Identification and Functional Characterization of Protein 4.1R and Actin-Binding Sites in Erythrocyte β Spectrin: Regulation of the Interactions by Phosphatidylinositol-4,5-bisphosphate†

Xiuli An,*,‡ Gargi Debnath,‡ Xinhua Guo,‡ Shuwen Liu,‡ Samuel E. Lux,§ Anthony Baines,|| Walter Gratzer,⊥ and Narla Mohandas‡

Red Cell Physiology Laboratory, New York Blood Center, New York, New York 10021, Division of Hematology/Oncology, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115, Department of Biosciences, University of Kent, Canterbury, Kent, CT2 7 NJ, United Kingdom, Randall Division of Cell and Molecular Biophysics, Kings College, Guy's Campus, London SE1 1UL, United Kingdom

Received December 20, 2004; Revised Manuscript Received April 22, 2005

ABSTRACT: The ternary complex of spectrin, F-actin, and protein 4.1R defines the erythrocyte membrane skeletal network, which governs the stability and elasticity of the membrane. It has been shown that both 4.1R and actin bind to the N-terminal region (residues 1−301) of the spectrin β chain, which contains two calponin homology domains, designated CH1 and CH2. Here, we show that 4.1R also binds to the separate CH1 and CH2 domains. Unexpectedly, truncation of the CH2 domain by its 20 amino acids, corresponding to its N-terminal α helix, was found to greatly enhance its binding to 4.1R. The intact N terminus and the CH1 but not the CH2 domain bind to F-actin, but again, deletion of the first 20 amino acids of the latter exposes an actin-binding activity. As expected, the polypeptide 1−301 inhibits the binding of spectrin dimer to actin and formation of the spectrin−actin−4.1R ternary complex *in vitro*. Furthermore, the binding of 4.1R to 1−301 is greatly enhanced by PIP_2, implying the existence of a regulatory switch in the cell.

Underpinning the survival of erythrocytes in circulation is a skeletal network attached to the plasma membrane (for reviews, see refs *1* and *2*). This roughly hexagonal lattice is made up of long $(\alpha\beta)_2$ tetramers of spectrin, linked at their ends to junctional complexes, that contain short filaments of F-actin. Spectrin β chains embody an actin-binding activity in their N-terminal regions, which, however, is weakly expressed in the absence of other proteins (*3*−*6*). Two proteins, human erythrocyte protein 4.1 (4.1R)[1] and adducin, that promote the binding of spectrin to actin are found in the junctional complexes (*7*). Spectrin, 4.1R, and F-actin together form a high-affinity ternary complex (*4*). The importance of this interaction is evident from the properties of red cells in which it is defective. Thus, inherited 4.1R deficiency leads to hereditary elliptocytosis and mechanical instability of the membrane, which can be relieved, and near-normal membrane properties restored, by addition of exogenous 4.1R to the ghosts (*8*−*10*). The Kissimmee variant of β spectrin, in which Trp-202 is replaced by arginine (*11*), with a concomitant disturbance of the spectrin−4.1R interaction, is marked by an autosomal dominant hereditary spherocytosis (*2*, *12*).

The binding region in 4.1R for spectrin and actin has been mapped in fine detail. Spectrin- and actin-binding sites are located in a 10-kDa spectrin−actin-binding (SAB) domain (*13*−*14*). Two spectrin-binding motifs have been identified, an N-terminal 21 amino acid cassette encoded by exon 16 and a region encompassing residues 27−43 within the C-terminal 59 amino acid stretch of SAB, encoded by exon 17 (*15*). The residues between the two spectrin-binding motifs are responsible for actin binding (*16*).

Much less is known about the manner in which spectrin binds actin and 4.1R. β Spectrin is one of a large number of actin-binding proteins with an N-terminal actin-binding region characterized by two calponin homology domains (CH domains) (*17*). A tryptic fragment of erythrocyte (βI) spectrin (residues 47−186), containing CH1 (residues 51−156), has been shown to bind actin *in vitro* (*6*), but recombinant CH2 from βII spectrin does not (*18*). Nothing has previously been determined regarding the 4.1R-binding site(s) in the N-terminal region of erythrocyte β spectrin.

In the present study, we have identified the 4.1R- and actin-binding sites in βI spectrin. We obtained the unexpected result that there are two 4.1R- and two actin-binding sites

† This work was supported in part by NIH Grants DK 26263 and DK 32094 and by Project Grant 96/18062 from U.K. Biotechnology and Biological Sciences Research Council and a Leverhulme Research Fellowship.

* To whom correspondence should be addressed: Red Cell Physiology Laboratory, 310 E. 67th St., New York, NY 10021. Telephone: 212-570-3247. Fax: 212-570-3195. E-mail: xan@nybloodcenter.org.

‡ New York Blood Center.

§ Harvard Medical School.

|| University of Kent.

⊥ Kings College.

[1] Abbreviations: 4.1R, human erythrocyte protein 4.1; CH domain, calponin homology domain; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP3, inositol-triphosphate; PS, phosphatidylserine; lyso-PS, lysophosphatidylserine; PC, phosphatidylcholine; GST, glutathione-*S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; CD, circular dichroism; PBS, phosphate-buffered saline.

10.1021/bi047331z CCC: $30.25
Published on Web 07/09/2005

in the N-terminal region of β spectrin, one of each in both the two CH domains. A remarkable feature is that both binding sites in the CH2 domain are largely masked by the first 20 residues of the domain. We have further found that the interaction of 4.1R with the spectrin N-termimal region is regulated by phosphatidylinositol-4,5-bisphosphate (PIP_2).

EXPERIMENTAL PROCEDURES

Materials. pGEX-4T-2 vector, glutathione-Sepharose 4B, fast-flow Q-Sepharose, Superdex-200, and PreScission proteinase were purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). pET31-b(+) vector was from Novagen (Madison, WI), and BL21(DE3) bacteria was from Stratagene (La Jolla, CA). Restriction enzymes were from New England BioLabs (Beverly, MA). Reduced form glutathione and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma (St. Louis, MO). Purified phosphatidylserine (PS), phosphatidylcholine (PC), PIP_2, PIP from brain and synthetic lysophosphatidylserine (lyso-PS), and inositol-triphosphate (IP3) were purchased from Sigma (St. Louis, MO) and Avanti (Alabaster, AL). Platelet actin was from Cytoskeleton Inc. (Denver, CO). Proteinase inhibitor cocktail set II was from Calbiochem (San Diego, CA). Rabbit anti-4.1R antibody was prepared by injecting rabbits with 4.1R purified from red blood cells as antigen. The antibody specifically recognizes the nonconserved 16-kDa domain of 4.1R, and it does not cross-react with other proteins of the 4.1 family. Monoclonal anti-glutathione-*S*-transferase (GST) antibody was from Oncogene (Boston, MA), and HRP-conjugated anti-mouse IgG and HRP-conjugated anti-rabbit IgG were from Jackson ImmunoResearch Laboratory (West Grove, PA). Renaissance chemiluminescence detection kit was from Pierce Biotechnology, Inc. (Rockford, IL). All other chemicals were reagent-grade products from standard sources.

Phospholipid vesicles were prepared by sonication as described before (*19*). Spectrin from erythrocytes was prepared according to the method of Tyler et al. (*20*).

Methods: Subcloning and Preparation of Recombinant β-Spectrin Polypeptides. The desired regions of human β spectrin were obtained by PCR amplification, using full-length β-spectrin cDNA (kindly provided by Dr. J. S. Morrow, Yale University) as a template, and subcloned into pGEX-4T-2 vector using *Eco*RI and *Sal*I cloning sites up- and downstream, respectively. The following primers were used to generate spectrin fragments: (1) 1−301FOR, GGAATTCCCATGATGACATCGGCCACAGAGTTTG-AAAATGTG; (2) 1−301REV, ACGGGTCGACTTCAGT-CTCAATGGCATGGTCAATAACC; (3) CH2 FOR, GG-AATTCCCATGGAAACACGCTCAGCCAAGGATGCG; (4) CH2 REV, ACGGGTCGACTTCACACCTTCATCTTG-GAGAAGTAGTGG; and (5) T-CH2 FOR, GGAATTC-CCATGTTAATGTCACCAACTTTACCTCCAGC.

Two primers used to insert a PreScission proteinase cleavage site between the CH1 and CH2 domains in construct 1−301 were FOR, CTCCGCTTCCAGATTCAGCTGGA-AGTTCTGTTCCAGGGGCCCGAAACACGCTCAGCC-AAG and REV, CTTGGCTGAGCGTGTTTCGGGCCCCTG-GAACAGAACTTCCAGCTGAATCTGGAAGCGGAG.

The fidelity of all of the constructs was verified by DNA sequence determination. Recombinant β-spectrin polypeptides were purified on a glutathione-Sepharose-4B affinity column. GST-1−160 (containing the CH1 domain) was obtained by cleaving the CH2 domain from the polypeptide 1−301 construct with a PreScission proteinase cleavage site between the domains. All of the recombinant polypeptides were expressed at 16 °C.

For circular dichroism (CD) measurements and analytical ultracentrifugation, GST was cleaved from 1 to 301 and the CH2 domain with thrombin, and the resulting proteins were dialyzed against phosphate-buffered saline (PBS). We were unable to recover enough of the 1−160 and T-CH2 for the above analyses after GST cleavage because of the low expression yield of these two peptides. Protein concentrations were determined spectrophotometrically, using absorptivities calculated from the tryptophan and tyrosine contents (*21*). For the binding assay, the proteins were dialyzed against the binding buffer (10 mM Tris at pH 7.4, 150 mM NaCl, and 0.05% Tween-20). All samples were centrifuged at 230000*g* for 30 min at 4 °C before use to remove any insoluble aggregates.

Subcloning and Preparation of 4.1R. cDNA encoding human 80-kDa 4.1R was cloned into the pET 31b(+) vector, using *Nsi*I and *Xho*lI cloning sites up- and downstream, respectively. A stop codon was introduced before the 6×His so that this tag could be eliminated. The cDNA was transformed into *Escherichia coli* BL21 (DE3) for protein production. Expression and purification of 80-kDa 4.1R were accomplished as follows: Bacteria were grown in 500 mL LB medium at 37 °C for approximately 10 h until the absorbance at 650 nm reached ~0.7. Expression of 4.1R was induced by 0.1 mM IPTG at 16 °C overnight. Bacteria were harvested by centrifugation and resuspended in 25 mL PBS-T (10 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, 150 mM NaCl, and 0.05% Triton X-100), and contents of one vial of proteinase inhibitor cocktail set II (dissolved in 1 mL of DMSO) were added. The bacterial lysate was passed through a gel-filtration column (Superdex-200, 2.5 × 125 cm) pre-equilibrated with gel-filtration buffer (10 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Triton X-100, and 1 mM NaN_3). The fractions containing almost pure 4.1R were pooled and dialyzed against ion-exchange buffer (10 mM Na_2HPO_4/NaH_2PO_4 at pH 7.4, 70 mM KCl, and 1 mM EDTA). The 4.1R was eluted from a Q-Sepharose ion-exchange column by a KCl gradient from 100 to 500 mM in the above buffer. The 4.1R concentration was determined spectrophotometrically, using a calculated specific absorptivity. The purified 4.1R was dialyzed against binding buffer (10 mM Tris at pH 7.4, 150 mM NaCl, and 0.05% Tween-20) and centrifuged at 230000*g* for 30 min at 4 °C before use.

CD Spectroscopy. UV CD spectra were recorded in a Jasco 700 spectropolarimeter, equipped with a thermostated cell-housing, in cells of 1 mm path length. The CD data are displayed as mean residue molar ellipticities. The α-helix content was estimated from the molar residue ellipticity, $[\theta]_{222}$ at the extremum at 222 nm, as $[\theta]_{222}/(-360\ 000)$ (*22*).

Mass Spectrometric Analyses. Mass spectrometric analyses were performed in an Applied Biosystems (ABI, Foster City, CA) Voyager DE MALDI mass spectrometer. Spectra were calibrated against an external standard. Samples at a concentration of 10 μM were mixed with sinapinic acid matrix solution, 1:1 (in 50% acetonitrile/0.1% trifluoroacetic acid,

from Sigma), and 1 to 2 μL from each mixture was spotted onto a sample plate and dried. The results were externally calibrated against aldolase (Sigma; $[M + H]^+ = 39\,212.28$) and bovine serum albumin (Sigma; $[M + H]^+ = 66\,430.09$).

Sedimentation Equilibrium Analysis. The apparent molecular weights of proteins were measured by sedimentation equilibrium in a Beckman Coulter ProteomeLab XL-A Analytical Ultracentrifuge, using ultraviolet scanning optics. Samples of 100 μL were introduced into 12 mm six-sector charcoal-filled Epon centerpieces. The cells were scanned at 280 and 235 nm at 0.001 cm intervals, and data points were averaged from 10 measurements. The rotor speed was 20 000 rpm. Molecular masses were determined by fitting the sedimentation equilibrium profile to a 1:1 self-association model, with the aid of Beckman XL-A/XL-I data analysis software (version 6.03). Partial specific volumes were calculated from amino acid compositions.

GST Pull-Down Assays. GST-tagged recombinant β-spectrin polypeptides were coupled to glutathione-Sepharose 4B beads at room temperature for 30 min. To study the effect of phospholipids on the spectrin polypeptide−4.1R interaction, the GST-tagged polypeptides were preincubated with phospholipid vesicles for 30 min at room temperature before mixing with beads. Beads were pelleted and washed. Various concentrations of 4.1R were added to the coupled beads in a final volume of 80 μL and coupled protein concentration of 1 μM, incubated for 1 h at room temperature, pelleted, washed, and eluted with 10% SDS. The supernatant was analyzed by SDS−PAGE, followed by the transfer to nitrocellulose membrane and exposure to an anti-4.1R antibody. GST was used as a negative control in all experiments, and correction was made for the vestigial binding of 4.1R to GST. The binding was quantified by densitometry, using known concentrations of 4.1R as standards. Binding profiles were expressed in terms of concentrations of the reactants in the assay mixtures.

Actin Pelleting Assay. G-actin (2.5 mg mL^{-1}) was polymerized in 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, and 1 mM EGTA for 30 min at room temperature. Recombinant GST-tagged spectrin polypeptide at various concentrations was incubated with aliquots of the resulting F-actin (7 μM with respect to the monomer) for 30 min at room temperature and then centrifuged at 25 °C for 30 min at 90 000 rpm (313000*g*) in a Beckman Optima TL ultracentrifuge with a TLA100 rotor. The pellet was dispersed in the original sample volume and analyzed by SDS−PAGE. The proteins were transferred to nitrocellulose membranes, probed with anti-GST antibody and quantified by densitometry, using known concentrations of spectrin fragments as standards. GST was used as a negative control in all experiments, and corrections were again made for any binding of GST to actin.

Falling Ball Viscometry. Gelation of spectrin/actin mixtures by protein 4.1 is a convenient assay for ternary complex formation between the three proteins (*23*). The formation of a ternary complex between F-actin, 4.1R, and spectrin in the presence of peptide 1−301 was assayed to measure the capacity of the peptide to compete with spectrin tetramer and thereby inhibit gelation. Spectrin polypeptide 1−301 at a series of concentrations was preincubated with F-actin (7 μM with respect to the monomer). Spectrin tetramer (0.2 μM) and 4.1R (0.2 μM) were then added, and the mixtures were incubated for 30 min at 4 °C in 50 μL microcapillaries. A steel ball, 0.025 in. (0.635 mm) in diameter, was introduced into the tube with a magnet at the solution interface, and the time taken by the ball to fall a given distance was recorded. The velocity of the ball was converted to the apparent viscosity with glycerol calibration standards. Apparent viscosities of solutions of spectrin/F-actin mixtures served as negative controls. When the ball remained at the interface, the mixture was assumed to have formed a gel.

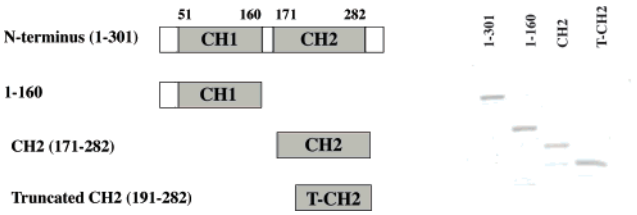

FIGURE 1: Scheme of β-spectrin fragments and Coomassie Blue-stained SDS gel of the purified GST-conjugated fragments. The boundaries of the N-terminal region and of the two CH domains were defined by SMART annotations and sequence alignment with known CH domain structures. The N-terminal region contains the first 301 residues of the β-spectrin chain. The CH2 domain construct corresponds to residues 171−282. Truncated CH2 lacks the first 20 residues of CH2.

RESULTS

Design and Characterization of Recombinant Spectrin Fragments. To locate the 4.1R- and actin-binding sites in the N-terminal region of β spectrin, we constructed three GST-tagged recombinant spectrin fragments depicted in Figure 1. These are (1) the N terminus (residues 1−301), (2) the CH1 domain comprising residues 1−160, and (3) the CH2 domain (residues 171−282). The domain boundaries were essentially as inferred from the SMART database (*24*) (http://smart.embl-heidelberg.de/). The N terminus and CH2 domain were well-expressed and soluble, but we encountered great difficulty in expressing the first half of the N-terminal region (CH1 domain-containing region). Many attempts (involving altering the chain length and using different expression vectors) failed. Eventually, a fragment corresponding to residues 1−161 was obtained by inserting a PreScission cleavage site between CH1 and CH2 in the complete N-terminal domain construct. Cleavage of the purified product with PreScission proteinase then led to recovery of the fragment encompassing the CH1 domain in a soluble form. A truncated CH2 domain, missing the first α helix, which would be expected from the three-dimensional structure (see below), to obstruct access to the other two helices, was also prepared.

The masses of recombinant polypeptides were confirmed by MALDI mass spectrometry. The N-terminal region (polypepide 1−301) and the CH2 domain were characterized by CD and sedimentation equilibrium. As shown in Figure 2, the CD spectra of both 1−301 and CH2 both revealed to have moderately high α helicities (ca. 40 and 50%, respectively). Figure 3 shows sedimentation equilibrium distributions of 1−301 (A) and CH2 (B). The measured apparent molecular weights for the above polypeptides were 36 000 and 14 300, respectively, and both polypeptides appeared

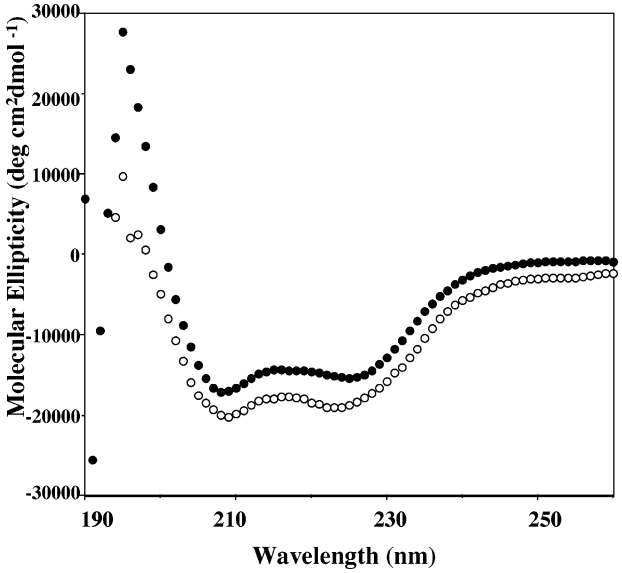

FIGURE 2: CD profiles of 1−301 and CH2. (●) 1−301; (○) CH2.

monodisperse. The experimental molecular weights are in good agreement with the calculated monomer values 35 226 and 13 474, respectively, showing that both proteins were monomeric in solution.

Characterization of 4.1R. Our initial attempts to express a His-tagged protein 4.1R (*19*) led to the appearance in the bacteria of insoluble inclusion bodies, which could only be dispersed in denaturing conditions (6 M urea). To overcome this problem, we expressed 4.1R without a His tag and at a lower bacterial growth temperature (16 °C). These two modifications in combination led to greatly improved solubility and consequently yield. Thus, 5 mg of active, soluble 4.1R could be recovered from 1 L of bacterial culture.

Binding of 4.1R to GST-Tagged Spectrin Fragments. A pull-down assay, with GST fusion proteins attached to glutathione-coupled beads, was used to study the binding of 4.1R to GST-tagged spectrin fragments. Figure 4A shows that 4.1R bound to 1−301, 1−160, and the CH2 domain in a dose-dependent manner. Under our experimental conditions, we were unable to obtain sufficiently high concentrations of protein for the reactions to approach saturation. Thus, we were unable to derive accurate affinities for the interactions. However, the finding that 4.1R not only binds to peptide 1−301 (which contains both the CH1 and CH2 domains) but also to individual CH1 and CH2 domains suggests that there are two 4.1R binding sites in the N terminus of β spectrin.

Binding of Spectrin Fragments to Actin. Binding of the GST-tagged β-spectrin polypeptides to F-actin was measured by the pelleting assay. Figure 4B shows that polypeptide 1−301 bound to F-actin in a saturable manner, with an association constant of about 2.5×10^5 M^{-1} and an apparent stoichiometry in the region of 7 actin subunits per peptide molecule. The precision of the data is insufficient to exclude the possibility of modest cooperativity. Binding of peptide 1−160 (the CH1 domain) could also be observed, but because of the difficulty in isolating adequate amounts of this peptide, we were unable to define the entire binding profile. These observations are consistent with the results of Karinch et al. (*6*), which placed an actin-binding site within the sequence of residues 47−186. The isolated CH2 domain did not bind actin to a significant extent in our assay (Figure 4B), as is also the case for CH2 of βII spectrin (*18*).

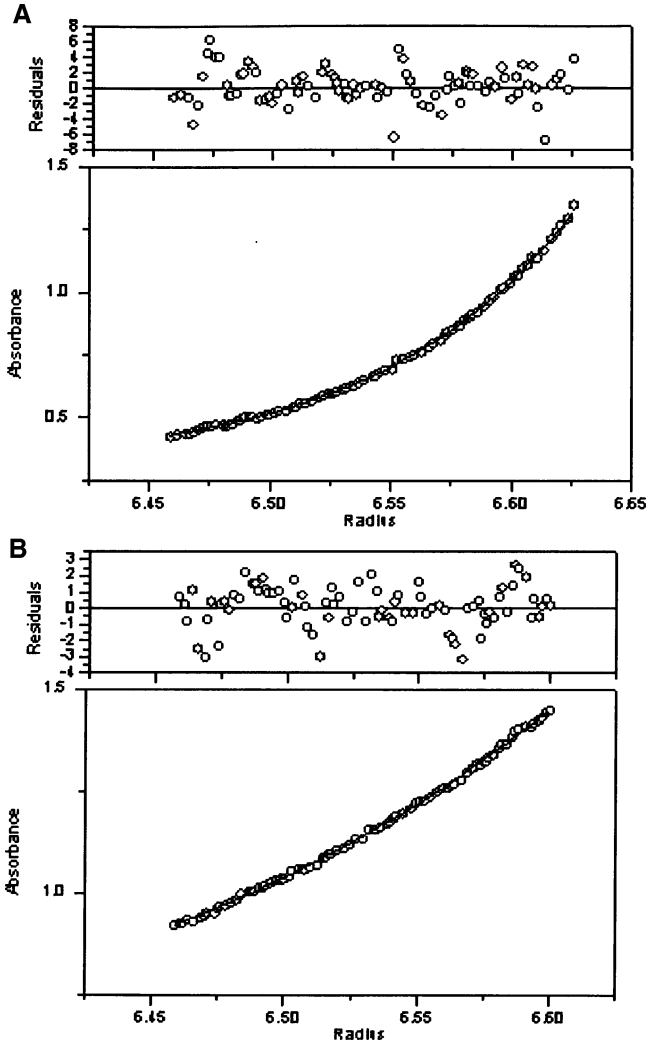

FIGURE 3: Sedimentation equilibrium profiles of recombinant spectrin fragments. Concentration distribution at equilibrium was determined by UV absorption optics in a Beckman XL-A analytical ultracentrifuge. The absorbance was set at 280 nm. The rotor speed was 20 000 rpm. The temperature was 4 °C in all cases. The curves show the global fits for an ideal monodisperse solute. Residuals are shown in the upper panels. (A) 1−301; (B) CH2.

Interaction of 4.1R and Actin with the Truncated CH2 Domain. Even though the isolated CH2 domain was found not to bind to actin (*18*), the suggestion has been made that an extended conformation might exist that does have actin-binding affinity, as reported for the CH domains of utrophin (*25*). To identify potential actin-binding sites in the spectrin CH2 domain, we generated a model of its three-dimensional structure with the aid of SWISSMODEL (*26*). The most closely related structure in the Protein Data Bank is that of the βII-spectrin CH2 domain (PDB 1AA2, with 76% identity to the β-spectrin sequence of residues 173−280). Templates for our model were 1AA2 and the other CH domain structures, 1BKR, 1DXX, 1QAG, and 1BHD. As shown in Figure 5, the CH2 domain contains several α-helical segments. Residues 171−190 account for the first helix and part of the first turn. A noteworthy feature is that the first α helix lies above two other helices. This led us to conjecture that the first 20 residues of the domain may occlude binding sites for 4.1R and actin. To test this, the binding of 4.1R and actin

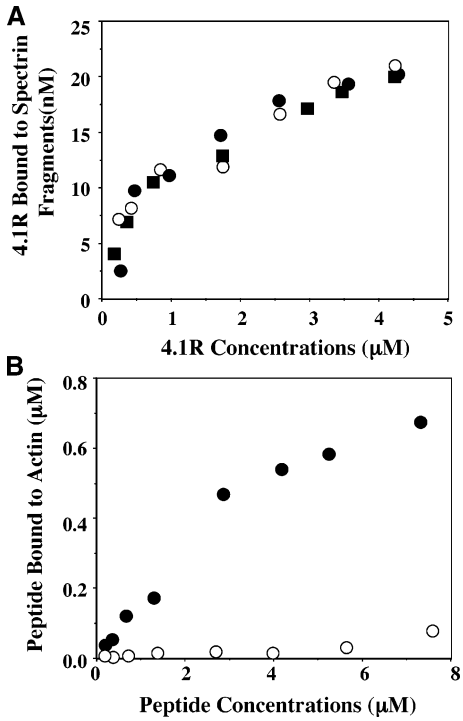

FIGURE 4: Interaction of 4.1R and actin to spectrin fragments. (A) Binding of 4.1R to βI-spectrin fragments. Binding of 4.1R to the indicated spectrin fragments (see Figure 1) was measured by GST pull-down assay. Equal molar concentrations of the fragments were coupled to the beads, and the amount of 4.1R carried down was quantified as described under the Experimental Procedures. The fragments were 1−301 (●), 1−160 (■), and CH2 (○). (B) Binding of βI-spectrin fragments to F-actin. Binding of GST-tagged βI-spectrin fragments to actin was evaluated by the actin-pelleting assay. The bound protein was detected by staining with monoclonal anti-GST antibody and peroxidase-conjugated secondary antibody. The 1−301 fragment but not the CH2 domain bound to actin. The fragments were 1−301 (●) and CH2 (○).

to the truncated CH2 domain (in which residues 171−190 have been deleted) was measured. Surprisingly, as Figure 6A shows, the truncated CH2 domain did bind actin. Thus, deletion of the first 20 residues from the CH2 domain unmasks an actin-binding activity. We conclude that, in addition to the actin-binding site in the CH1 domain, there is another cryptic site in the CH2 domain. Furthermore, 4.1R bound more extensively to truncated CH2 in comparison to intact CH2 (Figure 6B). The first 20 residues of CH2 must therefore be inhibitory of both actin and 4.1R binding.

Regulation of the 4.1R−Spectrin Interaction by PIP$_2$. If, as the above results indicate, the segment of 20 residues at the N-terminal side of the CH2 domain exerts an inhibitory effect on its binding to 4.1R and actin, the existence of a physiological regulatory switch must be surmised. The well-characterized activation of the actin-binding activity of α actinin by PIP_2 is one of several precedents for such a mechanism. In chick skeletal muscle α actinin, a PIP_2-binding site has been located within residues 168−184 (*27*). Alignment of this sequence with that of βI spectrin reveals a corresponding sequence at positions 187−203 in the latter, with an identity at 10 of the 16 positions (Figure 7). This clearly suggested that PIP_2 might regulate the 4.1R−βI spectrin interaction. Figure 8A confirms this inference: it shows that PIP_2 enhanced the binding of 4.1R to fragment 1−301 in a saturable manner, reaching a plateau at a PIP_2 concentration of 11 μM. PIP_2 neither induced binding of 4.1R to GST nor affected the 4.1R−CH2 interaction, implying that in βI spectrin the structural consequences of PIP_2 binding extend beyond the beginning of the CH2 domain. The effect of PIP_2 is evidently specific (Figure 8B). PIP caused only a slight perturbation of binding, while IP_3, PS, lyso-PS, and PC had no effect.

FIGURE 5: Model of the three-dimensional structure of erythrocyte βI-spectrin CH2 domain. The sequence was fitted to the SWISS-MODEL structure based on crystal structures of CH domains as templates. Residues 173−190 are colored in cyan. Note that these are deleted in the truncated CH2 domain constructs.

The binding of 4.1R to peptide 1−301 was examined by the pull-down assay in the absence and presence of PIP_2. As Figure 8C shows, binding in the absence of PIP_2 is sufficiently weak that, with the protein concentrations we were able to use, the affinity of the interaction could not be determined reliably. In contrast, in the presence of PIP_2, the interaction appears much stronger and clearly approaches saturation. We calculate K_A ca. 2.5×10^8 M^{-1}. Binding of the same spectrin fragment to F-actin, by contrast, was unaffected by PIP_2 (data not shown).

Inhibition of Spectrin Binding to Actin and of Spectrin−Actin−4.1R Ternary Complex Formation by Peptide 1−301. As further confirmation that peptide 1−301 possesses the activity of the parent protein, we examined its effect on spectrin binding to actin filament and on formation of the ternary complex between spectrin, F-actin, and 4.1R. Figure 9A shows that, with an increasing concentration of peptide 1−301, the binding of spectrin to F-actin was strongly inhibited. Figure 9B shows that ternary complex formation between spectrin, actin, and 4.1R was also inhibited by peptide 1−301, showing once again that the peptide competes with intact spectrin.

DISCUSSION

If we are to understand the molecular mechanisms that allow erythrocytes to survive in the circulation, we need to

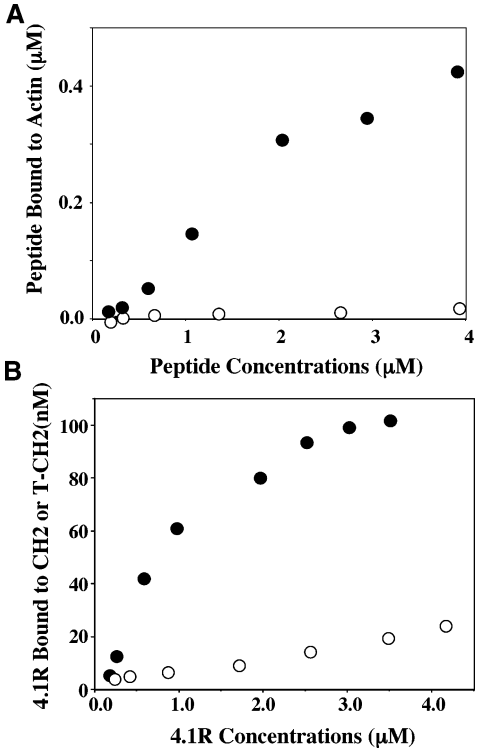

FIGURE 6: Interaction of 4.1R and actin to T-CH2 domain compared to intact CH2 domain. (A) Binding of CH2 or T-CH2 domain to F-actin. Binding of GST-tagged CH2 domain or T-CH2 domain to actin was evaluated by actin-pelleting assay. Note that T-CH2 (●) but not the CH2 domain (○) bound to actin. (B) Binding of 4.1R to CH2 or T-CH2 domain. Binding of 4.1R to CH2 or T-CH2 was measured by GST pull-down assay as described above. Note that the binding of 4.1R to T-CH2 (●) was greatly enhanced compared to CH2 domain (○).

define in full detail the interactions of the key components of the membrane skeleton. In this study, we have sought to identify the binding sites on spectrin recognized by protein 4.1R and actin. Our results give rise to the following conclusions: (1) binding sites for both 4.1R and actin are located in both of the βI-spectrin CH domains; (2) the 4.1R- and actin-binding sites in the CH2 domain are masked by the N-terminal α helix of this domain; and (3) the binding of the whole N-terminal domain to 4.1R is strongly regulated and is fully expressed only in the presence of PIP_2.

This study required fragments of spectrin containing postulated binding sites. We designed recombinant constructs, taking advantage of both sequence alignments with proteins of known structure and domain boundaries delivered by the SMART database (*24*, *26*) (Figure 1). The resulting constructs appear by all our criteria to possess the native structure.

In the effort to define the 4.1R- and actin-binding sites in the N-terminal region of β spectrin, we unexpectedly observed that, while 4.1R was able to bind to the whole N-terminal domain and to the CH1 and CH2 domains, it bound more extensively to a truncated CH2 domain, lacking the N-terminal α-helical segment of 20 residues. This suggests that this sequence (residues 171−190) obstructs access to a 4.1R-binding site. Similarly, these 20 residues prevent binding of CH2 to F-actin.

The model of the CH2 domain, which we have constructed on the basis of its homology with the crystal structure of the corresponding element in βII spectrin (Figure 5), offers a structural rationale based on the occlusion of the 4.1R- and actin-binding sites by a superimposed segment of α helix. The 17 amino acid PIP_2-binding motif discovered in the N-terminal region of α-actinin, a member of spectrin superfamily (*28*), is well-conserved in βI and βII spectrins. Our results, which reflect a relief by PIP_2 of inhibition of binding to 4.1R, imply that such a regulatory mechanism should operate physiologically.

Because the PIP_2-binding site in α-actinin has a closely similar counterpart in the βI-spectrin CH2 domain (Figure 7) and removal of the part of the CH2 that contains this presumptive site promotes 4.1R binding, we infer that in the absence of PIP_2 the N-terminal domain adopts a "shut" conformation, which "opens" upon PIP_2 binding. Evidently, however, the 20-residue sequence (171−191) does not contain all of the structural information required to effect

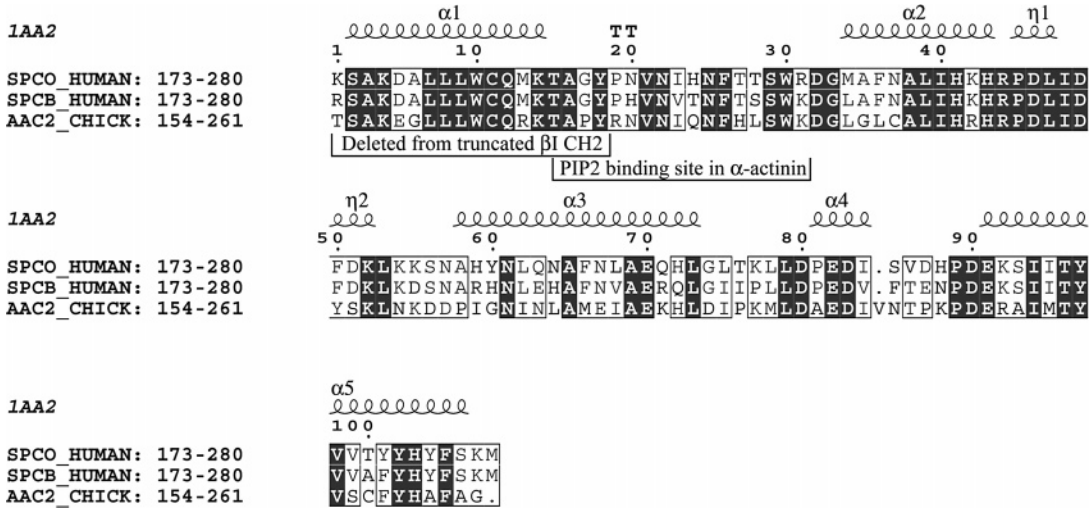

FIGURE 7: Alignment of sequence and structure in CH2 domains. The crystal structure of the CH2 domain of βII spectrin is given in PDB entry 1AA2. Amino acids in this structure are residues 173−280 of human βII spectrin (SwissProt, SPCO_HUMAN). The equivalent residues in βI spectrin (SwissProt, SPCB_HUMAN) are 173−280, and the equivalent residues in chick skeletal muscle α-actinin (SwissProt, AAC2_CHICK) are 154−261. Also indicated are secondary structure elements in 1AA2 aligned with the corresponding protein sequences. The PIP_2-binding site in α-actinin (residues 168−184) is indicated. Note that this overlaps the sequence deleted in the truncated CH2 and corresponds to the loop between the first and second α helices.

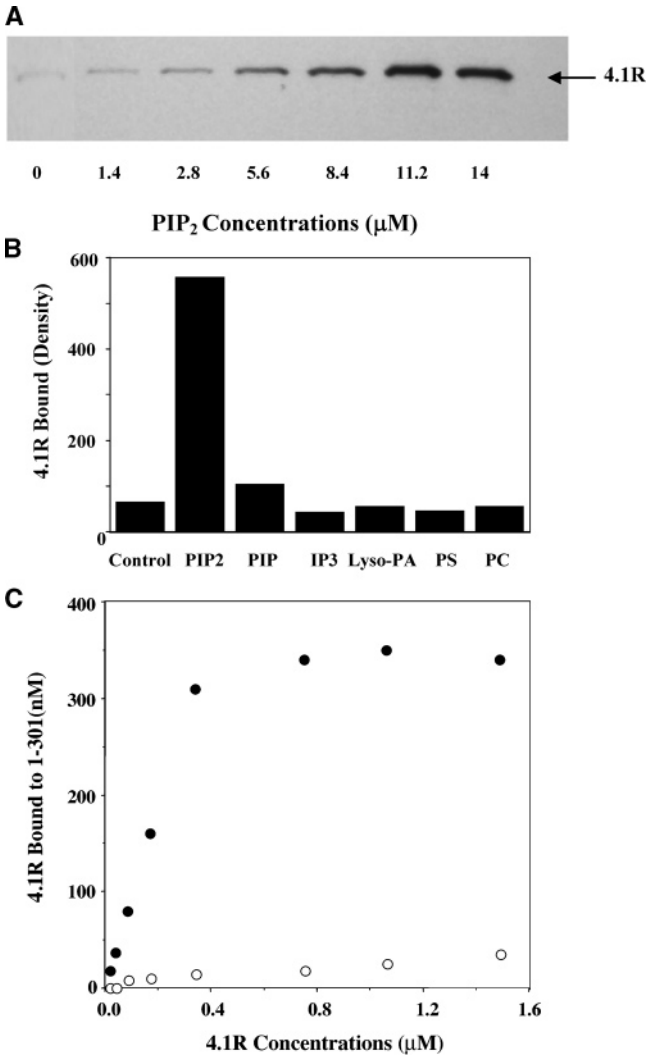

FIGURE 8: Effect of PIP_2 on the interaction of βI-spectrin fragments with 4.1R. (A) Binding of 4.1R to polypeptide 1−301 progressively increased with an increasing PIP_2 concentration, reaching a plateau at 11 μM. (B) PIP_2 greatly increased the binding of 4.1R to 1−301, while PIP showed a small effect. IP_3, PS, lyso-PS, and PC produced no detectable effects. (C) Binding of 4.1R to fragment 1−301 in the absence and presence of PIP_2 was quantified as described under the Experimental Procedures. (○ and ●) Binding of 4.1R to the fragment 1−310 in the absence and presence of PIP_2, respectively.

the PIP_2-dependent regulation of 4.1R binding, because interaction with the isolated CH2 domain does not respond to PIP_2.

In as much as PIP_2 strongly stimulates 4.1R binding to the N-terminal region, it might have been expected also to promote the binding of actin. The fact that it does not do so conflicts with the conventional view of PIP_2 as a positive regulator of the activities of muscle α-actinin (*27*−*29*). There have, on the other hand, been no reports of 4.1R binding to α-actinin, and it seems likely that the regulatory role of PIP_2 on spectrin will turn out to be multifactorial.

We also note the relative affinities of the interactions that we describe here in relation to earlier work on native proteins. We find that in the presence of PIP_2 4.1R binds β-spectrin 1−301 spectrin with a K_A of 2.5×10^8 M^{-1} (Figure 8C). Tyler et al. (*30*) found that spectrin dimers and 4.1R (both isolated from red cell membranes) bind with K_A close to 10^7 M^{-1}. Considering the difference in experimental approaches in their work and ours, the two values might be simply

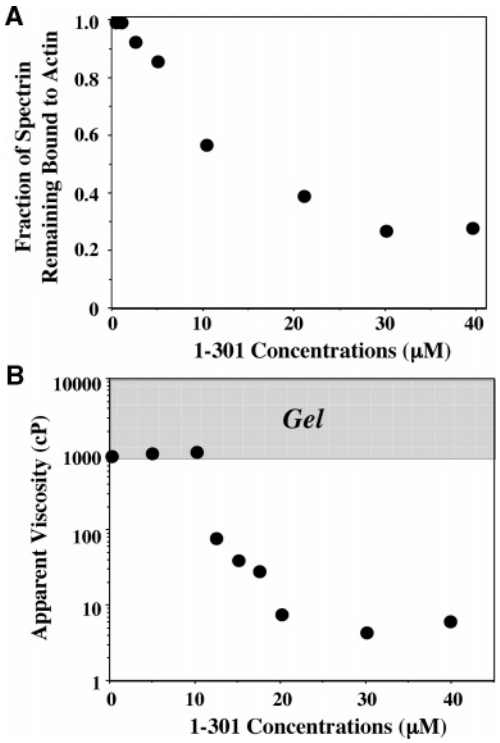

FIGURE 9: Inhibition of spectrin binding to actin and spectrin−actin−4.1R ternary complex formation by polypeptide 1−301. (A) Effect of 1−301 on spectrin binding to actin was evaluated by the actin-pelleting assay, and the binding of spectrin to actin was significantly decreased with the increasing concentration of peptide 1−301. (B) Effect of 1−301 on spectrin, actin, and 4.1R ternary complex formation was evaluated by falling ball viscometry. The shaded zone refers to immeasurably high viscosity because of gelation.

regarded as in reasonable agreement. On the other hand, PIP_2 was not added to their reactions, and a weaker interaction, similar to that which we find in the absence of added PIP_2, might have been found. Native spectrin/4.1 preparations are likely to contain some residual red cell membrane lipid; therefore, it is possible that their value reflects this.

We show in Figure 4b that β-spectrin 1−301 binds F-actin with a K_A of 2.5×10^5 M^{-1}. Li and Bennett (*31*) analyzed fragments of βII spectrin, the product of a closely related gene. They found that a fragment containing the N-terminal CH domains plus the first triple helical repeat bound actin with a K_A of 1.7×10^5 M^{-1}. It seems, therefore, that the actin-binding activities intrinsic to spectrin β subunits are very similar. On the other hand, Ohanian et al. (*4*) found that native erythrocyte spectrin dimers bind actin with a K_A of 5×10^3 M^{-1}. The difference between this value and our value for the affinity of the 1−301 fragment is large enough not to result simply from methodological differences. It seems likely that interactions of spectrin β chains with α in the native red cell dimer may influence affinity.

REFERENCES

1. Bennett, V., and Baines, A. J. (2001) Spectrin and ankyrin-based pathways: Metazoan inventions for integrating cells into tissues, *Physiol. Rev. 81*, 1353−1392.
2. Walensky, L. D., Mohandas, N., and Lux, S. E. (2003) in *Blood, Principles and Practice of Hematology* (Handin, R. I., Lux, S. E., and Stossel, T. P., Eds.) 2nd ed., Lippincott, Williams, and Wilkins, Philadelphia, PA.

3. Ungewickell, E., Bennett, P. M., Calvert, R., Ohanian, V., and Gratzer, W. B. (1979) *In vitro* formation of a complex between cytoskeletal proteins of the human erythrocyte, *Nature 280*, 811−814.
4. Ohanian, V., Wolfe, L. C., John, K. M., Pinder, J. C., Lux, S. E., and Gratzer, W. B. (1984) Analysis of the ternary interaction of the red cell membrane skeletal proteins spectrin, actin, and 4.1, *Biochemistry 23*, 4416−4420.
5. Gardner, K., and Bennett, V. (1987) Modulation of spectrin−actin assembly by erythrocyte adducin, *Nature 328*, 359−362.
6. Karinch, A. M., Zimmer, W. E., and Goodman, S. R. (1990) The identification and sequence of the actin-binding domain of human red blood cell β-spectrin, *J. Biol. Chem. 265*, 11833−11840.
7. Gilligan, D. M., and Bennett, V. (1993) The junctional complex of the membrane skeleton, *Semin. Hematol. 30*, 74−83.
8. Takakuwa, Y., Tchernia, G., Rossi, M., Benabadji, M., and Mohandas, N. (1986) Restoration of normal membrane stability to unstable protein 4.1-deficient erythrocyte membranes by incorporation of purified protein 4.1, *J. Clin. Invest. 78*, 80−85.
9. Takakuwa, Y., Ishibashi, T., and Mohandas, N. (1990) Regulation of red cell membrane deformability and stability by skeletal protein network, *Biorheology 27*, 357−365.
10. Discher, D. E., Winardi, R., Schischmanoff, P. O., Parra, M., Conboy, J. G., and Mohandas, N. (1995) Mechanochemistry of protein 4.1's spectrin−actin-binding domain: Ternary complex interactions, membrane binding, network integration, structural strengthening, *J. Cell Biol. 130*, 897−907.
11. Becker, P. S., Tse, W. T., Lux, S. E., and Forget, B. G. (1993) β-Spectrin kissimmee: A spectrin variant associated with autosomal dominant hereditary spherocytosis and defective binding to protein 4.1, *J. Clin. Invest. 92*, 612−616.
12. Tse, W. T., and Lux, S. E. (1999) Red blood cell membrane disorders, *Br. J. Haematol. 104*, 2−13.
13. Correas, I., Leto, T. L., Speicher, D. W., and Marchesi, V. T. (1986) *J. Biol. Chem. 261*, 3310−3315.
14. Correas, I., Speicher, D. W., and Marchesi, V. T. (1986) Identification of the functional site of erythrocyte protein 4.1 involved in spectrin−actin associations, *J. Biol. Chem. 261*, 13362−13366.
15. Schischmanoff, P. O., Winardi, R., Discher, D. E, Parra, M. K, Bicknese, S. E, Witkowska, H. E, Conboy, J. G., and Mohandas, N. (1995) Defining of the minimal domain of protein 4.1 involved in spectrin−actin binding, *J. Biol. Chem. 270*, 2143−2150.
16. Gimm, J. A., An, X., Nunomura, W., and Mohandas, N. (2002) Functional characterization of spectrin−actin-binding domains in 4.1 family of proteins, *Biochemistry 41*, 7275−7282.
17. Gimona, M., Djinovic-Carugo, K., Kranewitter, W. J., and Winder, S. J. (2002) Functional plasticity of CH domains, *FEBS Lett. 513*, 98−106.
18. Banuelos, S., Saraste, M., and Carugo, K. D. (1998) Structural comparisons of calponin homology domains: Implications for actin binding, *Structure 6*, 1419−1431.
19. An, X. L., Takakuwa, Y., Manno, S., Han, B. G., Gascard, P., and Mohandas, N. (2001) Structural and functional characterization of protein 4.1R−phosphatidylserine interaction: Potential role in 4.1R sorting within cells, *J. Biol. Chem. 276*, 35778−35785.
20. Tyler, J. M., Hargreaves, W. R., and Branton, D. (1979) Purification of two spectrin-binding proteins: Biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1, *Proc. Natl. Acad. Sci. U.S.A. 76*, 5192−5196.
21. Perkins, S. J. (1986) Protein volumes and hydration effects. The calculations of partial specific volumes, neutron scattering matchpoints, and 280-nm absorption coefficients for proteins and glycoproteins from amino acid sequences, *Eur. J. Biochem. 157*, 169−180.
22. Greenfield, N., Davidson, B., and Fasman G. D. (1967) The use of computed optical rotatory dispersion curves for the evaluation of protein conformation, *Biochemistry 6*, 1630−1637.
23. Fowler, V., and Taylor, D. L. (1980) Spectrin plus band 4.1 cross-link actin. Regulation by micromolar calcium, *J. Cell. Biol. 85*, 361−376.
24. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: Identification of signaling domains, *Proc. Natl. Acad. Sci. U.S.A. 95*, 5857−5864.
25. Galkin, V. E, Orlova, A., VanLoock, M. S., Rybakova, I. N., Ervasti, J. M., and Egelman, E. H. (2002) The utrophin actin-binding domain binds F-actin in two different modes: Implications for the spectrin superfamily of proteins, *J. Cell Biol. 157*, 243−251.
26. Schwede, T., Kopp, J., Guex, N., and Peitsch, M. C. (2003) SWISS-MODEL: An automated protein homology-modeling server, *Nucleic Acids Res. 31*, 3381−3385.
27. Fukami, K., Sawada, N., Endo, T., and Takenawa, T. (1996) Identification of a phosphatidylinositol 4,5-bisphosphate-binding site in chicken skeletal muscle α-actinin, *J. Biol. Chem. 271*, 2646−2650.
28. Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S., and Takenawa, T. (1992) Requirement of phosphatidylinositol 4,5-bisphosphate for α-actinin function, *Nature 359*, 150−152.
29. Fukami, K., Endo, T., Imamura, M., and Takenawa, T. (1994) α-Actinin and vinculin are PIP_2-binding proteins involved in signaling by tyrosine kinase, *J. Biol. Chem. 269*, 1518−1522.
30. Tyler, J. M., Reinhardt, B. N., and Branton, D. (1980) Associations of erythrocyte membrane proteins. Binding of purified bands 2.1 and 4.1 to spectrin, *J. Biol. Chem. 255*, 7034−7039.
31. Li, X. L., and Bennett, V. (1996) Identification of the spectrin subunit and domains required for formation of spectrin/adducin/actin complexes, *J. Biol. Chem. 271*, 15695−15702.

BI047331Z