Functional Characterization of Spectrin-Actin-Binding Domains in 4.1 Family of Proteins†

J. Aura Gimm,‡ Xiuli An,*,# Wataru Nunomura,§ and Narla Mohandas‡,#

UCSF/UCB Bioengineering Graduate Group and Life Science Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, New York Blood Center, New York, New York 10021, and Department of Biochemistry, School of Medicine, Tokyo Women's Medical University, Shinjuku-ku, Tokyo, Japan, 162-8666

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ABSTRACT: Protein 4.1R is the prototypical member of a protein family that includes 4.1G, 4.1B, and 4.1N. 4.1R plays a crucial role in maintaining membrane mechanical integrity by binding cooperatively to spectrin and actin through its spectrin-actin-binding (SAB) domain. While the binary interaction between 4.1R and spectrin has been well characterized, the actin binding site in 4.1R remains unidentified. Moreover, little is known about the interaction of 4.1R homologues with spectrin and actin. In the present study, we showed that the 8 aa motif (LKKNFMES) within the 10 kDa spectrin-actin-binding domain of 4.1R plays a critical role in binding of 4.1R to actin. Recombinant 4.1R SAB domain peptides with mutations in this motif showed a marked decrease in their ability to form ternary complexes with spectrin and actin. Binary protein-protein interaction studies revealed that this decrease resulted from the inability of mutant SAB peptides to bind to actin filaments while affinity for spectrin was unchanged. We also documented that the 14 C-terminal residues of the 21 amino acid cassette encoded by exon 16 in conjunction with residues 27−43 encoded by exon 17 constituted a fully functional minimal spectrin-binding motif. Finally, we showed that 4.1N SAB domain was unable to form a ternary complex with spectrin and actin, while 4.1G and 4.1B SAB domains were able to form such a complex but less efficiently than 4.1R SAB. This was due to a decrease in the ability of 4.1G and 4.1B SAB domain to interact with actin but not with spectrin. These data enabled us to propose a model for the 4.1R-spectrin-actin ternary complex which may serve as a general paradigm for regulation of spectrin-based cytoskeleton interaction in various cell types.

Red blood cell protein 4.1 (4.1R)[1] has multiple binding sites for transmembrane and membrane skeletal proteins and plays a critical role in maintaining cell morphology and membrane mechanical properties (*1*, *2*). More specifically, the spectrin-actin-4.1R ternary complex in the membrane skeleton has previously been shown to play an important role in regulating mechanical stability of the erythrocyte membrane (*3*, *4*). Hereditary defects in 4.1R result in abnormally shaped erythrocytes with decreased membrane mechanical stability manifested clinically as hemolytic anemia (*5*).

Three main structural/functional domains have been identified in the prototypical 80 kDa 4.1R. First, a 30 kDa N-terminal membrane binding domain possesses binding sites for the cytoplasmic tails of integral membrane proteins such as band 3 (*6*, *7*) glycophorin C (GPC) (*8*, *9*), and CD44 (*10*). This domain also binds to p55 (*11*, *12*), calmodulin (*13*−*15*), and phosphatidylserine (*16*−*19*). Second, an internal 10 kDa domain is responsible for the spectrin-actin-binding (SAB) activity, which plays a critical role in regulating membrane mechanical stability (*20*−*22*). Third, a 22−24 kDa C-terminal domain has recently been reported to bind to the immunophilin FKBP13 (*23*), to nuclear mitotic apparatus protein NuMA (*24*, *25*), to tight junction proteins ZO-1 and ZO-2 (*26*), and to eukaryotic translation initiation factor eIF3-p44 (*27*).

Detailed characterization of the 4.1R cDNA has shown that the SAB domain of 4.1R is encoded by an alternatively spliced exon, exon 16, which encodes the N-terminal 21 amino acids (aa) of SAB, and a constitutively expressed exon, exon 17, which encodes the C-terminal 59 aa of SAB (*28*). Functional characterization of 4.1R SAB domain enabled the identification of two spectrin-binding motifs: the N-terminal 21 amino acid cassette encoded by exon 16 and the region encompassing residues 27−43 within the C-terminal 59 aa of SAB encoded by exon 17 (*4*, *22*). However, the actin-binding motif in 4.1R remains to be defined.

Three novel 4.1R-like genes have been recently cloned and characterized (*29*−*31*). These include the widely expressed homologue 4.1G, the brain homologue 4.1B, and the neuronal homologue 4.1N. These three new 4.1 genes share a high degree of homology with prototypical 4.1R in the three main structural/functional domains: N-terminal

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* Address correspondence regarding this manuscript to Xiuli An. The New York Blood Center, 310 East 67th Street, NY, NY 10021. Phone: 212-570-3247. Fax: 212-570-3195. E-mail: xlan@lbl.gov.

‡ Lawrence Berkeley National Laboratory.

§ Tokyo Women's Medical University.

New York Blood Center.

[1] Abbreviations: 4.1R, human erythrocyte protein 4.1; SAB, spectrin-actin binding; aa, amino acid; PBS, phosphate-buffered saline; BSA, bovine serum albumin; $K_{(D)}$: dissociation constant from kinetic analysis.

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membrane binding domain, SAB domain, and C-terminal domain in 4.1R homologues being 72−74, 50−70, and 70−80% identical to corresponding domains in 4.1R, respectively (*31*). In contrast to the well characterized interaction between 4.1R SAB domain and spectrin-actin, very little is known about the interaction of the SAB domain of 4.1R homologues with spectrin and actin.

In the present study, we endeavored to define the actin-binding motif within 4.1R SAB domain. We were able to show that the ability of recombinant 4.1R SAB domain peptides with deletions or mutations in the putative 8 aa actin binding motif (LKKNFMES) to form ternary complexes with spectrin and actin was markedly diminished. The decreased ability of the mutant peptides to form ternary complex was the result of their failure to bind to actin but not to spectrin. Thus, the novel 8 aa motif we have identified plays a critical role in 4.1R binding to actin. Furthermore, we documented that the 14 C-terminal residues of the 21 aa cassette encoded by exon 16 in conjunction with residues 27 to 43 encoded by exon 17 constituted a fully functional minimal spectrin-binding motif. Finally, we investigated the ability of SAB domain of 4.1R homologues to interact with spectrin and actin. 4.1N SAB domain bound to neither spectrin nor actin, while 4.1G and 4.1B SAB domains could form a ternary complex with spectrin and actin but with a lower efficiency than 4.1R SAB domain. This resulted from the fact that 4.1G and 4.1B SAB domains had a decreased ability to bind to actin, while their binding affinity for spectrin was the same as that of 4.1R SAB domain. Taken together, these studies have enabled us to propose a model for the organization of spectrin-actin-4.1R ternary complex, which may serve as a general paradigm for regulation of spectrin-based cytoskeleton interaction in various cell types.

EXPERIMENTAL PROCEDURES

Materials. pET-28b(+) bacterial expression vector was purchased from Novagen Inc. (Madison, WI). The cobalt-based immobilized metal affinity chromatography resin (TALON) was purchased from Clontech (Palo Alto, CA). Nonmuscle actin monomer derived from human platelets was purchased from Cytoskeleton (Denver, CO). Silver stain plus kit was purchased from Bio-Rad (Hercules, CA). All other reagents were purchased from Sigma (St. Louis, MO) unless noted otherwise.

Methods. Preparation of Recombinant Proteins. Recombinant proteins corresponding to SAB domains of 4.1 family of proteins (4.1R, 4.1G, 4.1B, and 4.1N) were expressed as hexahistidine (6×His)-tagged fusion proteins. Site-directed mutagenesis of 4.1R SAB was performed using the QuikChange kit from Stratagene (La Jolla, CA) following the manufacturer's instructions. First, a 6×His-tag was inserted between the *Eag*I and the *Sal*I sites of the pGEX-KT 10 kDa 4.1R SAB construct previously described (*22*). Different variants of SAB bearing a 5′*Nco*I and a 3′*Sal*I site were prepared by polymerase chain reaction (PCR) amplification of the modified pGEX-KT 10 kDa 4.1R SAB construct. pET-28b(+) was altered by mutation of its *Eag*I site into a *Sac*II site to create the pET-28Q vector. This modification was necessary since an *Eag*I site existed within the amplified SAB PCR fragments. All constructs were cloned *Nco*I-*Sal*I into pET-28Q vector, thus allowing translation of minimal length peptides bearing a C-terminal 6×His tag. The pET-28Q constructs were transformed into *Escherichia coli* BL21 cells (Stratagene) and protein expression was induced by addition of 0.1 mM IPTG. His-tagged peptides were purified by metal affinity chromatography using cobalt resin, eluted with 200 mM imidazole, and then dialyzed against either phosphate-buffered saline (10 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.4) for resonant mirror detection assay or against F-buffer (100 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4 pH 7.4, 1 mM EDTA, 0.2 mM DTT, 0.05 mM PMSF, and 1 mM NaN_3) for falling ball and actin pelleting assays.

Preparation of Spectrin. Spectrin was purified from human red blood cells obtained from a local blood bank as previously described (*32*, *33*). The eluted spectrin fractions were pooled after fraction analysis by 7% SDS−PAGE (*34*). Spectrin was precipitated by addition of ammonium sulfate (50% saturation), and then resuspended and dialyzed against either PBS for resonant mirror detection analysis or against F-buffer for falling ball viscometry assays.

Falling Ball Viscometry Assay. Gelation of spectrin/actin by protein 4.1 has been well documented as a sensitive assay for this structural protein (*35*, *36*). F-actin was obtained after polymerization of a 5 mg/mL solution of monomeric actin for 30 min at room temperature in the presence of 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, and 1 mM EGTA. F-actin (14 μM) and spectrin (1.8 μM) were incubated with SAB recombinant proteins for 1 h at 4 °C in 50 μL microcapillary tubes. Using a magnet rod, a small steel ball of 0.025 inch in diameter was placed on top of the solution interface, and the time taken by the ball to fall a specific distance was recorded. Sedimentation velocity was converted to apparent viscosity using glycerol standards of known viscosity at 25 °C. Apparent viscosity of reactions containing only spectrin and actin was used as a negative control. When the ball did not move from its initial position on top of the solution interface, we surmised that the protein mixture formed a gel.

Actin Pelleting Assay. Monomeric actin was polymerized as described above at 2.5 mg/mL for 30 min at room temperature. SAB recombinant proteins (2 μM) were incubated with F-actin (15 μM) for 30 min at room temperature, and then centrifuged at 25 °C for 10 min at 90,000 rpm (313,000*g*) in a Beckman Optima TL ultracentrifuge using a TLA100 rotor. The pellet was reconstituted into the original sample volume, and equal volumes of resuspended pellet and supernatant were analyzed by SDS−PAGE. Silver stained gels were analyzed by densitometry using reactions containing SAB recombinant proteins alone as negative controls.

Binding Assay by Resonant Mirror Detection. Protein-protein interactions between recombinant SAB 4.1 proteins and spectrin were studied using the resonant mirror detection method (*37*−*39*) of the IAsys system (Affinity Sensors, Cambridge, UK). Wild type and mutant SAB 4.1 proteins were immobilized on the surface of aminosilane-coated cuvettes according to the manufacturer's instructions. All spectrin-binding assays were carried out in PBS at 25 °C with constant stirring. The procedures employed for kinetic analysis of the binding interactions has been previously described (*9*, *39*). The dissociation constant obtained from this form of kinetic analysis is calculated as: $K_{(D)} = k_d/k_a$ where k_a is the association rate constant and k_d is the dissociation rate constant (*10*, *40*). Spectrin binding to a

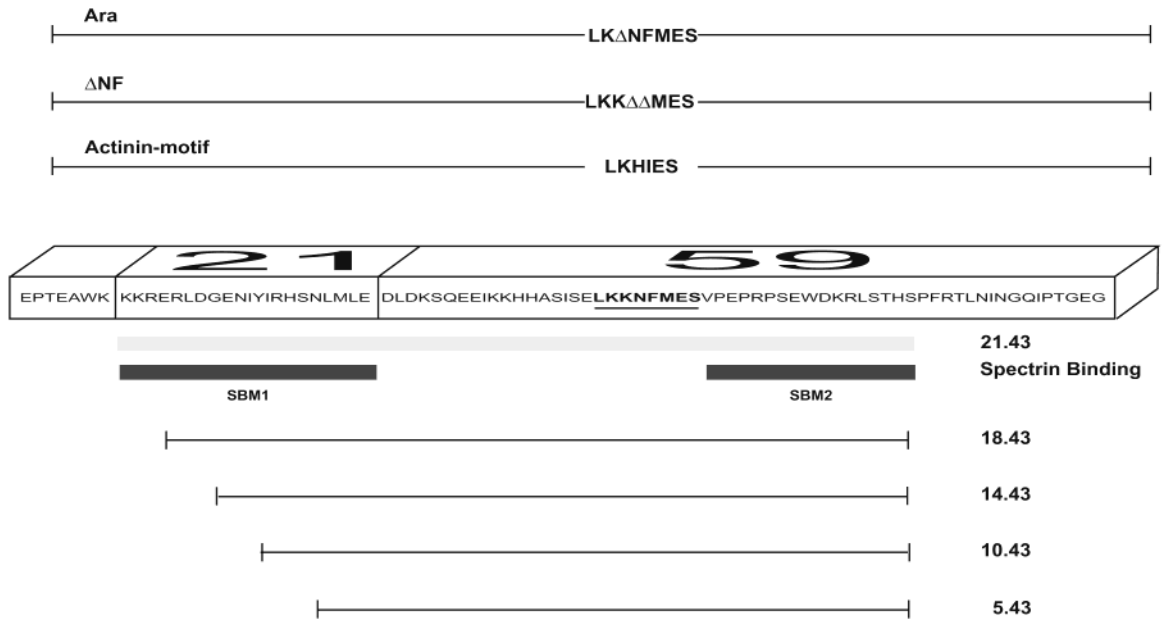

FIGURE 1: Primary structure of spectrin-actin binding (SAB) domain of 4.1R and of various mutants used in functional assays. The amino acid sequence of 4.1R SAB domain encoded by exons 16 and 17 is displayed in the middle of the figure. The bolded and underlined 8 aa sequence within the 59 residue cassette was targeted as a putative actin-binding site. At the top of the figure are shown the sequences of the various recombinant 4.1R SAB proteins mutated in the putative actin-binding motif. The Ara mutant (from 4.1 Aravis allele), previously found in patients with hereditary elliptocytosis, is missing one residue (K). The ΔNF mutant lacks two out of three residues (NF) in the spacer region. In the actinin-motif mutant, residues KNFM were changed to HI. At the bottom of the figure, are displayed the various SAB recombinant proteins used to map a minimal spectrin-binding motif in 4.1R SAB domain. Recombinant protein 21.43 represents the fully functional SAB domain, with the two spectrin-binding motifs (SBM1 and SBM2) highlighted as shaded boxes. The minimal spectrin binding motif within SBM1 region was characterized using a series of truncated constructs in exon 16-encoded region. These constructs are identified by two numbers separated by a period: the first number represents the number of C-terminal residues encoded by exon 16 (the 21 aa cassette), while the second number (43) indicates the number of N-terminal residues encoded by exon 17 (the 59 aa cassette).

cuvette immobilized with wild type SAB protein served as a positive control and a cuvette immobilized with recombinant protein missing the spectrin-binding motif 2 (SBM2) (Figure 1) served as a negative control.

RESULTS

Ternary Complex Formation of Recombinant 4.1R SAB Proteins with Spectrin and Actin. On the basis of similarities to various previously characterized actin binding motifs (*41*−*43*), a 8 aa sequence (LKKNFMES) encoded by exon 17 in the 10 kDa 4.1R SAB domain is a candidate actin-binding motif in 4.1R. This sequence consists of two conserved motifs LK(K/R) and E(S/T) found in other actin-binding proteins, separated by a three residue spacer sequence NFM. Three different mutant actin binding motifs were designed to test the functional effects of alterations in this region (Figure 1): (a) deletion of a single aa (K) in the conserved LKK motif (Ara mutant) previously identified in patients from a family with hereditary elliptocytosis (4.1R Aravis allele) (*44*); (b) deletion of two aa (NF) in the spacer sequence, reducing the spacing between the conserved LKK and ES sequences (2aa Δ mutant); and (c) mutating the sequence KNFM to HI to mimic the putative actin binding motif in actinin (actinin-motif mutant).

The ability of the wild type and the three mutant 4.1R SAB recombinant proteins to form ternary complexes with spectrin and actin was assayed by falling ball viscometry. The assay is based on previous observations showing that addition of 4.1R to a mixture of spectrin and actin results in the generation of a ternary protein complex and that the extent of ternary complex formation, which results in a gel formation, can be measured by monitoring changes in the viscosity of the solution.

Wild type 4.1R SAB protein induced a dose-dependent gelation of spectrin and actin at a concentration of 1 μM (Figure 2). In marked contrast, three of the mutants, with internal deletions resulting in a shorter putative actin-binding motif, were unable to induce gelation (Figure 2). Even at a concentration of 100 μM, neither the 2 aa deletion mutant nor the actinin-motif mutant had any effect on apparent viscosity of spectrin-actin mixture. At this concentration, the Ara mutant induced a modest increase in apparent viscosity but no gelation (Figure 2). Taken together, these data demonstrated that mutations in the putative actin-binding motif, LKKNFMES encoded by exon 17 of 4.1R, had significant effects on formation of the ternary complex with spectrin and actin.

Binary Interactions between Recombinant 4.1R SAB Proteins and Actin. To document that the putative actin binding motif in 4.1R SAB protein enabled actin binding and that the inability of the mutant recombinant proteins to form a ternary complex with spectrin and actin was due to their inability to bind to actin, we assayed interactions of these proteins with polymerized actin using the co-sedimentation assay. Wild type or the three mutant SAB domain peptides were incubated with filamentous actin, the mixture was then centrifuged, and the biochemical composition of the resulting pellets and supernatants was assayed. Wild type SAB protein pelleted with actin due to the binary interaction between 4.1R SAB domain and actin, a finding

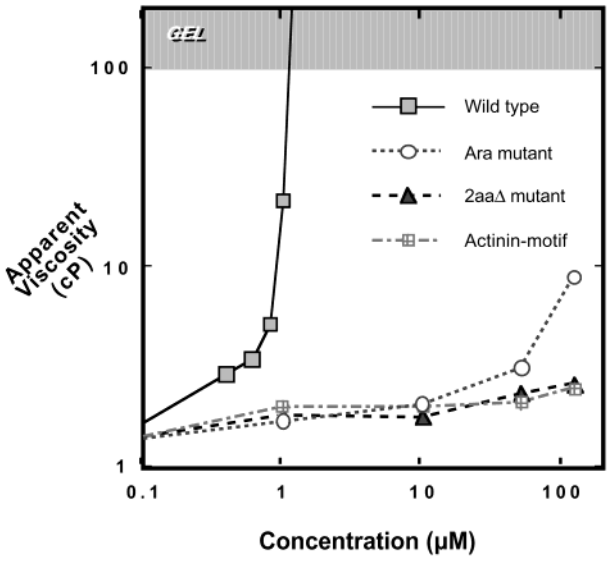

FIGURE 2: Ternary interaction between spectrin, F-actin, and 4.1R SAB recombinant proteins. F-actin (14 μM) and spectrin (1.8 μM) were incubated with recombinant 4.1R SAB proteins for 1 h at 4 °C in microcapillary tubes. Formation of a ternary complex was assessed by variations in sedimentation velocity of a steel ball placed in the microcapillary tubes as described in the methods section. Sedimentation velocity was converted to apparent viscosity using glycerol standards of known viscosity. Reactions with only spectrin and actin were used as baseline in all experiments. Deletions or mutations within the 8 aa sequence of the putative actin-binding motif resulted in a dramatic decrease in the ability of the mutant 4.1R SAB proteins to form ternary complexes with spectrin and actin.

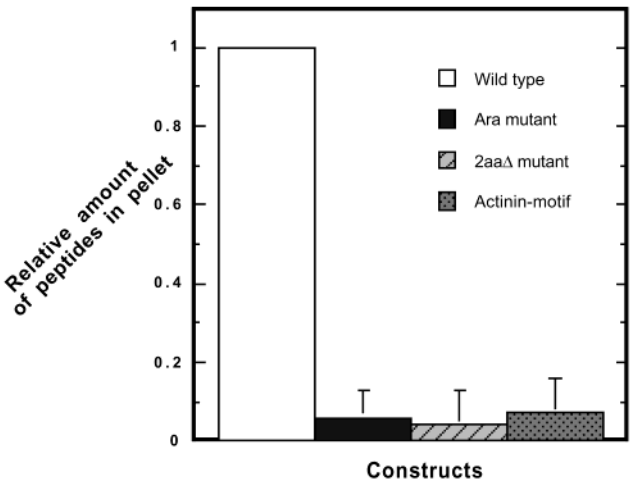

FIGURE 3: Binary interaction between F-actin and 4.1R SAB recombinant proteins. Binding of 4.1R SAB protein and various mutants was measured as described in the methods section. The amount of recombinant mutant proteins pelleted with actin was normalized relative to the wild type protein. The standard deviations are marked by the bars. Deletions within the 8 aa sequence of the putative actin-binding motif resulted in a dramatic decrease in the ability of the mutant 4.1R SAB proteins to bind to actin filaments.

consistent with earlier observations using native 80 kDa 4.1R (*45*). In marked contrast, all of the three recombinant proteins with mutations resulting in a shorter putative actin-binding motif showed markedly reduced binding to actin (Figure 3). These data implied that the LKKNFMES motif was responsible for actin binding.

Binary Interactions between Recombinant 4.1R SAB Proteins and Spectrin. To further document the specificity of the actin-binding motif, we measured the binary interaction between either wild type or the three mutated forms of 4.1R SAB domain proteins and purified spectrin using resonant mirror detection. Analysis of the binding response curves at varying concentrations of spectrin provided an apparent $K_{(D)}$ value, on the order of 2×10^{-7} M, for wild type 4.1R SAB domain peptide as well as for three recombinant peptides bearing deletions or mutations in the putative actin-binding motif (Table 1). Thus, deletions or mutations in the actin-binding motif, which had a marked effect on actin binding, had little or no effect on spectrin binding. These findings further implied that the observed decreases in the ability of the mutant proteins to form a ternary complex with spectrin and actin was due to their decreased ability to bind to actin. The ΔSBM2 construct, bearing a deletion of spectrin-binding motif 2 (Figure 1), failed to bind to spectrin, and was consequently served as a negative control.

Table 1: Binding Affinities for Binary Interactions between Spectrin and 4.1R Recombinant Proteins[a]

constructs	K_D [10^{-7} M]
wild type SAB	2.15 ± 0.79
ΔSBM2	no binding
Ara	2.54 ± 0.38
ΔNF	2.34 ± 0.98
actinin-motif	2.60 ± 0.60

[a] Resonant mirror detection was used to determine the dissociation constants (K_D) of the interaction between various 4.1R SAB recombinant proteins and spectrin as described in the methods section. Mutated 4.1R SAB proteins bound to spectrin with a similar affinity than wild type 4.1R SAB protein. The ΔSBM2 construct, lacking the spectrin-binding motif, was used as a negative control.

Ternary Complex Formation between SAB Domain of various 4.1 Homologues with Spectrin and Actin. The falling ball viscometry assay was also used to examine the ability of recombinant SAB domains of new members of protein 4.1 family to form a ternary complex with spectrin and actin. The amino acid sequences of 4.1 SAB domains of human 4.1R, human 4.1G, human 4.1B, and mouse 4.1N are shown in Figure 4A. It should be noted that human and mouse 4.1N SAB domains are 98.6% identical (*30*). Out of a total of 72 amino acids that constitute the SAB domain, there is only one amino acid difference between human and mouse SAB domain of 4.1N. Glutamic acid (56E) of human 4.1N SAB is replaced by aspartic acid (56D) in the mouse protein. The ability of each 4.1 homologue SAB domain to form a ternary complex with spectrin and actin correlated well with the degree of homology of its protein sequence with 4.1R SAB domain. Both 4.1G and 4.1B SAB proteins were able to form ternary complexes with spectrin and actin, while 4.1N was completely ineffective (Figure 4B). 4.1G SAB domain was approximately 2-fold less effective than the prototypical 4.1R SAB domain, an observation consistent with the recent finding that 4.1G SAB domain stimulates fodrin/actin association with less efficiency than 4.1R SAB domain (*46*), while 4.1B SAB domain was about 10-fold less effective.

Binary Interactions between SAB domain of Various 4.1 Homologue Proteins and Actin. The new members of protein 4.1 family were further studied by actin pelleting assay to examine their ability to interact with actin filaments (Figure 5). The 8 aa putative actin binding motifs in respective 4.1 homologues showed some minor conserved substitutions (Figure 4A), and some major changes which correlated with

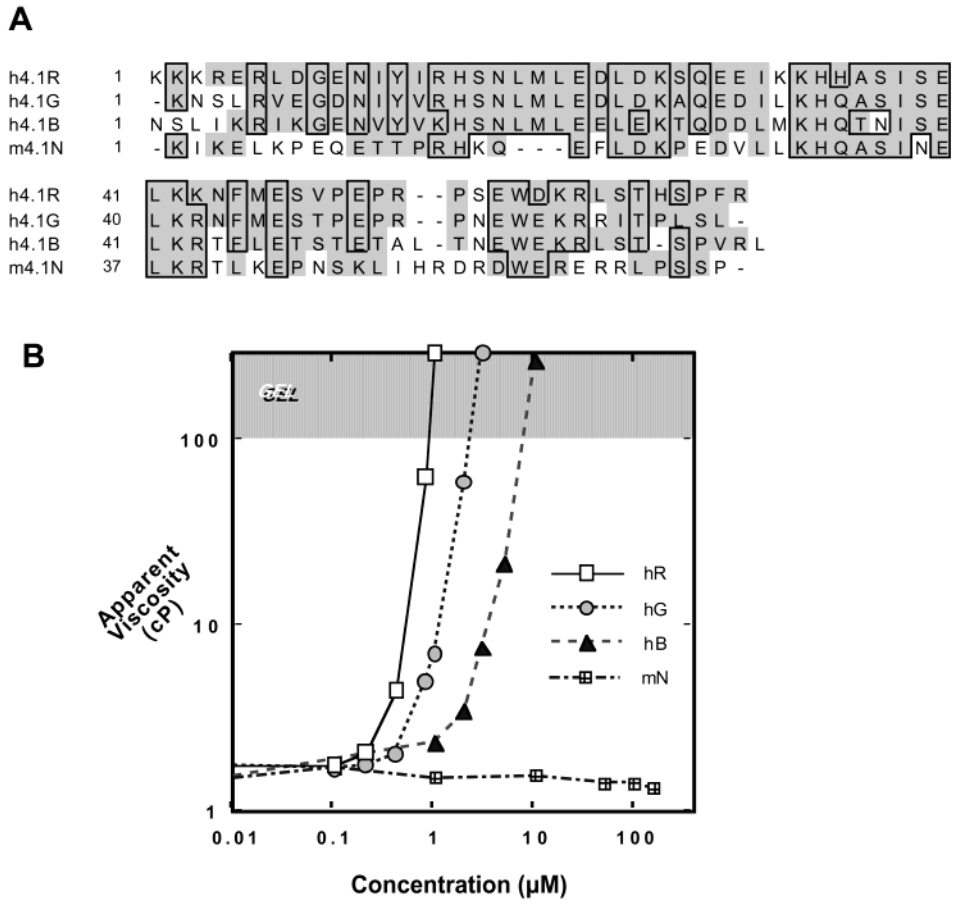

FIGURE 4: Ternary interaction between spectrin, F-actin, and different SAB domain of 4.1R homologues. Amino acid sequences of SAB domains of 4.1R homologues are shown in panel A. The ability of each 4.1 SAB domain to form ternary complexes with spectrin and actin correlates well with sequence homology to the prototypical 4.1R SAB domain. 4.1N, which lacks an SAB domain, is unable to form such ternary complexes, while 4.1G and 4.1B, which have similar sequences to 4.1R, are able to form ternary complexes although less effectively than 4.1R.

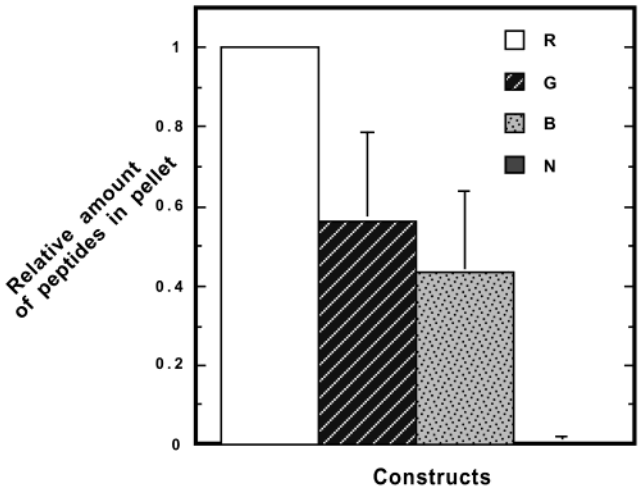

FIGURE 5: Binary interaction between F-actin and different SAB domains of 4.1R homologues measured by actin pelleting. SAB domains of 4.1G and 4.1B are able to pellet actin with 56 and 43% efficiency compared to 4.1R SAB domain, respectively. There is no detectable actin-binding activity of 4.1N SAB domain.

the extent of the decrease in these homologues' ability to bind to actin. SAB domains of 4.1G and 4.1B showed 56 and 43% of 4.1R SAB domain's ability to bind to actin filaments, respectively, while 4.1N SAB domain failed to bind actin. In both 4.1G and 4.1B, the integrity of LK and ES/T motifs was maintained, despite some amino acid substitutions. In 4.1N, the essential terminal hydroxyl-containing residue, serine, or threonine, was replaced by a proline residue, thereby significantly changing the ES/T motif. This major change in motif structure might explain why 4.1N could not bind to actin and furthermore why it was unable to form ternary complexes with spectrin and actin.

Binary Interactions between SAB Domain of Various 4.1 Homologue Proteins and Spectrin. To further understand the molecular basis for the inefficiency of 4.1G and 4.1B SAB domains to form ternary complexes with spectrin and actin, the ability of various 4.1 SAB domains to bind to spectrin was measured by resonant mirror detection assay. As shown in Table 2, while 4.1N SAB domain could not bind to spectrin, 4.1G, and 4.1B SAB domains interacted with spectrin as effectively as 4.1R SAB domain, suggesting that the alteration in 4.1G and 4.1B SAB domains' ability to form ternary complexes with spectrin and actin was due to a decrease in their ability to bind to actin but not to spectrin.

Spectrin-Binding Motifs of 4.1R. Earlier studies identified two distinct motifs—one encoded by exon 16 (residues 1−21) and one encoded by exon 17 (residues 27−43)—responsible for interaction of 4.1R with spectrin (Figure 1) (*4*, *22*). To define the minimal sequences involved in spectrin binding, we generated truncation constructs of sequences encoded by exon 16 (Figure 1, bottom). The ability of these N-terminal SAB truncation proteins to form ternary complexes with

Table 2: Binding Affinities for Binary Interactions between Spectrin and SAB Domain of 4.1R Homologues[a]

constructs	K_D [10^{-7} M]
4.1R	2.15 ± 0.79
4.1G	1.17 ± 0.23
4.1B	0.98 ± 0.10
4.1N	no binding

[a] Binding affinity of SAB domains of each 4.1 protein for spectrin was measured using resonant mirror detection as described in the methods section. 4.1G and 4.1B SAB domains bound to spectrin with similar affinity as that of 4.1R SAB domain. There was no detectable binding of 4.1N SAB domain to spectrin. Peptide sequences of each 4.1 protein SAB domain are shown in Figure 4A.

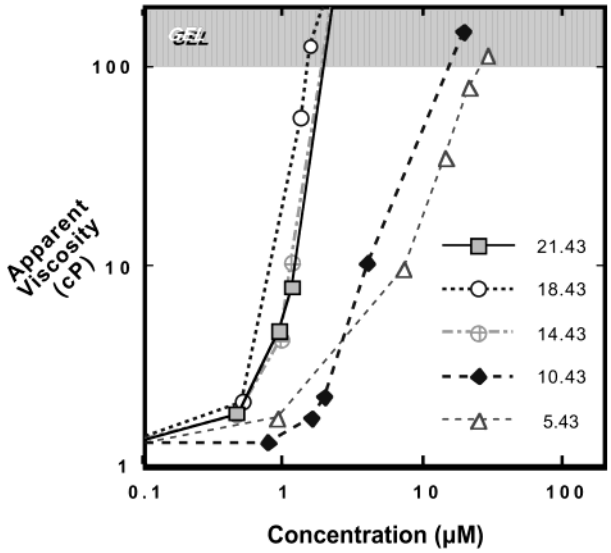

FIGURE 6: Ternary interaction between spectrin, F-actin and truncated 4.1R recombinant proteins assayed by falling ball viscometry. Recombinant proteins containing the 14 (14.43) or 18 (18.43) C-terminal residues encoded by exon 16 were as effective as the recombinant protein containing all 21 residues encoded by exon 16 (21.43) in forming a ternary complex with spectrin and actin. Recombinant proteins containing the sequences encoded by 10 (10.43) or 5 (5.43) C-terminal residues of exon 16 were 10- and 20-fold less effective in forming the complex, respectively.

spectrin and actin was evaluated using the falling ball viscometry assay. Recombinant proteins containing the sequences encoded by the C-terminal 14 (14.43) or 18 (18.43) residues of exon 16 were as effective as the recombinant protein encoded by full length exon 16 (21.43) in forming the ternary complex (Figure 6). All three of these proteins induced a dose-dependent increase in solution viscosity and induced gelation of spectrin and actin at a concentration of 1 μM. Recombinant proteins containing the sequences encoded by the 10 (10.43) or 5 (5.43) C-terminal residues of exon 16 were 10- and 20-fold less effective in forming a ternary complex (Figure 6). These findings implied that the 14 C-terminal residues in exon 16 in conjunction with residues 27−43 encoded by exon 17, which were previously shown to be involved in spectrin binding (*22*) constitute a fully functional minimal spectrin-binding motif.

DISCUSSION

In the present study, we showed that the 8 aa motif (LKKNFMES) within the 10 kDa spectrin-actin binding domain of 4.1R plays a critical role in binding of 4.1R to actin. The previously characterized actin-binding motifs in epsilon isoform of protein kinase C (*43*), and in actobindin (*42*) comprise a sequence of 6 aa, LKKQET and LKHAET, respectively. Other known actin-binding proteins, such as plastin and tropomyosin, also contain similar six amino acid sequences (*41*, *43*). These 6 aa sequence motifs have a conserved primary structure composed of a leucine residue at position 1, followed by two basic residues, a hydrophobic or uncharged residue, a conserved glutamic acid residue at position 5, and a terminal hydroxyl-containing residue. Interestingly, the 8 aa 4.1R actin-binding motif described in the present study is different. Although it starts with a leucine followed by two basic lysine residues and ends with a glutamic acid residue and a serine residue (a hydroxyl containing residue, characteristic of other actin binding sequences), the 4.1R motif contains three uncharged residues (NFM), instead of one found in six residue actin binding motifs, between the two basic residues (KK) and the conserved glutamic acid residue. The functional importance of this difference is revealed by the fact that 4.1R actin-binding motif mutants with only six residues (LKHIES and LKKMES) failed to bind to actin and to form ternary complexes with spectrin and actin. While the actin-binding motif, LKHIES, is able to bind actin in the context of actinin, it failed to bind actin when placed in the context of 4.1R. This finding strongly implies that the spacer length between LKK and ES motifs plays an important role in 4.1R binding to actin. The importance of the "spacer" sequence is further supported by our finding that the mutations of conserved residues LK and ES at the ends of the binding motif are less effective than deletions within the spacer region in reducing actin binding and in decreasing the ability to form ternary complexes (data not shown). The finding that various mutated 4.1R SAB domains can bind to spectrin with a similar affinity as that of wild type 4.1R SAB domain suggests that this 8 aa motif in 4.1R SAB domain is critical for actin binding.

Our observations with the Ara mutant (4.1 Aravis allele), in which one amino acid is missing in the putative actin-binding motif, can account for the red cell phenotype of hereditary elliptocytosis observed in patients carrying this allele (*46*). The inability of mutant 4.1R SAB domain to interact with actin and thereby failing to form a functionally effective junctional complex of spectrin-actin-4.1R can account for the decreased membrane mechanical stability of such mutant red cells and for generation of elliptocytic red cells. The in vivo findings with the naturally occurring mutant 4.1R lend support to our in vitro findings about the importance of 4.1R actin-binding motif in regulating red cell membrane function.

Previous studies demonstrated that two distinct motifs in 4.1R SAB domain were required for optimal binding to spectrin, one encoded by exon 16, "SBM1", and the other by exon 17, "SBM2" (*4*, *22*). In addition, the existence of a putative actin-binding motif encoded by exon 17 was postulated, a hypothesis that we confirm in the present study. Furthermore, we document that the 14 C-terminal residues encoded by exon 16 constitute a fully functional SBM1. Consistent with the functional importance of this domain, phylogenetic comparisons show that these 14 residues encoded by 4.1R exon 16 are evolutionarily conserved in a number of different species (*47*).

Recent cloning and characterization of three homologues of 4.1R (4.1G, 4.1B, and 4.1N) revealed that they share high homology with 4.1R in various functional domains including the spectrin-actin-binding domain. This compelled us to investigate the functional role of SAB domain of 4.1R homologues in stabilizing spectrin-actin interaction. Our finding indicates that, while 4.1N SAB domain is completely ineffective, 4.1G and 4.1B SAB domains possess the ability to interact with spectrin and actin, although with less efficiency than 4.1R SAB domain, an observation consistent with the recent finding that 4.1G SAB domain can promote fodrin-actin association (*46*). Binary interactions between 4.1 homologue SAB domains and actin or between 4.1 homologue SAB domains and spectrin indicate that the inability of 4.1N SAB domain to form a ternary complex with spectrin and actin is due to its inability to bind to both spectrin and actin. In contrast, the decreased effectiveness of 4.1G and 4.1B SAB domains to form ternary complex with spectrin and actin is mainly due to their decreased ability to bind to actin compared to 4.1R, while their ability to interact with spectrin was normal. Interestingly, the functionality of 4.1 homologue SAB domains correlates well with sequence homology to the prototypical 4.1R SAB domain. 4.1G, 4.1B, and 4.1N SAB domains share 73, 50, and 36% with 4.1R SAB domain, respectively (*31*). Of particular note, within the 8aa actin-binding motif (LKKNFMES), the integrity of both LK and ES/T motifs is maintained in 4.1G as well as in 4.1B SAB domain. Compared to 4.1R, the 8 aa actin-binding motif of 4.1G shows a single substitution of a lysine residue which is replaced by an arginine residue. This single conservative substitution minimally compromises the ability of 4.1G to interact with actin. 4.1B has the same substitution as 4.1G, but in addition displays two other residue changes in the spacer region, an asparagine replaced by a threonine and a methionine replaced by a leucine. The asparagine to threonine substitution changes the polarity of the spacer sequence in LKKNFMES, a change that may explain the significant decrease in the ability of this domain to promote gel formation in the presence of spectrin and actin. This observation emphasizes that not only the length of the spacer but also the nature of its residues is an important component of 4.1 protein actin-binding motif. Finally, analysis of 4.1N SAB domain sequence reveals numerous substitutions in the spacer region in addition to the substitution of the essential terminal hydroxyl-containing residue serine or threonine in proline, which results in a major alteration of the actin-binding motif, thus explaining the inability of 4.1N SAB domain to form a gel with spectrin and actin.

On the basis of these and earlier findings, we propose a model for the organization of the spectrin-actin-4.1R ternary complex (Figure 7). The last 14 of the 21 residues (GENIYIRHSNLMLE) encoded by exon 16, and residues 27−43 (VPEPRPSEWDKRLSTHS) encoded by exon 17, form the binding interface for the interaction of 4.1R with spectrin, while residues 19−26 encoded by exon 17, which encode the 8 aa motif LKKNFMES, form the binding interface for interaction of 4.1R with actin. Through its binding to both spectrin and actin, 4.1R significantly augments the overall affinity of the interactions within the ternary protein complex.

The model presented here for interaction of 4.1R with spectrin and actin may serve as a general paradigm for regulation of spectrin-based cytoskeletal interactions in various cell types in which this family of cytoskeletal proteins are expressed. The possibility therefore exists that 4.1 homologues can mediate formation of strong ternary complexes between skeletal proteins and thereby modulate important cytoskeletal functions in a wide variety of cells and tissues. Furthermore, since functional protein interactions involving 4.1 can be modulated both by phosphorylation and by calcium and calmodulin, it is highly likely that ternary complex formation and dissociation may be dynamically modulated in cells.

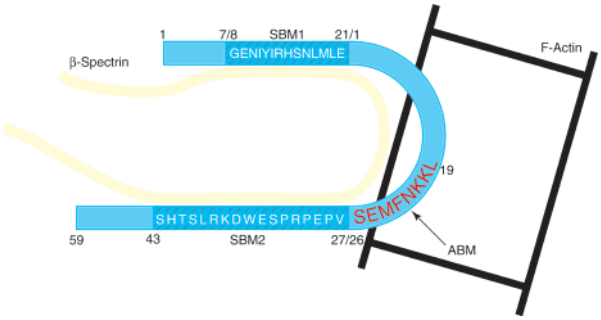

FIGURE 7: Schematic representation of 4.1 interaction with spectrin and actin. Residues 8−21 encoded by exon 16 (SBM1) and residues 27−43 encoded by exon 17 (SBM2) form the binding interface for the interaction of 4.1R with spectrin, while residues 19−26 encoded by exon 17 (ABM) form the binding interface for interaction of 4.1R with actin.

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