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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.

Cell count by automated hemocytometry of RBCs prepared by this technique, revealed that contaminant leukocytes and platelets were less than 0.002% and 0.02%, respectively. The purity of the RBCs preparation was further demonstrated by the β-profiling test, as recently proposed by Hoffman and co-workers [15, 16]. This powerful and definitive test exploits the fact that platelets and leukocytes, but not RBCs, express the mRNA for the β1 isoform subunit of the Na^+/K^+-ATPase, while the β2 isoform is exclusively expressed in RBCs but neither in leukocytes nor in platelets. Fig. 1A shows that mRNA for β2, but not β1 could be detected in RBCs, while the opposite was observed in purified MNC, thus confirming the high purity of our RBCs preparations. Based on the data from the β-profiling test, we confidently analyzed the expression of mRNA for the different Rap proteins. While all the five known Rap proteins were expressed in purified MNC, only the mRNA for Rap2b, but not that for Rap2a, Rap2c, Rap1a or Rap1b was present in highly purified RBCs (Fig. 1B).

The different expression of Rap proteins family members in RBCs was then investigated by immunoblotting. Currently available antibodies are able to discriminate between Rap1 and Rap2 proteins, but cannot distinguished among the highly homologous members within each subfamily. Fig. 1C(i) shows that, as previously reported [1], both Rap1 and Rap2 proteins are expressed in human platelets. In lysates of highly purified RBCs, however, only Rap2, but not Rap1, proteins were detected immunologically. In the light of the results from RT-PCR analysis, we may reasonably conclude that the Rap2 protein detected in RBCs by immunoblotting is most likely Rap2b.

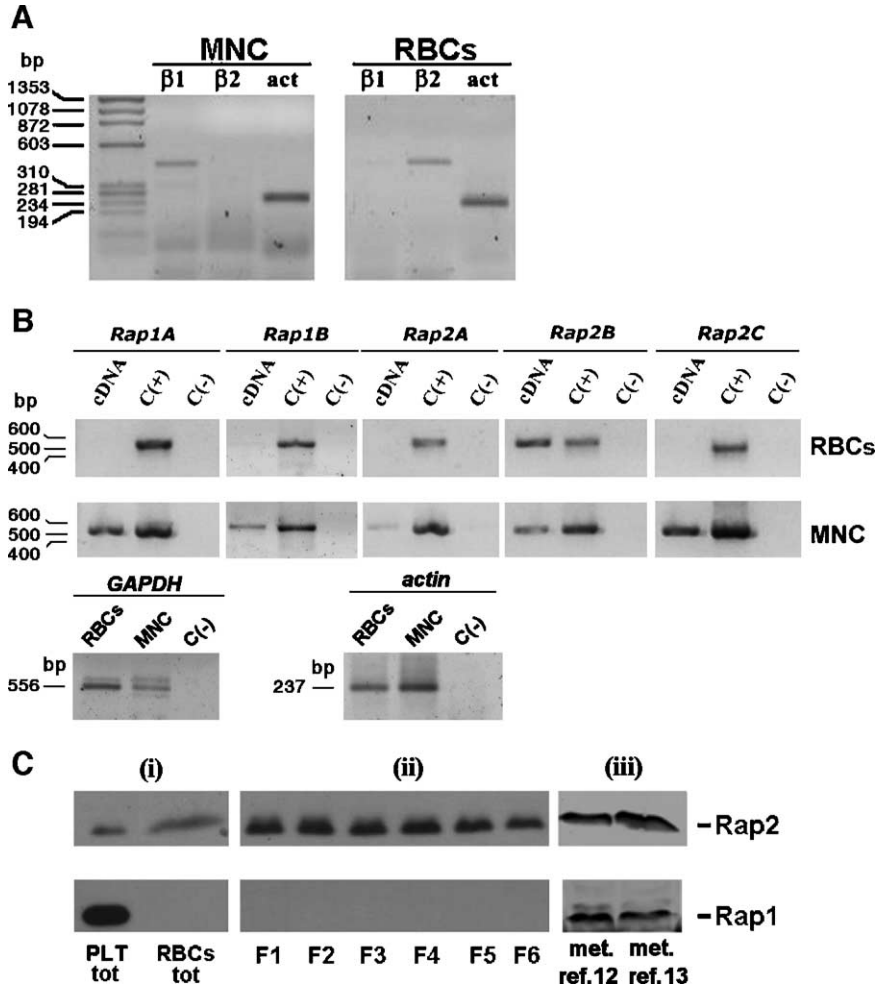

Fig. 1. Rap2, but not Rap1, is expressed in human RBCs. (A) β-profiling analysis of purified RBCs. Total RNA was extracted from a suspension of RBCs (RBCs) prepared by filtration through cellulose, and containing about 1% reticulocytes, as well as from purified MNC (MNC). First stranded cDNA was prepared and subjected to PCR amplification using pairs of primers for β1 and β2 isoforms of the β subunit of Na^+/K^+-ATPase, as well as β-actin, as a positive control, as indicated on the top. Products of amplification were identified after separation in 1.5% agarose gel and ethidium bromide staining. (B) RT-PCR was performed of cDNA from RBCs or MNC using primers for Rap1a, Rap1b, Rap2a, Rap2b, and Rap2c, as indicated on the top. In any reaction, primers were also tested with buffer, as negative control, C(−), and with the cDNA for the corresponding protein in *pET-16b*, C(+). As positive controls, RT-PCR reactions were performed with primers for GAPDH and β-actin, as indicated. (C) Proteins from 2×10^7 gel filtered platelets (PLT tot), from 10^7 RBCs prepared by filtration through cellulose (RBCs tot) (i) or as described by Low et al. [12] (met. ref. [12]) and by Kakhniashvili et al. [13] (met. ref. [13]) (iii), as well as membrane proteins from 5×10^7 RBCs of various subpopulations of increasing cell age (from F1 to F6) (ii) were separated in a 10–20% acrylamide gradient gel, transferred to nitrocellulose and probed with antibodies against Rap2 proteins (upper panel) or Rap1 proteins (lower panel). Note that the different lanes in panel (ii) correspond to an identical amount of cells (5×10^7) derived from the same RBCs preparation. Similarly, the amount of total RBCs loaded in the lanes shown in panels (i) and (iii) are identical (10^7).

The RBCs preparation analyzed represents a mixed population of cells of different age, and it has been previously reported that the protein endowment of these cells may be age-dependent. Therefore, by density-based fractionation of the purified RBCs, we separately analyzed Rap proteins expression in RBCs of different ages, which were prepared and characterized as previously described [18]. As shown in Fig. 1C(ii), Rap2 was found to be expressed in cells of different age, while Rap1 was never detected, even if, in these studies, proteins from a number of RBCs 5-fold higher than that analyzed in the experiments reported in Fig. 1C(i) (10^7 cells) were loaded on the gel. These observations definitively exclude the possibility of a selective expression of Rap1 in a subpopulation of RBCs, particularly in light/young subpopulations, which are highly enriched in reticulocytes.

Recently, two independent groups have reported detection of Rap1 in human RBCs [12,13]. In these studies, however, the blood samples were not subjected to rigorous leukodepletion, therefore a certain degree of contamination by white cells and platelets could have affected the RBCs population. In order to investigate whether the previously reported expression of Rap1 protein in RBCs was an artifact due to contaminating leukocytes and platelets, we prepared RBCs according to the procedure used by Low et al. [12], or by Kakhniashvili et al. [13]. By hemocytometer-based cell counting, we found that, in both RBC preparations, contamination by leukocytes and platelets resulted to be about 0.03% and 0.08%, respectively, thus much higher than that measured in RBCs prepared by filtration through cellulose (0.002% leukocytes and 0.02% platelets, see above). Immunoblotting analysis performed on proteins derived from an identical number of RBCs as shown in Fig. 1C(i) (10^7) revealed that not only Rap2, but also Rap1 was actually detectable in RBCs prepared according to Low et al. [12], or by Kakhniashvili et al. [13] (Fig. 1C (iii)). These results confirm the validity of the filtration through cellulose as a suitable and recommendable method for RBCs preparation. In addition, we can confidently conclude that Rap2, presumably Rap2b, but not Rap1, is expressed in RBCs, and that the previously reported expression of Rap1 proteins, based on proteomic studies, was to be ascribed to contaminating cells.

Rap1 has been recently proposed to promote sickle RBCs adhesion to laminin, thus implying its expression in these cells [14]. In that study, contaminating leukocytes were lowered to 0.01% by immunomagnetic depletion, but were still 5-fold higher than in our preparations. Moreover, the expression of Rap1 was not directly documented by immunoblotting or RT-PCR analysis, but, rather, deduced on the basis of a functional test showing that active, GTP-bound Rap1 could be precipitated from the RBCs preparation. Although it may sound reasonable to assume that the detection of the active form of the protein implies the expression of the protein itself, the approach is rather unusual, and, certainly, not as reliable as the direct detection of the protein or its mRNA. It cannot be excluded, for instance, that when mixed with RBCs, the low contaminant leukocytes produce enough active Rap1 to allow detection upon precipitation from a large volume of cell suspension. However, it should be considered that sickle cells often show an altered pattern of protein expression than normal RBCs [22], and, thus, it is still possible that in these cells, Rap1 is expressed and regulates cell adhesion, as proposed.

3.2. Rap2 is activated in human red blood cells and segregates into released vesicles

We next investigated the subcellular distribution and activation of Rap2 in human RBCs. Cells were fractionated into membranes (ghost) and cytosol. Moreover, cells were treated with Triton X-100 in order to isolate the membrane skeleton as detergent-insoluble fraction. Immunoblotting

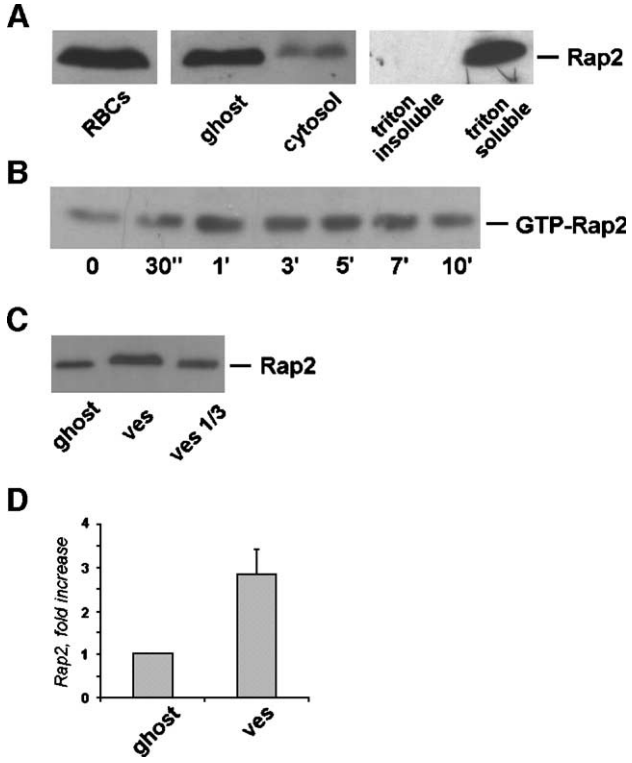

Fig. 2. Rap2 is membrane associated and segregates into vesicles released by Ca^{2+}-loaded RBCs. (A) RBCs were fractionated into membrane (ghost) and cytosolic (cytosol) fractions. Membrane skeleton was isolated from RBCs upon lysis with 2% Triton X-100, as detergent-insoluble materials (Triton insoluble) and separated from solubilized membrane components (Triton soluble) by centrifugation [18]. The distribution of Rap2 among these cellular fractions was analysed by immunoblotting with the specific antibody. In each lane, an equivalent of 10^7 RBCs was loaded. (B) A sample of purified RCBs was treated with 0.3 mM Ca^{2+}/4 μM A23187 (final concentrations) at 3% hematocrit in HEPES buffer. At the different times indicated in the figure, aliquots containing 10^8 cells were withdrawn from the RBCs suspension and lysed. Active, GTP-bound Rap2 was precipitated using GST-RalGDS-RBD, and identified by immunoblotting with a specific antibody. (C) Quantification of Rap2 enrichment in vesicles vs ghosts. Equal amounts of cholesterol (4 μg) for ghosts (ghost) and vesicles (ves) were loaded on a 10–20% acrylamide gradient gel, transferred to nitrocellulose and immunoblotted with anti-Rap2 antibody. For accurate quantification, an amount of vesicles corresponding to 1/3 of ghosts, in terms of cholesterol, was also loaded (ves 1/3), resulting in an integrated area approximately similar for ghosts and vesicles. The blot shown is representative of four independent experiments giving similar results. (D) The Rap2 enrichment factor in vesicles vs. ghosts was measured from the integrated area of Rap2-positive bands as described above, and was found to be: 2.90±0.58 (n=4).

analysis with anti-Rap2 antibody revealed that Rap2 was predominantly associated to the plasma membrane, but did not reveal a stable interaction with the membrane-skeleton (Fig. 2A). In circulating platelets, Rap2 can be activated in response to many physiological agonists through signaling pathways largely dependent on intracellular Ca^{2+} increase [21]. We found that treatment of RBCs with Ca^{2+}/A23187 induced a rapid and sustained increase of GTP binding to Rap2, which was evident after 30 s and maximal after 1 min (Fig. 2B). This indicates that Rap2 in RBCs can be regulated by Ca^{2+}-dependent exchange factors. It is known that treatment of RBCs with Ca^{2+}/A23187 induces the release of microvesicles. In vivo, membrane vesiculation is associated with RBC aging, and may have important physiological consequences, as released vesicles carry pro-coagulant activities [23,24]. RBCs were treated with Ca^{2+}/A23187, and the released vesicles were isolated. By immunoblotting analysis, we found that Rap2 segregated into microvesicles released from Ca^{2+}-treated RBCs. When the amount of Rap2 in RBCs and vesicles was normalized to the level of membrane cholesterol, which is equally represented, relative to total lipids, in the membrane of cells and vesicles [25], we measured that Rap2 was about 3-fold enriched in the released vesicles. Similar levels of enrichment are known only for the GPI-linked proteins of the erythrocyte membrane. Therefore, inclusion of Rap2 into the released vesicles is not merely a passive process due to membrane shedding, but reflects a specific segregation of this protein. Using the pull-down assay with GST-RalGDS-RBD, no active GTP-bound Rap2 was detected into the released vesicles (data not shown). This is probably due to the hydrolysis of the bound GTP by the intrinsic GTPase activity of Rap2 during the rather long time required to isolate the released vesicles, but it may also suggest that inclusion of Rap2 into the shedding vesicle is associated with the hydrolysis of protein-bound GTP.

3.3. Conclusions

In conclusion, by adopting the recently proposed "β-profiling" method as a screen to establish the purity of RBCs preparations, we have reported clear and definitive evidence that, in contrast to previous observations, Rap2, most likely Rap2b, is the only Rap protein expressed in human RBCs, and that Rap1 proteins are not present. Our study, therefore, recommends caution in the choice of the procedure used for RBCs preparation, and confirms the validity of the filtration through cellulose as a valuable, cheap, and reliable method to lower the amount of contaminating leukocytes to undetectable levels. Moreover, we have provided evidence that Rap2 can be activated in RBCs, and is selectively enriched in microvesicles released from Ca^{2+}-treated RBCs, thus suggesting its possible involvement in membrane shedding.

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