Mechanochemistry of the Alternatively Spliced Spectrin-Actin Binding Domain in Membrane Skeletal Protein 4.1*

Dennis Discher†, Marilyn Parra, John G. Conboy, and Narla Mohandas‡

From the Life Sciences Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720 and the ∗Joint Graduate Group in Bioengineering, University of California, Berkeley and San Francisco, California 94143

Protein 4.1's interaction with the erythroid skeletal proteins spectrin and actin and its essential role in regulating membrane strength are both attributable to expression of an alternatively spliced 63-nucleotide cassette. The sequence encoding 21-aa (21-aa) cassette is within the previously identified spectrin-actin binding domain (10 kDa molecular mass) of erythroid protein 4.1. This cassette is absent, however, in several isoforms that are generated by tissue- and development-specific RNA splicing. Four isoforms of the 10-kDa domain were constructed for comparative assessment of functions particularly relevant to red cells. In vitro translated isoforms containing the 21-aa cassette, denoted 10k[21] and 10k[21]19, were able to bind spectrin, stabilize spectrin-actin complexes, and associate with red cell membrane. Isoforms replacing or lacking the 21-aa cassette, 10k[19] and 10k[9], did not function in these assays. A bacterially expressed fusion protein with glutathione-S-transferase, designated GST-10k[19], coaggregated spectrin-actin into a network in vitro as found with purified protein 4.1. Additionally, incorporation of GST-10k[19] into mechanically weak, 4.1-deficient membranes increased mechanical strength of these membranes to normal. GST-10k[19] did not function in these assays. These results show that the 21-aa sequence in protein 4.1 is critical to mechanical integrity of the red cell membrane. These results also allow the role of protein 4.1 in membrane mechanics to be interpreted primarily in terms of its spectrin-actin binding function. Alternatively expressed sequences within the 10-kDa domain of nonerythroid protein 4.1 are suggested to have different, yet to be defined functions.

The erythrocyte's ability to deform reversibly is key to this cell's ability to cycle 100,000 times through the narrow passages and rapid flows of the circulation. Limits to the elasticity of the red cell membrane are particularly relevant to hemolytic anemias, and in a number of such diseases membrane protein defects have been identified (1). The molecular interactions that provide structure to the cell during its deformation are the essence of red cell mechanochemistry. By comparing the mechanical behavior and biochemistry of normal cells to pathological and artificially perturbed cells, it has been concluded, for example, that mechanical strength of the membrane is dependent upon interactions among skeletal proteins that include spectrin, actin, and protein 4.1 and also upon interactions between the skeletal network and the bilayer or its embedded proteins. In particular, previous results have clearly shown that red cell membranes lacking protein 4.1 exhibit an increased susceptibility to fragmentation both in vivo and in vitro (2). This phenomenon has motivated us to pursue the structure-function of protein 4.1.

Biochemical and ultrastructural studies indicate that protein 4.1 is a multifunctional structural element. Purified red cell 4.1 clearly associates with the membrane skeletal protein spectrin (3−5), and it may interact specifically with the skeletal element actin (6), the integral membrane proteins band 3 (7), and glycophorins (8, 9), as well as with tubulin (10), myosin (11), and calmodulin (12). The 4.1-spectrin interaction appears to stabilize the cross-linking of short actin oligomers in the topological plane of the network (13, 14), while interactions of 4.1 with membrane proteins have been thought to aid in anchoring the skeleton to the overlying bilayer (9).

Protein 4.1 is structurally diverse. Two major erythrocyte protein 4.1 isoforms, 4.1a (80 kDa) and 4.1b (78 kDa), have been shown to differ through a post-translational modification at Asn[292] (15). In addition, molecular cloning studies have shown that a number of closely related 4.1 isoforms are expressed in the erythroid lineage from a family of alternatively spliced mRNAs (16, 17). Isoforms of erythroid 4.1 have also been found in a number of other tissues including liver, intestine, and lymphocytes (18, 19). Some of these isoforms have well characterized differences in exon expression, and molecular masses of 4.1 isoforms range from 30 to 210 kDa (20). We are exploring the hypothesis that binding domains are spliced in and out of these various isoforms to potentiate a rich diversity of 4.1-derived mechanochemistry.

The spectrin-actin binding domain of red cell protein 4.1 has been mapped to an internal sequence of 67 amino acids (Lys[256]−Phe[272]) using high performance liquid chromatography-purified chymotryptic cleavage fragments in a spectrin-actin cosedimentation assay (21, 38). Furthermore, a canine subject with hereditary anemia and elliptocytic cells has recently been shown to have red cells with a protein 4.1 variant lacking the 21-aa cassette (Lys[256]−Glu[272]) in the 10 kDa domain (22). Interestingly, expression of the 21-aa cassette occurs only in the late stages of erythroid differentiation (23).

Cloning studies have indicated that eight structural variants of the 10-kDa domain may be generated in vivo by tissue-specific alternative-splicing of three exons encoding 19.

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†To whom correspondence should be addressed: Cell and Molecular Biology Div., Lawrence Berkeley Laboratory, 1 Cyclotron Rd., Bldg. 74-157, Berkeley, CA 94720. Tel.: 510-486-7029; Fax: 510-486-6746.

‡The abbreviations used are: aa, any amino acid; GST, glutathione-S-transferase; PAGE, polyacrylamide gel electrophoresis.
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Plasmid Constructs for Protein 4.1 Expression—Minigenes corresponding to the internal 10-kDa domains of four different splicing isoforms of protein 4.1 (approximately Glu<sup>400</sup> → Gly<sup>406</sup>) (Ref. 18) were prepared by polymerase chain reaction amplification of plasmid cloned cDNA isoforms. Polymerase chain reaction primers used for these studies incorporated restriction sites to allow cloning into both PBS-ATG (at NcoI and KpnI sites) and pGEX-KT (at EcoRI and SalI sites) vectors; the primers also contained N-terminal methionine (ATG) and C-terminal stop codons (TAG) required for proper translation of PBS-ATG. PBS-ATG constructs were expressed in vitro as independent peptides of 65–106 amino acids, while in pGEX-KT the same peptides were produced as fusion proteins linked to the N-terminal glutathione-S-transferase moiety of the vector. The oligonucleotide primers used had the following sequences: sense strand, 5’-GGATCCTGACGACGCTGATCTGCTTCGACGAAGGAG 3’; antisense strand, 5’-GACGTCGACCTTGGTACCCTAACGGCCGTCT CCCGTGGGGAT-3’. In Vitro Transcription and Translation—For in vitro expression, the PBS-ATG-10K plasmids were linearized with Sall, transcribed with T3 RNA polymerase, and translated in cell-free wheat germ extracts in the presence of [<sup>35</sup>S]methionine. RNA transcripts were analyzed on 7 M urea, 6% polyacrylamide gels; <sup>35</sup>S-labeled 4.1 peptides were run on 15% SDS-polyacrylamide gels and visualized by radiography. For the prototypical 10-kDa polypeptide, typical yields were 0.0125 ± 0.0025 pmol of [<sup>35</sup>S]methionine incorporated per µl of reaction. Molarities of the polypeptides (Fig. 2c) may be influenced by a net difference of five charges between the 21-aa cassette.

Bacterial Expression—Two of the 10-kDa isoforms constructed in the pGEX vector were expressed in Escherichia coli and designated GST-10K<sup>10</sup> and GST-10K<sup>19</sup>. Fusion peptides were purified from E. coli lysate by affinity chromatography using glutathione-agarose matrix (25, 26). Protein analysis by electrophoresis was carried out using 12.5% denaturing gels which were either stained with Coomassie (Fig. 3) or transferred to nitrocellulose for antibody probing. The bacterially expressed polypeptides produced the same electrophoretic pattern as found with the wheat germ translation system, but the bands were shifted up in molecular mass by approximately 26 kDa due to the fusion with GST (Fig. 3). Prominent bands occurring with both peptides were first identified as the 4.1 sequence using antibody against intact 4.1 (data not shown). An antipeptide antibody directed against a synthetic 21-amino acid peptide recognized only the fusion with T3 RNA polymerase, and translated in cell-free wheat germ extracts in the presence of [<sup>35</sup>S]methionine. RNA transcripts were analyzed on 7 M urea, 6% polyacrylamide gels; <sup>35</sup>S-labeled 4.1 peptides were run on 15% SDS-polyacrylamide gels and visualized by radiography. For the prototypical 10-kDa polypeptide, typical yields were 0.0125 ± 0.0025 pmol of [<sup>35</sup>S]methionine incorporated per µl of reaction. Molarities of the polypeptides (Fig. 2c) may be influenced by a net difference of five charges between the 21-aa cassette.
protein GST-10k\textsuperscript{Z1}. An antibody generated to a downstream sequence common to both GST-10k\textsuperscript{Z1} and GST-10k\textsuperscript{Z2} recognized both fusion peptides. These positively identified peptides were used for both in vitro and in situ analysis of function. For quantitative work with the fusion proteins, the 1.0 cm\textsuperscript{2}/mg absorptivity of equine liver glutathione S-transferase (Sigma No. 6511) at 280 nm was used.

**Purification of Spectrin, Protein 4.1, and F-actin—**Spectrin dimer was extracted by low ionic strength from ghosts at 37 °C, and then isolated by gel filtration (27). Protein 4.1 was subsequently extracted from spectrin-depleted vesicles with 1 M KCl and then purified by anion-exchange chromatography (5). Protease inhibitors diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride were used in the preparations. Actin was isolated from rabbit skeletal muscle (28) and stored at 4 °C in low salt buffer. Just prior to use, KCl was added in excess of 100 mM, the polymerized F-actin was pelleted by centrifugation, and then resuspended. Protein purity was verified by SDS-PAGE. Ultrafiltration was used to concentrate proteins or change solvent systems as needed. Protein concentrations were determined by spectrophotometry using 280-nm absorbivities of 1.0 cm\textsuperscript{2}/mg spectrin, 0.8 cm\textsuperscript{2}/mg protein 4.1, and 1.1 cm\textsuperscript{2}/mg actin (28-30). Proteins were stored in buffer A (10 mM Tris, 20 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.4) (Ref. 28) and appeared stable for several weeks.

**Analysis of Protein Associations by Sucrose Gradient Sedimentation—**In vitro translated polypeptides incorporating \([35S]\)Met were mixed with purified spectrin and diluted with buffer A (10 mM Tris, 20 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.4) to give final volumes of 50–60 μl. Incubations of the mixtures were typically done for 60 min at 4 °C, but results with longer incubations on ice were no different, indicating that reactions had reached equilibrium. Sucrose was dissolved in buffer A to various densities as verified by refractometry. Gradients were formed just prior to centrifugation by layering 600 μl of sucrose solutions, 10–25% by weight, in 5 × 41-mm ultracentrifuge tubes. Each reaction was layered on top of a gradient which was then centrifuged at 43 k rpm in an SW50.1 rotor at 4 °C for 6 h. Fractions were collected dropwise (14–20 fractions in total) from the bottom of the tube. Equal aliquots were counted for radioactivity.

**Incorporation of \([35S]\)-Labeled Peptides into Normal Red Cell Membranes—**The procedure for incorporating exogenous protein into red cell ghosts is slightly modified from that previously described (31). Normal red cells were hypotonically lysed on ice with 10 mM NaP, pH 7.4, and the membranes were collected by centrifugation at 13,000 × g within 3 min. Membranes, 0.5 ml, were combined with 10 μl of polypeptide (typically 10\textsuperscript{nM} M) and incubated on ice for 20 min. KCl and MgCl\textsubscript{2} were added to final concentrations of 100 and 1 mM, respectively, in order to resell the ghosted cells while incubating at 37 °C for 30 min. Ghosts were then lysed again for 20 min on ice, spun down, and washed once more with lysis buffer before membranes and final wash were counted.

**Falling Ball Viscometry and Network Composition—**Gelation of spectrin/actin mixtures by protein 4.1 has been well documented as a sensitive assay for this structural protein (32, 33). F-actin (15 μM) and spectrin (4.0 μM) were incubated with 4.1 or the fusion constructs for 60 min on ice in 100-μl capillary tubes. Using a magnet, a small steel ball (0.025 inch) was placed below the meniscus of a vertically oriented tube and released. The time for the ball to fall a specified distance was recorded. Samples were done in duplicate and results were normalized to those obtained with spectrin-actin alone.

Compositions of the networks were analyzed by first layering tube contents on top of 1 ml of 18.5% sucrose in buffer A. The samples were spun in a TL-55 for 90 min at 42 k rpm. The top three 100-μl fractions were then collected along with the bottom 100 μl, and these were analyzed by Coomassie staining after 12.5% SDS-PAGE.

**Incorporation of Fusion Proteins into 4.1-deficient Membranes—**In contrast to the procedure employed to assay association of the \([35S]\)-labeled peptides with normal membranes, fusion peptides at concentrations of about 1 mg/ml in lysis buffer were incubated with the same volumes (200–400 μl) of membranes which were approximately 50% deficient in protein 4.1. After the 20-min incubation on ice, the membranes were resedimented with KCl and MgCl\textsubscript{2}, as above, but with incubations of 60 min. The membranes were then kept on ice for up to an hour before quantitation of membrane strength by ektacytometry.

**Red Cell Membrane Fragmentation Assay—**The strength of membranes in a shearing flow has been quantified in vitro by ektacytometry which combines Couette rheometry as a method of deforming red cells with forward light scattering as a technique to quantitate deformation (31). In application here, the resedimented ghost samples were suspended in 35% dextran/phosphate-buffered saline and deformed to ellipsoids by a high shear laminar flow (nominal fluid shear stress of 750 dyn/cm\textsuperscript{2}). The large deformations lead to a progressive fragmentation of the intact membranes into small spherical vesicles. Fragmentation was indicated by the evolution of an initially elliptic diffraction pattern toward an axially symmetric pattern. The corresponding decay in the deformation index, elsewhere designated deformability index, was recorded graphically as the membranes fragmented.

**RESULTS**

**Binding of 10-kDa Isoforms to Spectrin**

Corresponding to four alternatively spliced isoforms of the 10-kDa domain (Fig. 1), \([35S]\)Met-labeled polypeptides were synthesized by in vitro transcription and translation. The ability of each isoform to bind purified red cell spectrin was then evaluated by sucrose gradient centrifugation. It was found that the 21-aa cassette in either the prototypal erythroid isoform 10k\textsuperscript{10} or the liver isoform 10k\textsuperscript{19,21} is requisite for cosedimentation with spectrin (Fig. 4, a and b). Neither the T-lymphocyte 10k\textsuperscript{9} nor the intestine and liver isoforms 10k\textsuperscript{10} appear to bind spectrin. Denaturing electrophoresis of the bound and free peaks showed identical compositions, indicating that a simple equilibrium between the 21-aa containing polypeptides and spectrin gives both peaks.

Since the yields from in vitro translation were on the order of nanomolar under a variety of translation conditions and because skeletal protein affinities are typically within orders of micromolar, it did not appear possible to demonstrate saturation in spectrin binding. Indeed, a 10-fold increase of 10k\textsuperscript{21} concentration in a reaction at fixed spectrin concentration did not alter the relative distributions of bound and free polypeptide (data not shown). Decreasing total spectrin concentration does, however, attenuate the bound peak and augment the free peak as shown in Fig. 4c. Competition for spectrin binding with purified unlabeled 4.1 also decreases the bound peak (Fig. 4d). Reversible binding could also be demonstrated, fractions from a bound peak were pooled, dialyzed to remove sucrose, and spun again through a larger volume gradient. The bound peak from the first centrifugation re-equilibrated to yield both bound and free peaks (data not shown). Nonspecific binding of peptides to centrifuge tubes was not a problem; typically, more than 80% of the initial radioactivity was recovered with fractionation.

Because the spectrin binding of the 10k\textsuperscript{21} construct is specific, reversible, and subject to competition, some simple
binding models were applied to the data represented in Fig. 4. All of the spectrin-binding data is consistent with a single bimolecular association constant $K_a \approx (0.5 \pm 0.1) \times 10^6 \text{M}^{-1}$. Values reported for purified full-length protein 4.1 range from $0.5 \times 10^6 \text{M}^{-1}$, as also determined by sucrose gradient sedimentation (3), through $10 \times 10^6 \text{M}^{-1}$ by an immunoprecipitation technique (29), to $86 \times 10^6 \text{M}^{-1}$ by resonance energy transfer supported by microcalorimetry (4). Neglecting the effects of sucrose on thermodynamic stability and neglecting artifacts possible in the other reported assays, it appears that association measure can increase by orders of magnitude when the speed of assay increases by orders of magnitude. Our reported $K_a$ is therefore likely to be a lower limit on the actual chemistry. Most importantly, our apparent $K_a$ agrees with that reported for full-length 4.1 when determined by a comparable method indicating that our 10k21 has the same spectrin affinity as purified full-length 4.1.

**Spectrin**-$\text{F-actin}$ Binding with the 21-aa Cassette

Ternary complex formation occurs when protein 4.1 is added to a mixture of spectrin and actin (34). At moderate protein concentrations, such mixing leads to extended three-dimensional networks in vitro suggestive of skeletal assembly in vivo. However, given the very low concentrations of 4.1 constructs available from in vitro translation, we again examined biochemical interactions by sucrose gradient centrifugation. Fig. 5 shows that the 10k21 construct is predominantly in a pellet at the bottom of the tube when both spectrin and F-actin are present. No stable binary interaction of actin with the 10k21 was detected under these experimental conditions (dotted line); the data will admit a very weak association $<0.5 \times 10^4 \text{M}^{-1}$. The 10k19 construct did not cosediment with any combination of spectrin and F-actin.

Consistent with the initial biochemical characterization of the chymotryptic 10-kDa fragment (21), Fig. 5 shows that the prototypical 10k21 construct complexes with spectrin and F-actin, but here we have further defined the site of interaction since the 10k19 is nonfunctional. In these assays, F-actin alone does not appear to interact energetically with any of the cassettes nor any part of the 10-kDa domain. All of these findings are consistent with a previous model applied to full-length 4.1 and ternary complex formation (34). As calculated below, the absence of a specific interaction between F-actin and the 10k21 may be incorporated in a ternary association constant $K_a \approx (0.9 \pm 0.3) \times 10^3 \text{M}^{-2}$ corresponding to a free energy change for in vitro ternary complex formation of $-62$...
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Fig. 5. Ternary complex assay, sucrose gradient sedimentation of 10k21 reacted with F-actin and spectrin. Typical distributions of counts when 10k1 (0.5 nM) and F-actin (230 µM) were mixed with or without spectrin (2.1 µM). Two distributions are illustrated for the spectrin reaction as an indication of experimental variation. Fractions were collected by pipet from the top of the gradients to avoid disturbing gelatinous pellets visible in all the samples. The 10k21 complexes with F-actin when spectrin is present; the 10k19, however, is inactive (data not shown).

Table I

Summary of association constants from sedimentation velocity studies

| Construct | Spectrin + construct | Actin + construct | Spectrin + actin + construct |
|-----------|----------------------|-------------------|-----------------------------|
| 10k16     | 0                    | 0                 | 0                           |
| 10k19     | 0                    | 0                 | 0                           |
| 10k21     | 0.5 x 10^6 M^-1      | 0                 | 0.9 x 10^-1 M^-2             |
| 10k19,21  | 0.2 x 10^6 M^-1      | 0                 | 0.3 x 10^-1 M^-2             |

The absence of a detectable association is indicated by a 0.

to ~70 kJ/mol. This is certainly comparable to the ~70 kJ/mol found previously for full-length protein 4.1 (34). It agrees with the original semiquantitative results that used chymotryptic fragments (21), and it also supports the previous finding that 4.1 does not interact strongly with F-actin.

Table I summarizes our calculated association constants. Most evident is the importance of the 21-aa cassette to interactions with spectrin and actin. Steric hindrance by the 19 additional residues may offer explanation for the lower affinity of the tandem 10k19,21 in comparison to the 10k21.

Effects of ionic strength, calcium, and pH on the 10k21 association with spectrin were also examined. Interactions were substantially the same over the range of 20-600 mM KCl, consistent with previous reports (34). Calcium up to 50 µM, as controlled with EGTA, also had little effect. The binding of 10k21 to spectrin appears to be weakened at both pH 5 and 12. The latter effect has been noted before (4). As further evidence for binding specificity, the 10k21 was incubated with urease (272 and 545 kDa) or a 500-kDa dextran and no cosedimentation was observed (data not shown).

Incorporation into Erythrocyte Membranes Requires the 21-aa Cassette

To test the ability of 10-kDa constructs to associate with structures in situ, 35S-labeled peptides were added to normal red cells using a hypotonic lysis/resealing protocol (31). In agreement with the above results using purified proteins, only polypeptides containing the 21-aa cassette integrate into the membranes of red cells (Fig. 6). Constructs lacking the 21-aa cassette do not bind sites in the membrane: residual counts in both the membranes and final wash have equilibrated at a minimal value. Since these red cells retain a normal complement of protein 4.1, the exogenous polypeptides may either exchange with endogenous 4.1 or bind to unoccupied sites generated by partial disruption of the membrane skeleton. The latter is suggested by observations of large lytic holes in red cell ghosts (35). Although the mechanism of membrane incorporation is not clear from the limited but highly repro-
ducible results, a rough calculation indicates that about 0.1 to 1% of endogenous 4.1 may be replaced or supplemented by the 10kZ1 construct. This is estimated from the polypeptide concentrations as given in Fig. 4, the initial total volume of cells, an average cell volume taken as 100 femtoliters, and 2 × 10^9 sites for protein 4.1 interaction with spectrin-actin per cell (36). Moreover, by making certain assumptions about the number of binding sites available to the 10kZ1, we have estimated that its affinity for red cell membranes is similar to its affinity for purified spectrin and actin, i.e. the observed membrane binding may be explained solely on the basis of ternary complex formation.

In Vitro Gelation Requires the 21-aa Cassette

All the binding studies above were performed with trace amounts of radiolabeled 10-kDa isoforms in the presence of unlabeled components of the wheat germ translation system. In order to obtain large amounts of pure polypeptide for use in biophysical studies, fusion proteins with glutathione-S-transferase (GST) were expressed in E. coli using the pGEX expression vector. For focusing on function of the 21-aa cassette, we have examined only GST-10kZ1 and GST-10k’ (Fig. 3). In preliminary experiments, the bacterially expressed GST-10kZ1 was found to compete with 35S-labeled 10kZ1 for spectrin-binding with about the expected affinity (data not shown).

A second, more sensitive assay for structural function utilized the well documented gelation of spectrin/actin mixtures by protein 4.1 (33, 37). As measured by falling ball viscometry (32), addition of either purified protein 4.1 or GST-10kZ1 to a mixture of spectrin and actin increased apparent viscosity with a strong concentration dependence (Fig. 7a). GST-10k’ and denatured GST-10kZ1 had no such effect. Gelation at about 3 μM appears to occur at molar ratios of spectrin:polypeptide:F-actin of 1:1:7.5 which approximates the 1:1:2.3 stoichiometry found in the red cell skeleton. The phase transition with GST-10kZ1 appears at a lower concentration than found with protein 4.1 because the GST-10kZ1 concentration may be slightly underestimated and because 4.1 aggregation may alter stoichiometry or effective concentration (e.g. Ref. 3).

Subsequent to viscometry, proteins and protein complexes in the viscometry sample were fractionated on the basis of sedimentation velocity and then analyzed by SDS-PAGE (Fig. 7b). Both protein 4.1 and GST-10kZ1 complexed with spectrin and F-actin to produce a thermodynamically stable and viscous network that rapidly sediments to the bottom of a gradient (Fig. 7b, i and iii). In contrast, GST-10k’ was found to be neither a gelation factor nor a binding factor (Fig. 7b, ii) in the presence of spectrin and actin. Note that composition analysis of the mixture with GST-10kZ1 showed some spectrin bound F-actin; this interaction is, however, greatly enhanced by either 4.1 or GST-10kZ1. This is completely consistent with our analysis of ternary complex formation which attributes about two-thirds of the ternary association energy to 4.1 function. As a final note, we have also found that 2,3-diphosphoglycerate reduces the viscosity of spectrin/actin/GST-10kZ1 mixtures, suggestive of ternary complex inhibition (39) which has been correlated with membrane destabilization by 2,3-diphosphoglycerate (40).

In Situ Membrane Strengthening Requires the 21-aa Cassette

Previous studies have shown that red cells deficient in protein 4.1 have decreased membrane strength (2), and that incorporation of exogenous 4.1 can increase membrane strength to normal (31). To test cellular function of our 10-kDa isoforms, mutant red cells with a 50% deficiency of 4.1 were lysed in the presence of either GST-10kZ1 or GST-10kZ1 at concentrations previously found sufficient for full 4.1 effect. Each membrane sample was resealed with salt, suspended in

![Fig. 7. Cross-linked network formation with the 21-aa construct. a, gel-formation assay for mixtures of spectrin (3.5 μM), F-actin (15 μM), and fusion proteins or purified 4.1. Viscosity is reported relative to spectrin/actin mixtures which had apparent viscosities of about 2-3 centipoise. A gel phase is indicated by a break in the axis above a relative viscosity of 100. b, network composition was analyzed by subjecting viscometry samples to sucrose gradient sedimentation in order to separate high molecular weight complexes from unbound protein. Three samples were examined: (i) spectrin, actin, plus 4.1; (ii) spectrin, actin, plus GST-10kZ1; and (iii) spectrin, actin, plus GST-10kZ1. SDS-PAGE 12.5% was done with the top 30% of the gradient’s volume (top three fractions as lanes 1-3, respectively) and the bottom fraction (pellet).](image-url)
with the 10-kDa construct demonstrate that only about one-
level of strength to the membrane
of protein 4.1 to 4.1-deficient membranes will impart a normal
deficient membranes. Previously it was shown that addition
It appears that GST-lOklg imparts normal strength to the
fragment and at roughly the same rate as the deficient cells.
Albumin has no effect either (data not shown). In contrast,
tation. GST-lOklg does not affect this fragmentation process.

After 25 s of deformation, however, the partially 4.1-deficient
membranes have clearly made a transition to steady fragmen-
tation assay employing ektacytometry (see "Materials and
Methods").

Representative fragmentation curves for protein 4.1-deficient red cell membranes, 4.1(-), and the range for normal membranes (stippled) along with 4.1-deficient membranes incubated with the fusion constructs. Briefly, 200 μl of lysed membranes, deficient by about 50% of normal protein 4.1 content, were combined with an equal volume of lysis buffer containing either GST-10k21 (1.0 mg/ml) or GST-10k19 (1.0 mg/ml). Resealed membranes were subjected to 750 dyn/cm² in the ektacytometer and their deformation plotted as a function of time. Purified 4.1 (1.0 mg/ml) was found to make the 4.1(-) membranes fragment within the normal range (data not shown), as previously reported (25).

To analyze data of the type illustrated in Fig. 4d, a simple competition model involving 10k21 is written as the simultaneous reactions,

\[ F + S \rightleftharpoons B, \quad K_s = [B]/([F][S]) \] (Eq. 1)

\[ 4.1 + S \rightleftharpoons S*4.1, \quad K_{4.1} = [S*4.1]/([4.1][S]) \] (Eq. 2)

where 4.1 denotes isolated protein 4.1 and "*" denotes macromolecular complex formation with spectrin. Again, \([S] \gg [F], [B],\) so that

\[ [S] = [S]_0 - [S*4.1] - [B] \approx [S]_0 - [S*4.1] \] (Eq. 3)

and also

\[ [4.1] = [4.1]_0 - [S*4.1] \] (Eq. 4)

Thus

\[ [B]/[F] = K_s([S]_0 - [S*4.1])/([4.1]_0 - [S*4.1]) \] (Eq. 5)

gives a quadratic equation for \([S*4.1].\) This is solved uniquely by requiring \([S*4.1] \leq [S]_0\) and using \(K_4 = 0.5 \times 10^6 \text{M}^{-1}\), the association constant determined for S*4.1 by sucrose gradient centrifugation (3). Therefore

\[ [S*4.1] = 1.1 \times 10^{-6} \text{M} \] (Eq. 6)

The calculations from 10 experiments with the 10k21 gave

\[ K_s = ([B]/[F]) + ([S]_0 - [S*4.1]). \] (Eq. 7)

Note that the competition data can alternatively be viewed as validating our sucrose gradient assay since we could have used \(K_s\) from our other binding experiments and calculated the same \(K_s\) above.

Spectrin-Actin Binding Model—To analyze data of the type illustrated in Fig. 5 it is assumed that no stable interaction occurs between F-actin and the 10k21 (34). Denoting spectrin again with \(S\), writing \(A\) for F-actin, and \(C\) for the 10k21 construct.

\[ A + S \rightleftharpoons A*S \quad K_1 = [A*S]/([A][S]) \] (Eq. 8)

\[ S + C \rightleftharpoons S*C \quad K_2 = [S*C]/([S][C]) \] (Eq. 9)

\[ A*S + C \rightleftharpoons A*S*C \quad K_3 = [A*S*C]/([A*S][C]) \] (Eq. 10)

\[ S*C + A \rightleftharpoons A*S*C \quad K_4 = [A*S*C]/([S*C][A]) \] (Eq. 11)

Note the absence of a specific complex between F-actin and
the construct. Also note $K_a K_4 = K_2 K_3 = K_0$ corresponds to a termolecular association.

$$A + S + C \rightarrow A^* S C \quad K_{AC} = [A^* S C]/([A][S][C]) \quad (\text{Eq. 11})$$

The experiments provide a measure of the bound-to-free ratio. The volume fraction cutoff for free counts is defined from the noninteracting case (taken as 500–650 μM inclusive); the rest of the counts are bound so that

$$[B] = [A^* S C] + [S*C]. \quad (\text{Eq. 12})$$

Dropping the concentration brackets, denoting initial concentrations of spectrin, actin, and construct with subscript zeros, and writing $s, a, c$ for the final free concentrations $[S], [A], [C]$, mass conservation gives the following.

$$A_0 = a + A^* S + B - S*C$$

$$C_0 = c + B$$

$$S_0 = s + B + A*S$$

With these, the expression for $K_1$ can be used to invert the free actin concentration $a$ as a function of $(S_0, B, K_1, s)$. This is used in the expression for $K_2$ to solve for the free spectrin concentration via a quadratic equation parameterized by $(K_1, S_0, A_0, K_2, c, B)$ and satisfying $s < S_0$. The expression for ternary complex association constant $K_3$ then becomes the following

$$K_3 = \frac{[B]}{[a - s - S_0 - A_0]}/[a ~s ~c] \quad (\text{Eq. 14})$$

This is implicitly a function of the spectrin-actin equilibrium constant $K_1 = 5000 ~M^{-1}$ (Ref. 34), the spectrin-construct constant $K_2 = 0.5 \times 10^{10} ~M^{-1}$ as determined previously, the initial concentrations $A_0 = 2 \times 10^{-5} ~M,$ $C_0 \approx 1 \times 10^{-5} ~M,$ $S_0 = 1.8 \times 10^{-4} ~M,$ and the experimentally determined bound-to-free ratio $(B/c) = 2.5-4$. The calculation from four experiments yields $K_3 \approx (0.9 \pm 0.3) \times 10^{11} ~M^{-2}$.

### Membrane Binding Model

The affinity of $10k^{21}$ for red cell membranes was estimated by applying the Langmuir equation to the binding results in Fig. 6. With $s$ as the mean number of occupied sites per membrane, $m$ as the total number of independent and equivalent available sites for the $10k^{21}$ per membrane, $\theta$ as the fraction of occupied sites, $C$ as the free concentration of $10k^{21}$ construct, and $K_s$ as the association constant.

$$\sigma = m^2 s = m K_s C/(K_s C + 1) \quad (\text{Eq. 15})$$

As estimated under “Results,” $s$ is 200–2000 spectrin-actin binding sites occupied by $10k^{21}$ in the membrane of the lysed cells. Given $C \approx 0.25 \times 10^{-10} ~M$ as the unbound concentration of construct (Fig. 6), we can give order of magnitude estimates for $K_s$ by assuming a range of values for saturation $\theta$ such that $m$ is significantly less than $2 \times 10^6$ which is the total number of spectrin-actin-4.1 sites per intact membrane (36). This constraint on $m$ is motivated by the observation that ghost membranes remain substantially intact during a 20-min lysis on ice. Thus, with $\theta$ varied from 0.1 to 1 and using the estimated range for $s$, $m$ ranges from $2 \times 10^{10} ~M$ to 200, and $K_s$ remains within an order of magnitude of $4 \times 10^{12} ~M^{-1}$. The corresponding range of binding energies is then $-55$ to $-66$ kJ/mol, which is within the range of the ternary complex energies calculated from $K_0$ for purified spectrin, actin, and 4.1. Our membrane binding data may therefore be viewed as consistent, within approximation, with binding of the $10k^{21}$ at spectrin-actin junctional sites not occupied by protein 4.1.

### DISCUSSION

Four alternative isoforms of the protein 4.1 10-kDa region have been expressed and assayed by several methods to elucidate functional differences. The primary conclusion consistent with all experiments is that an alternatively expressed 21-aa cassette is essential to spectrin-actin-4.1 interactions and is thereby key to red cell membrane strength. The major red cell isoform $10k^{21}$ was active in binding to spectrin, stabilizing spectrin-actin, and associating with red cell membranes; it also functioned as a gelation factor and it was able to increase the strength of 4.1-deficient red cell membranes.

The lymphocyte $10k^{22}$ and the nonerythroid $10k^{19}$ isoforms, prepared in parallel by exactly the same techniques, were completely inactive in all of the assays even though the C-terminal three-fourths of these polypeptides are identical to $10k^{21}$. Quantitatively, the $10k^{21}$ functioned in the biochemical assays to the same extent as intact protein 4.1, it exhibited an apparent affinity for spectrin of $0.5 \times 10^6 ~M^{-1}$, and a much higher degree of association in the presence of actin, $0.9 \times 10^{11} ~M^{-2}$. Consistent with prior results using full-length 4.1 (34), no direct interaction between the $10k^{22}$ and actin was observed, in contrast to previous qualitative efforts (6). The ternary complex affinity of the $10k^{21}$ also appeared to give quantitative explanation for membrane-association.1 Built from this ternary complex activity (Fig. 7b), both purified 4.1 and $10k^{21}$ structured spectrin-actin into a solid-like phase, representing in vitro the solidifying or strengthening role the $10k^{21}$ was found to have in the membrane.

Given the dramatically contrasting functions of the $10k^{21}$ and $10k^{19}$, it is insightful to first describe structural features of these isoforms. Primary structures of both cassettes show charged residues at either end of each cassette: nine on the 21 aa and four on the 19 aa (18). Several charges also occur on the constitutively expressed residues bordering the cassette region. These charges may be expected to place each cassette at the folded protein’s surface, accessible for protein-protein interactions. The upstream splice site of these cassettes (Lys$^{300}, X$) is, in fact, accessible to chymotrypsin which cleaves at Trp$^{300}, Lys^{306}$ (39). Secondary structure predictions of both cassettes indicate that $\alpha$-helix occurs at either end of each cassette with a central region of less-ordered structure (40). Such helical structures should sterically remove a number of the 19 or 21 amino acids from the folded protein surface; it is an interesting fact that 12-20 residues/protein are typically involved in the contacts of interprotein associations over the range $10^6$ to $10^{13} ~M^{-1}$ (41). These structural considerations strongly suggest that the 21-aa cassette is located at the protein surface, has a sufficient number of residues to mediate spectrin-actin binding, and hence is likely to be monofunctional in the red cell. Quantitative analysis of membrane binding (Fig. 6) also indicates that the apparent affinity of the $10k^{21}$ can be explained solely on the basis of a spectrin-actin association. This is consistent with the lack of association found with both $10k^{22}$ and $10k^{19}$ (Fig. 6). Interactions of the $10k^{21}$ with membrane proteins or structures additional to spectrin-actin need not be posited to fully explain our results. This would imply that the $10k^{21}$ does not “link” the skeleton and bilayer in situ, it only stabilizes the spectrin-actin complex.

Localization of the cassettes to the surface of protein 4.1 also supports the more general observation that intron-exon boundaries nearly always (>95%) occur at the surface of a folded protein (42). This generalization about protein and gene structure supports a current perspective on the micro-gene hypothesis (43) which, when applied to protein 4.1, would view each of the alternatively expressed cassettes as structur-
ally and functionally autonomous peptides. Unification of various exons into a single gene would then have evolved as the most efficient way to express a complex of functions. High homology among species would be expected of a conserved functional unit and is indeed found as far back as Xenopus. The 21-aa cassette in Xenopus differs from the human by 5 residues: four conservative changes plus one structure-preserving change (44) are all localized to the N terminus of this potential microgene.

Since the 10k21 is necessary and sufficient for the ternary complex formation manifested in membrane strength, we speculate that the other regions of the 80-kDa protein 4.1 may act in vivo to chemically or physically modulate this primary function. For instance, calmodulin binding to 4.1 in or near the domain upstream of the 10 kDa, appears to be a strong chemical modulator of both the spectrin-actin interaction (12) and membrane strength (45). Likewise, phosphorylation of 4.1 can result in 5- to 20-fold (46) or greater (47) reductions in spectrin-related affinity. Note that the large magnitude of these effects suggests that such post-translational modifications are not reflected in our results. In this paper we have shown that the 10k21 can impart normal strength to membranes (Fig. 8), even in the complete absence of the mutative bilateral region of 4.1, the 20-kDa domain at the N terminus of 4.1 (48). This finding implies that 4.1 does not mechanically link the skeleton to the overlying bilayer in the sense of providing resistance to large membrane deformations, i.e. 4.1 function does not require simultaneous binding to both spectrin-actin, and glycoprotein or band 3. In fact, it has been suggested previously that association of spectrin with the membrane is actually inhibited by protein 4.1 since calorimetry shows a 50% decrease in the association enthalpy (49). Ankyrin, in contrast, was found to be a very strong mediator of association, increasing the reaction enthalpy by 350%. Band 3 and glycoprotein may have compartmentalization roles, maintaining high membrane concentrations of 4.1 to drive association with the skeleton. This is consistent with the observation that high cell concentrations of protein 4.1 (several fold above normal) are required to impart normal membrane strength to completely 4.1-deficient cells (50) also lacking 90% of glycoprotein C (9).

With all of these insights as to why the 10k21 functions so well for the red cell in contrast to other isoforms, some understanding of how this structural protein functions may be gained through a quantitative comparison of biochemical and biophysical energetics. In this paper we have used ektacytometry as a technique to quantify cellular function of the 10k21. The measurements (e.g. Fig. 8) represent an “average” deformation response of cells suspended in a shearing field. A direct view of such cell deformations is provided by rheoscopy, and recent progress on these flow-induced deformations (51) has lead to formal connections with well established red cell mechanics worked out primarily from single cell micropipet manipulation experiments (52). Drawing from these theoretical efforts, an argument* may first be made that the

\[ \text{local work done on the membrane by the fluid tractions imposed in the ektacytometer is near the yield point energy (energy at which this material fails) in some region of the membrane.} \]

Failure in shear can be formulated for that part of the membrane, and with completely 4.1-deficient cells, membrane failure is immediate (31). Partially 4.1-deficient membranes begin to fragment after a short interval of sustained deformation (Fig. 8). Since incorporation of either protein 4.1 or GST-10k21 into deficient membranes gives the membranes normal strength in order to endure large deformations, we presume, as a simple model, that the membrane yield energy in shear is increased by the spectrin-actin interaction. By pulling membrane tethers from cells and considering this failure process also as a failure in shear (52), it can be estimated from measurement (53) that the typical yield energy for any part of the membrane is 65% higher for normal red cells versus completely 4.1-deficient cells. Given the skeleton’s connectivity (13), whereby failure at the end of a single spectrin cross-link should require failure of only one spectrin-4.1 bond (14), and given 200,000 molecules of protein 4.1 (36) per 100 µm² of membrane surface, the contribution of protein 4.1’s 21-aa cassette to the membrane failure threshold may be estimated to be about 4 kJ/(mol cross-link).

Our solution measurements of association between 10k21 and spectrin agree with previous reports for intact 4.1 and may be expressed energetically as -32 kJ/(mol of protein 4.1). However, association of two diffusing proteins in solution involves a significant loss of entropy estimated to be -16 to -28 kJ/mol for 50-kDa proteins (54). A “local” Gibbs’ free energy for spectrin-4.1 association, i.e. an inter-protein bond energy, may therefore actually be nearer -48 to -60 kJ/(mol of spectrin-protein 4.1). A similar estimate may be made from the ternary complex result. Ignoring transition state energies and bond strain, the inter-protein bond energy is about 12-15 times above protein 4.1’s energetic contribution to membrane integrity as determined mechanically. A possible molecular mechanism for initiation of membrane yield would then involve localization of the strain energy density, if just transiently, to one out of 12-15 cross-links. Thus, with some assumptions about cell mechanics in the ektacytometer, both our fragmentation data and biochemical results may be integrated into a single energetic view of membrane failure.

\[ F = \mu(2/3 + \lambda^2 - 2), \quad \mu_{\text{local}} = 6 \times 10^{-3} \text{dyn/cm} \] (Eq. 16)

The response parameter \( \mu \) is a generalized shear modulus that characterizes the membrane material in deformation. As a logarithmic function of the fluid shear stress from 10 to 100 dyn/cm², the membrane strain energy increases quasilinearly from a very small value to 0.004 dyn/cm². Elongation of the cell’s major axis has separately been shown to be linearly increasing up to 1000 dyn/cm² and so we expect the membrane strain energy at any material point along the contour to be of the order 0.01 dyn/cm² at the shear stress of 750 dyn/cm² used in the ektacytometry.

The energy estimate above can be compared to a mechanical determination of protein 4.1’s function as obtained by another method for stretching red cells to failure. In this method, cells are first attached to a coverslip mounted in a microscope flow chamber. A sufficiently high flow rate is then set which pulls the cell away from its point of attachment on the coverslip, drawing out a thin membrane tether in the process. Up to the instant of tether formation, hyperelasticity may still apply to cell deformation, with the transition to tether formation at the attachment point corresponding to a simple threshold or yield energy of the membrane. Normal cells yield, on average, when the strain energy in the region of attachment is about 0.016 dyn·cm², while completely 4.1-deficient cells yield at 0.010 dyn·cm² (53). The calculated difference of 0.007 dyn/cm is a mechanical measure of protein 4.1’s contribution, and presumably the 21-aa cassette’s, to the integrity of the stretched membrane.
The set of functions we have found with the 21-aa isofrom elucidates its importance to normal red cell physiology. Although formal connections between the chemistry and mechanics are not complete, to a first approximation it appears that interaction of the 10k$^2$ domain with spectrin and actin is necessary and sufficient to fulfill protein 4.1's role in maintaining membrane strength. Other domains and interactions of 4.1 may modulate this function, but they do not appear to have a direct role in maintaining the membrane strength so essential to a circulating red cell. Consistent with this view, red cells expressing mutant 4.1 species with deletions only in the 10k$^2$ retain 4.1 at the membrane (55) but exhibit easily fragmentable membranes. Finally, based on the striking functional differences demonstrated for the isoforms, our results also begin to offer an evolutionary explanation for the complexity of exon expression from the 4.1 gene.

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