Fractional Attachment of CD47 (IAP) to the Erythrocyte Cytoskeleton and Visual Colocalization with Rh Protein Complexes

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Abstract

Interactions of CD47 and RhAG and the Rh proteins are visualized between one another and with the cytoskeleton of intact erythrocytes. In a first study, CD47 is labeled with a phycoerythrin (PhE)-tagged antibody, which generates discrete spots that reflect induced clusters of CD47. Rh and RhAG colocalize with each other and to these induced clusters, whereas Band 3 and Glycophorin C remain more homogeneously dispersed on the cell periphery. In a second study, red cells are aspirated into a micropipette, and immuno-fluorescent maps of the surface gradients that develop for CD47 and RhAG determine cytoskeletal connectivity. CD47 and RhAG gradients on normal red cells prove to be nearly identical and also appear intermediate to those found for the fluid bilayer and network-linked Glycophorin C. Similar gradients are obtained for CD47 on Rhnull cells suggesting that linkage of CD47 to the spectrin-actin skeleton is independent of Rh or RhAG and is not affected by CD47’s reduced surface expression on these cells. The results show that CD47 colocalizes with Rh and RhAG but is fractionally attached to the red cell membrane skeleton independent of these and other major integral membrane proteins involved in cytoskeletal attachment. The results imply a homogeneous base distribution of CD47, restrained by cytoskeleton linkages, plus a smaller fraction of CD47, which is able to diffuse in the membrane.

Introduction

CD47, also known as IAP (integrin associated protein) or OA3, is a ubiquitously expressed five-span transmembrane protein with a single extracellular immunoglobulin domain and a short, multiform intracellular tail1. CD47 in non-erythroid cells appears to be involved in a variety of functions ranging from adhesion to signal transduction. In association with αvβ3 integrins, CD47 mediates leukocyte activation and chemotaxis2 as well as thrombospondin interactions with endothelium3. Interactions of CD47 with the tyrosine kinase signaling receptor protein SIRP-α result in multinucleation of macrophages4 and cellular signaling in neuronal cells5. CD47 has also been hypothesized to signal apoptosis in T cells6.

On mature erythrocytes, which lack integrins, CD47 appears to mediate cell-cell interactions with SIRP-α of splenic macrophages. This association is thought to inhibit a phosphorylation cascade that blocks phagocytosis and prevents erythrocyte clearance from the circulation7. CD47 interacts with soluble thrombospondin and, in sickle erythrocytes, appears
to generate an intracellular signal that ultimately increases sickle cell adhesiveness, perhaps contributing to vaso-occlusive events\textsuperscript{8}. Whether additional erythrocyte components contribute to these varied observations is not yet clear and motivates a better understanding of CD47’s associations within the erythrocyte membrane.

Four isoforms of CD47 have been found among various cell types: the isoforms differ only in their cytoplasmic tails, which range in length from twenty to forty amino acids. The haematopoietic form of CD47 found in bone marrow (form 2) contains twenty amino acids in its cytoplasmic tail\textsuperscript{9}. In many non-erythroid cell lines, attachment of CD47 to the cytoskeleton has been shown to occur via PLIC (Proteins Linking IAP to Cytoskeleton) linkage to intermediate filaments\textsuperscript{10}. However, neither intermediate filaments nor PLIC proteins are detectable in erythrocytes (E. Brown, personal communication).

Association of CD47 on erythrocytes with proteins of the Rh membrane complex is suggested by the observation that Rh\textsubscript{null} erythrocytes, which lack Rh and Rh-associated glycoprotein (RhAG), express significantly less CD47\textsuperscript{11}. A physical or functional association between CD47 and the Rh complex has not otherwise been directly demonstrated.

The major attachment sites between the erythrocyte spectrin-actin cytoskeleton and the lipid bilayer are widely understood to be Glycophorin C and Band 3\textsuperscript{12}. However, additional attachment sites are suggested by the observation that targeted deletion of Band 3 does not result in loss of membrane assembly\textsuperscript{13}. A loss of cytoskeletal attachment through Rh, RhAG, or associated proteins (e.g. CD47) might explain the altered morphology of Rh\textsubscript{null} cells\textsuperscript{14}. Indeed, previous studies employing statistical analysis of the distribution of Rh antigens on the erythrocyte membrane\textsuperscript{15} and resistance to Triton-X-100 extraction\textsuperscript{16,17} strongly suggest that Rh is connected to the cytoskeleton.

The present study was undertaken to visualize the interactions of CD47 with Rh, RhAG and with the cytoskeletonally-attached proteins Band 3 and Glycophorin C and to measure cytoskeletal connectivity of CD47 and RhAG. In a first study, we show colocalization of Rh and RhAG, but not Band 3 and Glycophorin C, to clusters of CD47 induced by a crosslinking antibody moiety on the surface of erythrocytes. In a second study, we use micropipette aspiration of cells labeled with fluorescent antibody to show that a partial yet significant fraction of both CD47 and RhAG are linked to the cytoskeleton. However, CD47 cytoskeletal attachment in Rh\textsubscript{null} cells, which lack Rh and RhAG and have reduced CD47, is comparable to normal erythrocytes, strongly suggesting that linkage of CD47 to the cytoskeleton can occur without Rh and RhAG. We conclude that CD47 colocalizes with Rh and RhAG but is fractionally attached to the red cell membrane skeleton independent of Rh, RhAG, Band 3 or Glycophorin C. Recent studies of protein 4.2 deficient cells indeed suggest a connection of CD47 and/or Rh with this cytoskeletal protein\textsuperscript{18}.
Materials and methods

Erythrocytes

Erythrocytes were collected at room temperature in phosphate buffered saline, PBS (Sigma-Aldrich, St. Louis, MO) and washed twice with PBS. Rh-negative (ce/ce) and Rh-positive (DcE/DcE) cells were used for most experiments. Glycerol frozen control cells and Rhnull cells were thawed in PBS at 37°C and washed twice with PBS. Rhnull cells showed no labeling with antibodies to RhAG or Rh(c and E).

Antibodies

The following mouse monoclonal antibodies were used: R-phycoerythrin (PhE)-labeled BRIC126 (IgG2b; IBGRL, Bristol, UK) and 6H9 (IgG1) anti-CD47; 2D10 (IgG1) anti-RhAG; BRIC6 (IgG3) anti-Band 3 (IBGRL); and FITC-labeled BRIC10 anti-Glycophorin C (IgG1; IBGRL). Rh was detected using a human polyclonal serum containing anti-c and anti-E. Binding of unlabeled mouse antibody was detected with FITC-labeled Fab fragment of goat, anti-mouse (Jackson Laboratories, West Grove, PA) or TRITC-labeled goat, anti-mouse (Sigma-Aldrich) as indicated, and human antibody binding was detected with FITC-labeled anti-human IgG Fab (Cappel, Aurora, OH). The secondary antibodies were tested to ensure no non-specific binding to human erythrocytes. For co-labeling experiments requiring the use of labeled anti-mouse with the PhE-BRICS6, the primary and secondary antibodies were followed by the addition of PhE-BRIC126. This stepwise sequence was used to avoid artifactual colocalization due to possible binding of the anti-mouse antibody to the murine PhE-BRIC126. Both positive and negative colocalization data were obtained validating the use of this approach.

For fluorescence imaged micro-deformation (FIMD) experiments, unlabeled BRIC126 was converted to Fab fragments (ImmunoPure Fab Preparation Kit, Pierce, Rockford, IL) and fluorescently tagged with TRITC (FluoReporter TetramethylRhodamine Protein Labeling Kit, Molecular Probes, Eugene, OR) per manufacturer’s instructions. All labeling was done at room temperature. Dilution curves were generated for the binding of each antibody to red blood cells. Antibody concentrations below the apparent KD of cell binding were used to ensure that the fluorescent signal was not overly saturated.

Membrane labeling

The lipid membrane was labeled by incubation with the fluorescent lipid FL-PE (N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt, Molecular Probes). For colocalization experiments the actin cytoskeleton was labeled with rhodamine tagged phalloidin (Sigma-Aldrich) or Alexa 488 tagged phalloidin (Molecular Probes) incorporated into erythrocytes reversibly permeablized by cold and low osmotic concentration and resealed with KCl and MgCl2 at 37°C.
Fluorescence Imaged Micro-Deformation (FIMD)

Central features of FIMD methods and analyses are described in the Results section. Further details can be found in Discher et al\textsuperscript{21} and Discher and Mohondas\textsuperscript{22}.

Analysis of colocalization

Images were acquired on a Nikon TE300 inverted microscope with a 60X (oil, 1.4 NA) objective using a liquid nitrogen cooled CCD camera (Roper Scientific, Trenton, NJ). Image acquisition and analyses were performed with Image Pro software (Media Cybernetics, Silver Spring, MD). Images of osmotically swollen cells were taken at the equatorial position of the cell, with one image per fluorophore. For cluster analyses, images were changed from gray scale to black and white to accentuate the areas of localized intensity. The area of peripheral pixels of positive intensity was counted in each image. The images were then mathematically overlaid such that common pixels were also counted, and the coincident intensity index was determined as the common pixels divided by the total pixels less the common. At least fifteen cells were used for each analysis. Statistical significance was assessed with a student t-test.

Results

Nano-scale colocalization of CD47 and Rh

![Figure 1. Equatorial sections of normal and Rh\textsubscript{null} erythrocytes labeled for CD47 with phycoerythrin-tagged monoclonal antibody BRIC126 (PhE-BRIC126). Spots on the membrane of these osmotically sphered cells are the result of both the size and intensity of the antibody probe, and the production of clusters of crosslinked CD47. Rh\textsubscript{null} cells labeled with the same concentration of probe clearly exhibit a lower overall fluorescence intensity as well as a decrease in the number of clusters on the cell. The bar represents 2 \textmu m.](Image)
Figure 2. Colocalization of membrane components to clusters of PhE-BRIC126 labeled CD47 or to polyclonal labeled Rh. (A) Positive colocalization: double labeling of CD47 with a non-competing monoclonal antibody, 6H9. Higher intensity spots of 6H9 (green) generally coincide with the clusters of CD47 produced by PhE-BRIC126 (red). The overlap image shows yellow colocalized regions. (B) Negative colocalization: uniform labeling of the lipid bilayer with fluorescein-phosphatidylethanolamine (FL-PE). Similar images were obtained with antibody labeling of Band 3 as well as Glycophorin C, as quantified in Figure 3. (C) Yellow spots in the colocalization image indicate significant overlap of antibody labeled RhAG (green) with PhE-BRIC126 (red). (D) A polyclonal antibody against Rh(c and E) also generates clusters to which RhAG colocalizes.

CD47 labeling of erythrocytes at sub-saturating concentrations of BRIC126 conjugated with R-phycoerythrin (PhE-BRIC126) leads to discrete surface spots (Figure 1). At the concentrations of PhE-BRIC126 used for these experiments the average cluster size on normal red cells appears to be $520 \pm 130$ nm in diameter spaced by $1017 \pm 248$ nm. Reduced expression of CD47 on Rhnull cells is reflected in a reduction in overall fluorescence intensity as well as a decreased number of PhE-BRIC126 spots. It is true that individual PhE-BRIC126 conjugates are sufficiently large and fluorescent to be visualized when simply adsorbed to a cover slip in the absence of any cells. These intense spots could thus be providing
visual tags of individual CD47 molecules. However, the large size (240 kDa) of the PhE probe also provides multiple sites for conjugation to primary antibodies. It is likely, as explained in the Discussion, that the resultant spots represent crosslink-induced clusters of PhE-antibody and CD47. Also sequestered into these clusters are membrane components that are physically associated with the cross-linked antigen. Colabeling and fluorescence colocalization of other membrane components with the PhE-BRIC126 can, therefore, visibly indicate surface association. Examples of antibody labeling and visual colocalization are shown in Figure 2, and quantitative analyses of the overlap images for all membrane components tested are shown in Figure 3.

Double labeling of CD47 with the monoclonal antibody 6H9 and the non-competing PhE-BRIC126 shows a punctate pattern of green-6H9 coincident with the spots of red-PhE-BRIC126 (Figure 2A). In addition to these intense spots being in the same regions of the cell when shown separately, the degree of colocalization is emphasized by the yellow-overlap image. In contrast, labeling of the membrane with the green lipid probe FL-PE and the red-PhE-BRIC126 shows a uniform green fluorescence at the periphery in the overlap image (Figure 2B). The lack of intense spots with this lipid probe in the presence of the cluster inducing PhE-BRIC126 is consistent with the idea that constraints on proteins have little to no effect on the bilayer. The double labeling of CD47 is used to establish an upper bound for the coincident intensity index, and lipid with CD47 provides a lower bound for the index (Figure 3). This coincident intensity index allows comparison for colocalization of various membrane components with a cluster inducer. At least 15 cells were examined for each case. Samples showing positive colocalization (denoted with *) are statistically similar to the positive control, double-labeled CD47 (p > 0.15), and statistically different than the negative control, lipid with CD47 (p < 0.0007). Conversely, samples showing no colocalization are statistically similar to the negative control (p > 0.08) and statistically different from the positive control (p < 0.0003).

PhE-BRIC126 labeled CD47 with phalloidin labeled cytoskeletal F-actin results in no significant coincidence. Band 3 and Glycophorin C, thought to be the major cytoskeletonally connected proteins in the red cell membrane, do not show fluorescence colocalization to CD47. However, RhAG appears coincident to the CD47 clusters suggesting that RhAG and CD47 colocalize and associate on the cell surface (Figure 2C and Figure 3). RhcE also colocalizes with CD47 (Figure 3).
**Figure 3.** Assessment of colocalization with induced clusters. The coincident intensity index is the area of coincident intensity (yellow in Figure 2) normalized by the area of non-coincident intensities (red + green). The average ± standard deviation for at least 15 cells is plotted. The maximum apparent range of this index is established from double labeling of CD47 with the non-competing antibody 6H9 and cluster-inducing PhE-BRIC-126. The minimum apparent range of this index is established from labeling CD47 with PhE-BRIC-126 together with the lipid bilayer. Cytoskeletal F-actin, Band 3 and Glycophorin C do not appear to be colocalized with CD47. In comparison, both RhAG and RhcE appear to colocalize in these clusters with CD47. Additionally, RhAG is colocalized to clusters induced by polyclonal anti-Rh(c and E).

Polyclonal antibodies directed against Rh(c and E) also exhibit intense localized fluorescence. This could be due to crosslinking of RhcE molecules. Colocalization of RhAG with crosslinked RhcE provides additional evidence of their membrane surface association (Figure 2D). Colocalization of both RhcE and RhAG to one another and to CD47 is quantitatively similar to the positive control (Figure 3).

**Micro-scale views of CD47 and RhAG connectivity to the spectrin-actin skeleton**

**Fluorescence Imaged Micro-Deformation**

Fluorescent labeling of almost any red cell component - bilayer, cytoskeleton, or integral membrane protein - appears homogeneous on a normal discocyte. Fluorescence imaged micro-deformation (FIMD)\(^2\) segregates structurally diverse membrane components on a micron scale. The method involves first fluorescently labeling a membrane component on intact cells or ghosts and then aspirating an individual cell or ghost into a micropipette of 1-2 µm diameter (Figure 4A). Analysis of the resulting fluorescent image focuses on the aspirated projection of the cell, which exhibits one of three responses or a combination thereof. (I) The lipid bilayer displays a completely uniform fluorescent intensity over the entire projection length and is consistent with a fluid membrane (Figure 5C). (II) The spectrin-actin network and connected proteins, such as Glycophorin C, resist the aspiration and appear concentrated at the entrance of the pipette and diminished at the cap (Figure 5D). This is consistent with an overall elastic resistance to deformation. (III) Mobile integral membrane proteins such as GPI-linked CD59 are sterically excluded from the entrance and produce a brightly fluorescent cap with an intensity profile that increases toward the cap\(^2\). Intermediate responses between I and II are indicative of proteins with a significant, but not complete, attached fraction\(^2\). Regardless of response (I, II, III or mixed), the fluorescence intensity on the minimally strained section of cell outside of the micropipette provides a means of normalizing the intensity of the projection to give a relative density, \(\rho\).
Figure 4. Fluorescence imaged micro-deformation (FIMD) analysis of RhAG on a normal erythrocyte. (A) Image of a micropipette-aspirated erythrocyte with fluorescently labeled RhAG. The schematic illustrates an aspirated cell of projection length $L$ in a pipette of radius $R_p$. The entrance, $e$, and cap, $c$, regions are identified. (B) Relative fluorescence intensities or densities averaged across the entrance and cap for nine cell projections of various lengths. See Methods for normalization method. (C) Ratio of entrance-to-cap densities as a function of projection length. This plot (with linear fit forced to intercept $\rho_e / \rho_c = 1$) illustrates the increasing component gradients that are characteristic of considerable cytoskeletal attachment$^{21}$. For typical bilayer probes, the slope, $m$, has a value of ~ 0.0, whereas freely diffusing proteins such as GPI-linked CD59 give significant negative slopes.
Figure 5. Fluorescence images of CD47, lipid and Glycophorin C on normal and Rhnull aspirated erythrocytes. (A) FIMD of CD47-labeled cells (using TRITC-BRIC126 Fab) shows gradient from entrance to cap, indicative of considerable cytoskeletal attachment. (B) FIMD of CD47-labeled Rhnull cells (using TRITC-BRIC126 Fab) shows similar cytoskeletal attachment. (C) FIMD of lipid bilayer-labeled Rhnull cells illustrates the absence of any significant gradient, consistent with a homogeneous fluid bilayer. (D) FIMD of Glycophorin C-labeled Rhnull cells illustrates the strong entrance to cap gradient, consistent with a cytoskeletally-linked protein.

Figure 6. Gradient slopes, \( \rho_\text{entrance} \) versus \( L/R_p \), from FIMD analysis (Figure 4C) of both normal and Rhnull cells. Slopes of FIMD analysis are indicative of cytoskeletal attachment. Results for CD47 and RhAG suggest a cytoskeletal attachment similar to one another and intermediate to Glycophorin C and the lipid bilayer. CD47 retains this attachment in the absence of the Rh proteins in Rhnull cells. The connectivity, \( f^* \), of CD47 and RhAG can be determined from these FIMD slopes (table 1).

Quantitative analyses of relative densities at both the entrance (\( \rho_\text{entrance} \)) of the pipette and the cap (\( \rho_\text{cap} \)) provide deeper insights into connectivity and mobility. When plotted as a function of the relative projection length (\( L/R_p \)) aspirated into the
micropipette, a progressive increase in entrance density and decrease in cap density are observed (Figure 4B); such results are typical of cytoskeletonally-linked populations of protein. More simply, the ratio \( \rho_y/\rho_c \) plotted versus \( LR_p \) yields a positive slope (Figure 4C). This indicates a significant level of cytoskeletal attachment since similar plots for phospholipid show near-zero slope and proteins such as CD59 produce a negative slope\(^{21} \). FIMD of RhAG (Figure 4) and CD47 (Figure 5A) visually show similar fluorescent gradients along the aspirated projection. Both gradients exhibit a higher fluorescence at the entrance than the cap, closer in form to the response of a spectrin-actin network (\( II \)) than that of either lipid (\( I \)) or freely diffusible membrane proteins (\( III \)). CD47 on \( \text{Rh}_{\text{null}} \) cells, shown to have no RhAG or Rh and reduced expression of CD47 by immunolabeling, exhibit a very similar FIMD gradient to CD47 on normal erythrocytes (Figure 5B). In comparison, FIMD analyses of Glycophorin C generally show a 2-3-fold steeper slope than CD47 or RhAG (Figure 5D). Normal red cells show similar projections for lipid and Glycophorin C as \( \text{Rh}_{\text{null}} \) cells. The slopes of the fluorescent gradients of the projections can be used to analytically determine the fractional connectivity of proteins to the underlying cytoskeleton (Figure 6).

**Connectivity of CD47 and RhAG from FIMD**

Membrane proteins are rarely completely attached or freely mobile; most exhibit a fractional attachment or connectivity, \( f^* \), to the underlying cytoskeleton. The slopes of the fluorescent projection gradients provided by FIMD allow a quantitative measure of this connectivity for the fluorescently labeled protein of interest (Figure 6). Proteins with a high mobile fraction or low connectivity have negative projection gradients closer in form to those of freely-diffusible CD59. Proteins that have a high degree of connectivity to the underlying cytoskeleton show fluorescent projection gradients similar to cytoskeleton-attached Glycophorin C. FIMD analysis of Glycophorin C suggests an \( f^* \) near 100\%\(^{21} \), which agrees with \( f^* \approx 90\% \) by Triton X-100 extraction\(^{23} \). Fluorescence recovery after photobleaching (FRAP), which examines diffusive processes over longer time scales, also shows the majority of Glycophorin C is cytoskeletonally-immobilized, indicative of agreement between the various measures\(^{24} \).

**Table 1. Fractional connectivity of various membrane components**

<table>
<thead>
<tr>
<th>Protein</th>
<th>( \text{erythrocyte type} )</th>
<th>( \text{normal} )</th>
<th>( \text{Rh}_{\text{null}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycophorin C</td>
<td>100%</td>
<td>88%</td>
<td></td>
</tr>
<tr>
<td>CD47</td>
<td>62%</td>
<td>58%</td>
<td></td>
</tr>
<tr>
<td>RhAG</td>
<td>60%</td>
<td>NP</td>
<td></td>
</tr>
<tr>
<td>CD59</td>
<td>0%</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Connectivity of various membrane components as described by:

\[
f^* = \frac{(m - m_{\text{CD59}})}{(m_{\text{GlyC}} - m_{\text{CD59}})}
\]

Where \( m \) is the slope of the FIMD projection, as given in Figure 6.

NP = not possible, ND = not done

Using Glycophorin C and CD59\(^{21} \) for calibration of the network-like gradients and fully mobile components, one can estimate the connectivity of RhAG and CD47 (Table 1).
Discussion

Colocalization to induced clusters of CD47 and Rh

Phycoerythrin (PhE) molecules have been used for tracking the diffusion of single proteins because of their high intensity. The large size of the PhE probe also provides sites for binding of multiple immunoglobulin molecules and a mechanism for crosslinking of CD47 on the cell surface. Particle tracking of individual PhE-BRIC126 spots on the erythrocyte membrane indicates a diffusion constant several orders of magnitude below that of lipid (data not shown); this diffusivity even appears lower than that of proteins with significant immobile fractions such as Glycophorin C. This apparent immobility of PhE-BRIC126 labeled CD47 is inconsistent with the FIMD data, which indicates a minor yet significant mobile fraction, unless CD47 is being crosslinked.

Immobilization of surface proteins has been shown by others to occur with the addition of crosslinking antibodies to red cells. CD47 has been shown on other cells to be clustered as recognized by the antibody CDw148, but this clustering does not occur erythrocytes. Here, crosslinking of the mobile fraction of CD47 to the immobile fraction would tend to nucleate localized concentrations of CD47. Further evidence of this is shown by the lack of colocalization of the actin cytoskeleton with PhE-BRIC126 indicating no deformation of the underlying network. Clusters of CD47 on Rh null cells also imply a mobile and immobile fraction, despite a significant reduction in overall number of CD47 proteins. Molecules physically associated with CD47, such as Rh and RhAG, would tend to be displaced into these induced clusters. CD47 is shown to colocalize with RhAG as well as with RhoE. Since the size of the clusters being analyzed is close to the limit of optical resolution, it cannot be shown that colocalized surface proteins are directly bound to one another. However, the active movement of RhAG and the Rh proteins with the clustering of CD47 shows that these proteins are physically associated in some way on the red cell membrane. The previous observation of reduced levels of CD47 on Rh null erythrocytes is consistent with these results, but here membrane interactions on normal red cells are shown.

By the same method, the colocalization of RhAG to RhoE clusters is highly suggestive of an Rh protein complex. This is consistent with previous, indirect results showing that Rh proteins co-immunoprecipitate with RhAG and exist as heterotetramers in the erythrocyte membrane. One significant advantage of the fluorescence colocalization method used here over co-immunoprecipitation, in addition to being more visually direct, is the minimal disruption of intact cells.

Band 3 is known to have a significant immobile fraction and has been suggested to be a component of the Rh complex since K562 erythroleukemia cells transfected with Band 3 have increased levels of detectable RhD and RhoE by flow cytometry. However, unlike Rh or RhAG, Band 3 and Glycophorin C are neither stoichiometrically coincident with CD47 nor are they displaced into the induced clusters. This suggests that there is little interaction of Band 3 or Glycophorin C with CD47 on the surface of the erythrocyte. Band 3 and Glycophorin C have been thought to represent the majority of cytoskeletal attachment sites of the red cell membrane. The fact that CD47 does not colocalize with either Band 3 or Glycophorin C together with the results of the FIMD studies showing cytoskeletal linkage of CD47 and RhAG suggests that the CD47-Rh complex must be cytoskeletally-linked either directly or via a novel set of associations. This appears consistent
with recent data on cells lacking Band 3 in which RhAG and CD47 are both resistant to TritonX-100 extraction\textsuperscript{16}. Protein 4.2 deficient human red cells have recently been shown to have a 75\% reduction in CD47 compared to normal red cells, implicating the cytoskeletal protein 4.2 in the network association of CD47\textsuperscript{18}.

**Fractional connectivity of RhAG and CD47 to the spectrin-actin cytoskeleton**

FIMD results for RhAG indicate significant (~60\%) cytoskeletal attachment. This association is either direct or via an associated complex, confirming data from nearest-neighbor statistical analysis of immuno-electron micrographs\textsuperscript{15} and Triton-X-100 extractions\textsuperscript{16,17}. Triton-X-100 results can be affected by the considerable hydrophobicity of the membrane spanning proteins involved. Nonetheless, the approaches here on the intact membrane also suggest that Rh proteins interact, directly or indirectly, with the erythrocyte spectrin-actin cytoskeleton.

The FIMD results for CD47 on normal cells also indicate significant (~60\%) cytoskeletal attachment, suggesting that RhAG and CD47 may be associated. This result supports the fluorescent colocalization data. The connectivity of CD47 is not significantly reduced in Rh\textsubscript{null} cells, which contain neither RhAG nor RhcE. This suggests that CD47 itself mediates a cytoskeletal connection of the Rh complex.

![Image of network-linked plus mobile CD47 molecules signaling red cell 'self' to macrophages](image)

**Figure 7.** Schematic of network-linked plus mobile CD47 molecules as they might function in signaling red cell 'self' to macrophages\textsuperscript{2}. Not shown are the many Rh proteins (and other components), so that the picture is most relevant perhaps to Rh\textsubscript{null} red cells. The network-linked CD47 ensures a uniform, if sparse, distribution of this putative 'marker of self', while the mobile fraction of CD47 allows for affinity-driven clustering which reinforces the signal to SIRP-\(\alpha\) on the macrophage.

The fact that CD47 has significant cytoskeletal attachment could suggest a novel contribution to cytoskeletal assembly. CD47 has been shown to be present throughout the entire erythrocyte maturation process in cultured progenitor cells\textsuperscript{31}. Rh\textsubscript{null} cells lack Rh protein and have a reduced level of CD47 and are often stomatocytic or spherocytic\textsuperscript{14} as are human 4.2 deficient red cells\textsuperscript{18}. Band 3 deficient cells are also spherocytes, and Glycophorin C deficient cells are elliptocytes. In contrast, no abnormalities in cell morphology are reported for deficiencies in abundant transmembrane proteins that are not network-linked\textsuperscript{32}. Since erythrocytes with deficient cytoskeletal linkage sites have altered morphology and since the results here
suggest CD47 is also a site for cytoskeletal linkage, we tentatively conclude that CD47 could have a role in cell morphology. Rh
null cells have ~60% cytoskeletally attached CD47, but the level of CD47 is severely reduced, and the overall number of
cytoskeletal connections via CD47 is therefore reduced. Rh proteins may contribute as well, but the cytoskeletal connection
of CD47 in Rhnull cells, despite its reduced expression level, indicates that CD47 connects independently to the red cell
cytoskeleton.

As introduced, erythrocyte CD47 has a putative role in binding macrophage SIRP-α and signaling “self”\(^7\). The
fractional connectivity (~60%) found here might be critical in this signaling process. A substantial degree of cytoskeletal
connection ensures that an immobile fraction of CD47 remains equally distributed around the red cell at all times. Moreover,
model systems show that freely diffusible receptors tend to accumulate in zones of adhesive contact\(^{33,34}\). CD47's mobile
fraction (~40%) could likewise be recruited to contact sites with macrophages. Such reinforcement is emulated here in the
antibody crosslinking of mobile to immobile proteins. We speculate that this accumulation could reinforce and amplify some
critical signal of “self” (Figure 7).

**Summary**

Utilizing the intensity and discrete separation of the PhE-BRIC126 and polyclonal Rh(c and E) antibodies binding on the cell
surface it is possible to visually confirm the presence of an Rh protein complex and the colocalization of CD47 to the Rh
protein complex. FIMD data suggests that a fraction of these complexes are anchored to the cytoskeleton. The attachment
appears to be largely independent of Glycophorin C and Band 3 suggesting novel attachment by a protein of the Rh complex.
Finally, FIMD analysis of CD47 on Rhnull red cells shows essentially no change in cytoskeletal attachment, suggesting that
the Rh proteins are not necessary for this novel attachment.

**Acknowledgements**

The authors thank A.E. von dem Borne, Netherlands Red Cross, Amsterdam for the monoclonal antibody 2D10, M. Telen,
Duke University, Durham, NC for the monoclonal antibody 6H9 and M.E. Reid, New York Blood Center for the polyclonal
anti-c and anti-E antibody. The authors also gratefully acknowledge important discussions with Mohandas Narla, New York
Blood Center.
References


33. Noppl-Simson DA, Needham D. Avidin-biotin interactions at vesicle surfaces: adsorption and binding, cross-

34. Maier CW, Behrisch A, Kloboucek A, Simson DA, Merkel R. Specific biomembrane adhesion -Indirect lateral
Fractional attachment of CD47 (IAP) to the erythrocyte cytoskeleton and visual colocalization with Rh protein complexes

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