Red Cell Membrane Remodeling in Sickle Cell Anemia

Sequestration of Membrane Lipids and Proteins in Heinz Bodies

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Abstract

In red cells from patients with sickle cell anemia, hemoglobin S denatures and forms Heinz bodies. Binding of Heinz bodies to the inner surface of the sickle cell membrane promotes clustering and colocalization of the membrane protein band 3, outer surface-bound autologous IgG and, to some extent, the membrane proteins glycophorin and ankyrin. Loss of transbilayer lipid asymmetry is also found in certain populations of sickle red cells. The lateral distribution of sickle cell membrane lipids has not been examined, however. In this report, we examine by fluorescence microscopy the incorporation and distribution of the fluorescent phospholipid analogues 7-nitro-2,1,3-benzoxadiazol-4-yl (NBD)-phosphatidylserine and NBD-phosphatidylcholine in sickle red cells. Both phospholipid analogues are observed to accumulate prominently at sites of Heinz bodies. Accumulation at sites of Heinz bodies is also shown by 1,′1-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, a fluorescent lipid analogue that readily crosses membranes, but not by fluorescein-phosphatidylethanolamine, an analogue that is localized to the outer leaflet of the membrane. Double labeling and confocal microscopy techniques show that NBD-lipids, band 3 protein, protein 4.1, ankyrin, and spectrin are all sequestered within sickle red cells and colocalized at sites of Heinz bodies. We propose that Heinz bodies provide a hydrophobic surface on which sickle red cell membrane lipids and proteins are sequestered. (*J. Clin. Invest.* 1996. 97:29–36.) Key words: sickle cell anemia • Heinz bodies • colocalization • confocal light microscopy • band 3 protein

Introduction

Red cells from patients with sickle cell anemia exhibit a wide range of membrane abnormalities (for review see reference 1). These changes include alterations in the asymmetric distribution of membrane phospholipids between the two lipid bilayer hemileaflets (2–6), weakened interactions among membrane skeletal proteins and integral membrane proteins (7), and clustering of band 3, the principal integral protein of the red cell membrane (8–12). Recent evidence suggests that some of these alterations are confined to distinct regions of the membrane. One such site is the spicule region of deoxygenated sickle red cells, where the membrane skeleton separates from the overlying lipid bilayer and is replaced by rigid polymers of deoxygenated hemoglobin S (13).

Another region of localized membrane injury is the site of attachment of precipitates of denatured hemoglobin S to the inner surface of the red cell membrane (8–11). The formation of such precipitates, called Heinz bodies, is caused by hemoglobin S instability leading to formation of insoluble hemichromes, release of reactive oxygen species, and release of iron from the heme moiety of hemoglobin (for review see reference 14). At such sites, both band 3 and ankyrin cluster into aggregates that are readily labeled by anti–band 3 and antiankyrin antibodies, respectively (10, 11). These band 3 clusters are thought to provide binding sites for autologous IgG which, in turn, targets such red cells for destruction by macrophages and other cells of the reticuloendothelial system (11, 15–18).

Altered transbilayer phospholipid asymmetry in the plasma membrane of sickle red cells has been documented by experiments in which red cells were treated with phospholipases and by studies of the distribution of spin-labeled phospholipids in the two lipid bilayer hemileaflets (2, 3, 19). In contrast, there are limited data examining alterations in the lateral distribution of phospholipids in the plane of the membrane. Labeling of membrane lipids by merocyanine, a probe of lipid packing (20–22), has demonstrated that the merocyanine fluorescence is preferentially localized in vesicles which are released from the spicules of deoxygenated sickled red cells upon reoxygenation suggesting that membrane lipid asymmetry is disrupted in the spicules (23).

In this report, fluorescent analogues of phosphatidylserine (PS)[1] and phosphatidylcholine (PC) are used to examine the lateral distribution of phospholipids in sickle red cell membranes. PS and PC are the principal phospholipids of the inner and outer hemileaflets of the lipid bilayer, respectively. Both PS and PC analogues are found to accumulate prominently at sites of Heinz bodies. Further, both phospholipid analogues

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1. *Abbreviations used in this paper:* APH, acetylphenylhydrazine; DiI, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; fluorescein-PE, *N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; NBD, 1-palmitoyl-2[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] caproyl]-*sn*-glycero-3; PC, phosphatidylcholine; PS, phosphatidylserine; RITC, rhodamine isothiocyanate.

are internalized within sickle red cells, since extracellular albumin is not able to remove the analogues from labeled sickle red cells. Double labeling and confocal microscopy techniques demonstrate that the phospholipid analogues, together with band 3, protein 4.1, ankyrin, and spectrin, are sequestered within sickle red cells and colocalized at sites of Heinz bodies.

Methods

Lipid vesicles. NBD (1-palmitoyl-2 [6-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] caproyl]-*sn*-glycero-3)-PC and NBD-PS were obtained from Avanti Polar Lipids (Birmingham, AL). DiI (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) and fluorescein-PE (*N*-(5-fluoresceinthiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt) were from Molecular Probes, Inc. (Eugene, OR). Lipid vesicles containing NBD-PC, NBD-PS, or fluorescein-PE were prepared by ethanol injection (24). Briefly, a stock solution containing 30 μg of NBD-lipid in chloroform or fluorescein-PE in ethanol was dried under a stream of nitrogen. The dried lipid was dissolved in 30 μl of ethanol and injected into 270 μl of PBS. The solution was spun in a microfuge at 13,000 rpm for 5 min, and the supernatant was dialyzed overnight at 4°C against 500 ml of PBS. The lipid vesicles were collected and stored at 4°C in the dark. Alternately, NBD-lipid and fluorescein-PE vesicles freshly prepared by ethanol injection were used to label red cells directly, without prior dialysis against PBS. The ethanol concentration in the labeling mixture was kept at $< 1\%$. Lipid vesicles containing DiI were prepared by a method similar to that used for preparation of NBD-lipid vesicles, except that methanol was used for DiI solubilization and subsequent injection into PBS.

Labeling of red blood cells (RBCs) with NBD-PS, NBD-PC, DiI, and fluorescein-PE. Blood was collected from normal individuals and from patients with homozygous (SS) sickle cell anemia into acid citrate dextrose-containing vacutainer tubes. Packed RBCs were prepared by centrifugation and removal of supernatant plasma and buffy coat. Packed RBCs were resuspended in 9 vol of ATP-maintaining buffer (50 mM glycylglycine, pH 7.4, 5 mM KCl, 0.54 mM adenine, 12.7 mM inosine, 2 mM $MgCl_2$, 2 g/liter glucose, 105 mM NaCl). NBD-lipid and fluorescein-PE vesicles (7.5–10.0 μg lipid in PBS) or DiI vesicles (0.05 μg in PBS) were diluted with 200 μl of ATP-maintaining buffer and then added to 200 μl of RBC suspension (20% hematocrit). The mixture was incubated at 37°C for 30 min. Labeled RBCs were washed with PBS and then hypotonically lysed with 5 mM NaPi, pH 8.0, or quenched with 4% delipidated BSA. Labeled cells and ghosts were examined by a fluorescence and phase-contrast light microscope (standard epifluorescence microscope; Carl Zeiss Inc., Thornwood, NY) equipped with the appropriate dichroic mirrors and filters for observation of NBD, fluorescein, and DiI fluorescence.

NBD-PC labeling of denatured hemoglobin in solution. Ghost-free hemolysate was prepared from sickle red cells by lysis in 30 vol of hypotonic buffer followed by centrifugation. The supernatant was incubated with acetylphenylhydrazine (APH, 15 mM) at 37°C for 2 h. The denatured hemoglobin was incubated with NBD-PC (1.25 μM, final concentration) in PBS at 37°C for 30 min. The reaction mixture was diluted with PBS and examined by light microscopy.

Immunofluorescence labeling of red cell membrane proteins. Anti–protein 4.1 antibodies were affinity purified from rabbit antisera as previously described (25). Anti–band 3, antiankyrin, and antispectrin antibodies were prepared from rabbit antisera by precipitation with ammonium sulfate. The specificity of these antibodies was tested by immunoblotting against red cell ghost proteins which were fractionated by SDS-PAGE and transferred onto nitrocellulose filters (26). RBCs in PBS were cytospun onto a glass slide, air dried, and then hypotonically lysed in 5 mM NaPi, pH 8.0, buffer. The slides were then washed with PBS containing 1% BSA and incubated with rabbit antibodies at room temperature for 30 min. After three washes with 5 mM NaPi, pH 8.0, containing 1% BSA, the bound IgG was labeled with rhodamine isothiocyanate (RITC)-conjugated goat antibodies to rabbit IgG (Cappel-Organon Teknika, Durham, NC) in PBS containing 1% BSA at room temperature for 30 min. After three washes with 5 mM NaPi, pH 8.0, the labeled ghosts were mounted in 5 mM NaPi with a coverslip. Photographs were taken using Tmax film (400 ASA; Eastman Kodak Co., Rochester, NY) using a microscope (Carl Zeiss, Inc.) equipped with the appropriate dichroic mirror and filters for observation of rhodamine fluorescence. As negative controls, ghosts were incubated with preimmune rabbit serum, washed, and labeled with the second (RITC-conjugated) antibodies.

APH treatment of normal red cells. To induce Heinz body formation, washed normal RBCs (20% hematocrit) were treated with 15 mM APH in PBS at 37°C for 15 min. RBCs were then washed twice with PBS to remove APH and incubated in ATP-maintaining buffer at 37°C for 48 h. Subsequently, the incubated cells were labeled with NBD-PS and anti–band 3 antibodies as described above.

Eosin maleimide labeling. Externally exposed band 3 was fluorescently labeled in intact RBCs using eosin-5-maleimide, as previously described (12). Briefly, fresh RBCs were washed in high potassium PBS (KPBS; 140 mM KCl, 15 mM NaPi, 10 mM glucose, pH 7.4) and mixed with 0.25 mg/ml (final concentration in KPBS) eosin-5-maleimide for 30 min at room temperature. Cells were then washed three times in KPBS with 1% BSA.

Confocal microscopy. Confocal laser scanning fluorescence microscopy was performed using a confocal microscope equipped with an argon ion laser (Leica Inc., Deerfield, IL). The microscope had excitation wavelengths and emission filters suitable for observation of rhodamine and fluorescein fluorescence. A ×100 water immersion objective lens was used throughout (Fluor; Olympus Corp., Lake Success, NY). Fluorescently labeled RBCs or ghosts were optically sectioned at intervals from 0.3 to 1.0 μm. Pseudocolor images were photographed directly from the monitor using Ektachrome Elite 100 film (Eastman Kodak Co.). The relative intensities of pseudocolor fluorescence were as follows: blue, > 240; white, 170–240; yellow, 120–170; orange, 80–120; red, 50–80; brown, 20–50; black, 0–20.

Results

Colocalization of NBD-PS and NBD-PC with sites of Heinz bodies. Exposure of intact normal red cells to either NBD-PS or NBD-PC resulted in uniform distribution of fluorescent phospholipid analogue over the entire surface of the membrane (data not shown). In contrast, labeling of intact sickle red cells by either NBD-PS or NBD-PC produced a distinct punctate pattern in addition to the membrane-associated "ring stain" (Fig. 1 *A*). This punctate pattern was detected after labeling at 37°C for 30 min with NBD-PS or NBD-PC at concentrations as low as 2 μg/ml (final concentration). At higher concentrations of NBD-PS or NBD-PC (e.g., 80 μg/ml), the punctate labeling pattern was detected after a 5-min incubation. Examination of ghosts of NBD-lipid–labeled sickle red cells revealed that the sites of punctate staining colocalized with morphologically identifiable Heinz bodies (Fig. 1 *B*). Although albumin treatment of intact NBD-lipid–labeled sickle red cells resulted in decreased plasma membrane fluorescence, neither NBD-PS nor NBD-PC was extracted by albumin from sites of punctate labeling (Fig. 1 *C*). These data suggested that the punctate-labeled sites represented either clustered phospholipid within the inner leaflet of the lipid bilayer or phospholipid that was internalized and sequestered within the sickle red cells.

Internalization of NBD-PS, NBD-PC, and Heinz bodies within sickle red cells. Several approaches were used to examine whether NBD-PS and NBD-PC were clustered at distinct regions in the inner lipid bilayer leaflet or internalized and se-

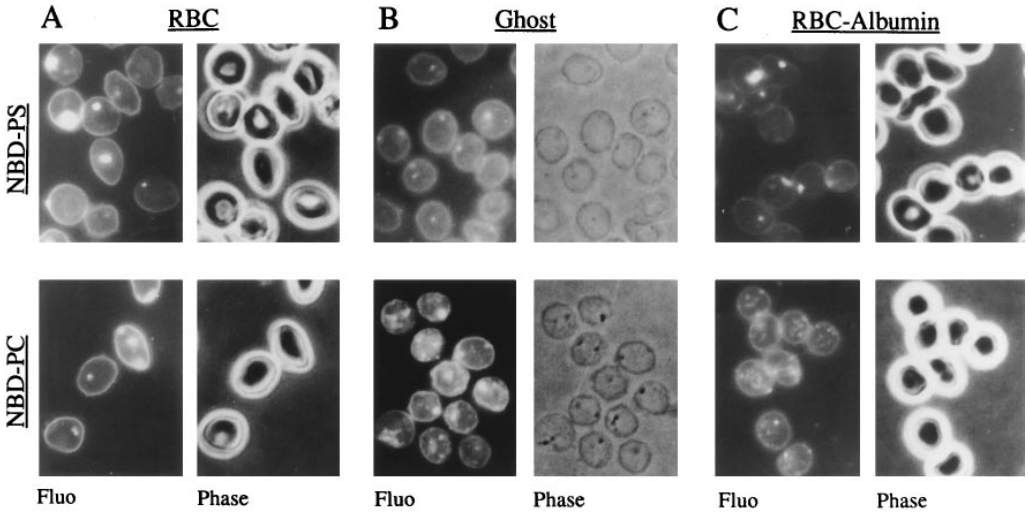

Figure 1. Phospholipid labeling of Heinz bodies in (*A*) sickle red cells, (*B*) sickle red cell ghosts, and (*C*) sickle red cells quenched using albumin. Sickle red cells were labeled with NBD-PC or NBD-PS (final concentration, 1.25 μM) at 37°C for 30 min. The labeled cells were lysed or quenched using 4% delipidated albumin and examined by fluorescence (*Fluo*) and phase-contrast microscopy. Note that NBD-lipids labeled Heinz bodies in both intact cells and ghosts with higher intensity than the plasma membrane. The labeling of Heinz bodies was not quenched by albumin treatment of intact cells.

questered at sites of Heinz bodies. First, we asked whether NBD-PS and NBD-PC were capable of interaction with denatured, cell-free hemoglobin S. Acetylphenylhydrazine was used to denature the ghost-free sickle cell hemolysate and to produce a hemoglobin S precipitate which was readily visible by phase-contrast microscopy. Exposure of the hemoglobin S precipitate to either NBD-PS or NBD-PC resulted in distribution of the fluorescent label over the entire surface of the precipitate (Fig. 2). This finding raised the possibility that, in intact cells, a fraction of bilayer lipids was sequestered within sickle red cells and deposited on the surface of intracellular aggregates of denatured hemoglobin S (Heinz bodies).

Second, we took advantage of the observation that some of the Heinz bodies in sickle red cell ghosts were detached from the membrane and exhibited distinct Brownian motion. Successive fluorescence and phase-contrast images of the same sickle red cell ghost labeled with NBD-PS (Fig. 3) or NBD-PC (data not shown) showed that the fluorescent phospholipid analogue comigrated with the Heinz bodies. These data directly suggested that a fraction of bilayer phospholipids was internalized and sequestered within the cells. Third, fluorescence imaging of optical sections of NBD-lipid labeled sickle red cells was used to define further the association between NBD-lipids and Heinz bodies. In these confocal microscopy images, fluorescently labeled phospholipids colocalized with the entire Heinz body in the cytoplasm of cells (Fig. 4 *A*). For Heinz bodies attached to the plasma membrane, NBD-PS or NBD-PC labeled the whole Heinz body but not the membrane site at which the Heinz body was attached (Fig. 4 *B*). Confocal sectioning through the mass of the Heinz body (*arrow*, Fig. 4 *A*) showed that the fluorescence intensity was greater in the center than at the edge of the particle, suggesting that the particles represented Heinz bodies and not endocytic lipid vesicles and that the NBD-lipid was incorporated within the Heinz bodies, probably on the hydrophobic surface of denatured hemoglobin.

Finally, two different membrane lipid analogues were used to study the molecular mechanism by which exogenously added NBD-lipids colocalized with Heinz bodies in sickle red cells. The first lipid probe was fluorescein-PE, a phospholipid analogue with a polar fluorescein moiety attached to the amine function of the PE head group. Fluorescein-PE has a much slower rate of exchange between membranes than do acyl chain–labeled lipid analogues, such as NBD-PS and NBD-PC, possibly because fluorescein-PE has a slow rate of "flip-flop" between lipid bilayer leaflets (27–30). Fluorescein-PE labeled the sickle cell surface but not the Heinz bodies (Fig. 5),

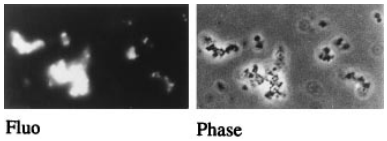

Figure 2. NBD-PC labeling of denatured hemoglobin in solution. Ghost-free hemolysate from sickle red cells was incubated with acetylphenylhydrazine (15 mM) at 37°C for 2 h. The denatured hemoglobin was then labeled with NBD-PC as described in the legend to Fig. 1. The mixture was then diluted with PBS and examined by light microscopy. Note that the hemoglobin precipitate was labeled intensely by NBD-PC. *Fluo*, fluorescence.

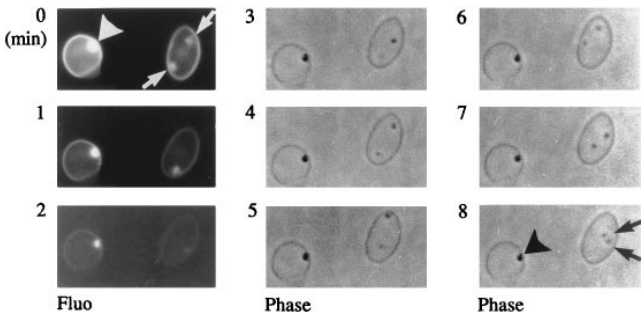

Figure 3. Brownian motion of NBD-PS–labeled Heinz bodies in sickle red cell ghosts. Fluorescence (*Fluo*) and phase-contrast light micrographs of the same ghosts were taken every minute for 8 min. Note that some Heinz bodies (*arrows*) exhibited Brownian movement, implying that they were not membrane bound, while other Heinz bodies (*arrowhead*) appeared to be membrane bound.

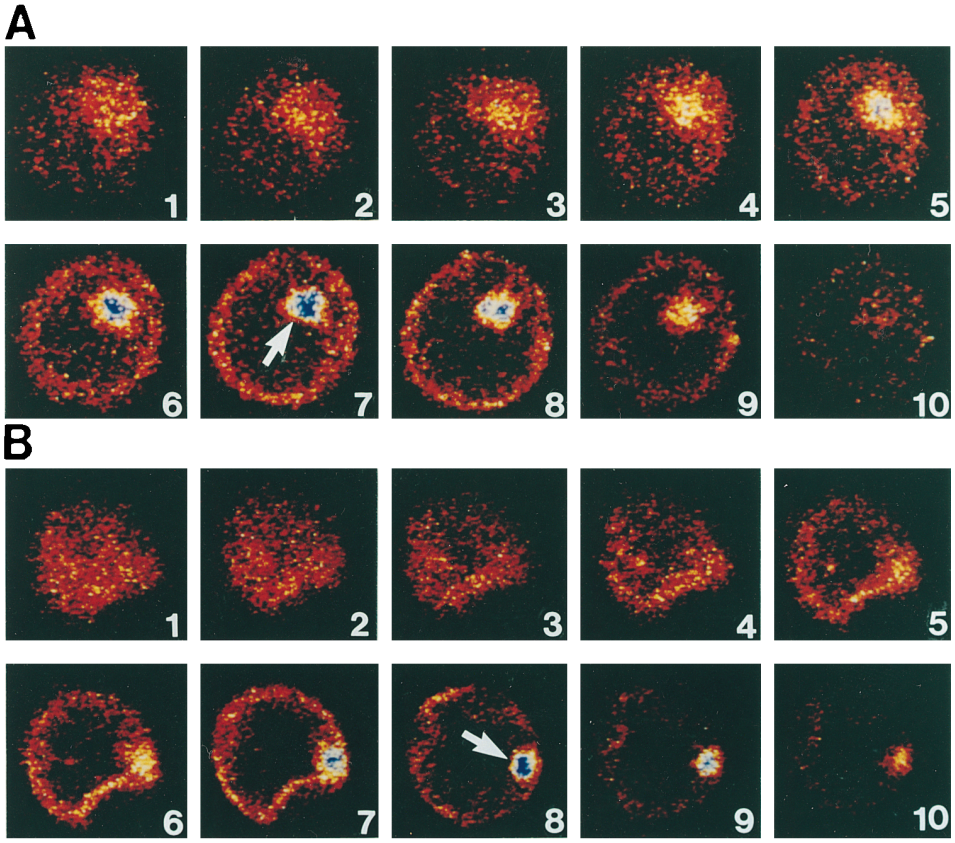

Figure 4. Confocal fluorescence images of optical sections (1–10, top to bottom) of NBD-PC–labeled intact sickle red cells. Optical sections of the labeled cell were taken from top to bottom at 0.3-μm increments using a confocal laser scanning fluorescence microscope. In cell *A*, the NBD-PC–labeled Heinz body (*arrow*) appeared as a dense particle that was entirely within the cell and not directly adjacent to the cell membrane. In cell *B*, NBD-PC labeled the membrane-bound Heinz body but not the membrane site where the Heinz body was attached (*arrow*).

suggesting that translocation of a lipid probe from the outer to the inner lipid bilayer leaflet was a prerequisite for subsequent lipid removal from the plasma membrane and sequestration in Heinz bodies. Consistent with this interpretation, albumin treatment of fluorescein-PE–labeled sickle red cells resulted in a $> 95\%$ decrease in plasma membrane fluorescence and did not reveal sites of punctate labeling (data not shown). The second membrane lipid probe was DiI, an amphiphilic carbocyanine with two long alkyl tails (28, 31) that translocates readily across plasma and internal membranes (28–30, 32). DiI-labeled Heinz bodies as well as the plasma membrane of sickle red cells (Fig. 5), suggesting that the surface of Heinz bodies provided a hydrophobic environment on which membrane lipids were internalized and sequestered.

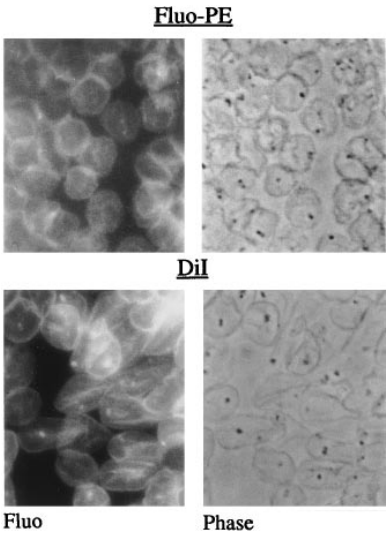

Figure 5. Fluorescein-PE and DiI labeling of sickle red cells. Sickle red cells were labeled with fluorescein-PE (*Fluo-PE*) (final concentration, 63 μM) or DiI (final concentration, 0.14 mM) at 37°C for 30 min. The labeled cells were lysed and examined by fluorescence (*Fluo*) and phase-contrast microscopy. Note that Fluo-PE labeled the plasma membrane but not the Heinz bodies. In contrast, DiI labeled both the plasma membrane and the Heinz bodies.

Internalization and colocalization of band 3 protein, spectrin, ankyrin, protein 4.1, and Heinz bodies within sickle red cells. We next examined whether the intracellular sequestration of sickle red cell membrane phospholipids was accompanied by a similar redistribution of membrane proteins. Cells were labeled with NBD-PS and lysed, and the resulting ghosts were double labeled with antibodies against band 3 protein, spectrin, ankyrin, or protein 4.1. Band 3, spectrin, ankyrin, and protein 4.1 were all observed to colocalize with the NBD-PS–labeled Heinz bodies (Fig. 6). No immunostaining was seen when the ghosts were incubated with preimmune rabbit serum and then labeled with the second (RITC-conjugated) antibody (data not shown).

Several approaches were used to verify that the bulk of the morphologically punctate band 3 protein staining, like that of NBD-PS and NBD-PC, was internalized and sequestered in cells rather than clustered in plasma membrane regions overlying membrane-associated Heinz bodies. First, sickle red cells were labeled with eosin-5-maleimide, which binds to Lys-430 at an exoplasmic loop of the band 3 protein and which does not permeate across the intact red cell membrane (33). Eosin-labeled cells showed a uniform distribution of band 3 protein over the entire surface of the membrane and a notable absence of clustering over membrane areas adjacent to Heinz bodies (Fig. 7). Second, confocal fluorescence microscopy and polyclonal anti–band 3 antibodies were used to visualize band 3 clusters in permeabilized sickle red cell ghosts. Band 3 protein in the plasma membrane of such cells was uniformly distrib-

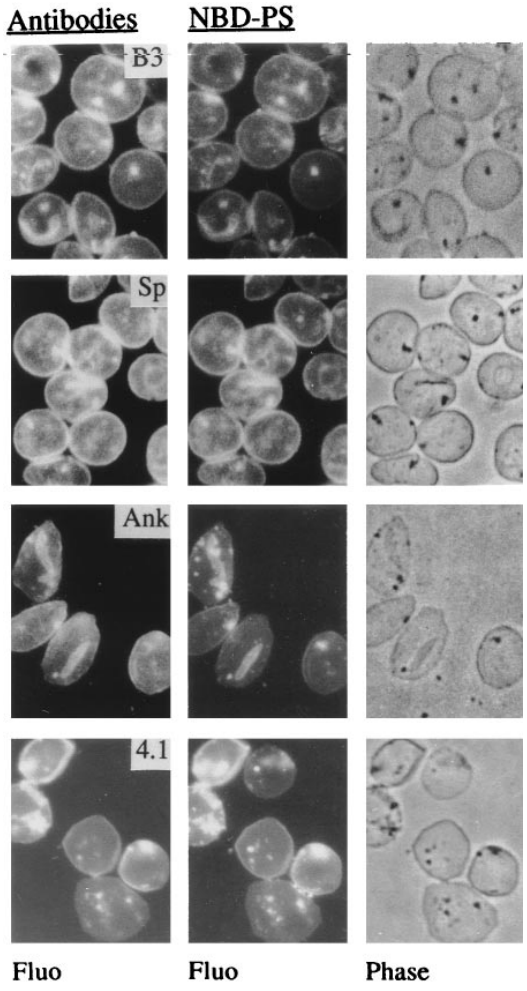

Figure 6. Colocalization of band 3 (*B3*), spectrin (*Sp*), ankyrin (*Ank*), protein 4.1 (*4.1*), and NBD-PS in Heinz bodies in sickle red cell ghosts. Sickle cells were labeled with NBD-PS, washed, and hypotonically lysed. The resulting ghosts were then labeled with antibodies against red cell membrane proteins, washed, and indirectly labeled with RITC-conjugated goat anti–rabbit IgG. Note that band 3, spectrin, ankyrin, and protein 4.1 colocalized with NBD-PS in Heinz bodies. (*Fluo*), fluorescence.

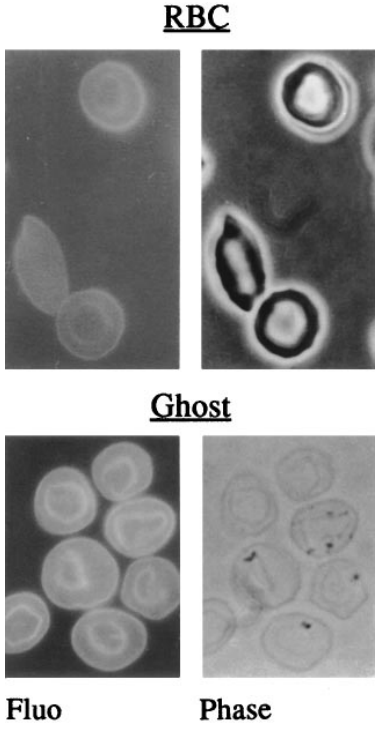

Figure 7. Eosin-maleimide labeling of band 3 in intact sickle red cells. Sickle red cells (*RBC*) were incubated with eosin-5-maleimide (0.25 mg/ml) in KPBS. Cells were then washed three times in KPBS with 1% BSA. Eosin-labeled cells and membrane ghosts, prepared by hypotonic lysis, were examined by fluorescence (*Fluo*) and phase-contrast microscopy. Note that the eosin label was distributed uniformly on the cell surface; there was a notable absence of clustering of eosin label over membrane areas adjacent to Heinz bodies.

uted and distinct from protein associated with Heinz bodies (Fig. 8). Again, there was a notable absence of band 3 clustering in plasma membrane regions adjacent to the underlying Heinz bodies.

Internalization and colocalization of NBD-PS, band 3 protein, and Heinz bodies within normal red cells pretreated with acetylphenylhydrazine. To investigate whether Heinz body formation was both necessary and sufficient for membrane lipid and protein sequestration, normal red cells were treated briefly with APH (15 mM, 37°C, 15 min), which denatures normal hemoglobin and induces Heinz body formation. APH-treated normal red cells were incubated in ATP-maintaining buffer for 48 h without APH, and the colocalization of NBD-PS, band 3, and Heinz bodies was examined. Punctate patterns of colocalized NBD-PS, band 3 protein, and Heinz bodies were detected, similar to those observed in sickle cells (Fig. 9). Although Heinz bodies were produced immediately upon brief treatment of normal red cells with APH (15 mM, 37°C, 15 min), the Heinz bodies were not readily labeled by NBD-PS or anti–band 3 antibodies until the APH-treated cells were subsequently incubated for 48 h in ATP-maintaining buffer (data not shown). Presumably, a period of hours was necessary for migration of membrane lipids and proteins to the surface of Heinz bodies.

Internalization and colocalization of NBD-PS, band 3 protein, and Heinz bodies within red cells containing unstable hemoglobin E. Red cells containing unstable hemoglobin E, which spontaneously denatures and forms Heinz bodies, were used to determine whether the sequestration of membrane phospholipids and proteins within cells was specific for sickle red cells. Heinz bodies were readily recognized in ghosts prepared from homozygous hemoglobin E red cells. Examination of hemoglobin E cells labeled with NBD-PS or anti–band 3 antibody revealed a punctate pattern of fluorescence suggestive of phospholipid and band 3 sequestration. Both NBD-PS and band 3 protein colocalized with Heinz bodies in hemoglobin E cells (Fig. 10). Internalization of membrane lipids and proteins may be a general feature of red cells in which hemoglobin denatures and forms Heinz bodies.

Discussion

We demonstrate in this report that, in red cells from patients with sickle cell anemia, a fraction of the plasma membrane phospholipids, together with integral membrane proteins and membrane skeletal proteins, is sequestered within the red cells. Further, the sequestered membrane lipids and proteins colocalize with Heinz bodies containing denatured hemoglobin S. This process also takes place in red cells containing other unstable hemoglobins, such as hemoglobin E, or normal hemoglobin denatured artifactually by oxidation. These conclusions are based on the following observations.

First, exposure of sickle red cells to the fluorescent phos-

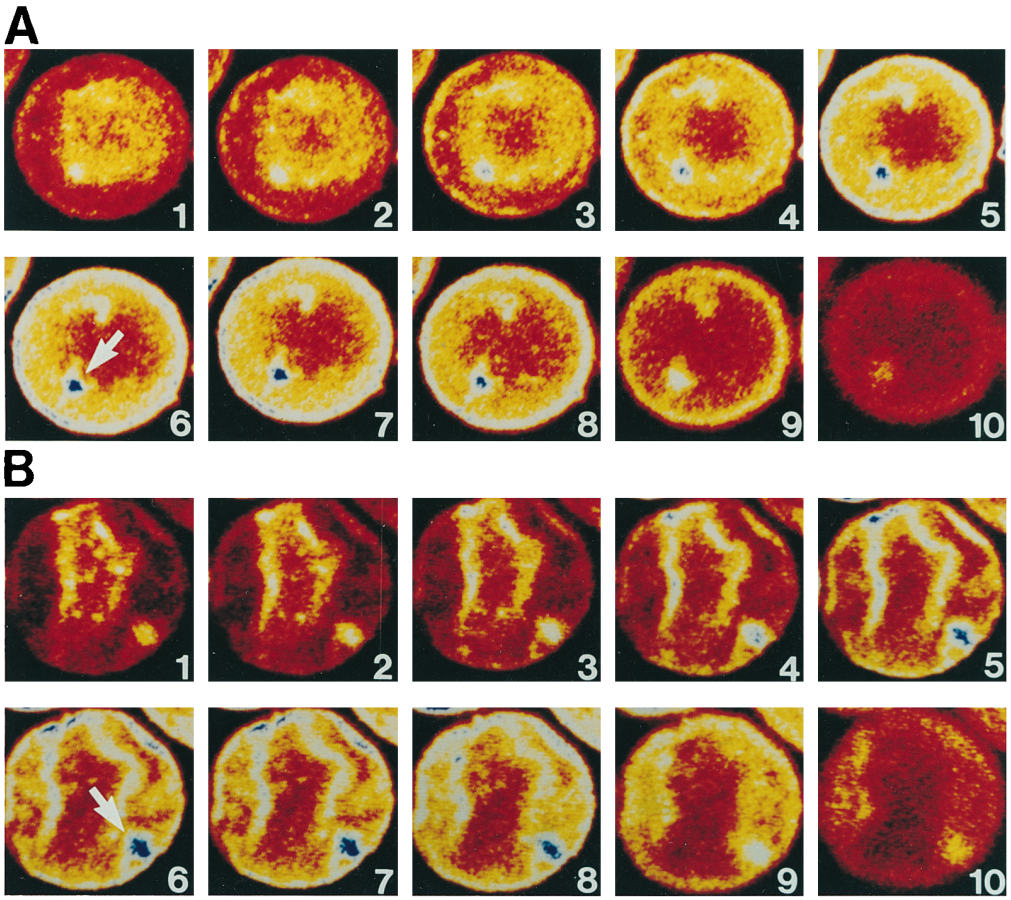

Figure 8. Confocal fluorescence images of optical sections (1–10, top to bottom) of anti–band 3 antibody–labeled sickle red cell ghosts. Ghosts were indirectly labeled with anti–band 3 antibodies using a protocol similar to that described in the legend to Fig. 5. Optical sections were taken using a confocal fluorescence microscope as described in the legend to Fig. 4. Note that anti–band 3 antibodies labeled both the plasma membrane and the Heinz bodies which were either trapped inside the ghost or attached to the inner surface of the membrane.

pholipid analogues NBD-PS and NBD-PC, and to the membrane lipid probe DiI, produces a punctate labeling pattern. Neither NBD-PS nor NBD-PC can be extracted by albumin from the sites of punctate labeling, suggesting that the phospholipid analogues are internalized within the cells. Second, NBD-PS, NBD-PC, and immunofluorescently labeled band 3 protein, spectrin, ankyrin, and protein 4.1 all colocalize with Heinz bodies in sickle red cells. Third, when visualized by confocal fluorescence microscopy, both the fluorescent phospholipid analogues and the labeled band 3 protein are found in association with the entire mass of the Heinz body. The latter finding can also be replicated by exposing a denatured precipitate of cell-free HbS to NBD-PS or NBD-PC. Fourth, the fluorescently labeled clusters of NBD-PS or NBD-PC comigrate with Heinz bodies during their Brownian motion.

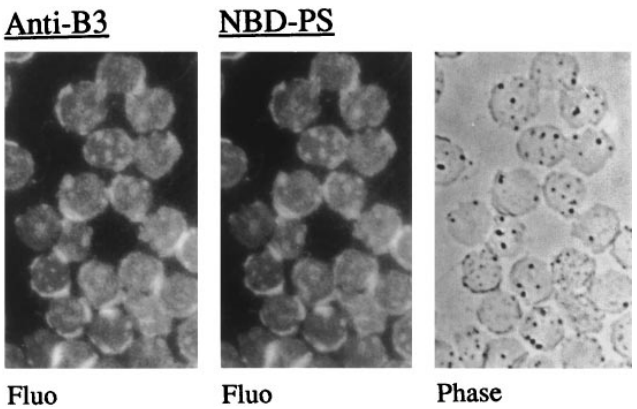

Figure 9. NBD-PS and anti–band 3 (*Anti-B3*) antibody labeling of Heinz bodies in ghosts from APH-treated red cells. Normal red cells were pretreated with APH (15 mM, 37°C, 15 min) and subsequently incubated at 37°C for 48 h without APH. The cells were then labeled with NBD-PS and hypotonically lysed, and the ghosts were indirectly labeled with anti–band 3 antibodies as described in the legend to Fig. 6. Note that band 3 colocalized with NBD-PS in Heinz bodies. *Fluo*, fluorescence.

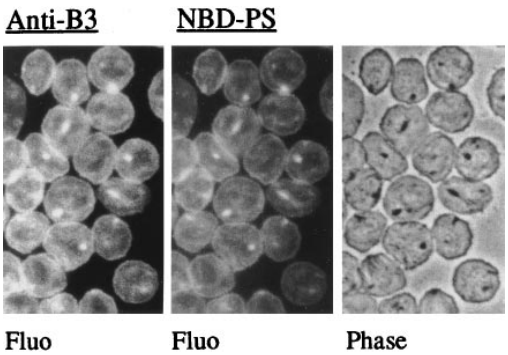

Figure 10. NBD-PS and anti–band 3 antibody labeling of Heinz bodies in ghosts from red cells containing unstable hemoglobin E. Red cells from a homozygous hemoglobin E individual were labeled with NBD-PS, washed, and hypotonically lysed. The resulting ghosts were indirectly labeled with rabbit anti–band 3 (*Anti-B3*) antibody and RITC-conjugated goat anti–rabbit IgG. Note that the Heinz body labeling by NBD-PS and anti–band 3 antibody was similar to that observed in ghosts from sickle red cells. *Fluo*, fluorescence.

Previous studies have shown that red cells containing Heinz bodies exhibit clustering of band 3 protein and of ankyrin at sites of attachment of the Heinz bodies to the membrane. Such sites are postulated to create a senescent red cell antigen that binds autologous IgG which, in turn, targets the red cells for premature destruction by cells of the reticuloendothelial system (10, 11, 15–18). Although our data are not inconsistent with the suggestions that microaggregates of band 3 are found at such sites, we show here that the band 3 redistribution in sickle red cells is considerably more profound than that associated with clustering within the plane of the membrane. It appears that a fraction of band 3 molecules is internalized and sequestered within the cells.

Red cells containing Heinz bodies exhibit a reduced surface area. The surface area deficiency has been attributed to the removal of Heinz bodies, together with the overlying plasma membrane, by cells of the reticuloendothelial system including splenic macrophages (34). Our data are consistent with the hypothesis that the loss of membrane surface area within the Heinz body–containing cells may also result from sequestration of a fraction of membrane lipids and integral membrane proteins. Reticuloendothelial remodeling of such cells is likely to involve removal of the entire Heinz body–associated lipid/protein complexes.

Surface area deficiency has also been demonstrated in irreversibly sickled red cells (35, 36). These permanently deformed cells are dehydrated, dense, and rigid (1, 36, 37), and the permanent deformation has been reproduced in irreversibly sickled red cells ghosts and membrane skeletons (38). We propose that the surface area deficiency of irreversibly sickled red cells is, at least in part, caused by membrane lipid and protein sequestration at sites of Heinz bodies. This process is likely to be compounded by a loss of membrane surface area mediated by the release of skeleton-free membrane spicules (13).

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