Small GTPase ARF6 Regulates Endocytic Pathway Leading to Degradation of ATP Binding Cassette Transporter A1

Nigora Mukhamedova, Anh Hoang, Huanhuan L. Cui, Irena Carmichael, Ying Fu, Michael Bukrinsky, Dmitri Sviridov

Materials and Methods

Cells

Mouse macrophage cell line RAW264.7 (ATCC, Manassas, VA) was used in the experiments described in this study, unless specified otherwise. The cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS). Twenty four hours after transfection, Abca1 expression was stimulated by adding compound TO901317 (final concentration 4 μM), which was retained in subsequent incubations.

Bone marrow derived macrophages (BMDM) were isolated from tibia and femur bones of 6-8 week-old C57BL/6 male mice as described by Shrestha et al [1]. In brief, bone marrow was flushed out from bones with IMDM containing 10% FBS. Cells were spun, and pellet was incubated for 10 min in red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). Cells were centrifuged and pellet was resuspended in IMDM containing 10% FBS and 15% of L929 cell conditioned media. Cells were plated in Petri dishes (BD, 351029) and left for 5 days.

Cells were loaded with cholesterol by incubating them with cholesterol/methyl- β-cyclodextrin complex (final concentration of methyl-β-cyclodextrin, 5 mM) for 3 h in serum-free RPMI-1640. Cholesterol/methyl- β-cyclodextrin complex was prepared as described by Klein et al [2]. Cells were depleted of cholesterol by 1 h incubation in 1 mM methyl-β-cyclodextrin (Sigma-Aldrich) solution in serum-free medium (5 mM in the experiments with BMDM).

Time-lines of all experiments described below are shown in Supplemental Figure S1

Transfections

Cells were seeded in antibiotic-free RPMI-1640 media supplemented with 10% FCS. Transfections were performed 18 h later when cells were 70-80% confluent. Transfection of cells with mouse siRNAs was done using RNAiMax Reagent (ThermoFisher Scientific). The siRNA target sequences are shown in the table below.

Target	Target sequence	Source	Cat#
Scrambled	ALLStars Negative Control	Qiagen	1027280
Clathrin	GGAAAGCAAUCCAUACAGA	Dharmacon	J-063954-05
Dynamin-2	ACCAUGAGCUGCUGGCUUA	Dharmacon	J-044919-05
Cdc42	AUAGAAACGCCUCGAUUAA	Dharmacon	J-043087-09
Arf6	CAAACGGGGUGGGGUAAUA	Dharmacon	J-043217-09
Arf6-2	CAAGTCTGTTTCATCTAGTAA	Qiagen	SI00166593

In brief, RAW264.7 were seeded in 12 well plates at the density $0.22x10^6$ cells per well in antibiotic free RPMI media containing 10% FBS. For each well to be transfected, RNAi duplex-Lipofectamine RNAiMAX complexes were prepared as follows. RNAi duplex were diluted in Opti-MEM Reduced Serum Medium. Lipofectamine RNAiMAX was diluted in Opti-MEM, mixed gently and incubated for 5 min. Diluted RNAi duplex was combined with the diluted Lipofectamine RNAiMAX, mixed gently and incubated for 20 min at room temperature. RNAi duplex-Lipofectamine RNAiMAX complexes were added drop wise to each well. The final siRNA concentration was 50 nM. When a double transfection was employed, half the amount of each siRNA was added to the incubation mixture. Transfection with scrambled siRNA is referred as Mock transfection.

BMDM were seeded on day 5 after isolation and tranbsfected next day when cells were 35-45% confluent. Silencing of ARF6 was done using INTERFERin reagent (Polyplus, France) according to the manufacturer instructions. In brief, both scrambled and ARF6 siRNAs were diluted in serum free OptiMEM and mixed with INTERFERin After 10 minutes incubation mixture was added drop wise to the cells growing in fresh added IMDM/10%FBS/15% L929 conditioned media. Final concentration of siRNA was 16nM. Cells were grown for 96 h after transfection.

Transfection with Arf6-tGFP cDNA plasmid (Origene) was done using Lipofectamine LTX Reagent (ThermoFisher Scientific) according to manufacturer's protocol. Transfection with the same vector without Arf6 gene is referred as Mock transfection.

When specified, cells were treated with cycloheximide (5 μg/ml) for 4 h.

Antibodies

Primary antibodies used for the Western blotting analysis were:

1. Monoclonal mouse anti-Abca1 antibody (Abcam , #ab18180). This antibody was characterized in our previous studies [3, 4].
2. Polyclonal rabbit anti-clathrin antibody, (Abcam, #ab21679). This antibody was characterized by the manufacturer; in our studies they stained a single band with predicted MW170kD.
3. Monoclonal mouse anti-alpha1 sodium potassium ATPase antibody (Abcam, #ab7671). This antibody was characterized by the manufacturer; in our studies they stained a single band with predicted MW 100kD.
4. Monoclonal mouse anti-Gapdh antibody (Merck, CB1001). This antibody was characterized in our previous studies [3, 4].
5. Monoclonal mouse anti-Arf6 antibody (Sigma-Aldrich, #A5230). This antibody was characterized by the manufacturer; in our studies they stained a single band with predicted MW 20kD.
6. Monoclonal mouse anti-Cdc42 antibody (BD Transduction Laboratories, #610929). This antibody was characterized by the manufacturer; in our studies they stained a single band with predicted MW 22kD [5].
7. Monoclonal mouse anti-dynamin-2 antibody (BD Transduction Laboratories, #610248). This antibody was characterized by the manufacturer; in our studies they stained a single band with predicted MW 98kD.

Secondary antibodies used for the Western blotting analysis were:

1. Horseradish peroxidase-conjugated anti-mouse IgG antibody, (Sigma-Aldrich, # A9044).
2. Biotin conjugated anti-mouse IgG antibody (Sigma-Aldrich, #B8520).
3. Horseradish peroxidise-conjugated anti-rabbit IgG antibody (Merck, #AP307P).

Primary antibodies used for confocal microscopy were:

1. Polyclonal antibody against Abca1 (Novus Biologicals, #NB400-105). This antibody was characterized in our previous studies [4].
2. Anti-Lamp-1 monoclonal antibody (Abcam, #ab25245). This antibody was characterized in our previous studies [6].
3. Anti-Rab11 monoclonal antibody (BD Transduction Laboratories, #610657).

Secondary antibodies used for confocal microscopy were:

1. AlexaFluor 633-labelled anti-rabbit IgG antibody (Invitrogen, #A-21070).
2. AlexaFluor 546-labeled anti-mouse IgG antibody (Invitrogen, A111030)
3. AlexaFluor 546-labeled anti-rat IgG antibody (Invitrogen, A11081)

Western blots

Cells were lysed with RIPA buffer, protein concentration in lysates estimated by Bradford assay followed by SDS-PAGE and transfer of proteins to PVDF membrane. Semi-quantitative analysis of western blots was performed by densitometry and presented as a proportion of control after normalization to loading controls.

Abundance of cell-surface and internalized Abca1

To assess abundance of the cell-surface Abca1, cells were washed three times with ice cold PBS and biotinylated with Sulfo-NHS-SS-Biotin as described previously [6]. Biotinylation reaction was quenched by washing cells twice with 50 mM Tris, 150 mM NaCl, 0.1 mM EDTA. Cells were lysed with RIPA buffer and biotinylated proteins were purified by incubating for 2 h at 4ºC with High Performance Streptavidin Sepharose (ThermoFisher Scientific). Bound biotinylated proteins were removed from resin by 30 min incubation in loading buffer supplemented with 50 mM DTT at 50ºC. Supernatants were subjected to SDS-PAGE, proteins were transferred to PVDF membrane and probed.

The abundance of the internalised Abca1 was assessed by biotinylation of the cell-surface proteins followed by quenching reaction and 30 min incubation at 37ºC. Biotinylated surface proteins were stripped with 50 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, Sigma-Aldrich) solution (2 x 15 min incubations at 4^0C). Cells were then washed three times with ice-cold Tris buffer and lysed with RIPA buffer. Biotinylated proteins were purified by incubation for 2 h at 4ºC with High Performance Streptavidin Sepharose and processed as described above.

Degradation of Abca1

Degradation of Abca1was estimated in pulse-chase experiments. Twenty four hours after transfection cells were activated with 4 μM TO-901317 for 18 h. Cells were washed with PBS and incubated in serum-free RPMI containing 4 μM TO-901317 for 3 h. Media was then replaced with methionine-and cysteine-free RPMI (Sigma-Aldrich) containing TO-901317 for 1 h incubation. The cells were pulsed with 3.7 MBq/ml of [^{35}S]methionine & cysteine (PerkinElmer, EasyTag EXPRESS Protein Labeling Mix) for 2 h, washed and chased in RPMI medium supplemented with 1.5 mg/ml L-methionine and 0.5 mg/ml L-cysteine for the indicated periods of time. Incorporation of ^{35}S-labeled amino acids into Abca1 was determined by immunoprecipitation of Abca1 with affinity-purified polyclonal anti-mouse Abca1 antibody followed by SDS-PAGE, transfer to PVDF membrane and exposure to the high performance autoradiography film (ThermoFisher Scientific).

Cholesterol efflux

Cholesterol efflux was measured as described previously [7]. In brief, cells were labelled by incubation in serum-containing medium supplemented with [^{3}H]cholesterol (75 kBq/ml, American Radiolabeled Chemicals (ARC), Inc.) for 48 h. Cells were washed and incubated for 18 h in serum-free medium in the presence of LXR agonist TO-901317 (final concentration, 4 μM). Human apoA-I (kind gift from CSL Behring) was added to the final concentration of 30 μg/ml, or methyl-β-cyclodextrin was added to the final concentration of 200 μg/ml, and cells were incubated for 2 h at 37^{o}C. The efflux was calculated as a proportion of radioactivity moved from cells to medium; non-specific efflux (i.e. the efflux to the medium without acceptor) was subtracted.

To measure the efflux of intracellular cholesterol, cells were incubated with [^{3}H]acetic acid (10 MBq/ml, ARC) for 2 h at 37^{o}C and simultaneously with apoA-I (30 μg/ml). Media and cells were then collected and lipids extracted using 2.5ml mixture of isopropanol: heptane: sulphuric acid (40:10:1, v/v/v). Samples were incubated at room temperature for 1 h before adding 1.5 ml heptane and 1 ml water and another 1 h incubation at room temperature. The organic phase was collected, supplemented with 100 μg of carrier cholesterol and dried under nitrogen steam. Dry pellets were dissolved in 100 μl of chloroform and lipids separated using thin layer chromatography as described previously [8]. Cholesterol bands were scraped and ^{3}H radioactivity counted on a β-counter.

Confocal microscopy

Cells were grown in μ-slide 8-well chambers (Ibidi, USA) and transfected as described above. Twenty four hours after transfection cells were treated with 4 μM TO-901317 for 18 h. Cells were then treated for 3 h in serum-free medium supplemented with 4 μM TO-901317 only or with the addition of 5 mM cyclodextrin-cholesterol complex for 3 h or 1mM methyl-β-cyclodextrin for 1 h followed by a 2 h incubation with apoA-I (30 μg/ml). Cells were then washed with ice-cold PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilised using 0.1% triton X-100 for 10 min at room temperature, washed and blocked with 10% goat serum for 30 min. Cells were then stained with a polyclonal antibody against Abca1 and AlexaFluor 633 anti-rabbit secondary antibody. Lamp-1 was stained with anti-Lamp-1 monoclonal antibody and AlexaFluor 546-labeled secondary antibody. Rab11 was stained with a monoclonal antibody and AlexaFluor 546 anti-mouse secondary antibody. Images were collected using a Nikon A1r+ confocal microscope equipped with 60x Water Immersion objective (Nikon 60x Plan Apo VC, WI NA 1.2). Images were collected with the 405, 488 and 568 lasers sequentially to minimise bleed through. Co-localisation was quantitated using Coloc2 in Fiji software on at least 50 to 100 cells.

The abundance of lipid rafts was assessed using the Vybrant lipid raft labelling kit (Life Technologies) according to manufacturer's instructions. Briefly, cells were incubated with AlexaFluor 488-labeled CT-B for 30 min on ice, washed, cross-linked by incubation with anti-CT-B antibody for 15 min at 4^{o}C and fixed with 4% paraformaldehyde for 15 min at room temperature. Methyl-β-cyclodextrin/BODIPY-cholesterol complex was prepared as described previously [5].

Statistics

All data is shown as mean ± standard deviation (SD) unless stated otherwise. Statistical significance of the differences was assessed in GraphPad prism software package by unpaired Student's *t*-test and one-way ANOVA when data followed normal distribution or Mann-Whitney U test on ranks. The experiments were conducted in quadruplicates and repeated 2-5

times, the exact number of experiments used for statistical analysis and the exact *p* value are shown in the figure legends or text. When normalization of data was difficult, and the experiments were performed in multiplicates, representative experiments out of 2-3 identical experiments are shown.

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