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Rap2, but not Rap1 GTPase is expressed in human red blood cells and is involved in vesiculation

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Abstract

Recent studies have suggested that Rap1 and Rap2 small GTP-binding proteins are both expressed in human red blood cells (RBCs). In this work, we carefully examined the expression of Rap proteins in leukocytes- and platelets-depleted RBCs, whose purity was established on the basis of the selective expression of the β2 subunit of the Na^+/K^+-ATPase, as verified according to the recently proposed "β-profiling test" [J.F. Hoffman, A. Wickrema, O. Potapova, M. Milanick, D.R. Yingst, Na pump isoforms in human erythroid progenitor cells and mature erythrocytes, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 14572-14577]. In pure RBCs preparations, Rap2, but not Rap1 was detected immunologically. RT-PCR analysis of mRNA extracted from highly purified reticulocytes confirmed the expression of Rap2b, but not Rap2a, Rap2c, Rap1a or Rap1b. In RBCs, Rap2 was membrane-associated and was rapidly activated upon treatment with Ca^{2+}/Ca^{2+}-ionophore. In addition, Rap2 segregated and was selectively enriched into microvesicles released by Ca^{2+}-activated RBCs, suggesting a possible role for this GTPase in membrane shedding.

Keywords: Red blood cells; Rap1; Rap2; Small GTPases; Vesiculation

1. Introduction

Rap proteins define a family of highly homologous small GTP-binding proteins, which includes five members, Rap1a, Rap1b, Rap2a, Rap2b, and the recently discovered Rap2c, which are grouped into two subfamilies, Rap1 and Rap2, based on the high sequence homology [1,2]. Like other small GTPases, Rap proteins function as molecular switches, as they are activated by exchange of GDP for GTP and inactivated through GTP hydrolysis stimulated by GTPase-Activating Proteins (GAP). Many physiological agonists are able to activate Rap proteins in a number of cell types, through the stimulation of exchange factors regulated by cAMP, Ca^{2+}, or tyrosine kinases [3]. Rap proteins represent important regulators of fundamental cellular functions, such as adhesion, secretion and vesicle trafficking [4,5]. In particular Rap1, but also Rap2 proteins have been recognized to mediate integrin activation and to regulate integrin-dependent cell adhesion [5]. In addition, different studies have suggested the involvement of Rap proteins in intracellular vesicle trafficking and release [6,7]. Rap proteins are widely expressed, but the relative amount of individual members may vary considerably, depending on the tissue or cell type considered. Among circulating cells, members of both Rap1 and Rap2 subfamilies have been found to be abundant in platelets and leukocytes [1].

Several small GTP-binding proteins have also been identified in red blood cells (RBCs), including RhoA and members of the Arf, Rab, and Ral families [8–11]. Recently, two independent studies based on proteomic approaches have reported the expression of both Rap1a and Rap1b in

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RBCs [12,13]. Moreover, on the basis of the responsiveness to the Rap-specific cAMP-sensitive guanine nucleotides exchange factor, EPAC, Rap1 proteins have been proposed to mediate sickle cell adhesion to laminin [14].

Analysis of protein expression in RBCs is often compromised by contaminating leukocytes and, to a lesser extent, platelets. The level of contaminating cells and their impact on the measurements performed is not always taken in due consideration. Recently, it has been demonstrated that RBCs are unique among circulating cells, as they express exclusively the β2 subunit isoform of the Na^+-pump, while leukocytes and platelets possess the β1 isoform [15]. The analysis of the β subunit expression profile (the so-called β-profiling) represents an excellent and definitive method to evaluate the purity of RBCs preparations [16].

By combining a cellulose-based method for RBCs purification, which allows efficient removal of contaminating cells, and a β-profiling analysis, we obtained highly purified RBCs, and we demonstrated that, in contrast to previously reported findings, Rap2, but not Rap1, is expressed in RBCs. Moreover, we found that this protein can be activated by GTP binding in RBCs treated with Ca^{2+} and Ca^{2+} ionophore, and segregates into the vesicles released by RBCs in a Ca^{2+}-dependent manner.

2. Materials and methods

2.1. Cell preparation

Blood was collected from healthy volunteers using 0.1 volume of 3.8% (w/v) tri-sodium citrate as anticoagulant. RBCs were recovered by centrifugation and further purified from leukocytes and platelets by filtration through cellulose, as described [17]. Subpopulations of RBCs of different age were prepared as detailed elsewhere [18]. Mononuclear cells (MNC) were isolated by Lympholite-H (Cedarlane, Hornby, Ontario, Canada) according to manufacturer's instructions. Human platelets were prepared from blood withdrawn in 0.1 volume of ACD (152 mM sodium citrate, 130 mM citric acid, 112 mM glucose). Platelet rich plasma was obtained by centrifugation at 120×*g* for 10 min, and platelets were then purified by gel-filtration on Sepharose 2B, as previously described [19].

2.2. Stimulation of red blood cells with Ca^{2+} and vesicles preparation

Purified RBCs at 3–5% hematocrit in HEPES buffer (5 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4, containing 0.2 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 5 μg/ml aprotinin, 50 μg/ml leupeptin), were incubated at 37 °C in the presence of 0.3 mM $CaCl_2$ and 4 μM calcium–ionophore A23187 for various times as specified. Vesicles released during 20 min incubation were isolated and purified as previously detailed [18]. Purified ghost membranes were prepared by hypotonic lysis of RBCs in 5 mM phosphate buffer, pH 8.0, containing 0.2 mM PMSF at 4 °C according to the method of Dodge et al. [20]. Cholesterol was assayed in RBCs ghost membranes and vesicles using a colorimetric assay kit (R-Biopharm Italia Srl, Milan, Italy).

2.3. RT-PCR analysis

RNA was extracted from reticulocytes present in RBCs preparations and from MNC using TRIzol reagent (Invitrogen Life Technology, Paisley, Scotland, UK), according to the supplier's recommendations. First strand cDNA was synthesized from 1 μg of total RNA using the High Capacity cDNA Archive Kit (Applera, Woolston, Warrington Cheshire, UK), according to manufacturer's instructions. Beta-profiling assay was performed using primers for β1 and β2 subunits of the human Na^+/K^+ ATPase, and RT-PCR conditions as described by Hoffman et al. [16]. Analysis of Rap proteins expression was performed using the following primers:

Rap1a: 5′-CGCGGATCCATGCGTGAGTACAAGCTAGTG-3′ (forward), and
5′-CGCGGATCCTCACTAGAGCAGCAGACATGATT-3′ (reverse)
Rap1b: 5′-GGAATTCCATATGAATTCCATGCGTGAGTATAAGCTA-3′ (forward), and
5′-CGCGGATCCTTATTAAAGCAGCTGACATGA-3′ (reverse)
Rap2a: 5′-CGCGGATCCATGCGCGAGTACAAAGTGGTG-3′ (forward), and
5′-CGCGGATCCTTACTATTGTATGTTACATGC-3′ (reverse)
Rap2b: 5′-CGCGGATCCATGAGAGAGTACAAAGTGGTG-3′ (forward), and
5′-CGCGGATCCTCATTATCAGAGGATCACGCAGG-3′ (reverse)
Rap2c: 5′-GGAATTCCATATGAATTCCATGAGGGAATACAAGGT-3′ (forward), and
5′-CGCGGATCCTTATTACTGGACGACACAAGT -3′ (reverse)

Reactions were cycled 40 times through the following program: the denaturation (94 °C for 1 min) was followed by the annealing (55 °C for 45 s), and the extension (72 °C for 45 s). To verify the specificity of the primers used, parallel PCR reactions were performed using as templates *pET-16b/rap1a, pET-16b/rap1b, pET-16b/rap2a, pET-16b/rap2b, pET-16b/rap2c.* The products of the PCR reaction were separated on 1.5% agarose gels and visualized with ethidium bromide.

2.4. Immunoblotting analysis

Samples of total RBCs were lysed as previously described [18], and aliquots corresponding to a given number of cells (specified in the figures) were separated by SDS-PAGE on 10–20% acrylamide gradient gels, and transferred to nitrocellulose. When RBCs ghosts were subjected to SDS-PAGE, they were also loaded on the basis of ghost membrane number or based on cholesterol content. Immunoblotting analysis was performed with anti-Rap1 and anti-Rap2 polyclonal antibodies (Santa Cruz Biotechnology, Tebu-Bio, Magenta, Italy) (1:1.000 dilution), essentially as previously described [19].

2.5. Rap2 activation assay

Activation of Rap2 was evaluated by a pull-down assay using purified recombinant GST-tagged Rap-binding domain of RalGDS (GST-RalGDS-RBD) to precipitate GTP-bound Rap proteins from a cell lysate, as described [21]. Precipitated, active Rap2 was then separated by SDS-PAGE on a 10–20% acrylamide gradient gel, transferred to nitrocellulose, and identified by immunoblotting with the specific polyclonal antibody.

3. Results and discussion

3.1. Rap2, but not Rap1 proteins are expressed in highly purified human red blood cells

Analysis of protein expression in human RBCs is often hampered by the presence of contaminating leukocytes and platelets, which may produce misleading results. Among the several techniques currently used to prepare isolated RBCs, filtration through cellulose has been proven to represent the most efficient strategy to significantly lower the level of contaminating cells. We prepared highly purified RBCs by filtration through cellulose in order to investigate the expression of different Rap proteins family members in these circulating cells.