Molecular Basis for Membrane Rigidity of Hereditary Ovalocytosis

A Novel Mechanism Involving the Cytoplasmic Domain of Band 3

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Abstract

Hereditary ovalocytic red cells are characterized by a marked increase in membrane rigidity and resistance to invasion by malarial parasites. The underlying molecular defect in ovalocytes remained a mystery until Liu and colleagues (*N. Engl. J. Med.* 1990. 323:1530–38) made the surprising observation that the ovalocytic phenotype was linked to a structural polymorphism in band 3, the anion transporter. We have now defined the mutation in band 3 gene and established the biophysical sequelae of this mutation. This mutation involves the deletion of amino-acids 400–408 in the boundary between the cytoplasmic and the first transmembrane domains of band 3. The biophysical consequences of this mutation are a marked decrease in lateral mobility of band 3 and an increase in membrane rigidity. Based on these findings, we propose the following model for increased membrane rigidity. The mutation induces a conformational change in the cytoplasmic domain of band 3, leading to its entanglement in the skeletal protein network. This entanglement inhibits the normal unwinding and stretching of the spectrin tetramers necessary for membrane extension, leading to increased rigidity. These findings imply that the cytoplasmic domain of an integral membrane protein can have profound effects on membrane material behavior. (*J. Clin. Invest.* 1992. 89:686–692.) Key words: red cells • band 3 • anion transporter • malaria

Introduction

Hereditary ovalocytosis, a red cell disorder, is widespread in Southeast Asia occurring in several different ethnic groups (1–3). Based on an epidemiologic study of Malayan aborigines from an area where malaria was endemic, Baer et al. (4) first proposed that the high incidence of hereditary ovalocytosis in this population (~ 30%) might represent yet another red cell variant genetically selected by its associated protection against malaria. Subsequent in vitro culture studies showed that these ovalocytes were indeed resistant to invasion by malarial parasites (5, 6). Further studies revealed that the resistance to parasite invasion was a consequence of a marked increase in the membrane rigidity of the ovalocytes (7, 8).

An extensive series of theoretical and experimental studies have shown that red cell membrane rigidity is primarily regulated by the organization of the spectrin-based membrane skeleton (9–12). It was therefore suggested that the increased membrane rigidity of ovalocytes was most likely to be a consequence of a mutation in one of the principal components of the skeletal network, namely spectrin, actin, ankyrin, and protein 4.1. However, detailed biochemical analysis failed to identify a defect in any of these skeletal proteins. The underlying molecular defect in ovalocytes remained a mystery until Liu et al. (13) made the surprising observation that the ovalocytic phenotype was linked to a structural polymorphism in band 3 protein, the red cell anion transporter. However, the mechanism by which a mutation in band 3 can induce increased membrane rigidity has yet to be defined.

In order to further our understanding of the molecular mechanism responsible for increased membrane rigidity of ovalocytes, we have defined the mutation in band 3 gene in several Malayan individuals, and established the biophysical sequelae of this mutation. The mutation involves the deletion of nine amino acids (residues 400–408) in the boundary between the cytoplasmic and the first transmembrane domains of band 3. The biophysical consequences of this mutation are a marked decrease in lateral mobility of band 3 in the membrane, and an increase in membrane extensional rigidity. Based on these findings, we propose the following model for increased membrane rigidity of the ovalocyte membranes. The mutation induces a conformational change in the cytoplasmic domain of band 3, leading to its entanglement in the skeletal protein network. This entanglement inhibits the normal unwinding and stretching of the spectrin tetramers necessary for membrane extension, leading to increased extensional rigidity. Previous studies have demonstrated that membrane deformability and mechanical stability are affected by interactions among the peripheral skeletal proteins of the membrane skeleton. Our current findings imply that the interactions between the cytoplasmic domain of an integral membrane protein and the skeletal network can likewise have profound effects on membrane material behavior.

Methods

Reagents. α-^{32}P-dCTP and α-^{35}S-dATP were obtained from Amersham Corp. (Arlington Heights, IL). Reverse transcriptase was supplied by

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Gibco-BRL (Bethesda, MD), Taq polymerase by Perkin Elmer-Cetus (Norwalk, CT), Sequenase by US Biochemical Corp. (Cleveland, OH) and the plasmid vector Bluescript KS(+) by Stratagene Inc. (La Jolla, CA). Eosin-5-maleimide was purchased from Molecular Probes, Inc. (Eugene, OR).

Blood was obtained from six unrelated Malayan individuals living in Kuala Lumpur who had been previously identified, on the basis of red cell morphology, as having hereditary ovalocytosis. Blood drawn into acid citrate dextrose was shipped on ice from Kuala Lumpur to San Francisco. The delay from the time the blood was drawn to the time of analysis was less than 72 h. Blood samples from normal volunteer donors from Kuala Lumpur and Berkeley served as controls.

Reverse transcription/Polymerase chain reaction (RT/PCR)[1] *analysis of band 3 mRNA.* Reticulocyte RNA was prepared as described previously, (14) from the blood of patients with ovalocytosis, normal individuals, and from a sickle-cell patient undergoing exchange transfusion. Approximately 1 μg total RNA was reverse-transcribed into first-strand cDNA using random hexanucleotide and M-MLV RTase, and 20% of this reaction mixture was amplified by PCR, using Taq DNA polymerase under the following conditions: (*a*) initial denaturation at 94°C for 5 min; (*b*) 35 cycles of denaturation at 94°C for 30 s; (*c*) annealing at 50°C for 30 s; (*d*) elongation at 72°C for 2 min; and (*e*) final extension at 72°C for 10 min. Amplified DNA fragments were analyzed by 5% PAGE. PCR primers used in this study and their positions on the nucleotide sequence are as follows:

			exon
p1	5′ AACTGGACACTCAGGACCACG 3′	(−23 to −3)	1 (non-coding)
p2	5′ GTACTGTTCTCCTAGACCTG 3′	(349 to 368)	6
p3	5′ CTGCTCACAGAAGAGCTGTG 3′	(608 to 589)	7
p4	5′ CTGAATGCATCTGTGATGTCAC 3′	(1205 to 1183)	11
p5	5′ TTCCGCATAGATGCCTACATG 3′	(879 to 899)	10
p6	5′ TGCAGTGGAGATCAGCAGCTC 3′	(1334 to 1314)	12

Nucleotide sequence analysis of band 3 cDNA. PCR-generated cDNA fragments were subcloned into Bluescript KS(+) vector. Plasmid DNA from several independent clones was spun-column purified (Pharmacia Fine Chemicals, Piscataway, NJ) and sequenced using Sequenase.

Lateral mobility measurement of band 3 in red cell membrane. The technique of fluorescence-recovery-after-photobleaching (FRAP) was used to measure lateral mobility of band 3. The FRAP apparatus used is similar to the one described earlier (15, 16). Briefly, an argon ion laser beam (476 nm at 350 mW; Coherent Optics Inc., Palo Alto, CA) is split into two beams and one beam is attenuated (10^{-3}). Computer-controlled shutter synchronization allows independent use of these two beams for illumination. The two beams are subsequently recombined and the superimposed beams are focused and spatially filtered at the back image plane of an epi-fluorescence microscope. Conjugate images are produced by the objective at the object plane. These images are diffraction-limited in size with a Gaussian intensity profile [$1/(e^2)$, radius = 0.55 μm]. The attenuated beam is used to excite fluorescence light from the object. The light is collected by the objective lens and imaged via an ocular onto a photomultiplier tube. The photomultiplier signal is amplified, discriminated, and counted. The data acquisition is computer-controlled. The experiment consists of measuring the fluorescence intensity of labeled band 3 in an area of interest using the attenuated beam, irreversibly bleaching the fluorophor in that area using the unattenuated beam, and measuring the subsequent fluorescence recovery in the bleached area due to lateral motion of labeled band 3 from unbleached areas of the membrane into the bleached area. For these measurements, band 3 was labeled in situ at its extracellular domain with eosin-5-maleimide, as originally described by Nigg and Cherry (17), with slight modifications. In brief, 20 μl of whole blood was washed three times in 1 ml of phosphate-buffered saline (290 mosM, pH 7.4) with 0.05 g% human serum albumin (PBS/HSA). A stock solution of eosin-5-maleimide at a concentration of 2 mg/ml in PBS/HSA buffer, was added to the washed cells to obtain a final concentration of 50 μg/ml. The red cell suspension was incubated at room temperature in the dark for 45 min, washed three times, and the labeled cells used for measurement.

Microrheological measurements. Micropipette aspiration technique was used to evaluate the membrane material properties of the red cell. In this study, we focused on the extensional deformation properties of the membrane. Details of the micromechanical system and analysis used to measure red cell mechanical properties has been described previously (18). Briefly, the membrane extensional rigidity (μ) was derived from observation of cell length (L) aspirated into a small micropipette (with radius R_p) in response to the increase in suction pressure (P). Analysis of this experiment has shown that μ is proportional to the derivative of the pressure with respect to length: $\mu \alpha\text{-}R_p^2 \times dP/dL$.

Results

Characterization of band 3 mutation. Although the human band 3 cDNA sequence has been reported (19, 20), the intron-exon boundaries of the human band 3 gene have not yet been completely defined. However, partial sequencing data of the human band 3 gene suggest that the intron-exon boundaries have a location similar to that of the murine gene, in which all of the boundaries have been defined (21, 22). These data enabled us to juxtapose the murine intron-exon boundaries onto human cDNA sequence and design exon-specific PCR primers to amplify both band 3 gene and transcript. Employing several sets of PCR primers, the coding region of band 3 cDNA (exons 1–12) corresponding to the whole cytoplasmic domain (nucleotide [nt] 1 to nt 1209) and part of the transmembrane domain (nt 1212 to nt 1334) were amplified (Fig. 1, *a* and *b*). Both normal and ovalocytic cDNAs gave bands of identical sizes following amplification with primer sets p1-p3 and p2-p4, spanning 1228 nucleotides encoding the entire cytoplasmic domain and indicating that the mutation, if located within the amplified region, was unlikely to be deletional or insertional (data not shown). Nucleotide sequence analysis of these PCR-amplified fragments revealed an A > G polymorphism at nt 166 known as "band 3 Memphis" (23–25), but no evidence of any other mutation. However, amplification of ovalocytic cDNAs with the primer set p5-p6, spanning nt 879 to nt 1334, gave not only the expected normal 455-bp fragment but, additional PCR products of 428 bp, ~ 700 bp, and ~ 800 bp (Fig. 1 *c*). While the nucleotide sequence of the 455-bp fragment was identical to that of normal band 3 cDNA, the nucleotide sequence analysis of the 428-bp fragment revealed a 27-nt deletion encoding amino acid residues 400–408 of band 3 protein (Fig. 2). All six Malayan individuals with the ovalocytic phenotype were heterozygous for the band 3 deletional mutation. Regarding the two high molecular weight DNA bands, control experiments showed that they arise by heteroduplex formation between normal and mutant cDNA strands (data not shown).

In another series of experiments, genomic DNA from normal individuals and individuals with ovalocytic phenotype was amplified with the primer set p5-p6. These primers are located within sequences provisionally defined, by comparison with the murine band 3 gene, as exons 10 (p5) and 12 (p6). Thus,

1. *Abbreviations used in this paper:* FRAP, fluorescence-recovery-after-photobleaching; PCR, polymerase chain reaction; RT, reverse transcription.

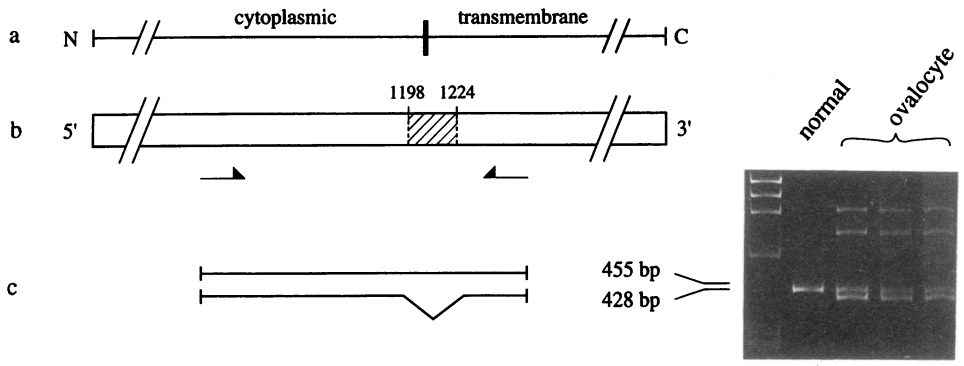

Figure 1. Scheme for band 3 mRNA analysis by PCR. (*a*) A schematic model of band 3 protein indicating the location of cytoplasmic and first transmembrane domain. (*b*) A model of band 3 cDNA. Arrow indicates the location of primer set (p5-p6) used in PCR reaction to define the deletion in mutant cDNA. Hatched area represents the location of the deletion identified in mutant cDNA from ovalocytes. (*c*) Polyacrylamide gel electrophoresis of amplified DNA products. The larger 455-bp band represents the normal allele and the smaller 428-bp band represents the mutant allele. Two higher molecular weight bands seen in ovalocytes represent heteroduplexes. Mol wt marker is HaeIII-digested ΦX174 DNA.

amplification of normal genomic DNA yielded a band of ~ 870 bp (including introns 10 and 11), whereas the ovalocytic DNA showed a doublet of ~ 870 bp and ~ 840 bp, plus their corresponding heteroduplexes (data not shown). The 27-nt deletion thus appears to be within exon 11.

Lateral mobility of band 3. Lateral mobility of band 3 in the plane of the membrane in normal and ovalocytic red cells was derived from the measurement of the fluorescence recovery after photobleaching (FRAP) of labeled band 3 molecules. Fig. 3 shows the fluorescence recovery curves for normal and ovalocytic red cells. In normal membranes, fractional recovery of labeled band 3 molecules in the bleached area following photobleaching was found to range from 23 to 45%. The diffusion constant for this mobile fraction was calculated to be (2.2±0.7)

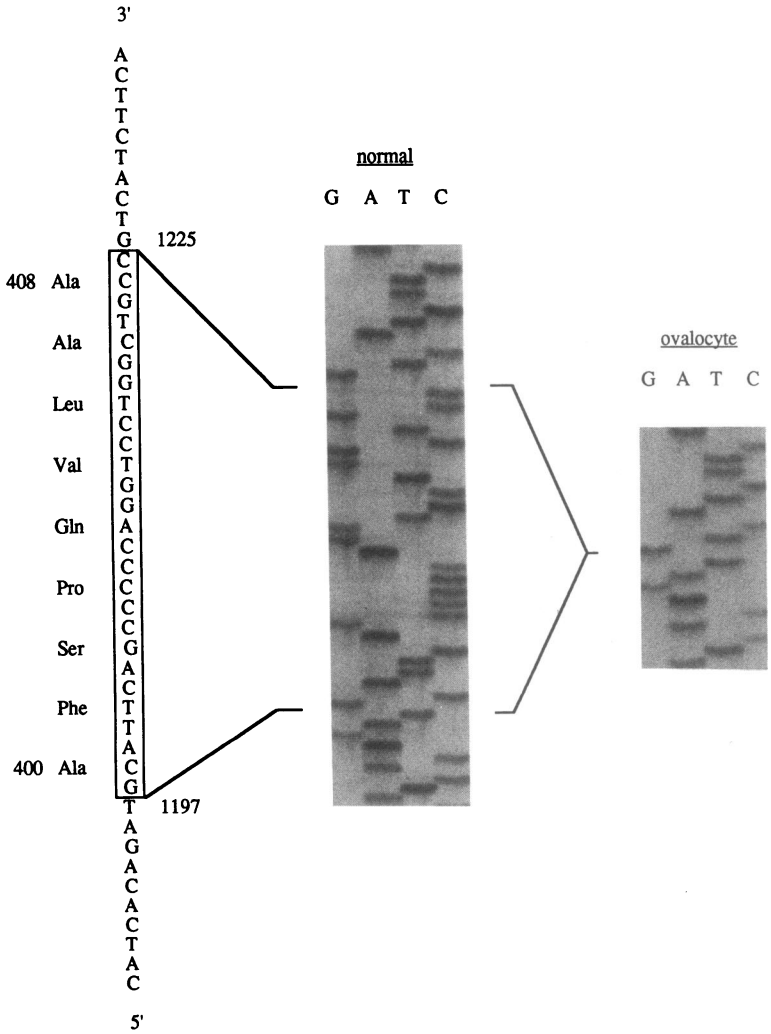

Figure 2. Nucleotide sequence of the normal and mutant alleles of band 3 cDNA from an ovalocytic individual. The 27 nucleotide deletion in the mutant allele is shown in the boxed area. Residues 400–408 deleted in the mutant band 3 protein are also indicated.

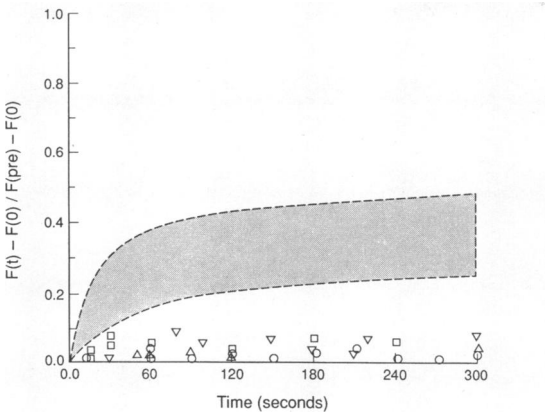

Figure 3. Fluorescence photobleaching recovery of labeled band 3 in normal and ovalocyte membranes. The ordinate is fractional recovery of fluorescence calculated from measured fluorescence counts just prior to photobleaching, F(pre); immediately after photobleaching, F(0); and at various times following photobleaching, F(t). Data for ovalocytic red cells from four different affected individuals (□, △, ▽, ○) is shown. Shaded area represents the range of data obtained with normal red cells.

$\times 10^{-11}$. In marked contrast, there was little or no diffusion of labeled band 3 into the bleached area of ovalocytic membranes during a period of observation that lasted 300 s. Interestingly, the lateral mobility of glycophorin A molecules in the ovalocytic membranes was similar to that observed in normal membranes (data not shown). These findings imply a specific reduction in the lateral mobility of band 3 in ovalocyte membranes.

Membrane extensional rigidity. Membrane extensional rigidity of normal and ovalocytic red cells was determined using a micropipette aspiration assay. Fig. 4 shows the relationship between membrane tension and aspirated cell length during both the loading phase, when the aspiration pressure was increased, and during the unloading phase when the aspiration pressure was decreased for an ovalocytic red cell (Fig. 4 *a*) and for a normal red cell (Fig. 4 *b*). Two features that distinguish the response of these two cells are (*a*) a much higher membrane tension was required to aspirate equivalent cell length of ovalocyte into the pipette compared to normal red cell; and (*b*) while the curves of aspirated cell length vs. suction pressure were observed to be nearly the same during both loading and unloading phases for the normal red cell, they were different for the ovalocytic red cell. For truly elastic behavior of the membrane, the curves during both phases should be identical; detection of hysteresis during aspiration and release of the ovalocyte indicates that the membrane underwent plastic deformation. Since the curves were not identical during loading and unloading phases, the membrane extensional rigidity was derived from the slope of the curve during the aspiration phase only. For 20 normal red cells the extensional rigidity was found to be $(7\pm2.4) \times 10^{-3}$ dyn/cm, while it ranged from 25 to 40×10^{-3} dyn/cm for 20 ovalocytic red cells from five affected individuals. Since we were unable to aspirate large lengths of the ovalocytic membranes into the micropipette, the calculated values for the extensional rigidity should be taken only as a lower limit of the actual values. Thus the two mechanical features that distinguish the ovalocytic red cell membrane are its markedly increased extensional rigidity, and its inability to undergo truly elastic deformation.

Discussion

We have characterized the molecular defect in erythroid band 3 protein in membranes of hereditary ovalocytosis by molecular cloning of mutant RNA sequences using RT/PCR techniques, and assessed the structural consequences of this mutation on red cell membrane material behavior using biophysical techniques. Following the seminal observation of Liu et al. (13) that the molecular defect in ovalocytes resides in band 3, and our own observation that marked changes in membrane material behavior can be induced by alterations in the cytoplasmic domain of an integral membrane protein (26, 27), we focused our attention on the cytoplasmic domain of band 3. PCR analysis on the first 1448 nucleotides of band 3 cDNA including the 5′ untranslated region and extending to the beginning of the second transmembrane domain, showed a deletion of 27 nucleotides (nt 1198 to nt 1224) in reticulocyte cDNA from blood of Malayan individuals with hereditary ovalocytosis. PCR amplification of genomic DNA suggested an intraexon deletion of 27 nucleotides in exon 11 of the band 3 gene. All six individuals with ovalocytosis were heterozygous for this mutation.

Lux et al. (20) proposed a model for band 3 in which the protein traverses the membrane 14 times. The cytoplasmic portion of band 3 protein has been assigned to amino acids (aa) 1–403, and the membrane spanning domains to aa 404–882. Thus, the nine amino acid deletion identified (aa 400–408) resides at the junction of the cytoplasmic and the first transmembrane domains (Fig. 1 *A*). Interestingly, the mutation lies within a phylogenetically conserved region of erythroid band 3. Amino-acid sequence comparison among human, chicken, and mouse band 3 indicated that the regions encompassed by aa 375–403 (cytoplasmic) and aa 404–550 (membranous) are the most highly conserved (20), suggesting that the region deleted in ovalocytes (aa 400–408) might be functionally important. The marked increase in membrane shear rigidity and the large decrease in lateral mobility of band 3 observed in association with this deletion imply that this region of band 3 is indeed critical in regulating membrane function.

Our observations, in addition to precisely defining the molecular basis for the ovalocytosis phenotype, also provide some unexpected insights into the role of integral membrane proteins in regulating membrane mechanical function. The observed decrease in lateral mobility of band 3 suggests that the deletion of nine amino acids at the junction of the cytoplasmic and the first transmembrane domain induces a conformational change in the cytoplasmic domain, and this change, in turn, leads to a marked increase in the association of this domain with the skeletal protein network. The biophysical basis for this suggestion is that the extent of decrease we observed in lateral mobility can only result from an increased interaction of the cytoplasmic domain of band 3 with the skeletal network, and not by conformational changes in the transmembrane domains (28, 29). Moreover, the thesis that the lateral mobility of membrane proteins is influenced by interaction with the underlying skeleton is supported by our recent studies of red cell glycophorin A. Following binding of extracellular ligands, the lateral mobility of glycophorin A was decreased in normal red cells,

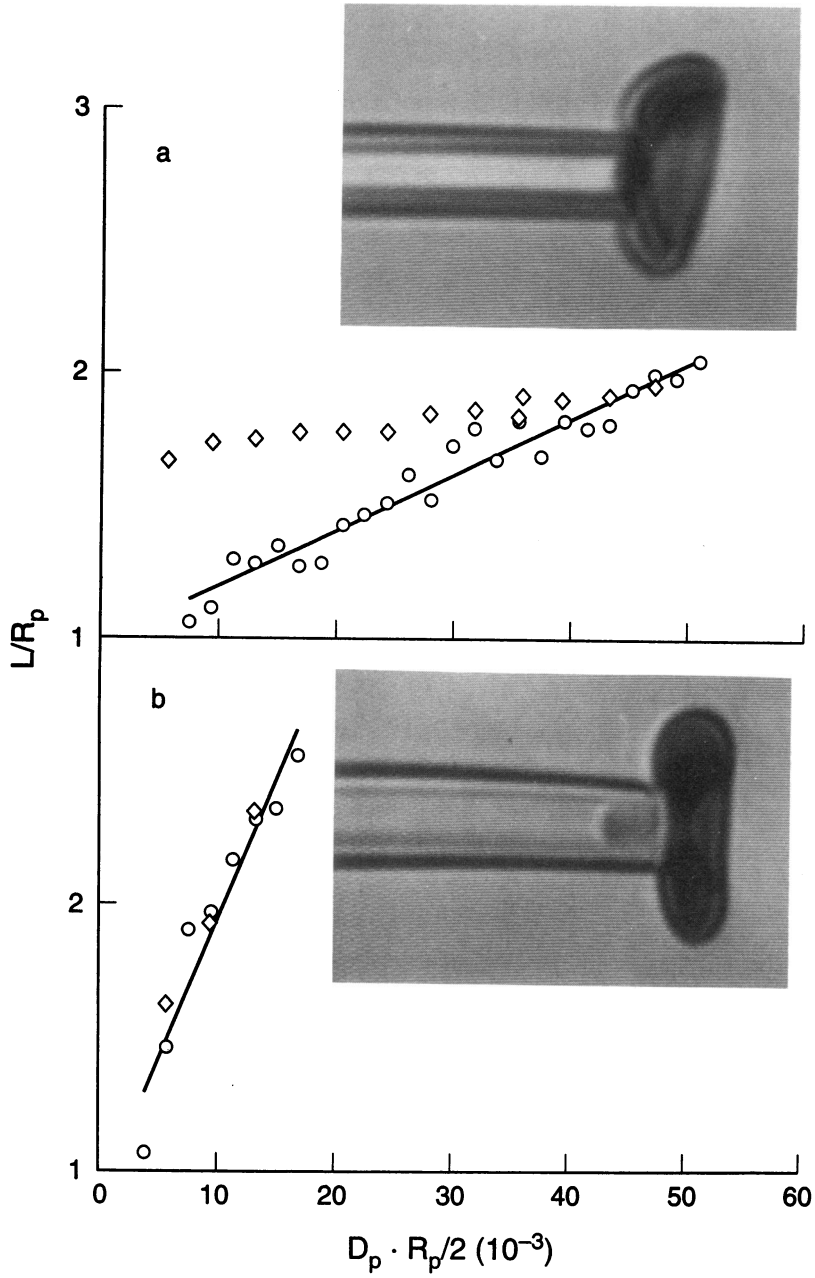

Figure 4. Videomicrographs of pipette aspiration of an ovalocytic (*b*) and (*a*) red cell at comparable aspiration pressures. Note the markedly decreased extension of the ovalocyte membrane compared to normal membrane. Plots of membrane tension $Dp \times Rp/2$ (dyn/cm, abscissa) vs the aspiration length normalized by the pipette radius (L/Rp) for a normal red cell (*b*) and an ovalocytic membrane (*a*) are also illustrated. The open circles (○) represent the aspiration or loading phase of the experiment while the open diamonds (◇) represent the release or the unloading phase.

but not in red cells expressing a variant glycophorin A with a truncated cytoplasmic tail (30). Thus, ligand-induced conformational change in the cytoplasmic tail of glycophorin A, and mutation-induced conformational change in the band 3 cytoplasmic tail alter membrane properties by changing their interaction with the membrane skeleton.

It should be noted that band 3 predominantly exists as a dimer in situ (31), and although the region responsible for the self association has not been defined, conformational change in the cytoplasmic tail may very well affect oligomerization of band 3. This alteration may in turn affect the binding affinity of the cytoplasmic domain of band 3 to ankyrin. At the present time we are unable to precisely define either the exact nature of the conformational change in the cytoplasmic domain of mutant band 3, or the nature of its altered interaction with the skeletal protein network. Secondary structure analyses of normal and mutant band 3 protein sequences, however, do suggest a possible conformational difference between the two proteins. A proline-rich region in the middle of normal band 3 cytoplasmic tail (aa 175–190) has been suggested to function as a flexible "hinge," which through pH-dependent conformation interconversion, modulates binding affinity for ankyrin (32, 33). A similar hinge has also been assigned to the region connecting the cytoplasmic and transmembrane domains (31). Prediction of normal band 3 secondary structure by both Chou-Fasman (34) and Garnier-Robson (35) algorithms indicate that an unstructured stretch of amino acid residues (aa 400–404) may be responsible for this conformational flexibility. The stretch includes pro 403 (prolines are known to disrupt ordered structure) which may produce either a β-bend or random coil between the cytoplasmic tail and the first transmembrane domain. Interestingly, the mutation in ovalocytic band 3 deletes

this putative "inter-domain hinge," resulting in a prediction of contiguous β-sheet through the cytoplasmic domain into the first membrane-spanning domain. This continuity in secondary structure can lead to a loss in conformational flexibility, thus altering the conformation of the cytoplasmic tail. This change in conformation could affect the interaction of the cytoplasmic domain of band 3 with the dense skeletal network.

While it is not difficult to rationalize how the mutation in band 3 can induce a marked decrease in its lateral mobility, it is difficult to conceptualize how this mutation can have such a profound effect on membrane shear rigidity. As most of the evidence accumulated to date suggests that shear rigidity of the red cell membrane is regulated by the spectrin-based skeletal network (9–12), it has been tacitly assumed that integral proteins like band 3 and glycophorin A contribute little to this membrane property. A widely accepted view is that in the nondeformed state the spectrin tetramers exist in a folded configuration, and during extensional deformation some of these tetramers become uncoiled and extended, while others assume a more condensed, folded form (11, 12, 36). The spectrin tetramers are thus considered to function as springs capable of storing energy during deformation and frictionally dissipating energy as they slide against each other, as well as against other skeletal proteins (12). Extensional rigidity is thus thought to depend primarily on energies required for uncoiling and extending spectrin tetramers during deformation. A significant weakness of this model is that it fails to take into account the potential for steric interference of spectrin tetramer uncoiling and extension by the cytoplasmic domains of various integral proteins protruding into the dense skeletal network. The potential contribution of these cytoplasmic domains to membrane material behavior was brought to the fore by the recent studies on glycophorin A (26–27). These studies unequivocally showed that the profound increase in red cell membrane extensional rigidity that accompanies binding of ligands to the exoplasmic domain of glycophorin A requires the presence of an intact cytoplasmic domain, and may be a direct consequence of increased entanglement of the cytoplasmic domain with the skeletal protein network. We suggest that a similar mechanism accounts for the observed extensional rigidity of the ovalocytes. This model is depicted in Fig. 5. Normal band 3, possessing the flexible hinge region, interacts specifically with skeletal proteins such as ankyrin and protein 4.1 (not shown), but otherwise remains untangled with the spectrin-based network. The cytoplasmic tail of mutant band 3 assumes a more rigid conformation and is envisioned to extend further into skeletal network, becoming physically entangled with skeletal proteins. This in turn leads to decreased ability of spectrin tetramers to uncoil and extend during induced deformation. Although we favor the hypothesis that the entanglement is purely steric in nature, we cannot rule out the possibility that specific associations with skeletal proteins are also involved. The model we have outlined provides a novel mechanism by which conformational changes in the cytoplasmic domains of integral proteins can increase membrane extensional rigidity.

The combined molecular biological and biophysical studies on ovalocytic red cells have enabled us to document an important role for the cytoplasmic domain of band 3 in regulating its lateral mobility and in determining membrane rigidity. These findings further imply that in addition to skeletal proteins, integral proteins such as band 3 can profoundly influence membrane material behavior. Exploring the biophysical consequences of interactions between cytoplasmic domains of various integral proteins with the underlying structural protein network in other cells may similarly shed important and unexpected insights into the role of these interactions in membrane function of nonerythroid cells.

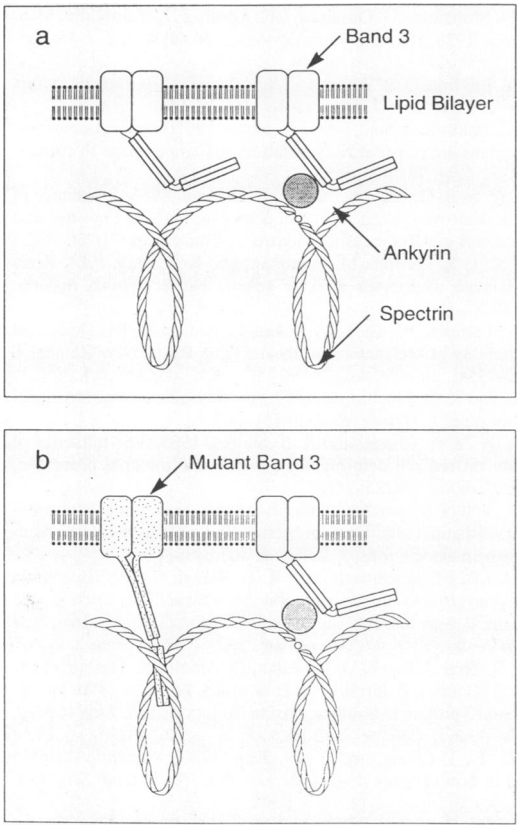

Figure 5. Model for role of cytoplasmic domain of band 3 in regulating membrane extensional rigidity. Conformation of the cytoplasmic domain of normal band 3 (*a*) offers little resistance to the extension of spectrin tetramers during membrane deformation. In contrast, the conformationally altered cytoplasmic domain of mutant band 3 (*b*), by getting entangled in the spectrin network, sterically hinders the extension of spectrin tetramers. For reasons of clarity, all the individual components of the skeletal network have not been delineated.

Acknowledgments

This work is dedicated to the memory of L. E. Lie-Injo, whose tireless efforts to understand the Ovalocytic phenotype was truly inspirational.

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