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Membrane raft actin deficiency and altered Ca^{2+}-induced vesiculation in stomatin-deficient overhydrated hereditary stomatocytosis

D. Katie Wilkinson [a], E. Jane Turner [b], Edward T. Parkin [c], Ashley E. Garner [a], Penny J. Harrison [b], Mark Crawford [b], Gordon W. Stewart [b], Nigel M. Hooper [a,*]

[a] *Institute of Molecular and Cellular Biology, Faculty of Biological Sciences, and Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds LS2 9JT, UK*
[b] *Department of Medicine, University College London, Rayne Institute, University Street, London WC1E 6JJ, UK*
[c] *Department of Biological Sciences, Lancaster University, Lancaster LA1 4YW, UK*

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Abstract

In overhydrated hereditary stomatocytosis (OHSt), the membrane raft-associated stomatin is deficient from the erythrocyte membrane. We have investigated two aspects of raft structure and function in OHSt erythrocytes. First, we have studied the distribution of other membrane and cytoskeletal proteins in rafts by analysis of detergent-resistant membranes (DRMs). In normal erythrocytes, 29% of the actin was DRM-associated, whereas in two unrelated OHSt patients the DRM-associated actin was reduced to <10%. In addition, there was a reduction in the amount of the actin-associated protein tropomodulin in DRMs from these OHSt cells. When stomatin was expressed in Madin–Darby canine kidney cells, actin association with the membrane was increased. Second, we have studied Ca^{2+}-dependent exovesiculation from the erythrocyte membrane. Using atomic force microscopy and proteomics analysis, exovesicles derived from OHSt cells were found to be increased in number and abnormal in size, and contained greatly increased amounts of the raft proteins flotillin-1 and -2 and the calcium binding proteins annexin VII, sorcin and copine 1, while the concentrations of stomatin and annexin V were diminished. Together these observations imply that the stomatin–actin association is important in maintaining the structure and in modulating the function of stomatin-containing membrane rafts in red cells.

Keywords: Actin; Detergent-resistant membrane; Erythrocyte; Membrane raft; Stomatin

1. Introduction

Overhydrated hereditary stomatocytosis (OHSt) is a rare, dominantly inherited haemolytic anaemia that shows three main features: (i) stomatocytic morphology, implying inward bending of the membrane [1]; (ii) a major leak to the monovalent cations Na^+ and K^+ across the plasma membrane [2]; and (iii) deficiency of the 32-kDa membrane raft protein stomatin [3,4]. The *STOM* gene that codes for this protein is almost certainly not mutated [5] but there appears to be a trafficking defect in developing erythroid cells, such that stomatin is synthesized but becomes obstructed in the secretory pathway [6].

Stomatin has emerged as the prototype for a superfamily of proteins, comprising stomatin, the stomatin-like proteins, the flotillins, prohibitins, the bacterial hflk and podocin [7]. The precise function of these proteins remains enigmatic. Eukaryotic examples all associate with cholesterol- and sphingomyelin-rich membrane rafts [8,9]. Many of the stomatin-type proteins show functional links to lipids and are prominently involved in trafficking. Some of these proteins may be important in the regulation of surface expression of other proteins [10] or may act as scaffolding proteins for membrane microdomains [7]. Stomatin was first discovered in erythrocytes that show a major abnormality in cation permeability [3] and some other members of the

Abbreviations: AFM, atomic force microscopy; DRM, detergent-resistant membrane; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionisation time-of-flight; MDCK, Madin–Darby canine kidney; PBS, phosphate-buffered saline; OHSt, overhydrated hereditary stomatocytosis; TBS, Tris-buffered saline

* Corresponding author. Tel.: +44 113 343 3163; fax: +44 113 343 3167.
E-mail address: n.m.hooper@leeds.ac.uk (N.M. Hooper).

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superfamily have been linked to ion channel function [11,12], although the exact link is not clear. In prokaryotes, genes showing strong homology to stomatin are linked in an operon with a gene that codes for a membrane-bound protease [13].

In several cell types rafts have been identified as sites for association of the actin cytoskeleton. For example, in UAC cells (which like the majority of cells have a three-dimensional actin skeleton), stomatin was clustered at sites of attachment of the actin filaments to the plasma membrane [14]. The stomatin homologue podocin is linked to the cytoskeleton via the adaptor protein CD2AP [15]. There are many reports which emphasise the attachment of actin microfilaments to rafts, where typically phosphatidylinositol-(4,5)-bisphosphate acts as a key signalling intermediate [16–20]. Recently, both raft and non-raft protein islands have been shown to be connected to the cytoskeleton by actin which plays a crucial role in island formation and/or maintenance [21].

Ca^{2+}-induced exovesicles derived from normal erythrocytes are known to be depleted in cytoskeletal proteins and enriched in raft proteins, an example of protein sorting [22,23]. In particular stomatin, but not the flotillins, is a prominent constituent of exovesicles released from normal human erythrocytes when the internal Ca^{2+} concentration is raised experimentally with the ionophore A23187 [22,24].

In this work we explored two aspects of OHSt red cells that are pertinent to the structure and function of membrane rafts in normal erythrocytes. We show that OHSt cells have a deficiency of actin associated with detergent-resistant membranes (DRMs) and that there are abnormalities in the actin-associated protein tropomodulin. The actin–stomatin link was further explored by showing that when stomatin is expressed in Madin–Darby canine kidney (MDCK) cells, actin association with the membrane is increased. To relate this actin–stomatin link to function, we show that Ca^{2+}-induced exovesicles derived from OHSt cells are increased in number and abnormal in size, and contain greatly increased amounts of the raft proteins flotillin-1 and -2 and the calcium binding proteins annexin VII, sorcin and copine 1, as compared to exovesicles from normal erythrocytes.

2. Materials and methods

2.1. Patients

Two previously described patients with OHSt have been studied. Patient A, whose mother was the subject of Lock's original paper [1] was A-II-1 in [5]. Patient B was B-II-1 in the same report, the daughter of the patient originally reported by [25].

2.2. Materials

Monoclonal antibodies against flotillin-1 and flotillin-2 were purchased from BD Biosciences (Oxford, UK). Antibodies against actin, annexins V and VI, band 3, adducin and clathrin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against spectrin, protein 4.1 and glycophorin A were gifts from Dr. J. Pinder (King's College, London, UK), while the anti-Glut-1 antibody was a gift from Prof. S.A. Baldwin (University of Leeds, Leeds, UK), and the anti-tropomyosin and tropomodulin were kindly donated by Dr. V. Fowler (Scripps Clinic, CA, USA). The anti-stomatin antibody has been reported previously [5]. Full-length human stomatin cDNA was purchased from OriGene Technologies Inc. (MD, USA). The protease inhibitor cocktail (catalogue no. P2714) and all other reagents were from Sigma-Aldrich (Poole, UK).

2.3. Isolation of red cell membranes

Erythrocytes were isolated from whole blood by centrifugation at 200×*g* for 10 min at 4 °C and washed 5 times with three volumes of 0.9% NaCl, 5 mM Na_2HPO_4, pH 8.0. Cells were then lysed in 5 mM Na_2HPO_4, pH 8.0, 1 mM dithiothreitol (DTT), 1 mM EDTA, 2 mM phenylmethylsulphonylfluoride and the membranes pelleted by centrifugation at 12,000×*g* for 10 min at 4 °C. The lysis step was repeated twice or until white ghosts were obtained [26]. When assessment of tropomyosin and tropomodulin was required, EDTA was replaced with 1 mM $MgCl_2$.

2.4. Isolation of detergent resistant membranes (DRMs) from red cells

Erythrocytes were isolated from whole blood by centrifugation at 200×*g* for 10 min at 4 °C and washed 5 times with Tris-buffered saline (TBS; 10 mM Tris/HCl, pH 7.5, 150 mM NaCl). DRMs were isolated as described by [9]. Following lysis in 9 volumes of ice-cold 0.5% Triton X-100 in TBS, incubation on ice for 20 min and centrifugation at 15,000×*g* for 10 min at 4 °C, the pellet was resuspended in 2 ml TBS and the protein concentration determined using the bicinchoninic acid protein assay with bovine serum albumin as standard [27]. The protein solution was diluted to 2 mg/ml with TBS and one volume of 90% (w/v) sucrose in TBS added. The protein solution with final sucrose concentration of 45% (4 ml) was placed at the bottom of a 17.5-ml centrifuge tube and overlaid with 5 ml 30% (w/v) sucrose in TBS and 5 ml 10% (w/v) sucrose in TBS. The sucrose gradient was then centrifuged using a SW-28 Beckman rotor at 140,000×*g* for 18 h at 4 °C and fractionated from the base upward. The insoluble pellet corresponding to the DRMs at the base of the tube was resuspended in TBS.

2.5. MDCK cell culture and transfection

MDCK cells were cultured in Dulbecco's Modified Eagle's Medium with 4.5 g/l glucose with L-glutamine supplemented with 10% fetal bovine serum and penicillin (50 U/ml) plus streptomycin (50 μg/ml). Cells were maintained at 37 °C in a 5% CO_2-humidified environment. The cDNA encoding stomatin was amplified using Accutaq LA DNA polymerase and cloned into the expression vector pcDNA3.1 TOPO (Invitrogen Ltd., Paisley, UK). Following transformation into *Escherichia coli* TOP10 competent cells the plasmid DNA was isolated and sequenced. MDCK cells were transiently transfected with 10 μg plasmid DNA using lipofectamine at 37 °C for 4.5 h. Media supplemented with 20% fetal bovine serum and penicillin/streptomycin (as above) was then added and the cells incubated at 37 °C for 19 h.

2.6. Isolation of lysates and membranes from MDCK cells

For the isolation of the total cell lysate, MDCK cells were washed twice with phosphate buffered saline (PBS), scraped from the flask and centrifuged at 1500×*g* for 5 min at 4 °C. The cells were then resuspended in lysis buffer (50 mM Tris/HCl pH 7.4, 1% (v/v) Triton X-100, 1% (v/v) protease inhibitor cocktail), sonicated for 30 s (full cycle, power setting 3, Branson Sonifier 450, Branson, Danbury, CT, USA) and centrifuged at 13,000×*g* for 5 min at 4 °C to remove insoluble material. For the isolation of membranes, MDCK cells were resuspended in 4 ml of ice-cold 100 mM Tris–HCl pH 7.4, sonicated for 1 min and centrifuged at 2000×*g* for 5 min at 4 °C. The supernatant was then centrifuged at 50,000×*g* for 1 h at 4 °C and the resultant membrane pellet resuspended in 100 mM Tris–HCl, pH 7.4.

2.7. SDS PAGE and western blot analysis

Samples were separated on 10% polyacrylamide SDS gels and then either stained with Coomassie Brilliant Blue or electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 h in TBS

containing 5% milk, 2% bovine serum albumin, 0.05% Tween 20 and then incubated with the relevant primary antibody. The membrane was then washed and incubated with the appropriate peroxidase-conjugated secondary antibody, followed by detection using the enhanced chemiluminescence detection kit (Amersham, UK). Densitometric analysis of the western blots was performed using NIH Image.

2.8. Ca^{2+}-induced vesiculation

Erythrocytes were isolated from whole blood by centrifugation at 200×*g* for 10 min at 4 °C and washed 5 times with TBS. Cells were resuspended in 9 volumes TBS containing 1 mM $CaCl_2$, 5 μM A23187 and then incubated at 37 °C for 30 min [22]. Following the incubation, EDTA was added to a final concentration of 5 mM and the cells pelleted by centrifugation at 15,000×*g* for 30 s at 4 °C. The supernatant was subjected to sequential centrifugation: 15,000×*g* for 10 min (microvesicles 1), 15,000×*g* for 20 min (microvesicles 2), 15,000×*g* for 30 min and finally 100,000×*g* for 1 h (nanovesicles) all at 4 °C. Each exovesicle pellet was resuspended in TBS.

2.9. Atomic force microscopy

Exovesicles were resuspended in 150 mM NaCl, 5 mM HEPES/NaOH, pH 7.3. The exovesicles were applied to freshly cleaved mica (Agar Scientific, Stansted, UK) and imaged at room temperature using a multimode atomic force microscope with a Nanoscope IIIa controller and an E-scanner (Digital Instruments, Santa Barbara, CA, USA). Images were recorded in intermittent tapping mode using oxide sharpened silicon nitride tips mounted on cantilevers with nominal spring constants of 0.32 newton/m, oscillating to a frequency between 7 and 9 kHz. The set point was adjusted during imaging to minimise the force whilst scanning at a rate of 1 Hz.

2.10. Sample preparation for MALDI-TOF mass spectrometry

From a Coomassie-stained one-dimensional SDS polyacrylamide gel, five 2-mm spots were cut from each band of interest. Each spot was destained in 40% EtOH, 50 mM NH_4HCO_3 overnight. Using the Investigator ProGest robot (Genomic Solutions, Huntingdon, UK), samples were washed in 50 mM NH_4HCO_3 then dehydrated in acetonitrile. This wash was repeated twice. Disulphide bonds were reduced by treating the spots with 10 mM DTT in 25 mM NH_4HCO_3 at 56 °C. Alkylation was then performed for 45 min in 100 mM iodoacetamide in 25 mM NH_4HCO_3. The NH_4HCO_3 washes and acetonitrile dehydrations were repeated thrice more and the samples dried briefly. Trypsin (100 ng) reconstituted in 50 mM NH_4HCO_3 was added to each spot and digestion carried out at 34 °C overnight. The peptides were extracted using acetonitrile, 1:1 acetonitrile:H_2O, acetonitrile again and 5% formic acid. The liquids were combined. For each band, the liquid from the 5 spots was combined and reduced in a Speedvac concentrator (Global Medical Instrumentation, Inc., Ramsey, MI, USA). Each digested peptide solution was deposited onto the target disc and allowed to air dry. Each sample was overlaid with matrix solution (1% w/v α-cyano-4-hydroxycinnamic acid in 33% v/v acetonitrile, 1% v/v trifluoroacetic acid). Spectra were obtained using an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltoniks Ltd., Coventry, UK) working under the FlexContol programme. Lists of mass-to-charge ratios of peptides for each spectra were acquired using the Bruker Data Analysis programme. Lists were compared to the ProFound database at www.prowl.rockefeller.edu/profound_bim/WebProFound.exe [28]. The maximum mass tolerance allowed was 200 p.p.m and proteins were identified according to the probability scores and percentage coverage.

3. Results

3.1. Actin and tropomodulin are deficient in DRMs from OHSt red cells

We used buoyant density gradient centrifugation in the presence of Triton X-100 [29] to investigate whether proteins other than stomatin were deficient in DRMs from two OHSt patients. In normal erythrocytes, less than half of the stomatin was present in the DRMs (fractions 6–8) as described previously [9] and, as expected [5], the total amount of stomatin was markedly reduced in the OHSt case (Patient A; Fig. 1). The raft proteins flotillin-1, flotillin-2 and Glut-1 were all present exclusively in the raft fraction in both the normal erythrocytes and in the OHSt cells from Patient A, while the non-raft proteins spectrin, ankyrin, protein 4.1 and band 3 were all located in the non-raft, lower regions of the sucrose gradients of both the

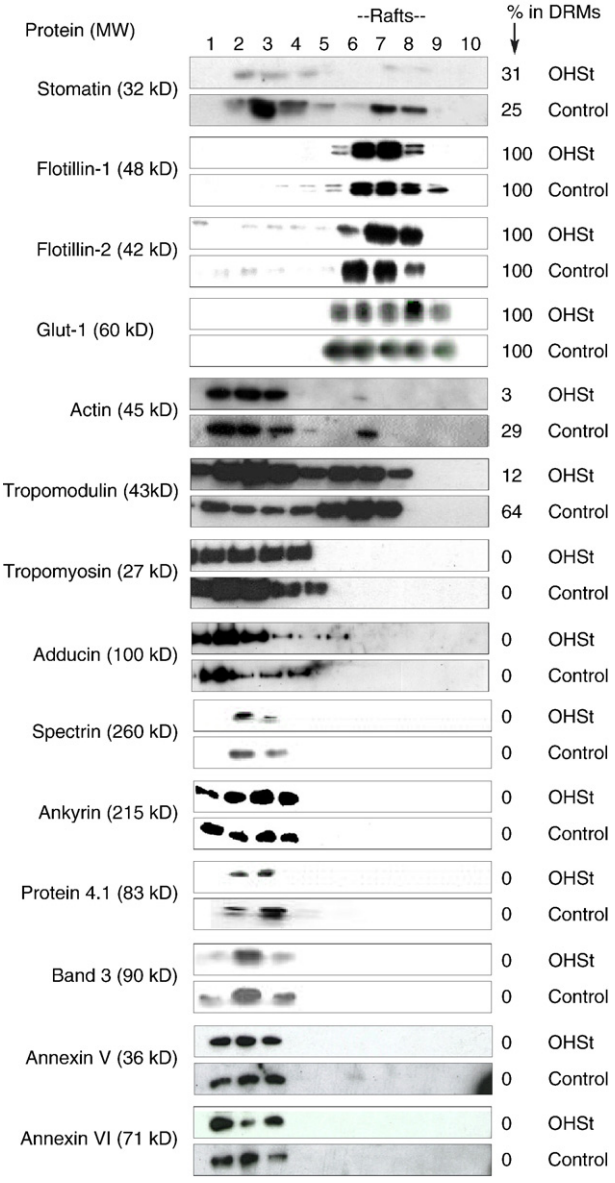

Fig. 1. Distribution of different proteins in DRMs from normal and OHSt erythrocytes. Normal (control) and OHSt erythrocytes from patient A were lysed in cold 0.5% Triton X-100 and subjected to buoyant sucrose density gradient centrifugation as described in Materials and methods. The gradients were fractionated from the base upwards (0=pellet; 10=top fraction). The fractions were subjected to SDS-PAGE (equal volume of each fraction loaded) and immunoblotted with the appropriate antibodies. Each immunoblot is representative of 2 separate isolations. The proportion of the immunoreactive protein that was present in the DRMs (fractions 6–9) is shown to the right of each immunoblot.

normal erythrocytes and OHSt cells from Patient A (Fig. 1). Likewise with the OHSt cells from Patient B, virtually all (98%) of the flotillin-1 was in the raft fraction, while 100% of the band 3 was in the non-raft fractions of the sucrose gradient (data not shown). Neither the amounts of these proteins nor their distribution in the sucrose gradients differed significantly between the OHSt and normal cells. However, in normal erythrocytes, 29% of the actin was found in the DRMs, whereas in both OHSt patients it was repeatedly found that a much lower quantity (patient A, 3%, Fig. 1; patient B, 10%, data not shown) was associated with the DRMs.

We also examined the distribution of the actin-associated proteins tropomodulin and tropomyosin in the sucrose gradients. In normal erythrocytes, the tropomodulin was largely associated with DRMs (64%), while in the OHSt cells from patient A only 12% was associated with the DRMs and the amount of tropomodulin in the lower, non-raft fractions of the gradients was clearly increased above that seen in the sucrose gradients from the control erythrocytes (Fig. 1). Interestingly, no tropomyosin was found in the DRMs in either normal or OHSt cells, although there did appear to be a reduction in the total amount of tropomyosin in the sucrose gradient (Fig. 1).

The DRM distribution of other proteins was studied. Adducin was excluded from DRMs and there was no difference between patient and control cells (Fig. 1). Neither annexins V and VI (Fig. 1) nor glycophorin A and clathrin (data not shown) were present in DRMs in either cell type and there was no difference in the total amount of these proteins between normal and OHSt cells.

To confirm that the total amount of actin and tropomyosin in the OHSt cells were reduced relative to the control cells, membranes were isolated from normal and OHSt cells and subjected to western blotting. This confirmed that the total amount of actin and tropomyosin in the membranes was slightly reduced (10% and 30%, respectively; Fig. 2). This was supported by a detectable reduction in total actin on Coomassie-stained SDS gels (quantitated by densitometry as a 15% reduction; data not shown). Together these data indicate that, of multiple membrane proteins, only actin and tropomodulin, along with stomatin, are reduced in DRMs from OHSt erythrocytes compared to normal erythrocytes.

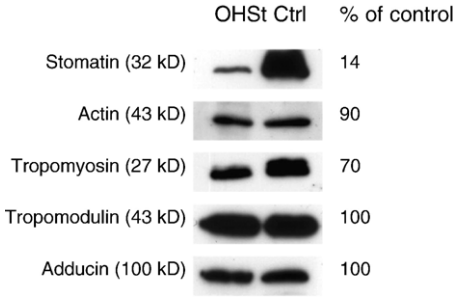

Fig. 2. Distribution of selected cytoskeletal and raft proteins in red cell membranes. Membranes were prepared from control erythrocytes and OHSt cells from patient A in Mg^{2+}-containing lysis buffer as described in Materials and methods. Samples were subjected to SDS PAGE and then immunoblotted for stomatin, actin, tropomyosin, tropomodulin and adducin. For each protein, the percentage that was present in the patient membranes as compared with the control membranes is shown to the right of the immunoblots. Coomassie stained gels of the membranes confirmed that equal quantities of protein were loaded (data not shown).

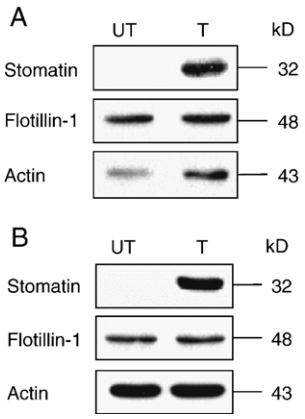

Fig. 3. Heterologous expression of stomatin in MDCK cells increases the association of actin with membranes. MDCK cells either untransfected (UT) or transiently transfected (T) with the cDNA encoding stomatin were fractionated into a total membrane fraction (A) and a whole cell lysate (B) as described in Materials and methods. Following SDS PAGE and western blotting, stomatin, flotillin-1 and actin were detected with the appropriate antibodies. The immunoblots are representative of two separate experiments.

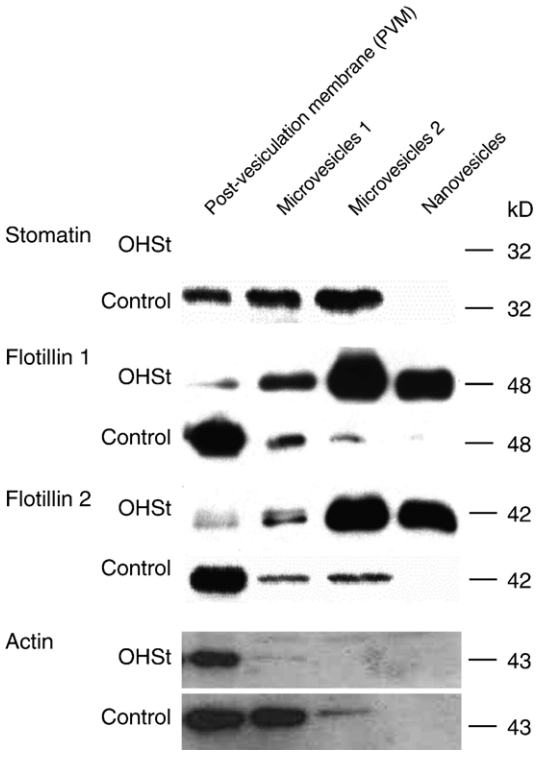

Fig. 4. Calcium-induced shedding of exovesicles from normal and OHSt erythrocytes. Normal (control) and OHSt erythrocytes from patient A were incubated with Ca^{2+} and A23187 to induce shedding of exovesicles as described in Materials and methods. Post-vesiculation membranes and exovesicles (5 μg protein) were subjected to SDS PAGE followed by immunoblot analysis using antibodies against stomatin and flotillin-1 and -2, and actin. The immunoblots are representative of three separate experiments.

3.2. Heterologous expression of stomatin in MDCK cells increases the amount of actin in membranes

To investigate whether variation in expression of stomatin might influence the level of actin associated with the membrane of another cell type, human stomatin was heterologously expressed in MDCK cells, which express this protein endogenously at only very low levels. Membranes isolated from untransfected cells and cells transfected with stomatin were immunoblotted for stomatin, flotillin-1 and actin. At the exposures used, no endogenous stomatin was detected in the membranes from the untransfected cells, but the protein was clearly present in the membranes from the transfected cells (Fig. 3A). While the quantity of flotillin-1 in the membranes from the untransfected cells was the same as that in the transfected cells, there was a 50% increase in the amount of actin in the membranes from the stomatin-transfected cells as compared with the untransfected cells (Fig. 3A). In contrast to this increase in membrane-associated actin, the amount of actin in the whole cell lysates (Fig. 3B) was not increased, suggesting that the increased membrane-associated actin was not due to an increased synthesis of actin.

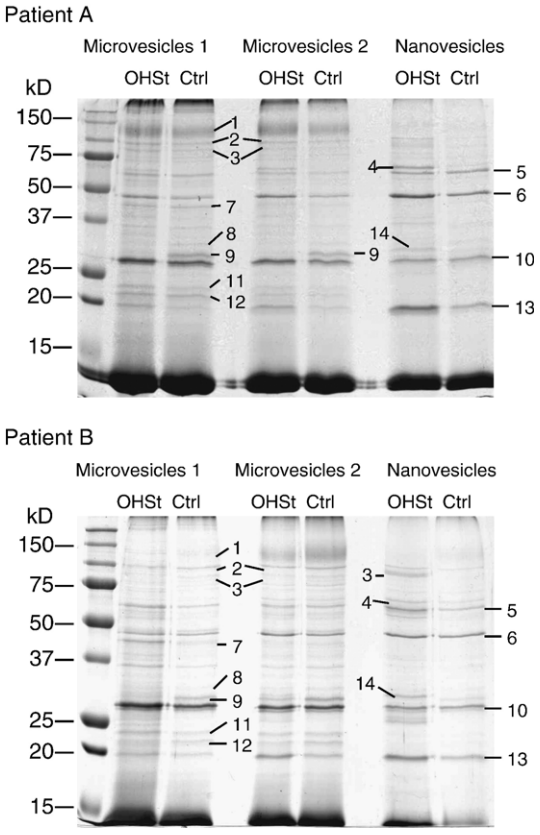

Fig. 5. SDS PAGE analysis of exovesicles derived from normal and OHSt cells. Normal (control) and OHSt erythrocytes from patients A and B were incubated with Ca^{2+} and A23187 to induce shedding of microvesicles and nanovesicles as described in Materials and methods. The exovesicle samples were subjected to SDS PAGE and stained with Coomassie Blue. The numbered proteins were subject to MALDI-TOF mass spectrometry (see Table 1).

Table 1
Proteins identified by MALDI-TOF mass spectrometry in the exovesicles from normal and OHSt erythrocytes

No.	MALDI-TOF identification	Probability of identification	Estimated *Z*-score	% coverage	kDa	OHSt vs. normal
1	Band 3	1.000	2.35	26	102.06	=
2	Band 3	1.000	2.33	17	102.06	=
3	Band 4.2	1.000	2.28	13	77.81	=
4	Copine I	1.000	2.38	25	59.67	↑↑
5	Catalase	1.000	2.21	24	56.71	=
6	Annexin VII	1.000	1.80	18	53.01	↑
7	Actin	1.000	2.12	27	40.83	↓
8	Annexin V	1.000	2.43	41	35.84	↓
9	Stomatin	1.000	3.27	60	31.88	↓
10	Carbonic anhydrase I	1.000	2.14	45	28.78	=
11	Flavin reductase (NADPH)	1.000	1.64	37	22.22	=
12	Thioredoxin 2peroxidase	1.000	2.16	43	21.68	=
13	Sorcin	1.000	2.01	50	21.94	↑
14	Unidentified				~35	↑

Protein no. refers to the numbering on Fig. 5. The 'probability of identification' and 'estimated *Z*-score' are derived from ProFound software [28]. '% coverage' denotes the percentage of the protein that was definitively identified in peptide fragments seen on MALDI-TOF mass spectrometry. 'kDa' refers to the molecular mass of the protein. 'OHSt vs. normal' denotes the relative level of expression of the protein in OHSt samples relative to normal cells: =, no change; ↑, increased in OHSt; ↓, decreased in OHSt.

3.3. Ca^{2+}-induced vesiculation is abnormal in OHSt cells

As calcium-dependent release of exovesicles from erythrocytes has been shown to involve stomatin-specific membrane rafts [22], we investigated whether this process was altered in cells deficient in stomatin. Erythrocytes from healthy donors and from an OHSt patient (patient A) were incubated with Ca^{2+} and A23187 to induce vesiculation. The resulting exovesicles (microvesicles and nanovesicles), along with the post-vesiculation membranes, were subjected to SDS-PAGE followed by immunoblot analysis (Fig. 4). Stomatin was present in significant amounts in the exovesicles from the control erythrocytes, but was absent from the OHSt exovesicles, as expected. Flotillin-1 and -2 remained predominantly in the post-vesiculation membrane of the control erythrocytes, but both were massively increased in the OHSt exovesicles: virtually all of both of these proteins was found in the exovesicles, while only a small fraction (less than 10%) was found in exovesicles derived from normal cells. Actin was present in the exovesicles from the normal cells, but was essentially absent from the exovesicles derived from the OHSt cells. These differences in exovesicle protein content were consistently seen whenever an OHSt patient was compared with one of four different normal controls.

To analyse further the protein composition of the exovesicles, samples from normal erythrocytes and from OHSt cells (both patients A and B) were analysed by SDS PAGE and stained with Coomassie Blue (Fig. 5). Proteins of interest on the gels were identified by MALDI-TOF mass spectrometry and the identification data are shown in Table 1. A number of

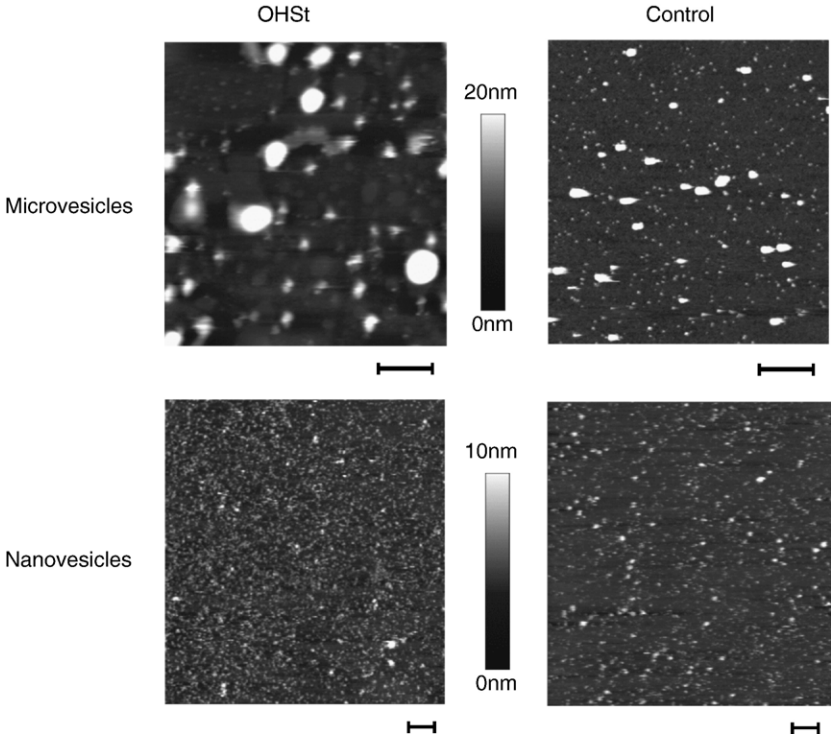

Fig. 6. AFM images of exovesicles from normal and OHSt erythrocytes. Normal (control) and OHSt erythrocytes from patient A were incubated with Ca^{2+} and A23187 to induce shedding of exovesicles. Microvesicles 1 (upper panels) and nanovesicles (lower panels) were imaged by AFM on mica-coated surfaces as described in Materials and methods. Bar = 1 μm.

differences in exovesicle protein content were observed. As seen in Fig. 4, the OHSt exovesicles lacked stomatin at 32 kDa (protein no. 9 in Fig. 5). There was a deficiency of a 35-kDa protein (protein no. 8) and MALDI-TOF mass spectrometry showed this to be annexin V. Three proteins were seen to be present in excess in the OHSt exovesicles: no. 4 at 60 kDa, identified as copine 1; no. 6 at 50 kDa, annexin VII; and no. 13 at 20 kDa, sorcin. Both the normal and OHSt vesicles contained a number of abundant soluble cytoplasmic proteins: carbonic anhydrase (no. 10), flavin reductase (no. 11) and thioredoxin peroxidase (no. 12). A prominent globin band was visible at the dye front below the 15-kDa marker. A further unidentified protein of about 35 kDa, no. 14, was increased in OHSt nanovesicles.

The morphology of the exovesicles derived from the normal and OHSt erythrocytes was studied by atomic force microscopy (AFM). Cells were incubated with Ca^{2+} and A23187 to induce vesiculation and equivalent volumes of each exovesicle sample were deposited on freshly cleaved mica and imaged using AFM (Fig. 6; Table 2). The images showed that in OHSt, the number of both the large and small microvesicles was reduced and both populations were larger in size than those from the normal erythrocytes. In contrast, the number of nanovesicles was greater in the OHSt case, and they were smaller in diameter than those derived from normal cells. Together these results indicate that in the absence of stomatin, calcium-induced vesiculation is perturbed, involving flotillin-containing membrane rafts, and that stomatin has a role in regulating calcium-induced vesiculation.

Table 2
Analysis of exovesicles from normal and OHSt erythrocytes by AFM

Exovesicle	Sample	Average vesicle diameter±S.E. (nm)	Average no. vesicles per mm^2
Microvesicles	OHSt	354.1±9.8	1.4
		192.4±3.2	7.8
	Normal	135.4±4.7	6.0
		58.8±0.8	34.0
Nanovesicles	OHSt	24.4±0.4	220.0
	Normal	39.0±1.1	162.0

Size and number of vesicles from the AFM images shown in Fig. 6.

4. Discussion

Although the fundamental genetic abnormality in OHSt erythrocytes is still not understood, the present work extends the spectrum of pathophysiology in these cells. Hitherto, it has been understood that OHSt cells have an abnormal, invaginated shape [1]; a major leak to the monovalent cations Na^+ and K^+; and a trafficking abnormality [6] that results in the deficiency of the 32-kDa protein stomatin [2], named after this disease [4,30]. The present work extends this knowledge to show additional structural and functional abnormalities, namely a reduction in the association of the cytoskeletal proteins actin and tropomodulin with membrane rafts and an alteration in the process of Ca^{2+}-induced exovesiculation.

Our work shows that in normal erythrocytes, a significant fraction of the actin is associated with the DRMs and that in the stomatin-deficient OHSt cells, the amount of actin associated

with the DRMs is reduced. In normal cells, a significant proportion of the tropomodulin is DRM-associated, but the proportion in the DRMs is decreased in the stomatin-deficient cells. In addition the total amount of tropomyosin, which is not DRM-associated in either normal or abnormal cells, is decreased in the stomatin-deficient cells. Previously it has been assumed that, in erythrocytes, the actin cytoskeleton is not associated with the detergent-insoluble, membrane rafts, although this may reflect differences in the protocols used, particularly the concentration of detergent [31]. The suggested association between stomatin and the raft-associated actin is supported by the heterologous expression in cultured MDCK cells, in which expression of stomatin led to a major increase in membrane-associated (but not total) actin. Although there has been some discussion [32], it is generally accepted that buoyant sucrose density gradient centrifugation in the presence of Triton X-100, as used here, does reflect lateral separation in the membrane [29,33]. Thus, these data agree with previous reports [8,9,34] that the actin cytoskeleton in erythrocytes has a raft-based link which probably involves stomatin. In contrast to a previous report [34] we found no evidence for spectrin being associated with DRMs. This may reflect differences in methodology used to isolate the DRMs between that study and ours (for example, the inclusion of EDTA in the buffer used by Ciana et al. [34] which is known to alter the distribution of proteins in DRMs [35], and differences in detergent:protein ratio in the two studies).

In other cell types, the key actin-related process that occurs at rafts is nucleation of the actin bundles. Tropomodulin is a player in this process, serving to cap pointed (slow-growing) ends [36]. It is possible that the raft tropomodulin seen here in normal erythrocytes may act as a reservoir of available tropomodulin accessible for control of actin turnover. Further studies would be required to confirm this. Quite why the total tropomyosin level in the OHSt cells should be reduced is difficult to understand, but it is possible that there exists some kind of bundling/remodeling process, that relies on a stomatin raft.

We detected a series of abnormalities in the protein content of OHSt exovesicles. Among the raft proteins, stomatin was greatly reduced, as expected, while flotillin-1 and -2 were much increased. The other differences were found in calcium-binding proteins. In OHSt cells, the exovesicle content of annexin VII, sorcin and copine 1 were all increased, while annexin V was decreased. It is known that annexin VII and sorcin are mutual binding partners [37], so their coordinated increase is not surprising. The actin content of the exovesicles, not expected to be high [38], was decreased in the OHSt exovesicles.

This work shows that although exovesicles derived from normal erythrocytes contained little of the raft proteins flotillin-1 and -2, they were abundantly present in the exovesicles derived from OHSt cells, to the extent that there was little of either flotillin left in the post-vesiculation erythrocytes. It is difficult to envisage a mechanism that would release virtually all of these proteins in the exovesicles but is consistent with there being a gross defect in exovesiculation from the OHSt cells. The fact that the flotillins were excluded from normal exovesicles, while stomatin was present, is consistent with stomatin and the flotillins being laterally separated in the normal membrane, otherwise they would both be expected to be either included or excluded from the exovesicles, and that there are two different kinds of membrane microdomains in the red cell membrane: those containing stomatin and those containing flotillin. This suggestion is consistent with the observation that during the course of malarial parasitisation, flotillin is carried into the erythrocyte with the parasite whereas stomatin is not [31].

The two aspects of this work can be drawn together. If the bleb of membrane that buds off when the internal [Ca^{2+}] is raised is composed primarily of raft proteins, then it can be suggested that the triggering event that releases the exovesicle is the breakage of a vertical link between the cytoskeleton and the bilayer at the raft. The first part of this work, in which it is suggested that actin and stomatin are associated at rafts in normal erythrocytes, could suggest that a putative actin–stomatin bridge is the weak link that is broken by increased [Ca^{2+}], possibly by competition from calcium-dependent proteins such as annexin VII, sorcin [39] or copine 1 [40]. In OHSt, we suggest that this link is abnormal, due to the lack of stomatin, and that this is one possible reason why the exovesicles are abnormal in shape, size and number. Extrapolating to the erythropoietic stages where stomatin is mistrafficked [6], we suggest that an abnormality in raft-based membrane-to-cytoskeleton bridging could explain the trafficking abnormalities seen in developing OHSt cells.

In conclusion, our data indicate that in erythrocytes stomatin is, in part, a structural protein tethered to the actin cytoskeleton that has a regulatory function in Ca^{2+}-induced vesiculation. A more complete understanding of these complex protein–protein interactions and the functional abnormalities in OHSt cells will be greatly facilitated by the understanding of the fundamental genetic defect in this red cell disease.

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