Guanine nucleotide-dependent translocation of RhoA from cytosol to high affinity membrane binding sites in human erythrocytes

Andrey A. BOUKHAROV* and Carl M. COHEN*†[1]

*Department of Biomedical Research, St. Elizabeth's Medical Center of Boston, Boston, MA 02135, U.S.A., and †Departments of Medicine and of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111, U.S.A.

The translocation of the small GTP-binding protein Rho from the cytosolic to membrane-bound form is an early step in many cellular signal-transduction events, but little is known regarding the mechanism of Rho association with the plasma membrane. We have used membranes from human erythrocytes to uncover a novel class of integral membrane components involved in the Rho-membrane association. Membranes of human erythrocytes contain several proteins of the Ras superfamily. Using specific antibodies and C3 exoenzyme of *Clostridium botulinum* we have identified one of them as RhoA. This protein was detected in both cytosol and membrane fractions of hypotonically lysed erythrocytes. We found that cytosolic Rho bound specifically to the cytoplasmic surface of the erythrocyte membrane and that the translocation of Rho to the membrane was absolutely dependent on the prior incubation of the cytosol with guanosine 5′-[γ-thio]triphosphate (1–50 μM) at low Mg^{2+} concentration. Rho binding sites could not be extracted from the membrane using conditions that extracted all other peripheral proteins and were unaffected by heat treatment and protease digestion. Rho binding was saturable, with a K_d in the range 1–5.0 nM, and the number of binding sites was estimated to be approx. $(1–2) \times 10^3$ sites per cell. This is the first report of Rho binding to integral membrane components. The identity of these components may reveal novel aspects of the mechanism by which Rho exerts its multiple biochemical effects.

INTRODUCTION

The small GTP-binding protein, Rho, plays a key role in numerous cellular processes. Rho proteins have been shown to control the activity of several protein kinases [1–6], phosphoinositide kinases [7,8] and phospholipase D [9–11] in mammalian cells. They have also been implicated in the regulation of various actin-containing structures, including stress fibres in fibroblasts [12] and astrocytes [13,14], actin filaments of tight junctions in polarized epithelia [15], and actomyosin complexes of the contractile ring in dividing eggs of sand dollar and *Xenopus laevis* [16,17]. There are three closely related Rho proteins found in mammalian cells, RhoA, RhoB and RhoC. While RhoB is associated with intracellular membranes of early endosomes and the prelysosomal compartment [18], RhoA and RhoC are present mostly in the cytosol with a small proportion found on the plasma membrane [18–20]. One pathway through which Rho is thought to act depends on the association of Rho with the plasma membrane after it is released from cytosolic Rho GDP dissociation inhibitor (Rho-GDI) and is converted into a GTP-bound active form [21–23]. Although the translocation of activated Rho from the cytosol to the membrane has been shown to occur both *in vivo* [21] and *in vitro* [23], the mechanism by which Rho associates with the plasma membrane is unknown. Although several targets for activated Rho have been identified, including Rho kinase, PKN and 4-phosphatidylinositol 5-kinase [3,8,24], it is unclear whether these proteins are involved in the recruitment of Rho to plasma membranes or whether other membrane proteins are also involved.

It is also unknown what role is played by post-translational modifications of Rho in its association with membranes. It has been shown that the CAAX C-terminal motif of Rho is post-translationally processed so that the cysteine residue is thio-esterified by geranylgeraniol (and farnesol in the case of RhoB) followed by cleavage of the last three residues and carboxy-methylation of the cysteine residue [25,26]. These modifications are required for interactions between Rho-GDI and Rho [25]. They also confer substantial hydrophobicity to otherwise hydrophilic proteins and could promote Rho association with membranes. However, it is unknown what role, if any, the polyprenyl moiety plays in directing the binding of Rho to membranes.

Membranes of human erythrocytes contain several types of small GTP-binding proteins. One has been immunochemically identified as Ras, while others have been shown to be ADP-ribosylation substrates of C3 exoenzyme from *Clostridium botulinum*, which identifies them as possible members of the Rho subfamily [27,28]. However, neither the function of these proteins in red blood cells nor the mechanism of their association with the membrane is known. The integrity of the erythrocyte membrane depends on the existence of an underlying hexagonal lattice of short actin filaments cross-linked by long, flexible spectrin molecules. Structural changes of this lattice affect erythrocyte membrane deformability and its resistance to shear stress and would, therefore, be vital for the survival of the cell in the circulation. The factors that influence the organization of the erythrocyte cytoskeleton are only partly understood, but could involve alterations in membrane skeletal protein phosphorylation, which has been shown to affect their association with the membrane as well as the association of actin filaments with spectrin and other actin-binding proteins [29,30]. The presence of Rho proteins in erythrocytes may suggest their involvement in regulation of the cytoskeleton organization of these cells.

In order to elucidate the mechanism by which Rho associates with plasma membranes, we have established an assay system *in vitro* to study nucleotide-dependent association of Rho proteins

Abbreviations used: Rho-GDI, Rho GDP dissociation inhibitor; IOV, inside-out vesicles; ROV, right-side-out vesicles; GTP[S], guanosine 5′-[γ-thio]-triphosphate; ECL, enhanced chemiluminescence; DTT, dithiothreitol.

[1] Present address and address for correspondence: Creative Biomolecules, 45 South Street, Hopkinton, MA 01748, U.S.A.

with the membrane of the erythrocyte. Here we show that membranes from human erythrocytes contain integral membrane components that bind Rho with high affinity. These membrane binding sites apparently represent a novel mode for the association of Rho with the plasma membrane and may play a key role in its regulatory or signal-transduction functions.

EXPERIMENTAL

Materials

Human RhoA coding sequence, cloned into pGEX-2T expression vector, was kindly provided by Dr. A. Hall (University College, London, U.K.). The pGEX vector with cloned C3 ribosyltransferase was kindly provided by Dr. L. Feig (Tufts University, Boston, MA, U.S.A.). Anti-peptide polyclonal antibodies specific for RhoA, RhoB and Rac were from Santa Cruz Inc.; anti-Rho-GDI antibodies were kindly provided by Dr. G. Bokoch (The Scripps Research Institute, La Jolla, CA, U.S.A.). [^{32}P]NAD was from ICN, nitrocellulose membranes were from Bio-Rad, chemiluminescence reagents were from NEN, and X-ray film (Hyper-Film-MP) was from Amersham. Sigmacell type 50 and α-cellulose were from Sigma, DE52 was from Whatman and glutathione-Sepharose was from Pharmacia. Protease inhibitors and thrombin were from Boehringer-Mannheim. All other reagents were the purest grade available.

Preparation of erythrocyte membranes

Erythrocyte ghosts, inside-out vesicles (IOV) and stripped IOV were prepared as described below.

Washed erythrocytes

Erythrocytes from freshly drawn whole blood were centrifuged at 2000 ***g*** for 5 min. The buffy coat of the white blood cells was carefully removed and the pelleted erythrocytes were resuspended and washed 4 times with 10 volumes of ice-cold isotonic buffer A [5 mM sodium phosphate (pH 7.5)/150 mM NaCl/0.5 mM EGTA]. The washed erythrocytes were used either directly in the preparation of the membrane and cytosol fractions or were treated as follows to remove any remaining leucocytes. A slurry, made with Sigmacell type 50 (0.25 g), α-cellulose (1 g) and 30 ml of buffer A, was used to pack the column and the matrix was washed with 30 ml of buffer A. Packed erythrocytes were diluted tenfold with buffer A and approx. 60 ml of the erythrocyte suspension was loaded on to the column. The erythrocytes were eluted with 15–20 ml of buffer A. The erythrocytes obtained were pelleted by centrifugation (2000 ***g*** for 5 min) and were washed 2–3 times with buffer A.

Preparation of erythrocyte ghosts

The washed erythrocytes were lysed by the addition of 20 volumes of ice-cold hypotonic buffer B [5 mM sodium phosphate (pH 7.5)/0.5 mM EGTA] containing 20 μg/ml of PMSF. Membranes were pelleted by centrifugation at 30000 ***g*** for 15 min and were washed 3–4 times with the same buffer until white.

Preparation of IOV

The erythrocyte-ghost pellet was resuspended in 30 vol. of low ionic strength buffer C (0.5 mM EGTA, pH 8.2), containing 20 μg/ml of PMSF, incubated for 30 min at 37 °C and centrifuged at 30000 ***g*** for 25 min, to pellet the IOV. The vesicles were then washed once with the same buffer.

Preparation of stripped IOV

IOV (prepared as decribed above) were incubated in 30 vol. of buffer D (0.5 mM EGTA, pH 11) containing 20 μg/ml of PMSF for 30 min at 37 °C. The membranes were pelleted by centrifugation at 30000 ***g*** for 25 min and washed once with buffer C.

Preparation of right-side-out vesicles (ROV)

A dense suspension of erythrocyte ghosts was passed ten times through a 1 in (2.54 cm), 28-gauge needle affixed to a 1 ml plastic syringe, resuspended in 10 vol. of buffer C and centrifuged at 30000 ***g*** for 25 min to pellet the membranes. Vesiculation was confirmed by phase-contrast light microscopy.

All vesicular membrane preparations were resuspended in buffer C so that the volume of the suspension was the same as the volume of packed ghosts from which they were prepared.

Proteolytic digestion and heat denaturation of membranes

IOV or stripped IOV membranes were digested with 20 μg/ml of proteinase K or chymotrypsin in buffer B for 1 h at 37 °C. The reaction was stopped by the addition of PMSF (40 μg/ml). The proteolysed membranes were then washed twice with 50 vol. of buffer B supplemented with PMSF. For heat denaturation, membranes were incubated at 80 °C for 30 min and cooled on ice before use.

Erythrocyte cytosol preparation

All preparations and buffers were kept on ice and all procedures were at 4 °C.

Crude cytosol

Washed erythrocytes were lysed by the addition of 10 vol. of buffer B containing a protease inhibitor cocktail [10 μg/ml aprotinin, 20 μg/ml bestatin, 40 μg/ml leupeptin, 3.5 μg/ml pepstatin, 25 μg/ml 7-amino-1-chloro-3-L-tosylamidohepatan-2-one (TLCK), 40 μg/ml 1-chloro-4-phenyl-3-L-toluene-*p*-sulphonamidobutan-2-one (TPCK) and 20 μg/ml PMSF]. The lysate was supplemented with 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) and cleared by centrifugation at 30000 ***g*** for 20 min. The membranes were discarded and the supernatant (crude cytosol) was used either directly for nucleotide loading and incubation with membranes or to prepare haemoglobin-depleted cytosol.

Haemoglobin-depleted cytosol

The crude cytosol was mixed with 0.1 vol. of 10 × DE52 binding buffer [200 mM Tris/HCl (pH 7.5)/200 mM NaCl/5 mM EGTA] and loaded on to a DE52 column pre-equilibrated with equilibration buffer (1 × binding buffer supplemented with 5 mM $MgCl_2$ and 1 mM DTT). The cytosol was loaded (6 ml of cytosol/1 ml of DE52 matrix) and the column was washed with 1.5 bed vol. of the binding buffer to remove haemoglobin. Cytosolic proteins bound to the column were eluted with 0.4 M NaCl in binding buffer. The eluate was dialysed overnight with 2–3 changes of the equilibration buffer and concentrated on a Centricon-10 spin concentrator (Amicon) to the same volume as that of the packed erythrocytes before lysis.

Concentrated crude cytosol

Washed erythrocytes were mixed with an equal volume of buffer B, briefly sonicated (5–10 s) and supplemented with 5 mM

$MgCl_2$ and 1 mM DTT. The dense suspension was cleared by ultracentrifugation at 100000 ***g*** for 20 min and the supernatant was recovered and used in subsequent assays.

ADP-ribosylation of erythrocyte Rho

Detection of Rho proteins in erythrocyte lysate fractions by C3 exoenzyme-mediated labelling with [^{32}P]NAD was carried out as described previously [31,32]. Erythrocyte-ghost membranes to be used for ADP-ribosylation were first solubilized with a buffer containing 30 mM Tris/HCl (pH 7.9), 5 mM $MgCl_2$, 5 mM DTT, 10 mM EGTA, 0.5 % (v/v) Triton X-100 and 0.05 % (w/v) SDS. Haemoglobin was removed from the erythrocyte cytosol to be used for labelling as described above.

Preloading cytosol with nucleotides

An aliquot of a cytosol preparation was first supplemented with 1–100 μM guanosine 5′-[γ-thio]triphosphate (GTP[S]) or 0.5–1 mM GDP and then mixed with a one tenth vol. of EDTA containing buffer N [300 mM sodium phosphate (pH 7.5)/600 mM NaCl/100 mM EDTA] and incubated for 25 min at 37 °C. After incubation, the cytosol was supplemented with 20 mM $MgCl_2$ and centrifuged at 20000 ***g*** for 25 min to remove Rho aggregates. The supernatant was used in Rho translocation assays. When preincubation of the cytosol with the nucleotides was not required, the nucleotide, buffer N and $MgCl_2$ were added to the cytosol at the same time.

Translocation assay

Aliquots of nucleotide-supplemented cytosol (0.5–1 ml) were mixed in Eppendorf tubes with 40–50 μl of an erythrocyte membrane preparation and incubated either on ice or at 37 °C for times ranging from 1–60 min. After incubation, the assay mixtures were centrifuged at 20000 ***g*** for 50 min and aliquots of the supernatants and entire pellets were solubilized in SDS/PAGE sample loading buffer S [10 mM Tris/HCl (pH 6.8)/1 mM EDTA/40 mM DTT/4 % (w/v) SDS/5 % (w/v) sucrose/0.01 % Bromophenol Blue] and analysed for the presence of Rho by Laemmli SDS/PAGE [33], followed by Western immunoblotting (see below).

Densitometry of Western blots

Samples separated by SDS/PAGE were blotted on to a nitrocellulose membrane and the membrane was probed, first with rabbit anti-RhoA antibodies and then with anti-rabbit horseradish-peroxidase-conjugated antibodies. The bands were detected by enhanced chemiluminescence (ECL) (Amersham). The exposed films of the blots were scanned using an EagleEyeII still-video system (Stratagene) and the images obtained were processed using densitometry software from Scanalytics. Samples of serially diluted, purified recombinant RhoA were analysed on the same blot to serve as quantification standards and to ensure that the quantities of other samples loaded were below the saturation limit for this detection system.

Binding experiment

Serial dilutions [2–55 % (v/v)] of undiluted erythrocyte cytosol (either concentrated, unprocessed cytosol or haemoglobin-depleted cytosol) were made with buffer B. Each dilution (1.2 ml) was individually loaded with GTP[S] or GDP, as described above, and then divided equally between two binding assay tubes containing the same amount of IOV (30 μl). The binding assay mixtures were incubated at 37 °C for 15 min and centrifuged at 20000 ***g*** for 50 min. Aliquots of the supernatants and entire pellets were solubilized in buffer S and analysed by SDS/PAGE and Western immunoblotting. The concentration of Rho in each pellet and supernatant sample was determined by blot densitometry using serial dilutions of purified recombinant RhoA as a quantification standard. To minimize variability, which might have been introduced by comparing standards with samples loaded on to different gels, the recombinant RhoA dilutions were loaded on to the same lanes as the binding-assay samples 15 min after the start of electrophoresis (Figure 6A, inset). The standard curve of integrated absorbance versus recombinant Rho concentration was used for quantitation.

Preparation of recombinant RhoA and C3 ribosyltransferase

Recombinant RhoA and C3 ribosyltransferase proteins were expressed in an *Escherichia coli* system as fusions with glutathione S-transferase, isolated by affinity purification on a glutathione–Sepharose column and cleaved by thrombin according to published procedures [31,34,35]. The RhoA protein thus obtained was used for immunization of rabbits and as a quantification standard in Western-blot densitometry. The C3 protein was used for [^{32}P]ADP ribosylation of erythrocyte Rho.

Preparation of polyclonal anti-RhoA antibodies

Rabbits were immunized with the glutathione S-transferase–RhoA fusion protein, then boosted with purified RhoA and bled every 4–5 weeks. The IgG fraction from the serum collected was prepared using a Protein A-affinity column. Anti-RhoA specific IgGs were isolated using an affinity matrix with covalently linked recombinant RhoA protein. In the both affinity purification steps Pierce Immunopure© buffer system for gentle IgG binding and elution was used.

RESULTS

Detection of the small GTPase RhoA in human erythrocytes

In our initial studies, we determined the relative distribution of Rho proteins between erythrocyte cytosol and membranes. There are two techniques commonly used to detect Rho in cellular lysate fractions, a [^{32}P]ADP-ribosylation assay with *C. botulinum* C3 exoenzyme and Western immunoblotting with anti-Rho specific antibodies. Neither technique could be used to test for the presence of Rho proteins directly in the crude cytosol fraction of erythrocyte lysate, because haemoglobin, which comprises about 99 % of its protein content, interfered with the assay. To make the detection possible, the crude cytosol was passed through a DE52 column and, under the conditions described in the Experimental section, haemoglobin did not interact with the matrix and was eluted in the void volume, whereas other proteins bound to the matrix and were subsequently eluted with buffer containing 0.4 M NaCl. The eluate was further dialysed and concentrated to the volume of the erythrocytes originally used for lysis. We refer to this preparation as haemoglobin-depleted cytosol.

For RhoA detection and quantification, two different antibodies were used. One was prepared in our laboratory using recombinant RhoA protein for immunization (see the Experimental section) and the other (obtained from Santa Cruz Inc.) was an anti-peptide antibody specific for RhoA. Western-blot analysis of human erythrocyte lysate fractions revealed that both the membrane and the cytosol contained a protein of 21 kDa,

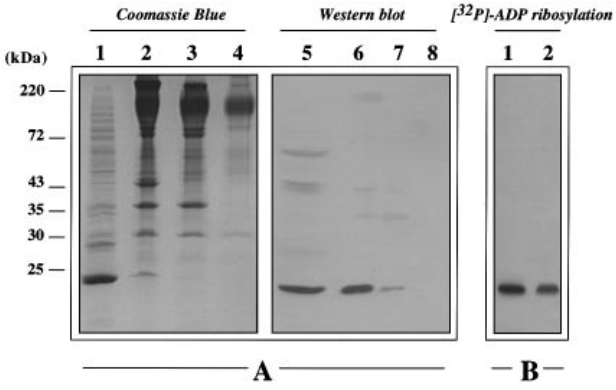

Figure 1 RhoA protein in human erythrocytes

Cytosol and membrane fractions were prepared from human erythrocytes as described in the Experimental section. (**A**) An aliquot of each fraction subjected to SDS/PAGE. The amount of protein loaded per lane was from same number of erythrocytes (approx. 1.3×10^8 cells) in each case. Lanes 1–4 were stained with Coomassie Blue. Lanes 5–8 were transferred to nitrocellulose and probed with polyclonal anti-RhoA antibodies and revealed using ECL. The samples were: haemoglobin-depleted erythrocyte cytosol (lanes 1 and 5); erythrocyte ghost membranes (lanes 2 and 6); IOV membranes (lanes 3 and 7); pH 11-stripped IOV (lanes 4 and 8). (**B**) Aliquots of haemoglobin-depleted cytosol (lane 1) and ghost membranes (lane 2) were used in a [^{32}P]ADP-ribosylation reaction with *C. botulinum* C3 ribosyltransferase and were analysed by SDS/PAGE followed by autoradiography.

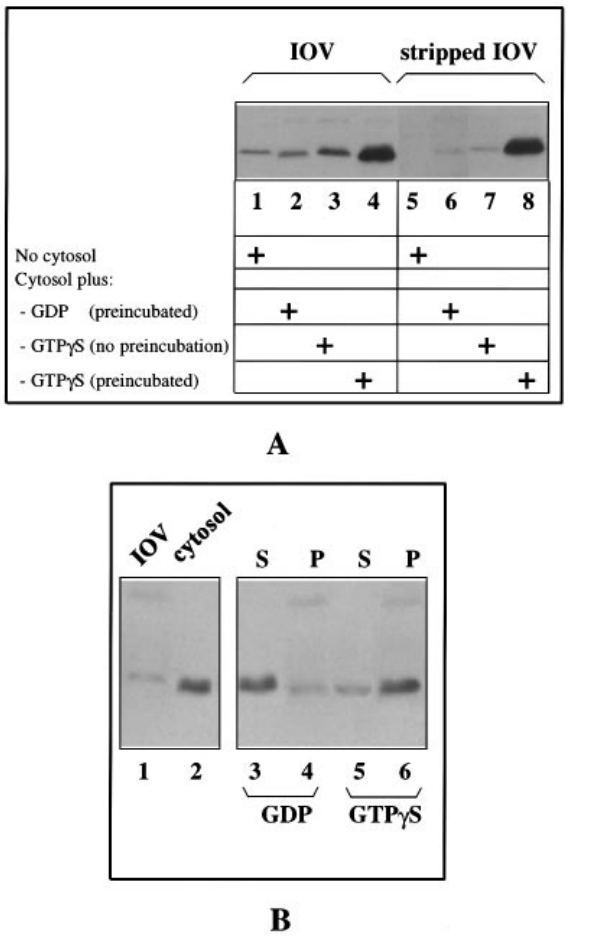

Figure 2 GTP[S]-stimulated translocation of cytosolic Rho protein on to erythrocyte membranes *in vitro*

(**A**) Rho translocation from unprocessed cytosol. A crude cytosolic fraction of erythrocyte lysate was preloaded with either GTP[S] (GTPγS) (50 μM) or GDP (1 mM) and incubated for 10 min at 37 °C with erythrocyte membranes as described in the Experimental section. Membranes were pelleted and analysed by Western immunoblot with polyclonal anti-RhoA antibodies. The membranes used in these assays were IOV (lanes 1–4) or pH 11-stripped IOV (lanes 5–8). They were incubated with either GTP[S]-containing buffer (lanes 1 and 5); GDP-preloaded cytosol (lanes 2 and 6); cytosol with GTP[S] added at the moment of mixing with membranes (no preloading) (lanes 3 and 7) or GTP[S] preloaded cytosol (lanes 4 and 8). The amount of membranes used in the assays and amounts of sample loaded on to each lane were adjusted to correspond to the same number of erythrocytes in each case. (**B**) Rho translocation from haemoglobin-depleted cytosol. The assay was carried out as in (**A**) except that haemoglobin-depleted erythrocyte cytosol was used. The cytosol was preloaded with GDP (lanes 3 and 4) or GTP[S] (GTPγS) (lanes 5 and 6), mixed and incubated with IOV for 20 min at 37 °C and centrifuged at 20 000 **g** for 45 min. Aliquots of the supernatants (S) and solubilized pellets (P) and of IOV (lane 1) and cytosol (lane 2) were subjected to SDS/PAGE, followed by Western immunoblot with polyclonal anti-RhoA antibodies. Rho was detected using ECL.

which was specifically recognized by the anti-peptide antibody against a sequence unique for RhoA (Figure 1A). The Western blot suggested that Rho was distributed approximately equally between these two fractions (Figure 1A, lanes 5 and 6). The presence of Rho in both fractions was further confirmed by ADP-ribosylation of the protein using *C. botulinum* C3 exoenzyme (Figure 1B). Of relevance to our later results, we note here that we were also able to detect Rho-GDI on Western blots of erythrocyte cytosol (results not shown).

Several lines of evidence led to the conclusion that erythrocytes contain primarily RhoA and not other common Rho subfamily members. First, Western blots of erythrocyte cytosol and membranes were consistently negative when probed with anti-peptide antibodies specific for RhoB and Rac (results not shown). Secondly, although our polyclonal anti-RhoA antibodies might be expected to cross-react with other Rho subfamily members, quantification of membrane and cytosolic Rho using our polyclonal anti-RhoA antibodies and recombinant RhoA standards gave results that were identical with those obtained using commercial anti-RhoA anti-peptide antibodies. This would not have been the case if our polyclonal antibodies also recognized other Rho-family members (for example RhoC) which co-migrated with RhoA in erythrocyte fractions. Taken together, these results suggest that there are no immunologically detectable Rac, RhoB and RhoC proteins in erythrocytes or that their levels are much lower than that of RhoA.

Using purified, recombinant RhoA as a quantification standard for Western-blot densitometry, the total erythrocyte Rho concentration (cytosolic plus membrane-associated Rho) was estimated to be 0.5 ± 0.1 μg/ml of packed erythrocytes (mean $\pm$ S.D., $n = 6$ independent measurements), i.e. 46 ± 9 pg/10^6 cells or 1.5 ± 0.3 ng/mg of total protein. This corresponds to $(1.3 \pm 0.3) \times 10^3$ molecules per cell. This estimate represents the minimum value, since some Rho may have been lost from the membranes and cytosol during preparation.

Extraction of membrane-associated Rho proteins

The incubation of ghost membranes at 37 °C in low-ionic-strength buffer is known to result in disintegration and extraction of the spectrin–actin cytoskeletal framework, followed by spontaneous vesiculation of the erythrocyte membrane. The resulting membranes are referred to as IOV [36]. This treatment led to a 90–95% decrease in the amount of membrane-associated Rho (Figure 1A, lanes 3 and 7). Further incubation of IOV in low-ionic-strength buffer at pH 11, which resulted in total extraction

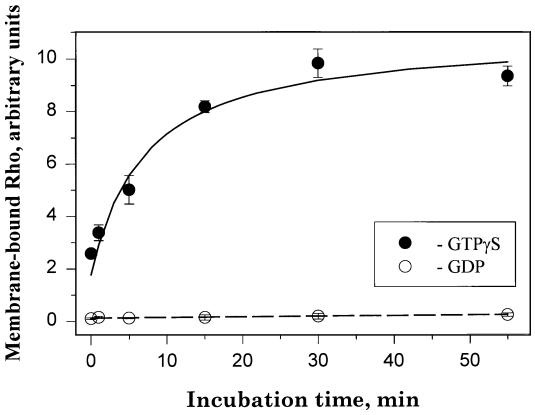

Figure 3 Amount of cytosolic Rho translocated to membranes is dependent on the time of cytosol preincubation with GTP[S]

Aliquots of crude cytosol were supplemented with either 25 μM GTP[S] (GTPγS) or 0.5 mM GDP and 10 mM EDTA to initiate nucleotide exchange on cytosolic Rho protein (cytosol preloading with nucleotide as described in the Experimental section). After incubation at 37 °C for various times, $MgCl_2$ (20 mM) was added to terminate the exchange reaction, the samples were centrifuged as described in the Experimental section and the supernatants were mixed with IOV (the same amount in each case). After 10 min incubation at 37 °C, translocation assay mixtures were centrifuged and the pellets were analysed by SDS/PAGE followed by Western immunoblot with polyclonal anti-RhoA antibody and densitometry. Each value is the mean ± S.D. of three independent determinations. This experiment is representative of three independent experiments, each giving similar results.

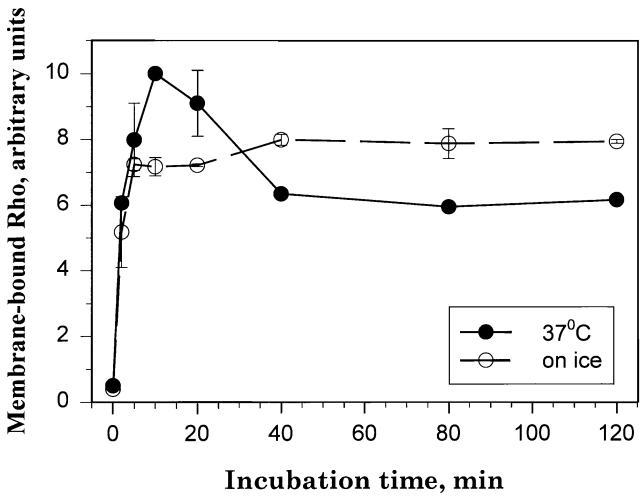

Figure 4 Kinetics of cytosolic Rho translocation on to membranes

Aliquots of crude erythrocyte cytosol (600 μl) preloaded with 25 μM GTP[S] were mixed with IOV (30 μl) and incubated for 2–120 min, either on ice or at 37 °C. The amount of Rho bound to membranes was estimated as described in the legend to Figure 3. Each value is the mean ± S.D. of two independent determinations. The results are representative of three independent experiments.

of all peripheral membrane proteins [37], led to complete removal of Rho from the membrane (Figure 1A, lanes 4 and 8).

GTP[S]-dependent binding of cytosolic Rho to Rho-depleted erythrocyte membranes

We have found that mixing erythrocyte cytosol with Rho-depleted erythrocyte membranes (IOV or pH 11-stripped IOV) in the presence of micromolar concentrations of the non-hydrolysable guanine analogue, GTP[S], resulted in the translocation of cytosolic Rho to membranes (Figure 2A, lanes 4 and 8; Figure 2B, lanes 5 and 6). This phenomenon was observed when either haemoglobin-depleted or unprocessed crude cytosol of erythrocytes was used (compare Figures 2A and 2B), which gave quantitatively identical results (not shown). This process was nucleotide-specific, since very little Rho became membrane-associated in the presence of GDP (Figure 2A, lanes 2 and 6; Figure 2B, lanes 3 and 4), ATP or ADP (results not shown). In the presence of GTP (as opposed to GTP[S]), the extent of Rho translocation varied from one experiment to another. The amount of the membrane-bound Rho was usually equal to or somewhat higher than that found in the presence of GDP, but was substantially lower than that in the presence of GTP[S] (results not shown). This is similar to an observation made for Rac proteins in neutrophil lysates [23] and is probably an indication of the intrinsic GTPase activity that renders Rho inactive and prevents (or disrupts) its interaction with the membrane.

Characteristics of GTP[S]-stimulated Rho binding

To achieve maximal binding of Rho to membranes it was necessary to preincubate the cytosol with GTP[S] under conditions known to promote nucleotide exchange in Rho in the absence of nucleotide exchange factors [38]. Without preincubation, when the nucleotide was added simultaneously with the cytosol and membranes (see the Experimental section for details), only a small amount of Rho was translocated (Figure 2A, lanes 3 and 7). This was further confirmed by the results of the experiment presented in Figure 3. It shows that the time of cytosol incubation with GTP[S] before mixing with membranes determines the amount of cytosolic Rho that will subsequently bind to membranes.

The preincubation of cytosol with GTP[S] appears to be the rate-limiting step for Rho binding to membranes. Following preincubation, binding was very rapid both on ice and at 37 °C (Figure 4). The amount of Rho bound to membranes reached a maximum in less than 10 min of incubation. Longer incubation at 37 °C resulted in some decrease in the amount of membrane-associated Rho (Figure 4). This decrease could be interesting, since it suggests that there may be a mechanism for release of activated Rho from membranes other than by GTP hydrolysis, but this was not investigated further.

In another experiment, the dependence of the nucleotide concentration on binding was investigated and it was found that maximal translocation of Rho to membranes occured at GTP[S] concentrations of 2 μM and higher (Figure 5).

The number of Rho binding sites on the membranes was unaffected by conditions that extracted all peripheral membrane proteins. This was demonstrated by the fact that the amount of Rho translocated on to equal amounts of IOV (Figure 1, lane 3) and stripped IOV (Figure 1, lane 4) was the same (Figure 2A, lanes 4 and 8), and suggests that Rho binding sites are integral membrane components.

We have also found that Rho binds specifically to the inner surface of the erythrocyte membrane. This was demonstrated in an experiment in which GTP[S]-preloaded cytosol was mixed with the same amounts of either IOV or ROV. Essentially no cytosolic Rho bound to ROV (note that ROV contain endogenous membrane-associated Rho that is lost from IOV), whereas substantial translocation on to IOV was observed (Table 1).

Surprisingly, exhaustive proteolytic digestion of erythrocyte

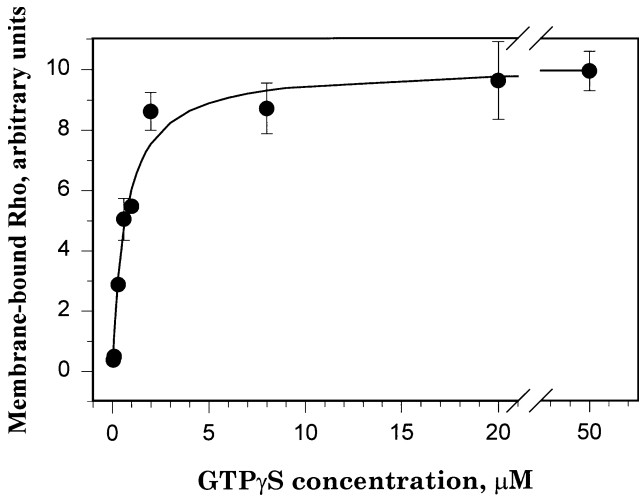

Figure 5 Binding of cytosolic Rho to membranes depends on the concentration of GTP[S]

Aliquots of crude erythrocyte cytosol (600 μl) were preloaded with the concentrations of GTP[S] (GTPγS) shown, mixed with IOV (30 μl) and incubated at 37 °C for 10 min. The amount of Rho bound to membranes was estimated as described in the legend to Figure 3. Each value is the mean ± S.D. of two independent determinations and the results are representative of three independent experiments.

Table 1 GTP[S]-bound cytosolic Rho preferentially translocates to the inner surface of erythrocyte membranes *in vitro*

IOV and ROV were prepared from the membranes of erythrocyte ghosts as described in the Experimental section. The vesicles were mixed with crude erythrocyte cytosol preloaded with either 0.5 mM GDP or 25 μM GTP[S], incubated and pelleted by centrifugation as described in the legend to Figure 2. Aliquots of solubilized pellets and of vesicles not incubated with the cytosol were analysed by SDS/PAGE followed by Western immunoblot with polyclonal anti-Rho antibodies. The relative amounts of Rho bound to membranes was quantified by densitometry of the blots. The amount of membranes used in the assays and the amounts loaded on to the gel were adjusted to correspond to the same number of erythrocytes in each case. Note that untreated ROV contain endogenous membrane-associated Rho, which is practically absent from IOV. Each value is the mean ± S.D. of three independent determinations and the results are representative of three independent experiments.

	Membrane-bound Rho (arbitrary units)	
	IOV	ROV
Membranes (no cytosol added)	0.08 ± 0.02	5.4 ± 0.7
Membranes incubated with cytosol and GDP	0.52 ± 0.1	4.8 ± 0.4
Membranes incubated with cytosol and GTP[S]	10.23 ± 0.2	5.5 ± 0.7

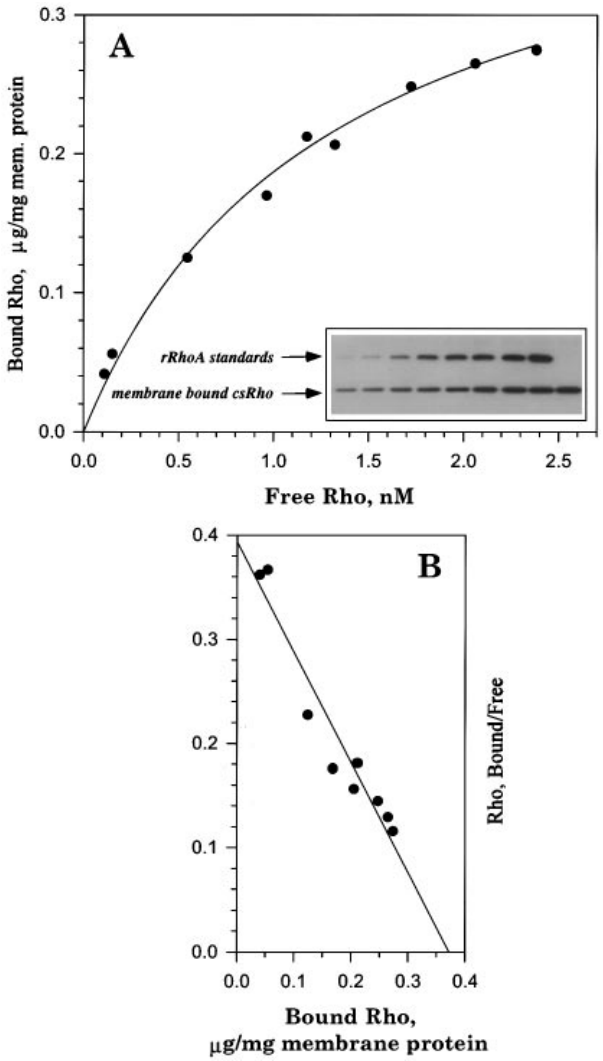

Figure 6 Cytosolic Rho interaction with erythrocyte membranes *in vitro* exhibits saturation of binding

Serial dilutions of haemoglobin-depleted cytosol ranging from 4% to 46% (v/v) of the original undiluted erythrocyte cytosol were preloaded with GTP[S] and mixed with the same amounts of IOV. Aliquots of supernatants and solubilized pellets of each of the translocation assay mixtures were immunoblotted with polyclonal anti-RhoA antibody. The amounts of Rho bound to membranes in the pellets or remaining free in the supernatants were quantified using serial dilutions of the standard sample of purified recombinant RhoA. (**A**) Inset: a blot with samples of translocation assay pellets containing bound cytosolic Rho (csRho) and serial dilutions of recombinant RhoA (rRhoA) loaded on to the same lanes of the gel as described in the Experimental section. The amounts of rRhoA loaded were (left to right): 0.5, 1, 2, 3, 4, 5, 6 and 7 ng. The cytosolic dilutions used for incubation with IOV were (left to right): 4, 6, 10, 14, 18, 24, 30, 38 and 46%. (**A**) Binding curve. Each value is the amount of Rho determined in the supernatants and pellets of an individual translocation assay mixture. The results are representative of three independent experiments. (**B**) Scatchard plot [44] of bound Rho versus bound/free Rho.

membranes with chymotrypsin or proteinase K had no effect on the subsequent GTP[S]-dependent binding of cytosolic Rho to the membranes. Moreover, pretreatment of the membranes for 30 min at 80 °C did not lead to decreased GTP[S]-dependent Rho binding (results not shown). These results are in contrast with findings reported previously that GTP[S]-dependent binding of Rho, Rac and CDC42 proteins to neutrophil membranes *in vitro* required heat- and trypsin-labile membrane components [23]. These results agree, however, with the reported observation that RhoB binding to various membranes was not inhibited by boiling or tryptic digestion of the membranes [39].

The membrane-associated Rho translocated from GTP[S]-preloaded cytosol could be released from membranes by incubation at 37 °C in a buffer without Mg^{2+}. The efficiency of the extraction of Rho increased with decreasing ionic strength and increasing pH of the buffer (results not shown). In particular, the extraction buffer used for preparation of IOV (buffer C) was very effective in extracting translocated Rho. This suggests that the loss of membrane-associated Rho upon conversion of ghosts to IOV (Figure 1A, lanes 6 and 7) is due to the release of Rho from membrane binding sites rather than to its association with

cytoskeletal proteins. Thus it appears that Rho binds to integral membrane elements of erythrocytes, and this binding is stabilized by electrostatic interactions.

Erythrocyte membranes contain a limited number of binding sites for Rho

To further characterize Rho binding and to estimate the number of binding sites on erythrocyte membranes, we measured binding at various Rho concentrations. Various dilutions of GTP[S]-preloaded erythrocyte cytosol were incubated with equal amounts of membrane and the Rho proteins that became membrane-associated or remained in the cytosol were quantified. The cytosol dilutions were in the range 2–55 % of the original non-diluted cytosol and either haemoglobin-depleted or crude, unprocessed cytosol preparations were used in these experiments. In either case the binding was saturatable, although when crude cytosol was used a moderate decrease in the amount of membrane-associated Rho was observed at cytosol concentrations greater than 40 % (where 100 % is the cytosol concentration in the intact erythrocyte; results not shown). Since we could not accurately measure the concentration of Rho in crude cytosol, we used the haemoglobin-depleted preparation to generate data for the binding curve (Figure 6A).

In the experiment shown, the K_d of the binding derived from the Scatchard plot (Figure 6B, correlation coefficient $r = 0.93$) was 1.3 ± 0.2 nM and the estimated binding capacity was 0.37 μg/mg of membrane protein, which is equivalent to $(1–2) \times 10^3$ binding sites per cell. This experiment is representative of three independent experiments in which the observed K_ds ranged from 1 to 5 nM. The data suggest a single class of binding sites for Rho on erythrocyte membranes, and the number of the sites appears to be quite low, which makes it unlikely that a major integral membrane protein is the binding site.

DISCUSSION

We have demonstrated a high-affinity association between Rho and an integral membrane component present in approx. 10^3 copies per erythrocyte. While the identity of this component remains to be determined, its resistance to harsh alkaline extraction suggests that it is a low-copy-number transmembrane protein, a low-abundance lipid or a combination of both. None of the proteins previously described, with which Rho interacts, falls into this category, suggesting that this is a novel type of interaction.

Unlike plasma-membrane-localized Ras proteins, the small GTP-binding proteins of the Rho family are found in various mammalian cells, mostly in the cytosol, with just 2–3 % of the molecules being plasma-membrane-associated [18–20]. This distribution is believed to be indicative of their function in the cell. Thus, for Rac proteins, it was demonstrated that their cycling between cytosolic and membrane partners regulates the assembly of NADPH oxidase complexes on the plasma membrane of phagocytic cells [40,41]. Rho proteins, which are implicated in regulation of focal adhesion complexes on plasma membranes and actin polymerization in response to extracellular stimuli, may also perform their function by translocating on to a membrane target upon activation [18]. Indeed, it has been shown that, *in vivo*, RhoA is translocated to regions of plasma membrane ruffling and cell–cell adhesion sites when cells are stimulated with phorbol ester or peptide growth factor [21].

Rho translocation to the plasma membrane takes place in conjunction with release of Rho from cytosolic Rho-GDI which keeps Rho in an inactive GDP-bound form and inhibits nucleotide exchange [42,43]. By Western-blot analysis we have found Rho-GDI in the cytosol fraction of human erythrocytes (results not shown); therefore, in these cells, some fraction of Rho may also be held in the cytoplasm by interaction with Rho-GDI. Despite the identification and characterization of cytosolic Rho-GDI, membrane-associated molecules with which Rho interacts in its active GTP-bound state remain uncharacterized. Although several proteins have been found to interact specifically with activated Rho [1–3,6–8], most are found in cytosol and are possibly translocated to the plasma membrane together with Rho. This co-translocation has been demonstrated for several of them [1,3].

We found that most of the Rho on erythrocyte ghosts was released at 37 °C in mildly alkaline buffer of low ionic strength and without Mg^{2+} (Figure 1A, compare lanes 6 and 7). Under these conditions the components of the actin–spectrin cytoskeleton are also released, which leads to membrane vesiculation. As a result, small vesicles of almost exclusively inside-out orientation are formed, which, as we have shown (Figure 2A), can bind soluble Rho found in erythrocyte cytosol. Further extraction of the vesicles with a buffer at pH 11 removed all peripheral membrane proteins together with the 3–5 % of the membrane-associated Rho that remained after the first extraction. The resulting vesicles had only a few proteins detectable by Coomassie Blue (Figure 1A, lane 4), but apparently retained all of the binding sites for Rho (Figure 2A, compare lanes 4 and 8). This shows that Rho binds to some integral component of the erythrocyte membrane. We found that most of the Rho present in haemoglobin-depleted erythrocyte cytosol associated with IOVs following incubation of the cytosol with GTP[S] under conditions that favoured nucleotide exchange in small GTP-binding proteins (Figure 2B).

We observed that the presence of GTP[S] stimulated Rho binding to membranes most efficiently when the cytosol was preincubated with the nucleotide before mixing with the membranes. The addition of nucleotide to the cytosol at the moment of mixing with membranes had a lesser effect (Figure 2A, compare lanes 3 and 4, 7 and 8). Moreover, we observed a positive correlation between the time of cytosol preincubation with GTP[S] and the amount of cytosolic Rho subsequently bound to the membrane (Figure 3). One possible explanation for this dependence is that the preincubation time reflects increasing incorporation of GTP[S] into cytosolic Rho, which activates Rho and allows it to bind to its membrane partner(s). We have not, however, measured this incorporation directly, and we cannot exclude that another cytosolic protein binds GTP[S] and is involved in stimulation of the binding of Rho to membranes.

The dependence between the time of cytosol preincubation with GTP[S] and the amount of Rho bound to membranes (Figure 3) strongly suggests that the nucleotide acts on a cytosolic protein rather than on some membrane-bound initiator of Rho translocation. It also suggests that the membranes in our assays serve only as a source of Rho-binding sites rather than being required for stimulating nucleotide exchange, as was reported for neutrophil membranes [23].

In a translocation system *in vitro* involving neutrophil membranes and Rac, it was shown that the presence of membranes was critical for GTP[S]-dependent nucleotide exchange on Rac followed by its association with the membrane [23]. It was proposed that two distinct events occurred upon association of Rac with the plasma membrane. First, the Rac-GDP–Rho-GDI complex interacted with a membrane-bound nucleotide-exchange stimulating factor, which resulted in exchange of GDP for GTP[S]. Then, activated and freed of GDI, Rac bound to its second membrane-localized partner, an 'effector'.

In our experiments, the effect of cytosol preincubation with GTP[S] on subsequent Rho binding (Figure 3) and the fact that the binding to membranes is a very rapid process both on ice and at 37 °C (Figure 4) make it unlikely that there is a membrane-associated nucleotide exchange stimulator involved in the translocation. Thus there is an apparent difference in the role played by membranes in GTP[S]-dependent translocation of Rho proteins observed in erythrocyte and neutrophil lysates *in vitro*.

There are several possible explanations for this difference. First, we incubated the cytosol (cytosolic Rho) under conditions which have been shown to be sufficient to initiate nucleotide exchange in Rho in the absence of nucleotide-exchange stimulating proteins. Thus, when cytosol that has been preincubated with GTP[S] is mixed with membranes, Rho may already be complexed with GTP[S] and thus can interact with its membrane target. Secondly, we used membranes that were stripped of some (IOV) or all (pH 11-stripped IOV) peripheral membrane proteins and thus might have already lost certain Rho-binding sites. Therefore it is possible that in preparing IOV we might have extracted the nucleotide exchange stimulating factor, whereas the 'effector', which interacted with activated Rho, was retained. If this is the case we may be dealing with a simplified system that gives us the opportunity to study Rho association with membranes as a consequence of a single molecular event, rather than looking at a superimposition of two reactions with separate kinetics.

This hypothesis might also explain another difference between our results and those reported for Rac/Rho translocation in neutrophil lysate. Although binding of Rac and Rho to plasma membranes was reported to be abolished by pretreatment of membranes with trypsin [23], in our studies Rho binding appeared to be unaffected, even by exhaustive proteolytic digestion of membranes with proteinase K (results not shown). In the neutrophil studies Rac binding to membranes was proposed to depend on the interaction with a membrane-bound GDP/GTP exchange stimulating factor. If there was a similar factor present on erythrocyte membranes which was extracted during preparation of IOV, this would mean that it was a peripherally associated protein which would be easily accessible to proteolytic enzymes. The 'effector', on the other hand, appeared to be an integral membrane protein which could be rather resistant to proteolysis, especially if the Rho binding site was embedded in the lipid bilayer.

In conclusion, our results show that Rho forms a high-affinity association with a low-abundance integral component of erythrocyte membranes. Identification of this component, currently in progress, should reveal another facet of the complex pathway through which Rho performs its multiple cellular functions.

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