Biochimica et Biophysica Acta 1803 (2010) 865–871

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Identification of importin α1 as a novel constituent of RNA stress granules

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ARTICLE INFO

Article history:
Received 4 December 2009
Received in revised form 24 March 2010
Accepted 26 March 2010
Available online 31 March 2010

Keywords:
Importin alpha
Stress granule
Stress response

ABSTRACT

Importin α is a nuclear transport receptor well established for its ability to mediate importin β-mediated nuclear import of proteins that possess classical nuclear localization signal (cNLS). Previously, we reported that importin α rapidly accumulates to the nucleus in response to H_2O_2-induced oxidative stress, which implies a role for this protein in stress response. In this study, we show that importin α1 (also known as KPNA2 or Rch1), a major subtype of the importin α family, localizes to RNA stress granules (SGs), large cytoplasmic bodies that are thought to function as RNA triage sites during stress response. The recruitment of importin α1 to SGs was compatible with its nuclear accumulation during heat shock. Depletion of endogenous importin α1 using siRNA showed that importin α1 regulates the dynamics of SG assembly, and that it promotes cell survival in arsenite-treated cells. These data revealed, for the first time, the involvement of importin α in the assembly of RNA granules and its pro-survival role during stress response.

1. Introduction

Importin α (or Karyopherin α) has long been investigated for its involvement in nuclear transport. It has now become clear that nuclear import of proteins bearing classical nuclear localization signal (cNLS) is initiated by importin α, which recognizes and binds cNLS and functions as an adaptor that allows access to importin β-mediated translocation through nuclear pores [1]. The importin α family is comprised of three major subtypes, which perform both distinct and overlapping functions in nuclear transport [2,3]. Aside from its role in nuclear transport, importin α has been shown to participate to scores of physiological processes, including spindle formation, reassembly of nuclear envelope at the end of mitosis, and ubiquitin-mediated protein degradation [4–6]. One of the unexplored cellular processes that importin α may play a role is stress response. Recently, importin α was found to rapidly accumulate to the nucleus upon exposure to H_2O_2-induced oxidative stress and heat shock by us and others [7,8]. This nuclear accumulation of importin α could be explained by the stress-induced depletion of ATP and subsequent collapse of Ran distribution [9]. However, the stress-specific functions of importin α that are likely to arise as a result of this redistribution have not been fully addressed.

In this paper, we report that importin α1 (alternately known as Rch1, KPNA2), a major subtype of the importin α family, localizes to RNA stress granules (SGs) in stressed mammalian cells. SGs are large cytoplasmic aggregates that are assembled upon exposure to stress, and have been implicated in global translational arrest and mRNA metabolism during integrated stress response [10]. We found that importin α1 is recruited to SGs by exposure to arsenic stress and by overexpression of proteins that nucleate SGs, and that its recruitment to SGs is compatible with nuclear accumulation during heat shock. We further show that importin α regulates the dynamics of SG assembly and promotes cell survival in arsenite-treated cells.

2. Materials and methods

2.1. Reagents, plasmids and antibodies

Sodium arsenite and nocodazole were purchased from Sigma, and used at the final concentration of 0.5 mM and 6.6 μM, respectively. Treatment with arsenite and nocodazole was performed for 30 min and 2 h, respectively, unless otherwise indicated. Hippuristanol was a kind gift from Dr. Junichi Tanaka (Ryukyu University). Plasmids encoding GFP-tagged G3BP, TIA1, PABP and cNLS were described previously [7,11,12]. The plasmid encoding HA-tagged Chk2 is a kind gift from Dr. Stephen J. Elledge (Harvard Medical School). Annexin V staining was performed using the Annexin V-FITC Apoptosis Detection Kit (BioVision)

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doi:10.1016/j.bbamcr.2010.03.020

as instructed by the manufacturer. The following antibodies were used for Western blotting and immunofluorescence: mouse monoclonal anti-G3BP, anti-importin α1, anti-Ran antibodies (BD Biosciences), rat monoclonal anti-importin α1 antibody (MBL), rabbit polyclonal anti-phospho-eIF2α (Cell Signaling Technology), goat polyclonal anti-TIA1, mouse monoclonal anti-lamin A/C (Santa Cruz Biotechnology), rabbit polyclonal anti-Dcp1a (a kind gift from Dr. Jens Lykke-Andersen, University of California, San Diego), mouse monoclonal anti-β-tubulin (Sigma), mouse monoclonal anti-GAPDH (Abcam) and rat monoclonal anti-HA (Roche) antibodies. The secondary antibodies used for immunofluorescence and Western blotting were purchased from Molecular Probes and Chemicon, respectively. Antibody dilution was 1:200 for immunofluorescence (except for anti-Dcp1a and anti-β-tubulin antibodies, which were used at the dilution of 1:800), and 1:1000 for Western blotting for primary antibodies; 1:400 for Alexa-conjugated secondary antibodies and 1:1000 for HRP-conjugated secondary antibodies.

2.2. Cell culture and transfection

HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Transfection of plasmids was performed using Effectene (Qiagen). For siRNA transfection, 40 pmol of siRNA was transfected using RNAiMAX (Invitrogen) and cultured for 48 h. siRNA directed against GFP (purchased from Nippon EGT), or alternatively, commercially available non-targeting siRNA (Ambion; Cat. No. 4390843) were used in control experiments. Where indicated, plasmids were transfected into cells treated with siRNA 48 h after and then further cultured for 24 h before used for experiments. The siRNA sequence for importin α1 was as follows: 5′-UGAACCUCUUAACUG-CAAATT-3′.

2.3. Western blotting

Western blotting was performed essentially as described previously [11], using ECL Western Blotting Detection Kit (GE Healthcare). Subcellular fractionation was performed essentially as described in [24]. Briefly, cells cultured in 6-cm dishes were trypsinized, rinsed with PBS and lysed in 200 μl of lysis buffer (10 mM Tris–HCl, 50 mM NaCl, 1.5 mM $MgCl_2$, 0.2% NP-40). Nuclei were pelleted by gentle centrifugation (500×*g*, 5 min) and cytoplasmic fractions were obtained by subjecting the supernatants to further centrifugation to eliminate any residual insoluble materials (15,000×*g*, 10 min). For preparing nuclear fractions, pelleted nuclei were washed twice with lysis buffer, and then incubated in 200 μl of nuclear extraction buffer (20 mM Tris–HCl, 500 mM NaCl, 1 mM EDTA) for 10 min on ice, and clarified by centrifugation (15,000×*g*, 10 min).

2.4. Immunofluorescence microscopy

Immunofluorescence was performed as described in [11]. Briefly, cells grown on glass coverslips were fixed with 3.7% formaldehyde after rinsed with PBS, and permeabilized with 0.5% Triton X-100. Subsequently, cells were incubated in 3% skim milk in PBS, and treated sequentially with primary and secondary antibodies. Microscopic observation was performed using a confocal laser scanning microscope (LSM510META, Carl Zeiss). For quantitative analysis on the kinetics of SG assembly, HeLa cells transfected with siRNAs were treated with arsenite for the indicated periods, immunostained for G3BP and the percentage of cells with discrete SGs was calculated. This procedure was performed in triplicate for at least 75 cells in each experiment.

2.5. FRAP (Fluorescence Recovery after Photobleaching) experiments

FRAP experiments were performed as described previously [11]. Briefly, HeLa cells were sequentially transfected with siRNA and the plasmid encoding GFP-tagged PABP and cultured for 24 h. Subsequently, cells were exposed to arsenite, and a single SG visualized by GFP-PABP was chosen as a region of interest (ROI). A single SG was bleached by scanning for 60 iterations at 100% of laser power, and the fluorescence recovery of the ROI was monitored for 15 cells in three independent experiments. For quantitative analysis, background intensity was subtracted and intensities of selected SGs was measured over time and normalized using intensities of a region of interest (ROI) in the cytoplasm of a neighboring non-bleached transfectant.

2.6. Cell viability assay

Cell viability assay was performed using CellTiter-Glo Cell Viability Assay kit (Promega), which allows the determination of the number of metabolically active cells by quantitation of ATP through luciferin–luciferase reaction. Briefly, 1.5×10^4 HeLa cells transfected with siRNA were seeded onto 24-well plates, and cultured for 48 h. Cells were then treated with 0.5 mM arsenite for 2 h, washed twice with growth medium and further cultured for 3 and 5 h in normal growth medium. Subsequently, CellTiter-Glo reagent was directly added to each well (so that the analysis would include detached cells) and cells were lysed by repeated pipetting, and incubated at room temperature for 10 min. Finally, luminescence signals were measured using GloMax luminometer (Promega). The experiments were performed in triplicate.

3. Results

3.1. Importin α1 is recruited to RNA stress granules (SGs)

Previously, we reported that importin α1 rapidly accumulates to the nucleus upon heat shock and H_2O_2-triggered oxidative stress, and that this is likely to be caused by the collapse of the Ran distribution as a result of stress-induced depletion of ATP levels. To gain more insight into the possible functions of importin α1 in stressed cells, we studied its subcellular localization in cells treated with a variety of toxic agents. When treated with sodium arsenite, a compound known to induce oxidative stress accompanied by the phosphorylation of eIF2α and subsequent integrated stress response (ISR) [13], we did not observe the massive nuclear accumulation of importin α1 but instead discovered that it was recruited to a number of large cytoplasmic foci. Since sodium arsenite has been described to induce the formation of RNA stress granules (SGs), cytoplasmic aggregates that have been considered to play a role in global translational arrest and determination of the fate of individual mRNA during ISR, we tested whether importin α1 accumulates to SGs during arsenic stress. As shown in Fig. 1A, importin α1 was efficiently recruited to SGs, as verified by double immunostaining with a SG marker TIA-1. This SG localization of importin α1 was further validated by immunostaining with another anti-importin α1 antibody (Supplementary Fig. 1A) and immunofluorescence using human osteosarcoma U2OS cells (Supplementary Fig. 1B). Importin α1 was recruited to SGs induced by hippuristanol treatment, a translational inhibitor that does not trigger eIF2α-phosphorylation [14], or by overexpression of TIA-1 or G3BP, which leads to the formation of spontaneous SGs in the absence of stress (Fig. 1B, C and Supplementary Fig. 1C). Thus, SG localization of this protein did not seem to be dependent on a specific stressor, such as arsenite, or on phosphorylation of eIF2α. Meanwhile, importin α1 was not concentrated in processing bodies (P-bodies), as assessed by double immunostaining with anti-Dcp1a antibody (Fig. 1D). We then examined whether heat shock would cause the accumulation of importin α1 to SGs. A severe heat shock (44 °C, 60 min) efficiently induced SGs in almost 100% of cells, in which did we observe the recruitment of importin α1 to SGs, concomitant with its nuclear accumulation (Fig. 1E). Thus, we concluded that SG localization of importin α1 could take place simultaneously with stress-induced nuclear accumulation, and might contribute to the inhibition of