Experimental Cell Research 357 (2017) 181–191

Contents lists available at ScienceDirect

Experimental Cell Research

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

RIC8A is essential for the organisation of actin cytoskeleton and cell-matrix interaction

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ARTICLE INFO

Keywords:
Actin cytoskeleton
Focal adhesions
RIC8A
G protein
Integrin

ABSTRACT

RIC8A functions as a chaperone and guanine nucleotide exchange factor for a subset of G protein α subunits. Multiple G protein subunits mediate various signalling events that regulate cell adhesion and migration and the involvement of RIC8A in some of these processes has been demonstrated. We have previously shown that the deficiency of RIC8A causes a failure in mouse gastrulation and neurogenesis – major events in embryogenesis that rely on proper association of cells with the extracellular matrix (ECM) and involve active cell migration. To elaborate on these findings, we used *Ric8a*$^{-/-}$ mouse embryonic stem cells and *Ric8a*-deficient mouse embryonic fibroblasts, and found that RIC8A plays an important role in the organisation and remodelling of actin cytoskeleton and cell-ECM association. *Ric8a*-deficient cells were able to attach to different ECM components, but were unable to spread correctly, and did not form stress fibres or focal adhesion complexes. We also found that the presence of RIC8A is necessary for the activation of β1 integrins and integrin-mediated cell migration.

1. Introduction

Resistance to inhibitors of cholinesterase 8 A (RIC8A) protein regulates the activity of heterotrimeric G protein α subunits and is functionally coupled to $G\alpha_{12/13}$, $G\alpha_{q/11}$ and $G\alpha_{i/o}$ families [9,33]. It was first recognised as a guanine nucleotide exchange factor [24,33], but recent evidence has shown that it may also act as a chaperone for the Gα subunits promoting their abundance [9,6]. This is supported by experiments showing that in the absence of RIC8A the levels of Gα subunits are reduced [9,18] and their recruitment to the plasma membrane is impaired [9,14,29,36]. RIC8A has a role in a variety of biological processes, such as asymmetric cell division and synaptic transmission. However, recent evidence indicates that RIC8A may also be involved in the regulation of cell adhesion and migration [9,17,36,8].

In vivo studies in mice have shown that the absence of RIC8A leads to a failure in gastrulation and neurogenesis, processes in embryonic development that involve major cell migration processes, which require functional communication of cells with the ECM [18,35]. Accordingly, in mice lacking RIC8A, the basement membrane was fragmented in both developing embryos and cerebral cortices [18,35]. Similarly, a study focusing on the development of mouse cerebellum reported that in the absence of RIC8A, cell adhesion to laminin was impaired and therefore the specialised astrocytes called Bergmann glia were unable to attach to the basement membrane [23]. Furthermore, experiments with *Xenopus laevis* demonstrated that xRic8A deficit causes defective migration, reduced adhesion to and spreading on fibronectin, and also reduced formation of focal complexes in the cranial neural crest cells [8]. In addition, RIC8A has been functionally linked to growth factor-induced cell migration of mouse embryonic fibroblast (MEF) cells, where downregulation of RIC8A inhibited platelet-derived growth factor (PDGF)-initiated cell migration and slowed down the PDGF-induced dorsal ruffle turnover [36]. Dorsal ruffles are structures that contain polymerised cortical actin, which localises to the dorsal plasma membrane upon cell stimulation by a variety of growth factors [3]. The involvement of RIC8A in actin remodelling was also reported in another study on gastrulation of *Drosophila melanogaster* where the mutation of *ric-8* resulted in perturbation of the organisation of cortical actin [17]. In addition, the amount of total and polymerised actin was reduced in *Ric8a*$^{-/-}$ ES cells, which was due to the impaired activation of RhoA GTPase, a known organiser of the actin cytoskeleton [9,5]. In addition, *Ric8a*$^{-/-}$ ES cells had reduced number of actin-rich filopodia-like structures, however, a constitutively active RhoA restored the normal levels of F-actin and actin-rich structures in these cells [9]. The reduced overall levels of F-actin were also observed in RIC8A-depleted B-cells [2]. These pieces of information indicate that RIC8A activity might be required for the functional interaction of cells with the ECM and actin remodelling. In the above-mentioned studies, most of the

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http://dx.doi.org/10.1016/j.yexcr.2017.05.012
Received 4 January 2017; Received in revised form 9 May 2017; Accepted 14 May 2017
Available online 16 May 2017

observed cytoskeletal defects caused by RIC8A deficiency were associated with disturbed G protein function.

Among the heterotrimeric G proteins, $G\alpha_{12}/G\alpha_{13}$ are involved in the regulation of cell morphology and movement via direct activation of RhoGEF's that activate RhoA GTPase. RhoA has been shown to regulate the organisation of actin cytoskeleton and activate the downstream targets such as formin (mDia) and ROCK [13]. G-protein-coupled receptors (GPCRs) that transmit signals via the $G\alpha_{12}/G\alpha_{13}$ proteins have invariably been shown to interact with one or more other Gα subunits [27]. For example many $G\alpha_q/G\alpha_{11}$-coupled receptors are reported to induce stress fibre assembly in the absence of $G\alpha_q$ and $G\alpha_{11}$, and this process has been suggested to involve either $G\alpha_{12}$ or $G\alpha_{13}$ and their respective signalling pathways [11]. Recently, it was reported that in addition to GPCRs, $G\alpha_{13}$ also interacts with integrins, a family of adhesion molecules that mediate cell adhesion to, and migration on, the ECM. Integrins transmit signals bidirectionally: signalling cascade that is activated upon ligand binding is called “outside in” signalling and high-affinity ligand binding to integrins is achieved by “inside out” signalling. In particular, $G\alpha_{13}$ has been reported to associate with platelet integrin β3 subunit [12] and a ubiquitously expressed β1 integrin subunit [32]. These interactions are believed to be necessary for integrin-mediated “outside-in” signalling, transient inactivation of RhoA, and activation of Src, both required for the initial cell spreading and migration [12,32]. Therefore it seems that $G\alpha_{13}$ plays a dual role in the regulation of RhoA: by stimulating it through GPCR-activated pathways, and, on the other hand, inhibiting it via integrin outside-in signalling [32].

In order to assess the possible role of RIC8A in these $G\alpha_{13}$-mediated processes, and to gain more insight into the role of RIC8A in cell-ECM adhesion and actin cytoskeleton remodelling, we utilised two RIC8A-deficient primary cell lines: mouse embryonic stem (mES) cells and mouse embryonic fibroblasts (MEF). We demonstrate that RIC8A-deficient cells cannot assemble stress fibres, dorsal ruffles or focal adhesion complexes. In addition, we provide evidence for the novel role of RIC8A in activation of β1 integrins and integrin-mediated cell migration.

2. Materials and methods

2.1. Reagents and antibodies

Antibodies: rabbit polyclonal to AKT1,2,3 (Santa Cruz Biotechnology, #sc-8312, WB dilution 1:200), rabbit polyclonal to pAKT Ser473 (Cell Signaling, Technology (company name) #4058S, WB dilution 1:1000), rat monoclonal CD29 clone 9EG7 (BD Pharmingen™, #553715, ICC and FACS dilution 1:100), rabbit monoclonal to $G\alpha_{13}$ (Santa Cruz, #sc-410, WB dilution 1:200), mouse monoclonal to GAPDH (Abcam, #ab8245, WB dilution 1:10 000), mouse monoclonal to RIC8A (Abcam, #ab118151, WB dilution 1:800, ICC dilution 1:100), rabbit polyclonal to vinculin (Bethyl laboratories Inc, #IHC-00700, ICC dilution 1:250).

Substrates used for adhesion and migration assays: Collagen I (Corning, #354236), Collagen IV (Santa Cruz Biotechnology, #sc-29010), Fibronectin (Millipore, #FC010), Laminin 521 (BioLamina, #LN-521TM).

2.2. Cell culture

mES cells The wild-type (wt) E14.1 ES cell line is derived from the 129/Ola mouse strain and has been described previously [19]. *Ric8a*$^{lox/lox}$ mES cells were created for the generation of *Ric8A*$^{lox/lox}$ mice and have been described previously [28]. *Ric8a*$^{-/-}$ line was generated by electroporating 5 μg of Cre recombinase expressing vector pPGK Cre ppA (a kind gift from Prof. A. Karis) into *Ric8a*$^{lox/lox}$ cells from one confluent 60 mm plate. Electroporation was carried out in FBS-containing media using BioRad electroporation equipment at 250 mV and 500 μF with a 4 mm cuvette. After electroporation the cells were seeded sparsely, single cells were picked and the *Ric8a* deletion was determined by genotyping. mES cells were cultured in Dulbecco's modified Eagle's medium containing L-glutamine and pyruvate (Gibco, LifeTechnologies) with 10–15% Foetal Bovine Serum (FBS), ES cell qualified (Gibco, LifeTechnologies), 10 ng/ml Leukaemia Inhibitory Factor (Gibco, LifeTechnolgies), 100 μM Mercaptoethanol, 100 μg/ml Penicillin/Streptomycin and 1% Non-Essential Amino Acids (Gibco, LifeTechnologies) on inactivated feeder cells (mouse embryonic fibroblasts). Cells that were cultivated without feeders were grown on 0.1% gelatine (Millipore).

MEF cells Mouse embryonic fibroblasts (MEFs) were derived from decapitated E12.5 embryos. Offspring of the *Ric8a*$^{lox/lox}$ mice (no phenotype) were used. Cells were cultured in DMEM containing L-glutamine and pyruvate (Gibco, LifeTechnologies), penicillin/streptomycin and 10% FBS. All materials were from Gibco, LifeTechnologies. Primary cells were frozen in aliquots after the second passage. In order to achieve a RIC8A-deficient population, ~ 4×10^6 *Ric8a*$^{lox/lox}$ MEFs were electroporated with 40 μg vector pPGK Cre ppA, in 400 μl electroporation solution (10 mM HEPES, 135 mM KCl, 2 mM $MgCl_2$, 20% FBS, pH 7,5), at 360 mV and 1000 μF in a 4 mm cuvette. As a positive control for estimating the efficacy of the electroporation, the plasmid expressing fluorescent pEF1+dTomato (Clontech) was transfected into *Ric8a*$^{lox/lox}$ MEFs together with Cre recombinase. Control cells received the pEF1+dTomato vector but not the Cre expressing plasmid. After the electroporation, MEFs were allowed to recover in regular media for 24 h followed by the enrichment for neomycin-resistant populations using 1 mg/ml G418 (geneticin) for 48 h. The absence or presence of RIC8A in individual analysed cells was verified by immunostaining with an anti-RIC8A mAb. Primary MEFs were cultured for maximum of 7 passages.

2.3. Genotyping

The deletion of first four exons of *Ric8a* was assessed with forward primers 1P1 5′-GGTAGGGCTCAATGTTGG-3′ and P2 5′-CTTTTCCA CGGGTGTTCTTC-3′ and a reverse primer P3 5′-GCCAAACAATCTC TCGAACC-3′. The annealing sites of primers onto *Ric8a* gene are shown on Fig. 1A.

2.4. Quantitative RT-PCR

RNA was extracted from cells with Trizol® Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer′s instructions. Reverse transcription was performed using SuperScript™ First-Strand Synthesis System (Life Technologies) following the manufacturer's protocol. 1 μg of cDNA from cells was used for Quantitative RT-PCR (Life Technologies Applied Biosystems StepOnePlus Real-Time PCR instrument) in 40 cycles of 15 s at 95 °C and 1 min at 60 °C using a qPCR mastermix HOT FIREPol® EvaGreen® qPCR Mix Plus [ROX] (Solis BioDyne) with the following primers: *Ric8a* 5′-GAGGAGTTCCACGGCCACA-3′ and 5′ -CTTCAGCCTGTGGGTCTGGT G-3′; *Hprt* 5′-CACAGGACTAGAACACCTGC-3′ and 5′-GCTGGTGA AAAGGACCTCT-3′. Each experiment was run 3 times with each sample in triplicates with *Hprt* chosen as a reference. The relative *Ric8a* mRNA expression was calculated using the ΔΔCt method.

2.5. Western blot

Standard Western blotting procedures were performed using a Bio-Rad electrophoresis and transfer apparatus. Proteins were separated in 10%, 12% or 14% SDS-PAGE gels and transferred onto low fluorescence polyvinylidene difluoride membrane (Amersham, GE Healthcare Life Sciences). The membranes were blocked with 10% nonfat dry milk and incubated with primary antibodies diluted in 1% nonfat dry milk. For visualisation, secondary antibodies conjugated to Alexa Fluor 555