Band 3 mutations, renal tubular acidosis and South-East Asian ovalocytosis in Malaysia and Papua New Guinea: loss of up to 95% band 3 transport in red cells

Lesley J. BRUCE*, Oliver WRONG†, Ashley M. TOYE*, Mark T. YOUNG*, Graham OGLE‡§, Zulkifli ISMAIL‖, Ashim K. SINHA§, Paddy McMASTER¶, Ilomo HWAIHWANJE**, Gerard B. NASH††, Sarah HART*, Evelyn LAVU§, Ronald PALMER‡‡, Ainoon OTHMAN§§, Robert J. UNWIN† and Michael J. A. TANNER*[1]

*Department of Biochemistry, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, U.K., †Centre for Nephrology, Royal Free and University College Medical School, London W1N 8AA, U.K., ‡HOPE *worldwide* (PNG), Boroko, National Capital District, Papua New Guinea, §Department of Clinical Sciences, University of Papua New Guinea, Boroko, Papua New Guinea, ‖Department of Paediatrics, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia, ¶9 McCardle St, Ermington, NSW 2115, Australia, **Wewak Hospital, East Sepik Province, Papua New Guinea, ††Department of Physiology, University of Birmingham, B15 2TT, U.K., ‡‡Department of Haematology, Bristol Royal Infirmary, Bristol BS2 8HW, U.K., and §§Haematology Unit, Department of Pathology, Universiti Kebangsaan, Malaysia

We describe three mutations of the red-cell anion exchanger band 3 (*AE1*, *SLC4A1*) gene associated with distal renal tubular acidosis (dRTA) in families from Malaysia and Papua New Guinea: $Gly^{701} \rightarrow Asp$ (G701D), $Ala^{858} \rightarrow Asp$ (A858D) and deletion of Val^{850} (ΔV850). The mutations A858D and ΔV850 are novel; all three mutations seem to be restricted to South-East Asian populations. South-East Asian ovalocytosis (SAO), resulting from the band 3 deletion of residues 400–408, occurred in many of the families but did not itself result in dRTA. Compound heterozygotes of each of the dRTA mutations with SAO all had dRTA, evidence of haemolytic anaemia and abnormal red-cell properties. The A858D mutation showed dominant inheritance and the recessive ΔV850 and G701D mutations showed a pseudo-dominant phenotype when the transport-inactive SAO allele was also present. Red-cell and *Xenopus* oocyte expression studies showed that the ΔV850 and A858D mutant proteins have greatly decreased anion transport when present as compound heterozygotes (ΔV850/A858D, ΔV850/SAO or A858D/SAO). Red cells with A858D/SAO had only 3% of the SO_4^{2-} efflux of normal cells, the lowest anion transport activity so far reported for human red cells. The results suggest dRTA might arise by a different mechanism for each mutation. We confirm that the G701D mutant protein has an absolute requirement for glycophorin A for movement to the cell surface. We suggest that the dominant A858D mutant protein is possibly mis-targeted to an inappropriate plasma membrane domain in the renal tubular cell, and that the recessive ΔV850 mutation might give dRTA because of its decreased anion transport activity.

Key words: erythrocyte, membrane, anion exchanger.

INTRODUCTION

Distal renal tubular acidosis (dRTA) results from the impaired secretion of hydrogen ions from the distal nephron, causing metabolic acidosis, often with nephrocalcinosis, hypokalaemia and metabolic bone disease [1]. dRTA is usually characterized by a patient's inability to acidify the urine to $pH < 5.5$. Patients of European extraction with autosomal dominant dRTA have recently been shown to have mutations in the red-cell anion exchanger band 3 (*AE1*, *SLC4A1*) gene [2–5]. A form of red-cell band 3, truncated at the N-terminus, described here as kidney band 3 (kB3), is present on the basolateral membrane of the α-intercalated cells of the renal collecting duct, and is involved in renal tubular acid secretion [6–8]. Although initial studies suggested that recessive dRTA did not result from band 3 mutations [5], recessive dRTA associated with mutant band 3 has recently been reported [9,10]. Mutations in the B1 subunit of the H^+-ATPase also give rise to recessive dRTA associated with nerve deafness [11].

The red-cell abnormality South-East Asian ovalocytosis (SAO) is common in the Malay Archipelago and Papua New Guinea (PNG), where it affects up to 40% of the population in some coastal areas [12,13]. The condition affords some protection against cerebral malaria in children [14,15] and causes increased red-cell rigidity [16,17]. SAO results from the heterozygous presence of a 9-amino-acid deletion (residues 400–408) in band 3 (B3 SAO) and is linked to the relatively common B3 Memphis mutation (K56E) [18–20]. B3 SAO is inactive in anion transport and does not bind some anion transport inhibitors [21–24].

There have been several reports of an association between dRTA and SAO [10,25–27]. To explore the relationships between band 3 gene mutations, dRTA and SAO, we studied 13 Malaysian and PNG dRTA patients from 9 families with dRTA or both dRTA and SAO, and 8 other subjects with SAO alone. We describe here three different band 3 mutations associated with dRTA in families from Malaysia and PNG: $Ala^{858} \rightarrow Asp$ (A858D), $Gly^{701} \rightarrow Asp$ (G701D) and deletion of Val^{850} (ΔV850). The A858D and ΔV850 changes have not previously been

Abbreviations used: DIDS, 4,4′-di-isothiocyanostilbene-2,2′-disulphonate; dRTA, distal renal tubular acidosis; GPA, glycophorin A; kB3, kidney band 3; PNG, Papua New Guinea; SSCP, single-strand conformation polymorphism; SAO, South-East Asian ovalocytosis.

[1] To whom correspondence should be addressed (e-mail m.tanner@bristol.ac.uk).

reported. G701D and ΔV850 have autosomal recessive inherit ance but segregate as though dominant when the B3 SAO allele is present, whereas A858D has autosomal dominant inheritance. Studies of the expression and function of the abnormal proteins in red cells and *Xenopus* oocytes suggest that the mutations exert different effects on the biosynthesis, structure and function of band 3 that lead to defective acid secretion in the kidney.

MATERALS AND METHODS

Patients

Three families with dRTA and SAO from Malaysia (Table 1, families A, B and H) and six families from PNG (Table 1, families C–G and J) were studied. The family trees are shown in Figure 1. The Malaysian families were racial Malays from separate states in central peninsular Malaysia; PNG families were all from the malarious northern coastal region of PNG. Family histories gave no evidence of consanguinity, nor were any of the families known to be related.

The diagnosis of dRTA was usually made in children under 5 years of age, who presented with failure to thrive and commonly with rickets. Growth milestones were delayed and most children had body weights and heights under the third percentile. Thirst and tachypnoea were common features. dRTA was not diagnosed until adult life in three patients: E:II:1 (ΔV850/SAO) aged 19 with hypokalaemic paresis, systemic acidosis and symptoms of renal stone; and H:I:2 aged 29 and J:I:1 aged 36 (both A858D/N), each of whom was the symptomless parent of a severely affected dRTA infant (see below). Blood studies of acidotic patients showed hyperchloraemia and hypokalaemia; two patients (C:II:1 and E:II:1) had episodes of hypokalaemic paralysis. Plasma bicarbonate, when this could be measured, was decreased to 5–15 mmol/l. Nephrocalcinosis was shown in six patients, by renal ultrasound or radiography (Table 1).

The complete dRTA syndrome was diagnosed in subjects with a urinary pH over 6.0 (usually more than 6.5) despite systemic acidosis. Normal urinary acidification was diagnosed in subjects whose early morning random urine pH was under 6.0 [28] and in subjects who lowered their urine pH to less than 5.5 after challenge with frusemide/fludrocortisone or NH_4Cl [29–31]. Subjects H:I:2 and J:I:1, symptomless and non-acidotic parents of children with the complete syndrome, did not lower their urinary pH below 6.5 on frusemide/fludrocortisone challenge, and were diagnosed as incomplete dRTA [29,32].

Most dRTA children were anaemic, with haemoglobin values below 10.8 g/dl (Table 1). Two of the three Malaysian children (A:II:3, B:II:2; G701D/SAO) had more profound anaemia, with haemoglobin values down to 5 g/dl despite repeated transfusions; both had splenomegaly, reticulocyte counts up to 5% and nucleated red cells in their blood films, suggesting that

Table 1 Clinical details of dRTA and band 3 mutations present in selected family members

Origins: Mal, Malaysia, PNG, Papua New Guinea. Band 3 mutations: N indicates a normal allele; SAO indicates the presence of both the K56E and ΔSAO (Δ1198–1224) mutations. An asterisk indicates that all exons of the band 3 genes in these individuals were examined by DNA sequencing or SSCP; for all other subjects the presence of the mutations was confirmed but not all exons of the band 3 gene were examined. The DNA sequence alterations corresponding to each amino acid sequence change were: M31T, t92c; D38A, a123c; K56E, g166a; G701D, g2102a; ΔV850, Δ2548–2550; A858D, c2573a. Blood films: SAO indicates the presence of stomatocytic ovalocytes characteristic of SAO. See the text for a description of films described as abnormal. Ages shown are the current age or the age at presentation for affected subjects (fractions represent the ages of children who are 12 months old or younger). Normal plasma K^+ values are 3.5–5 mmol/l. Abbreviations: n.t., not tested; M, methionine; T, threonine; K, lysine; E, glutamic acid; G, glycine; D, aspartic acid; A, alanine; c, cytosine; a, adenine; t, thymine; g, guanine.

dRTA mutation	Subject no.	Origin	Sex/age (years)	Band 3 mutations present	Blood film	Hb (g/dl)	dRTA	Rickets	Nephrocalcinosis	Initial plasma K^+ (mmol/l)
G701D/SAO	A:I:1	Mal	M/42	M31T,K56E,G701D/N	N	14.0	Absent	—	n.t.	n.t.
	A:I:2	Mal	F/43	SAO/D38A	SAO	12.6	Absent	—	n.t.	n.t.
	A:II:3	Mal	F/4	M31T,K56E,G701D/SAO*	SAO	5.2	Complete	+	Present	2.2
	B:I:1	Mal	M/30+	M31T,K56E,G701D/N	N	14.2	Absent	—	n.t.	n.t.
	B:I:2	Mal	F/30+	SAO/D38A	SAO	12.2	Absent	—	n.t.	n.t.
	B:II:2	Mal	$F/1^{10}/_{12}$	M31T,K56E,G701D/SAO*	SAO	9.8	Complete	+	Present	3.3
	B:II:3	Mal	$M/^{7}/_{12}$	M31T,K56E,G701D/SAO	SAO	10.4	Complete	—	Present	2.9
ΔV850	C:I:1	PNG	M/43	ΔV850/N	N	n.t.	n.t.	n.t.	n.t.	n.t.
	C:I:2	PNG	F/35	ΔV850/K56E	N	n.t.	n.t.	n.t.	n.t.	n.t.
	C:II:1	PNG	M/2	ΔV850/ΔV850*	N	15.0	Complete	+	Absent	2.3
	C:II:2	PNG	M/14	ΔV850/K56E	N	n.t.	n.t.	n.t.	n.t.	n.t.
	C:II:3	PNG	F/10	K56E/N	N	9.4	n.t.	n.t.	n.t.	n.t.
ΔV850/SAO	D:I:1	PNG	M/29	SAO/N	SAO	14.3	n.t.	—	Absent	n.t.
	D:I:2	PNG	F/27	ΔV850/D38A	N	12.8	Absent	—	n.t.	n.t.
	D:II:1	PNG	$M/^{12}/_{12}$	ΔV850/SAO*	SAO	11.1	Complete	—	Absent	2.5
	D:II:2	PNG	M	SAO/D38A	SAO	9.5	n.t.	—	n.t.	n.t.
	E:II:1	PNG	M/19	ΔV850/SAO*	SAO	11.5	Complete	—	Present	2.3
	F:I:1	PNG	M	SAO/N	SAO	11.9	n.t.	—	n.t.	n.t.
	F:I:2	PNG	F	ΔV850/N	N	11.7	n.t.	—	n.t.	n.t.
	F:II:1	PNG	F	ΔV850/N	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	F:II:2	PNG	$M/^{12}/_{12}$	ΔV850/SAO	SAO	8.1	Complete	+	n.t.	1.6
	G:II:1	PNG	M/2	ΔV850/SAO	SAO	9.2	Complete	+	n.t.	3.2
A858D/SAO	H:I:1	Mal	M/30+	SAO/N	SAO	13.7	Absent	—	n.t.	n.t.
	H:I:2	Mal	F/29	A858D/N	Abnormal	12.7	Incomplete	—	Absent	3.9
	H:II:2	Mal	$F/^{7}/_{12}$	A858D/SAO*	Abnormal	8.5	Complete	+	Present	2.9
ΔV850/A858D	J:I:1	PNG	M/36	A858D/N	N	13.8	Incomplete	—	Absent	4.0
	J:I:2	PNG	F/35	ΔV850/N	N	11.5	Absent	—	Absent	3.8
	J:II:1	PNG	F/4	ΔV850/A858D*	Abnormal	10.7	Complete	+	Present	2.4
	J:II:2	PNG	F/4	ΔV850/N	N	10.9	n.t.	—	n.t.	n.t.
	J:II:3	PNG	$F/^{5}/_{12}$	ΔV850/A858D	Abnormal	7.7	Complete	—	n.t.	3.6

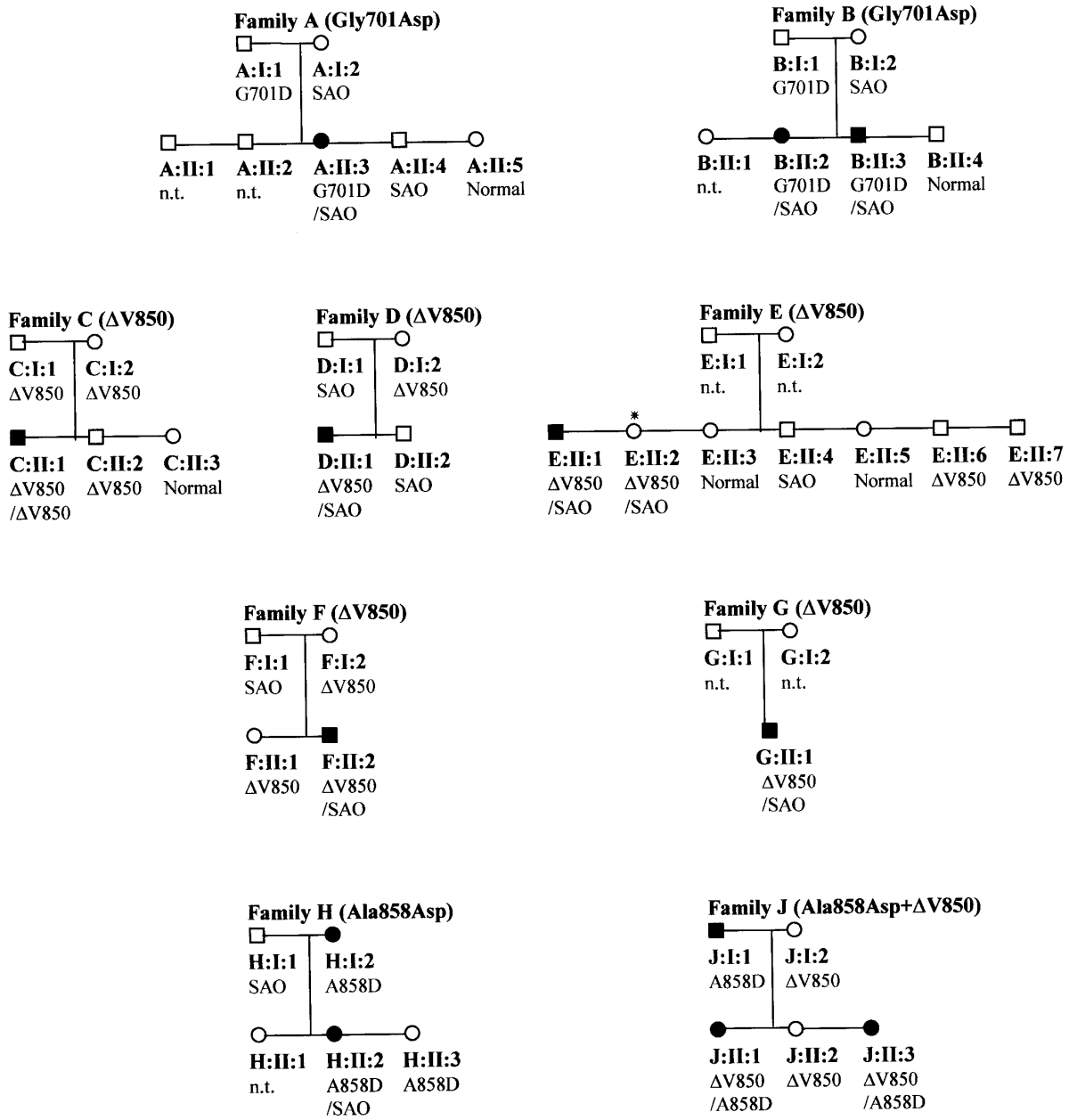

Figure 1 Family trees of dRTA patients examined in this study

The inheritance of the SAO deletion and the dRTA-associated band 3 mutations (G701D, ΔV850 and A858D) are shown. Where only one allele is shown, the other allele is normal (K56E [18–20], D38A [40] or the most common form of band 3 [36]). Families A, B and H were from Malaysia; families C–G and J were from PNG. Malaysian families were from separate states in peninsular Malaysia; PNG families were all derived from the malarious northern coastal region of PNG. Family histories gave no evidence of consanguinity, nor were any of the families known to be related. Filled symbols (■,●) denote individuals affected by dRTA. *E:II:2 is almost certainly affected by dRTA but this individual was not assessed for an acidification defect and is no longer traceable. Abbreviation: n.t., not tested.

haemolysis contributed to this anaemia. Red-cell morphology was normal in G701D/N, ΔV850/N, A858D/N and ΔV850/ΔV850 band 3 genotypes. All other genotypes showed SAO or abnormal blood films (Table 1, and discussed below).

Micropipette assay of red-cell membrane rigidity

The red cells in whole blood diluted 1:1000 in PBS containing 5% (v/v) autologous plasma were studied. The membrane shear elastic modulus was measured by aspirating a membrane tongue from the flattened side of the red cell into a micropipette (internal diam. 1.5 μm) by hydrostatic pressure [33]. Eight cells were measured from each sample at room temperature.

Red-cell membrane protein analysis

Membranes were prepared from untreated or chymotrypsin-treated red cells [34] and treated with peptide N-glycosidase F [3]. The membrane proteins were separated by SDS/PAGE and stained with Coomassie Blue. The amount of band 3 N-terminal chymotryptic fragment was assessed by scanning densitometry. Membranes were immunoblotted with murine monoclonal anti-

bodies: BRIC 163, directed against glycophorin A (GPA), or BRIC 170 and BRIC155, directed against band 3 [35].

Analysis of the band 3 gene by single-strand conformation polymorphisms (SSCPs) and DNA sequencing

Genomic DNA was isolated from blood samples by using Isocode Stix (Schleicher and Schuell, Dassel, Germany) or buffy coats. The coding regions of exons 2–20 [36], the putative kidney promoter (in intron 3), and kidney translation initiator codons of the human band 3 gene [8], were analysed for SSCPs and by DNA sequencing as described previously [3].

Anion transport studies

Sulphate transport studies

The number of 4,4′-di-isothiocyanostilbene-2,2′-disulphonate (DIDS)-binding sites was determined by titration of $^{35}SO_4^{2-}$ influx into red cells, using equal numbers of dRTA and control cells (determined with a cell counter) at 10% haematocrit [3,23]. $^{35}SO_4^{2-}$ efflux was measured from red cells that had been pre-equilibrated with 4 mM SO_4^{2-}; the flux (J_A) was calculated [37].

Chloride efflux assay

$^{36}Cl^-$ efflux was measured [38] from red cells that had been pre-equilibrated with 165 mM Cl^-.

Preparation of mutant constructs and expression in *Xenopus* oocytes

The cDNA clones encoding human band 3 (BSXG1.B3), the kidney isoform, kB3 (BSXG1.KB3) and glycophorin A (BSXG.GPA) have been described previously [3,39]. The BSXG1.B3 clone was used to construct the G701D, A858D and ΔV850 mutants by using the Seamless Cloning Kit (Stratagene, La Jolla, CA, U.S.A.). The kB3 mutants were prepared by substituting the *Xba*I–*Sal*I fragments of the BSXG1.B3 mutants into BSXG1.KB3. The kB3-SAO construct was derived from BSXG1.B3 SAO [24]. The methods used for the preparation of band 3 cRNA, expression in oocytes, assay of $^{36}Cl^-$ uptake, and estimation of cell-surface expression of band 3 in oocytes have been described previously [3,39].

RESULTS

SAO alone does not cause dRTA

Eight individuals with simple SAO (SAO/N) were examined: three from Malaysia, four from PNG and one from South Africa. The SAO status of all subjects was ascertained from the characteristic red-cell morphology in blood films. The presence of the SAO deletion was confirmed by PCR, SSCP or DNA sequencing in six of the subjects.

Urine pH was tested with different methods on different occasions. Four individuals produced early morning random urine pH values of 5.87, 5.71, 5.49 and 5.94. (Normal urinary pH < 6.0 in individuals tested in this way [28].) Three individuals produced a minimum urine pH of 5.12, 5.10 and 4.82 after frusemide/fludrocortisone treatment and one individual produced a minimum urine pH of 4.63 after NH_4Cl treatment. (Normal urinary pH < 5.5 in individuals tested by frusemide/fludrocortisone or NH_4Cl [29–31]). All these subjects with simple SAO therefore had normal ability to acidify their urine and did not have dRTA. These findings confirm previous observations by ourselves [3] and others [10,15] that the heterozygous presence of B3 SAO is not sufficient to cause dRTA.

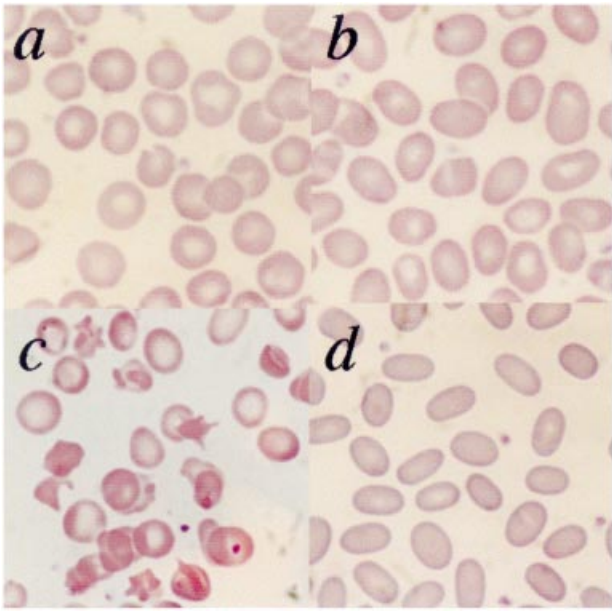

Figure 2 Morphology of red cells

(**a**) Normal discoid red cells ΔV850/ΔV850 (C:II:1). (**b**) SAO/N red cells (D:I:1). (**c**) Abnormal red cells ΔV850/A858D showing microcytes, elliptocytes and poikilocytes (J:II:3). (**d**) Small, elliptocytic red cells A858D/SAO (H:II:2).

Table 2 Shear elastic modulus of red cells from families A, B and H

The normal control value was 6.5×10^{-3} dyn/cm [20,33] (1 dyn $= 10^{-5}$ N). Results are means ± S.E.M. for measurements on eight red cells from each donor. $^{**}P = 0.02$, $^{*}P = 0.04$, †$P = 0.06$ for comparisons between the compound heterozygotes and SAO/N subjects in each family by Student's *t* test.

Subject	Mutation	$10^3 \times$ Shear elastic modulus (dyn/cm)
Control		6.5 ± 0.8
A:I:1	G701D/N	8.4 ± 1.7
A:I:2	SAO/N	18.1 ± 5
A:II:3	G701D/SAO	29.6 ± 10.1*
B:I:1	G701D/N	6.2 ± 0.6
B:I:2	SAO/N	14.8 ± 3.7
B:II:3	G701D/SAO	22.5 ± 9.45†
H:I:2	A858D/N	5.4 ± 0.65
H:I:1	SAO/N	16.3 ± 7.5
H:II:2	A858D/SAO	25.5 ± 6.8**

Mutations in the band 3 gene associated with dRTA

To explore the relationships between mutations in the band 3 gene, dRTA and SAO we studied 13 Malaysian and PNG dRTA patients from 9 families with dRTA, or both dRTA and SAO (Figure 1). Three different band 3 mutant alleles (G701D, ΔV850 and A858D) were found in the families with dRTA, either in association with each other or with the SAO allele (Table 1 and Figure 1). The D38A (Darmstadt) band 3 mutation [40] was present in families A, B and D but did not segregate with the

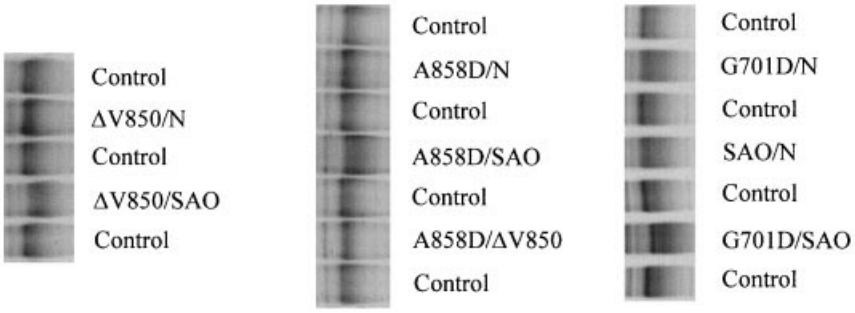

Figure 3 SDS/PAGE of band 3 in red cells of each genotype

Membranes were prepared and separated by SDS/PAGE [8% (w/v) gel], then stained with Coomassie Blue. Compound heterozygotes (G701D/SAO, A858D/SAO and ΔV850/SAO) show broader bands of lower mobility than the band 3 in normal (control) or SAO (SAO/N) red cells. The compound heterozygote (A858D/ΔV850) has a slightly lower mobility than the band 3 in normal red cells (control). The top of each gel is to the right of the figure.

Table 3 Studies on red cells

SO_4^{2-} influx and DIDS-binding sites were measured [23] and influx per band 3 was calculated. The S.E.M. for three replicates is shown for SO_4^{2-} influx. Cl^- efflux (marked *): $^{36}Cl^-$ efflux was measured from red blood cells pre-equilibrated with 165 mM Cl^- and the flux, J_A, was determined [38]. SO_4^{2-} efflux (marked †): $^{35}SO_4^{2-}$ efflux was measured from red blood cells pre-equilibrated with 4 mM SO_4^{2-} and J_A was determined [37]. The amount of band 3 N-terminal chymotryptic fragment was assessed by scanning densitometry of Coomassie-stained SDS/PAGE gels.

		DIDS titration of SO_4^{2-} influx (% of control)			Efflux experiments (% of control)			Band 3 protein component (%)	
Sample	Band 3 mutations	SO_4^{2-} influx	DIDS sites per cell	Influx per Band 3 molecule	SO_4^{2-} efflux	Cl^- efflux	Efflux per band 3 molecule	63 kDa fragment	60 kDa fragment
A:I:1	G701D/N	98 ± 7	95	103				45	55
A:I:2	SAO/D38A	43 ± 1	55	78				44	56
A:II:3	G701D/SAO	51 ± 6	51	100				100	
D:I:1	SAO/N	42 ± 2	56	75		42	75*		
D:I:2	ΔV850/D38A	79 ± 2/82 ± 2	76/84	104/98		82	103*		100
D:II:1	ΔV850/SAO	20 ± 3/19 ± 1	38/33	53/58		14	40*	51	49
D:II:2	SAO/N	42 ± 1	54	78				45	55
H:I:1	SAO/N	43 ± 2	55	75	42		76†	43	57
H:I:2	A858D/N	65 ± 3	82	80	71		86†		100
H:II:2	A858D/SAO	6 ± 1	36	17	3		8†	63	37
J:I:1	A858D/N	62 ± 2	79	78		63/68	80/86*		100
J:I:2	ΔV850/N	81 ± 2/80 ± 1	85/80	95/100					100
J:II:1	ΔV850/A858D	32 ± 1	59	54		26/27	44/46*		100
J:II:2	ΔV850/N	76 ± 1	80	95					100
J:II:3	ΔV850/A858D	28 ± 1	51	55					100

disease. The K56E (B3 Memphis) mutation [18–20] was always found linked to the SAO allele but was also present independently in families A, B and C. Both D38A and K56E polymorphisms are apparently innocuous and are common in oriental populations [9,40,41].

G701D mutation

Two Malaysian families (A and B) have the same mutant band 3 alleles transmitted in the same fashion. In both families dRTA is associated with inheritance of the mutations M31T, K56E and G701D (described here as the G701D allele) together with the transport-inactive SAO allele (G701D/SAO). Because kB3 lacks residues 1–65 of band 3, the kidney proteins carry only either the G701D mutation or the SAO deletion. The G701D/SAO compound heterozygotes had features of haemolytic anaemia but the red cells had the usual SAO morphology (Table 1).

ΔV850 deletion

The mutation ΔV850 (in which the three nucleotides corresponding to Val^{850} of band 3 are deleted) was present in six dRTA families from PNG. In family C, the affected individual (C:II:1) was homozygous for the ΔV850 mutation, whereas in families D, E, F and G the disease was present only in individuals who also had the SAO allele (ΔV850/SAO). Although dRTA associated solely with the ΔV850 mutation shows autosomal recessive inheritance, when the ΔV850 mutation is present as a compound heterozygote with the transport-inactive SAO allele it shows pseudo-dominant inheritance, like the G701D mutation. The dRTA individual homozygous for ΔV850 (C:II:1) had normal blood films and was not anaemic (Figure 2). The ΔV850/SAO compound heterozygotes had red cells with typical SAO morphology; two of the children (F:II:2 and G:II:1) had low haemoglobin values (Table 1). In family J, compound

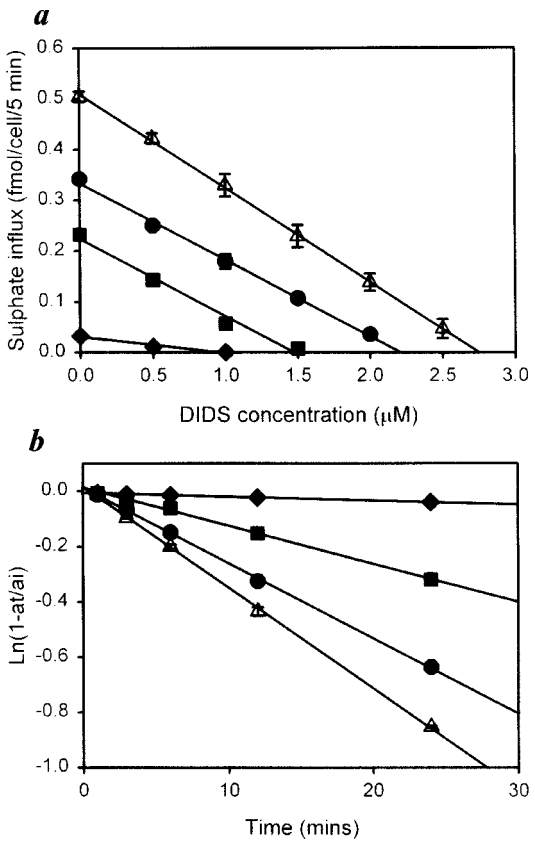

Figure 4 Anion transport studies

Representative examples of the SO_4^{2-} influx (**a**) and efflux (**b**) experiments showing the data from the Malaysian family H: ■, father of family H (SAO/N) (H:I:1); ●, mother of family H (A858D/N) (H:I:2); ◆, child of family H (A858D/SAO) (H:II:2); △, normal control red cells. Each data point was derived from measurements in triplicate. The lines show the results of linear regression analysis of the data. (**a**) DIDS titration of SO_4^{2-} influx. The influx of $^{35}SO_4^{2-}$ into the cells was measured at 10% haematocrit in isotonic citrate buffer [84 mM sodium citrate/1 mM EGTA (pH 6.5)] containing 4 mM Na_2SO_4. Influx was determined after 5 min at 30 °C in the presence of different concentrations of DIDS as described previously [23]. (**b**) SO_4^{2-} efflux. Red cells were equilibrated in citrate buffer containing 4 mM Na_2SO_4 and tracer $^{35}SO_4^{2-}$; the efflux of SO_4^{2-} was determined over 24 min at 30 °C as described previously [37].

heterozygotes of the ΔV850 and A858D mutations had the complete form of dRTA (see below).

A858D mutation

The Malaysian family H carried the A858D mutation and the SAO allele, whereas the PNG family J carried both the A858D and ΔV850 mutations. The simple heterozygotes (A858D/N; H:I:2 and J:I:1) had incomplete dRTA (they were unable to acidify their urine when tested with frusemide/fludrocortisone but had no other feature of dRTA). This demonstrates the autosomal dominant nature of the A858D mutation. Complete dRTA was present in the compound heterozygote A858D/SAO (H:II:2) and in the two children who were compound heterozygotes for A858D/ΔV850 (J:II:1 and J:II:3). The compound heterozygote A858D/SAO did not have the usual SAO red-cell morphology; instead, the cells were small and elliptocytic (Figure 2). However, this might not simply be due to the band 3 mutations present because the mother (H:I:2, A858D/N) had acanthocytic red cells. It is possible that these acanthocytes were an artifact of the blood film, relating to the condition of the blood sample when the film was made; equally, the mother (H:I:2, A858D/N) might have a further red-cell abnormality because an unrelated A858D/N subject (J:I:1) had normal red cells. The compound heterozygote A858D/ΔV850 patients both showed bizarre red-cell morphology with microcytes, elliptocytes and poikilocytes (Figure 2). All three A858D/SAO and A858D/ΔV850 patients (H:II:2, J:II:1 and J:II:3) had low haemoglobin values, suggesting increased haemolysis.

DNA sequences of the region of the kB3 promoter

The DNA sequence of the kidney promoter [8] (from the 5′ end of erythroid intron 3 to the 3′ end of kidney exon 3A [36]) was also examined. Polymorphisms c87t, c242t, g259a and a580g (numbered from the 5′ end of intron 3) were found but were not associated with dRTA. However, the c242t and g259a changes were correlated with the K56E Memphis polymorphism (and SAO).

Studies on red cells

Red-cell studies were performed where suitable fresh blood samples were available.

Red-cell membrane rigidity

The shear elastic modulus of red-cell samples from families A, B and H was measured with a micropipette method (Table 2). The simple heterozygotes G701D/N (A:I:1, B:I:1) and A858D/N (H:I:2) gave normal values, similar to the value of 6.5×10^{-3} dyn/cm (1 dyn = 10^{-5} N) obtained for a normal control and reported previously [20,33]. The SAO/N samples from the families gave significantly higher values [$(14.8–18.1) \times 10^{-3}$ dyn/cm], indicating increased membrane rigidity, as reported previously [20]. Interestingly, the red cells of A:II:3 and B:II:3 (G701D/SAO) and H:II:2 (A858D/SAO) had an even higher shear elastic modulus [$(22.5–29.6) \times 10^{-3}$ dyn/cm], and thus even greater membrane rigidity, than their SAO parents.

Analysis of red-cell membrane band 3 in the families

SDS/PAGE of the band 3 in the red cells of all the individuals with complete dRTA gave bands of slightly lower mobility than the band 3 of normal individuals or simple SAO/N heterozygotes (Figure 3). This shift in mobility was more evident in the 35 kDa C-terminal fragment of band 3 that was obtained after the treatment of red cells with chymotrypsin (results not shown). Treatment of the membranes with peptide N-glycosidase F resulted in deglycosylated 35 kDa fragments with the same mobility, suggesting that the altered mobility was due to altered N-glycosylation (results not shown). Similar N-glycan-dependent changes in mobility have been observed in band 3 with mutations at Arg^{589} that result in dRTA [3]. Immunoblotting with an anti-GPA antibody showed that GPA was present in all the red-cell samples (results not shown).

Band 3 proteins carrying the K56E mutation (B3 Memphis) can be distinguished from other band 3 proteins by SDS/PAGE of chymotrypsin-treated red cells [42]. B3 Memphis yields a 63 kDa fragment, whereas normal band 3 yields a 60 kDa fragment. In appropriate heterozygotes the relative expression of B3 Memphis (in K65E, SAO and G701D alleles) was measured and compared with the expression of band 3 with normal mobility (in normal, D38A, ΔV850 and A858D alleles) (Table 3). As noted previously [20] in SAO heterozygotes, the SAO protein

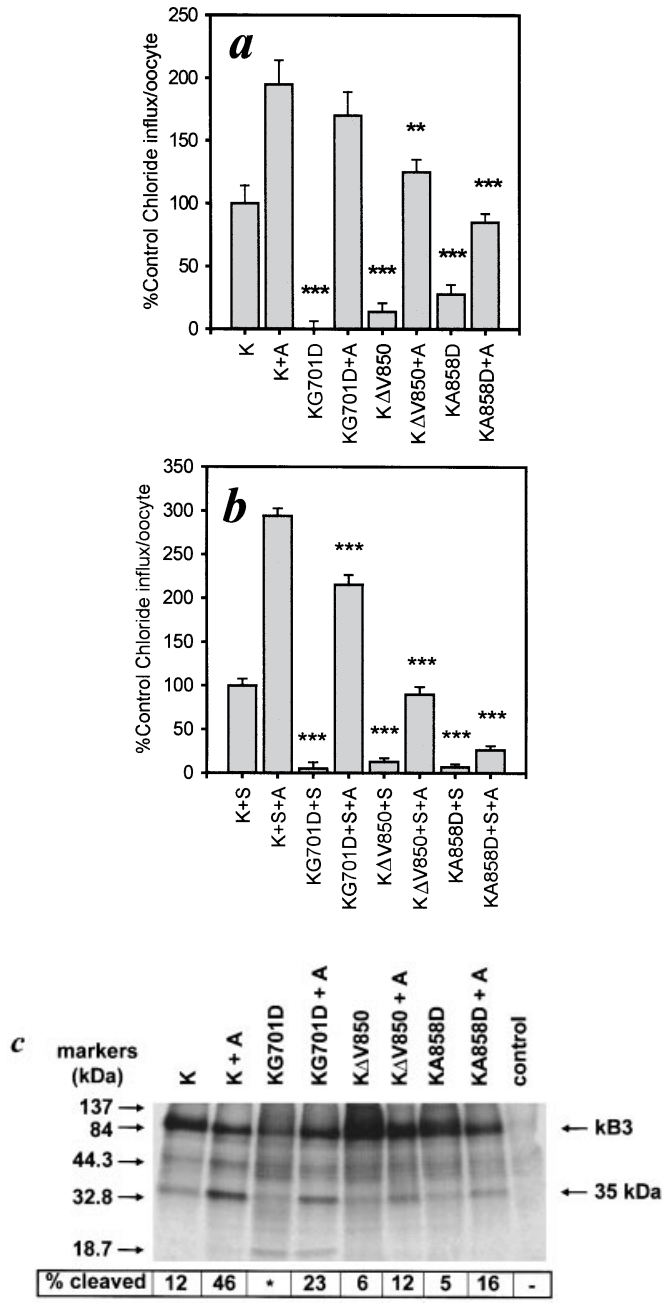

Figure 5 Expression of normal and variant kB3 constructs in *Xenopus* oocytes

(**a**, **b**) Cl^- influx studies. Oocytes were injected with cRNA species as indicated. After 24 h the 4,4′-dinitrostilbene-2,2′-disulphonate-sensitive Cl^- influx was measured for 1 h. Results are expressed as percentages of the Cl^- influx obtained with normal kB3, and are shown as means ± S.E.M. (12–15 oocytes). $^{**}0.002 < P < 0.005$; $^{***}P < 0.001$ for comparisons between normal and variant proteins by Student's *t* test. Where GPA (A) or SAO (S) cRNA species were co-injected, the variant is compared with the equivalent control. (**a**) Cl^- influx induced by 1.5 ng of kB3 (K) or variant kB3 cRNA (KG701D, KΔV850 or KA858D) with or without 1.5 ng of GPA (A). (**b**) Cl^- influx induced by co-expression of 0.75 ng of normal (K) or variant kB3 cRNA (KG701D, KΔV850 or KA858D) with 0.75 ng of kB3-SAO (S), with or without 1.5 ng of GPA (A). (**c**) Immunoprecipitation of kB3 from chymotrypsin-treated oocytes. Oocytes were injected with 1.5 ng of kB3 (K) or variant kB3 cRNA (labelled as above) and 0.15 ng of GPA (A) cRNA as indicated. Control cells were injected with water. Oocytes were pulse-labelled with ^{35}S-labelled amino acids for 24 h and chased for 24 h, then treated with chymotrypsin [39]. The protein was immunoprecipitated with antibody BRIC 155 (against the C-terminus of band 3) and separated by SDS/PAGE [10% (w/v) gel]. The proportion of kB3 cleaved by chymotrypsin (35 kDa fragment) was determined [39] and is shown below the gels. The asterisk indicates that intracellular degradation of the mutant protein did not allow meaningful quantitative comparison.

was consistently present at a lower abundance (approx. 80%) than normal band 3 or the D38A protein. The G701D protein was present at approx. 80% of normal band 3 in G701D/N cells (A:I:1). The ΔV850 protein was expressed at a level similar to that of the SAO protein in ΔV850/SAO red cells (D:II:1) but the A858D protein was much less abundant (55%) than the SAO protein in A858D/SAO cells (H:II:2) (Table 3).

Red-cell anion transport studies

Titration of red-cell SO_4^{2-} influx with DIDS [23] was used to measure anion transport and the DIDS-binding sites (which can be used to estimate the amount of band 3 protein present) in the mutant red cells (Table 3 and Figure 4a). The SO_4^{2-} influx per DIDS site (relative to normal control cells) was used to estimate the specific anion transport activity of the mutant proteins. The SAO protein does not bind DIDS or perform anion transport [23]. Although normal band 3 comprised 55% of the band 3 in SAO/N cells, these cells consistently showed 42–43% of normal anion transport, as noted previously [23]. Thus, under the conditions of the transport assay, the activity of the normal band 3 in SAO red cells is decreased to 75–78% of its activity in normal cells. In contrast, the G701D band 3 had 100% of the activity of normal band 3, whether present with normal band 3 (A:I:1) or with B3 SAO (A:II:3).

The ΔV850/N (J:I:2, J:II:2) and ΔV850/D38A (D:I:2) red cells had total band 3 decreased to approx. 80% of that of normal red cells but the activity of the protein was normal. The activity of ΔV850 band 3 was markedly decreased (to 55% of the normal protein) when present with B3 SAO (D:II:1). The A858D/N heterozygotes (H:I:2, J:I:1) also had a decreased amount of band 3 (80% of normal), and the activity of band 3 per molecule was decreased to 80% of normal (Figure 4a). When the SAO protein was present, the A858D protein had an anion transport activity per molecule that was only 17% of that of the normal protein. A858D/SAO red cells (H:II:2) showed only 6% of the SO_4^{2-} influx activity (Figure 4a) and 3% of the SO_4^{2-} efflux activity of normal red cells (Figure 4b). The difference in the influx and efflux activities of these cells might be due to the different conditions of the assays. The ΔV850/A858D patients (J:II:1, J:II:3) had much less total band 3 (55%) than normal cells and the overall activity per molecule of band 3 was also decreased to 55% of normal. These red cells had only 30% of the SO_4^{2-} transport activity of normal red cells. SO_4^{2-} efflux and Cl^- efflux studies, where done, gave results that were in broad agreement with the SO_4^{2-} influx studies (Table 3 and Figure 4b).

Chloride transport activity of the expressed mutant kB3 proteins and effects of GPA

We examined the stilbene disulphonate-sensitive Cl^- transport activity of kB3 constructs carrying the G701D, ΔV850 and A858D mutations expressed in *Xenopus* oocytes (Figure 5a) in the presence and the absence of GPA. GPA enhances the Cl^- transport activity of both red-cell band 3 and kB3 in oocytes by increasing their expression at the cell surface [3,39]. Without GPA, no Cl^- transport activity was found with the G701D construct, but a low Cl^- uptake was consistently obtained with the ΔV850 construct (14 ± 7% of normal kB3) and slightly more with the A858D construct (28 ± 8% of normal kB3). Transport activity was not detected with the G701D construct, even when 10-fold more cRNA was used (15 ng/oocyte) and the oocytes were allowed to express the protein for 48 h (results not shown). When GPA was present, the transport in the G701D mutant increased almost to the level of normal kB3, whereas the transport

in the ΔV850 and A858D mutants was increased by the presence of GPA to 64±5% and 43±3% of that obtained with the normal protein.

To mimic the situation in the red cells of patients who were compound heterozygotes with the SAO allele, we co-expressed kB3 containing the SAO deletion with an equal amount of each of the other kB3 mutants and GPA (Figure 5b). Expressed with GPA, the G701D+SAO mixture was almost as active as the normal kB3+SAO (73±4% of the normal kB3+SAO); the ΔV850+SAO combination also gave some activity (31±3% of the normal kB3+SAO). In contrast, the A858D+SAO mixture had very little activity even in the presence of GPA. This result suggests that the enhancement of the activity of the A858D mutant by GPA (and to a smaller extent the ΔV850 mutant) is suppressed by kB3 SAO. When equal amounts of the ΔV850 and A858D mutants were co-expressed, the results were not significantly different from the average of the measured transport activity when an equal amount of each mutant was expressed alone, whether GPA was present or not (results not shown).

Cell-surface movement of the mutant proteins in oocytes

We examined whether the reduced transport observed with the mutant proteins in oocytes resulted from their impaired movement to the cell surface (Figure 5c), with the use of a proteolysis-based assay [39]. Extracellular band 3 cleavage by chymotrypsin yields a C-terminal 35 kDa fragment that can be used to assess the proportion of kB3 at the oocyte cell surface [39]. Metabolically radiolabelled oocytes expressing kB3 were digested with chymotrypsin and the intact kB3 and 35 kDa fragment were immunoprecipitated with an antibody that binds to the band 3 C-terminus [39]. In the absence of GPA, much less ΔV850 or A858D than normal kB3 was present at the cell surface. There was also little or no G701D expressed at the cell surface but quantitative comparison was not possible because there was a substantial decrease in the total amount of G701D protein expressed in the cells (found in two independent experiments). GPA substantially enhanced the expression of normal kB3 and G701D at the cell surface but gave less enhancement of the ΔV850 and A858D expressed at the cell surface. Co-expression of GPA also resulted in an increase in the total amount of G701D protein expressed in the cells.

DISCUSSION

Band 3 mutations in oriental populations associated with dRTA

The oriental dRTA families studied here contained three different band 3 mutations, the properties of which are summarized in Tables 4 and 5. The ΔV850 and A858D mutations have not been described previously but two dRTA families carrying the G701D mutation have been found in Thailand [9,10]. The G701D and ΔV850 mutations show recessive inheritance of dRTA, which presents as pseudo-dominant inheritance in the presence of the SAO allele. The A858D mutation is dominant and the simple heterozygotes studied here have incomplete dRTA, an inability to acidify their urine but no accompanying features of dRTA [29,32]. None of our patients was homozygous for the A858D mutation, but the compound heterozygotes of ΔV850 and A858D showed full-blown dRTA.

Role of the SAO allele in dRTA in oriental populations

The association between SAO and dRTA has been noted several times [10,25–27]. It is clear from our own [3] and other studies [10,14] that the SAO allele does not itself give dRTA. It is evident that the association between SAO and dRTA exists because the transport-inactive SAO allele gives recessive dRTA mutations a pseudo-dominant phenotype when they are present as compound heterozygotes with the SAO allele. All three dRTA mutations were found as compound heterozygotes with SAO where they presented with complete dRTA.

Haematology of the dRTA mutants

Anaemia is not a recognized feature of dRTA. The ΔV850/ΔV850 phenotype showed normal red-cell morphology and haematology. In contrast, all the other phenotypes associated with complete dRTA showed anaemia, probably haemolytic in origin, especially in the children. These phenotypes also showed changes in the rigidity and morphology of the mutant red cells (see below). Although these changes could result in anaemia, other possible causes of anaemia in these populations, such as chronic infection, nutritional iron deficiency and hookworm infestation, cannot be ruled out.

The G701D/SAO red cells had the usual SAO morphology but were much more rigid than SAO cells; the condition was associated with anaemia. The A858D/SAO red cells were also more rigid than SAO red cells and were elliptocytic, although it is possible that there was another red-cell abnormality in this family. The reason for the increased rigidity is unclear but in both cell types the increased rigidity might contribute to the anaemia. We were unable to test the rigidity of the ΔV850/SAO red cells but this condition was also associated with anaemia. The ΔV850/A858D compound heterozygotes had red cells of bizarre shape (microcytes, elliptocytes and poikilocytes) and anaemia. The much-reduced band 3 content of these cells might decrease red-cell stability and cause haemolytic anaemia in a manner similar to band 3-deficient hereditary spherocytosis [43]. Homozygous G701D/G701D red cells have been reported to have abnormal shapes and associated haemolytic anaemia [9].

Anion transport properties of mutant red cells

The presence of the B3 SAO protein decreased the specific SO_4^{2-} influx activity of normal band 3 compared with its activity in normal red cells. A similar but more marked effect was observed when B3 SAO was present with the ΔV850 and A858D proteins. In contrast, the specific SO_4^{2-} influx activity of the G701D protein was the same in normal and in G701D/SAO red cells. This suggests that the normal, ΔV850 and A858D proteins interact with B3 SAO but the G701D protein might not.

The G701D protein was expressed in red cells at a level close to that of the normal protein. However, there were substantially smaller amounts of the ΔV850 and A858D proteins when B3 SAO was present. This, in combination with the lower activity of the proteins, resulted in very low anion transport activity in the ΔV850/SAO and A858D/SAO red cells. Indeed, the 6% of normal SO_4^{2-} influx activity and 3% of normal SO_4^{2-} efflux activity in the A858D/SAO red cells is the lowest anion transport activity so far reported for human red cells. The red cells of the ΔV850/A858D individual also had very low anion transport.

Activity of the expressed mutant proteins

Although red cells express GPA, there is no evidence for the presence of GPA in the kidney. GPA facilitates the movement of band 3 and kB3 to the oocyte cell surface [3,39]. The mutant kB3 proteins were expressed in *Xenopus* oocytes to assess their transport activity in the absence and the presence of GPA. The G701D protein did not induce any anion transport in the absence

Table 4 Summary of results in mutant red blood cells

Outline of significant properties of the dRTA mutant phenotypes compared with the properties of normal control (N/N) and SAO red cells (SAO/N). Rigidity studies: +, ++ and +++ indicate increasing rigidity of the red cell membrane. SDS/PAGE studies: +, ++ and +++ indicate increasing retardation of mobility of band 3 compared with the normal control. Red-cell transport properties: the SO_4^{2-} influx and DIDS sites per cell are the average, for each genotype, of the results shown in Table 3. Abbreviation: n.t.: not tested.

Genotype	dRTA	Red-cell morphology	Anaemia	Rigidity	SDS/PAGE band 3 mobility	SO_4^{2-} influx (% of normal)	DIDS sites per cell (% of normal)
N/N	Absent	Discoid	Absent	normal	normal	100	100
SAO/N	Absent	Stomatocytic ovalocytes	Absent	++	+	42	55
G701D/N	Absent	Discoid	Absent	normal	+	98	95
G701D/SAO	Complete	Stomatocytic ovalocytes	Present	+++	+++	51	51
ΔV850/N	Absent	Discoid	Absent	n.t.	+	80	81
ΔV850/ΔV850	Complete	Discoid	Absent	n.t.	n.t.	n.t.	n.t.
ΔV850/SAO	Complete	Stomatocytic ovalocytes	Present	n.t.	+++	20	35
A858D/N	Incomplete	Discoid (acanthocytes)*	Absent	normal	+	64	81
A858D/SAO	Complete	Elliptocytes	Present	+++	+++	6	36
A858D/ΔV850	Complete	Poikilocytes, microcytes, etc	Present	n.t.	++	30	55

* One A858D/N individual had normal discoid red cells and the other individual had acanthocytic red cells; in this case the acanthocytosis was possibly caused by a second red-cell abnormality.

Table 5 *Xenopus* oocyte expression studies on kidney band 3 mutant constructs

The amounts of Cl^- transport measured in *Xenopus* oocytes expressing each of the different mutant gene constructs are shown. For experimental details see the legend to Figures 5(a) and 5(b). Transport is expressed as a percentage of the relevant control for each of the four conditions. Abbreviations: kB3, kidney band 3; SAO, SAO band 3.

Construct	Cl^- transport (% of control)	
	−GPA	+GPA
kB3	100	100
kB3G701D	0	87
kB3ΔV850	14	64
kB3A858D	29	43
kB3 + SAO	100	100
kB3G701D + SAO	0	73
kB3ΔV850 + SAO	15	31
kB3A858D + SAO	10	10

of GPA but the transport activity was almost completely rescued by GPA. The A858D protein showed decreased but substantial transport activity in the absence of GPA, which was only partly rescued by GPA. The ΔV850 mutant behaved in a manner intermediate between the G701D and A858D proteins, showing a small but consistent transport activity when expressed alone, which was substantially but incompletely rescued by GPA. These results with GPA are consistent with the observations on the red-cell anion transport of the mutants.

There was clearly little or no cell-surface expression of the G701D protein in oocytes without GPA; the low recovery of intact protein in the oocyte immunoprecipitates suggests that the G701D protein was rapidly degraded by the oocytes in the absence of GPA. When GPA was present, the oocytes contained more G701D protein; a substantial proportion was present at the oocyte cell surface. These results are similar to those reported previously [9] and suggest that the cell-surface movement of band 3 occurs by distinct pathways in the presence and the absence of GPA. The structural change resulting from the G701D mutation clearly abolishes this GPA-independent pathway.

Interactions of the mutant proteins with band 3 SAO

Normal band 3 forms heterodimers with the SAO protein [44]; this is reflected in the decreased anion transport activity of the normal band 3 in SAO red cells. The ΔV850 and A858D mutant proteins also interact with the SAO protein because these both show diminished red-cell anion transport when present with the SAO allele. This lower activity is particularly evident in the A858D/SAO red cells. When co-expressed with kB3 SAO, the A858D protein showed very little enhancement of transport activity by GPA. In contrast, the anion transport activity of the G701D mutant was unaffected by the presence of the SAO protein, either in red cells or when co-expressed in oocytes, suggesting that G701D does not interact with the SAO protein to form heterodimers. This finding suggests that the G701D mutation might alter the structure of the band 3 membrane domain at the dimer interface.

Molecular basis for dRTA in the families studied

It is clear that the transport-inactive SAO gene behaves as a band-3-negative allele as far as kidney acid secretion is concerned. Our results confirm the suggestion that dRTA in the G701D/SAO individuals occurs because G701D protein cannot reach its functional site in the renal intercalated cell [9]. The dRTA in ΔV850/SAO and ΔV850/ΔV850 individuals probably arises from the very low anion transport activity in the basolateral membrane of the renal α-intercalated cells. The dominant A858D mutation might cause the disease by mis-targeting of the mutant protein to the apical instead of the basolateral membrane of the α-intercalated cells. This hypothesis has been suggested to account for the dominant phenotype of other dRTA mutations [2,3]. More severe complications of the disease seem to arise in the A858D/SAO and A858D/ΔV850 compound heterozygotes, in which the other mutant kB3 protein product (SAO and ΔV850, which are presumably expressed in the basolateral membrane) is inactive or has very little activity in the absence of GPA.

dRTA associated with band 3 mutations in oriental patients

The familial dRTA of our oriental patients was of greater clinical severity than reported occidental cases of familial dRTA. It is

not clear whether this is the result of the different mutations causing the disease, case selection, or other environmental or genetic factors. The incidences of nephrocalcinosis in the two groups are similar (approx. 70%) but metabolic bone disease and severe hypokalaemia were more a feature of the oriental patients; thus 73% of the oriental patients with complete dRTA had rickets, and their mean plasma [K^+] at presentation was 2.7 ± 0.6 mmol/l, whereas a recent survey of occidental patients (most subsequently shown to have band 3 mutations) found that only 25% had bone disease; mean plasma [K^+] at presentation was 3.6 ± 0.6 mmol/l [45]. Two of our oriental patients were sufficiently hypokalaemic to have general paralysis, which has not been reported in occidental familial dRTA [45].

The three different band 3 mutations reported here from oriental dRTA patients, affecting band 3 residues 701, 850 and 858, have not so far been reported in occidental cases of the disease, in whom most mutations have affected residue 589, usually with histidine substituting for arginine. No band 3 mutation, causally related to dRTA, has yet been reported in either occidental or oriental subjects. Our findings suggest that there might be a clustering of these newly discovered band 3 mutations in the orient: the 701 mutation has now been described in four families with dRTA from Malaysia and the immediately adjacent area of Thailand [10] and in a fifth family in the northeast part of Thailand [9], which is 700 miles distant; the ΔV850 mutation was found by us in six families in PNG; and we found the A858D mutation in two families in Malaysia and PNG, sites 3000 miles apart. All these areas have until very recent times been affected by endemic *Plasmodium falciparum* malaria, against which the band 3 mutation that gives rise to SAO affords some clinical protection [14,15]. There is also a high frequency of SAO in conjunction with the dRTA mutations (seven of the nine families that we studied). Our results raise the intriguing possibility that these newly discovered band 3 mutations might have evolved locally because they provide some protection against the clinical effects of *P. falciparum* malaria, as occurs with the band 3 mutation that gives rise to SAO [15].

We thank the Papua New Guinea Paediatric Surveillance Unit for its assistance in the collection of PNG cases; D. J. Anstee for monoclonal antibodies (BRIC 163, anti-GPA; BRIC 170 and BRIC 155, anti-band 3); R. Beckmann for the kB3 cDNA; R. C. Morris and Church & Dwight Co. (Princeton, NJ, U.S.A.) for travel support; C. S. Keng for help with photographic facilities and supplies; and Tan Sri Abubakr, R. Taufa and I. Kevau for their interest and support. This work was supported in part by grants from the National Kidney Research Fund and the Wellcome Trust. M.T.Y. was the recipient of a Wellcome Prize Studentship.

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Received 18 January 2000/14 April 2000; accepted 23 May 2000