Lungs from eight-to-ten weeks old males were digested according to Seluanov et al. [14]. In brief, lungs were washed with PBS with Ca^{2+}/Mg^{2+}, cut into fragments of approximately 1 cm^2 and placed in DMEM medium containing 1% penicillin/streptomycin, and 0.14 units/mL Liberase (Sigma). Lung fragments were digested for 1 h at 37°. Isolated cells were disseminated and cultivated.

2.5. Immunocytochemistry and confocal fluorescence microscopy

For cholesterol staining using filipin, cells were fixed with 4% paraformaldehyde (PFA) in PBS^{++} (PBS with Ca^{2+}/Mg^{2+}) for 10 min at room temperature, and further incubated with 1.25 mg/mL filipin (filipin complex from Streptomyces filipinensis, Sigma, F9765) for 2 h. For AnxA8-GFP localization, cells were fixed and simultaneously permeabilized with 4% PFA containing 0.2% Triton X-100 for 5 min. Mouse monoclonal TRITC-conjugated anti-CD63 antibody (sc-5275 TRITC, Santa Cruz) or Lysotracker Red DND-99 (Thermo Fischer Scientific) was used to visualize endosomal/lysosomal compartments. Confocal microscopy was performed using an LSM 780 or LSM800 microscope (Carl Zeiss, Jena, Germany) with a Plan-Apochromat 63 × / 1.4 oil immersion objective. To quantify LEL cholesterol contents, z-Stacks of individual cells were thresholded, and Manders' colocalization coefficients of the respective endosomal/lysosomal marker with the filipin signal were calculated using the ImageJ plug-in Jacop [15].

2.6. NPC1 phenotype induction and quantification of unesterified cellular cholesterol

To mimic the NPC1 loss-of-function phenotype, cells were incubated with the hydrophobic polyamine U18666A, a NPC1 small-molecule inhibitor (2 µg/mL, Enzo Life Sciences) for 16 h. The cholesterol content in the cellular fractions was determined as described previously [16] using the Amplex Red Cholesterol Assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For LDL uptake experiments, cells were starved overnight in DMEM with 0.2% BSA, 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and then pulsed for 30 min with 100 µg/mL human LDL (Millipore), followed by a 2 h chase period.

2.7. Subcellular fractionation by sucrose step gradient centrifugation

LEL membrane fractions were enriched via sucrose density gradient centrifugation as described previously [9]. Protein concentration in the fractions was measured by the Bradford assay, and the LEL enrichment was verified by Western blotting and detection of the early endosomal (Rab5), endolysosomal (Rab7) and Golgi (GM130) marker proteins with appropriate primary antibodies (mouse monoclonal anti-GM130, 610,823, BD Biosciences; rabbit monoclonal anti-Rab5, 3547, anti-Rab7, 9367, Cell Signaling) and secondary antibodies labeled with IRDye 680CW and IRDye 800CW and the Odyssey infrared imaging system (LI-COR).