Defining of the Minimal Domain of Protein 4.1 Involved in Spectrin-Actin Binding*

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The spectrin-actin-binding domain of protein 4.1 is encoded by a 21-amino acid alternative exon and a 59-amino acid constitutive exon. To characterize the minimal domain active for interactions with spectrin and actin, we functionally characterized recombinant 4.1 peptides containing the 21-amino acid cassette plus varying portions of the 59-amino acid cassette (designated 21.10 to 21.59). Peptide 21.43 was shown fully functional in binary interactions with spectrin (by cosedimentation and coimmunoprecipitation experiments) and in ternary complex formation with spectrin and actin (by an in vitro gelation assay). Further truncation produced peptides incapable of binary interactions but fully competent for ternary complex formation (peptides 21.36 and 21.31), shorter peptides with reduced ternary complex activity and altered kinetics (21.26 and 0.59), and inactive peptides (21.20 and 21.10). Binding studies and circular dichroism experiments suggested that residues 37–43 of the constitutive domain were directly involved in spectrin binding. These data indicate that 4.1-spectrin binary interaction requires the 21-amino acid alternative cassette plus the 43 N-terminal residues of the constitutive domain. Moreover, the existence of two possible ternary complex assembly pathways is suggested: one initiated by 4.1-spectrin interactions, and a second by 4.1-actin interactions. The latter may require a putative actin binding motif within the 26 N-terminal residues of the constitutive domain.

The erythrocyte membrane mechanical properties are determined by a protein skeletal network underlying the lipid bilayer composed mainly of spectrin, actin, protein 4.1, protein 4.9, and adducin (1). The ternary interaction between spectrin tetramer, actin protofilament, and protein 4.1 forms a highly stable complex (Ka = 10^{12} M^{-2}) which imparts mechanical integrity to the membrane (2–4). While the different binary interactions between spectrin, actin, and protein 4.1 have been extensively studied, little is known about the mechanism of the ternary complex formation. Binary interaction between spectrin and actin is characterized by a weak binding (K_a = 5 \times 10^3 M^{-1}) (3, 5). While the interaction between protein 4.1 and F-actin was initially found to be weak (5), more recent studies suggest a cooperative mechanism for this interaction (6). In contrast, protein 4.1 binds strongly to spectrin (K_a ranging from 0.5 to 86 \times 10^6 M^{-1}) (7–10). Although both subunits of spectrin are required to form a ternary complex with protein 4.1 and actin, only β-spectrin determines the sensitivity of the spectrin-actin interaction with protein 4.1 (11, 12).

Alternative pre-mRNA splicing generates a number of protein 4.1 isoforms in a tissue- and development-specific manner (13–15). A 10-kDa domain of protein 4.1 has been shown to be required for spectrin-actin interactions (16). This domain is encoded by an alternatively spliced exon (encoding the N-terminal 21-amino acid (aa)) and a constitutive exon (encoding the C-terminal 59 aa) (17). Expression of the 21-aa alternative exon occurs late in erythroid development and is concomitant with major changes in red cell membrane properties (18, 19). The 21-aa cassette is required for binding of protein 4.1 to spectrin, for stabilization of spectrin-actin complex, and for imparting mechanical strength to the membrane (20, 21).

The aim of the present study is to determine if the 21-aa cassette by itself constitutes the minimal functional domain required for spectrin-actin interactions. To achieve this objective, we have expressed, characterized, and functionally assayed a nested set of truncated peptides designated 21.10 to 21.59, containing the 21-aa peptide plus variable portions of the C terminus of the spectrin-actin-binding domain. Using both cosedimentation and coimmunoprecipitation methods, we have shown that binding of recombinant peptides to spectrin requires the 21-aa cassette plus at least 43 residues of the 59-aa domain; no interaction with spectrin was detected using shorter peptides. This minimum sequence of 64 aa was also required for the formation of a ternary complex manifested by gelation at physiologically relevant conditions. However, peptides further truncated from the N terminus (e.g. 0.59) or from the C terminus (21.26) could promote gelation of spectrin-actin mixtures at much higher concentrations. These truncated peptides may initiate ternary complex formation via an interaction with actin mediated by a motif within the first 26 residues of the 21-aa domain.

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1 Morris, M. B. and Lux, S. E. (1995) Eur. J. Biochem., in press.

2 The abbreviations used are: aa, amino acid; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonylfumyl fluoride; GST, glutathione S-transferase; SAB, spectrin-actin binding domain; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; TFE, trifluoroethanol. 21243
the 59-aa domain. The peptides 21.20 and 21.10 had no function whatsoever, suggesting that the alternative 21-aa cassette in itself is inactive. We have therefore characterized the sequences in protein 4.1 involved in its interactions with spectrin and actin.

MATERIALS AND METHODS

Reagents and Equipment—Plasmid expression vector pGEX-KG was provided by Dr. J. Dixon (Purdue University). [35S]Methionine and protein standards for electrophoresis were purchased from Amersham Corp. Polyclonal antibodies anti 21 and anti 10b were provided, respectively, by Drs. J. Chasis (Lawrence Berkeley Laboratory) and S. Marcus (Yale University). Disopropylphosphorothioate (DPP), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, glutathione-agarose beads, formaldehyde-treated insoluble protein A, Sepharose G-50, myoglobin, cytochrome c, aprotinin, blue dextran, trifluoroacetic acid, acetonitrile, acetone powder of rabbit skeletal muscle, and 100-μl capillary tubes were obtained from Sigma. Red cell proteins were purified from blood obtained from healthy volunteer donors.

Plasmid Constructs with Progressive C-terminal Deletion of the Spectrin-Actin-Binding Domain—The GST fusion constructs were designed in GST-YY.XX (YY refers to the alternatively spliced cassette of the construct, XX indicates the number of aa from the N terminus of the constitutive cassette). Construction of peptides GST-21.59, GST-0.59, and GST-21.26, GST-21.31, GST-21.20, and GST-21.10 was described previously (20). Truncated constructs GST-21.26, GST-21.31, and GST-21.36 were prepared by PCR amplification of a cDNA clone encoding the human erythroid SAB of protein 4.1 (residues 407–487) as a template. To generate the desired deletions, the sense primer (5 '-GCGAATTCCCATGGAGCCCACAGAAGCTTG-3') and antisense primer with the following sequences: 1) GST-21.36, 5'-GGGGCGGCCGCTAATCATCATTCACTAGG-3'; 2) GST-21.31, 5'-GGGGCGGCCCTACCCGGTTGTCTCGGATC-3'; and 3) GST-21.26, 5'-GGGGCGGCGCTAAGACCTGCTGAAGT-3'. The amplification reaction conditions were: denaturation, 94°C, 30 s; annealing, 50°C, 30 s; extension, 72°C, 1 min; for 35 cycles. The cDNAs and antisense primers incorporated a C-terminal stop codon. Amplified fragments were cloned into a modified pGEX-KG vector at EcoRI and Eagl restriction sites.

The constructs GST-21.43, GST-21.20, and GST-21.10 were generated by unidirectional deletion of the GST-21.59 plasmid linearized at the 3'-end of the insert (Exo III, Mung Bean Nuclacase Kit, Stratagene, La Jolla, CA).

Bacterial Expression—The deletion constructs were overexpressed by IPTG induction in Escherichia coli. Recombinant peptides were purified from bacterial lysate supernatant by glutathione-agarose affinity chromatography (22, 23). Protease inhibitors DFP (1 mm) and PMSF (1 mm) were used in all peptide preparations. Metabolic labeling of GST constructs was achieved by growing transformant E. coli in the presence of [35S]methionine. For some experiments the GST carrier was removed from the fusion peptide using thrombin (24). Thrombin-cleaved peptides were designated YY.XX. GST constructs and cleaved peptides preparations were subjected, respectively, to 12 and 20% SDS-PAGE (25), visualized by either Coomassie Blue staining or transferred to nitrocellulose for antibody probing using two polyclonal antisera: 1) an anti 21 raised against a synthetic peptide corresponding to the 21-aa cassette, and 2) an anti 10b recognizing the amino acid sequence MES-VPERPSEWDK in the 59-aa cassette (26). Fusion proteins concentrations were quantified using the 280-nm absorbivity of GST (1.0 cm2/mg).

Thrombin-cleaved peptides 280-nm absorbivities were calculated according to their composition in tryptophan and tyrosine residues (27).

Reversed-phase High Performance Liquid Chromatography (HPLC)—Analysis of HPLC chromatography was performed with a 4.6 × 250-mm, C-4, Vydac 214 TP 54 reversed-phase column (Vydac, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid, pH 2.0 (26). Elution of the peptide was achieved using a gradient of acetonitrile in 0.1% trifluoroacetic acid, pH 2.0: 0–25% from 10 to 20 min, and 25 to 45% from 20 to 90 min, with a flow rate of 1 ml/min. The effluent was monitored at 214 nm and peptide identities were analyzed for structural composition by mass spectrometry, or immediately neutralized, partially dried by Speed-Vac, and finally dialyzed against appropriate buffer prior to the analysis of their functional properties.

Amino Acid Sequence Analysis—Automated Edman degradation of HPLC-purified 21.43 and detection of the phenylthiohydantoin derivatitives were performed on an automatic sequencer (model 477 A, Applied Biosystems, Foster City, CA) at the Microchemical Facility at the University of California at Berkeley.

Mass Spectrometry Analysis of HPLC Purified Fractions—HPLC-purified peptides were analyzed at the Children's Hospital Oakland Research Institute Facility using electrospray ionization mass spectrometry on a VG Bio-Q quadrupole mass spectrometer (VG Biotech/Fisons, Altrincham, United Kingdom). The instrument was controlled, and data were analyzed using a Mass Lynx® software (VG Biotech/Fisons, Altrincham, U.K.). The extraction cone voltage was 55–65 V, and the source temperature was 60°C. 10-μl aliquots of HPLC fractions were deposited into a stream of solvent (50% acetonitrile, 0.1% trifluoroacetic acid) at a flow rate of 4 μl/min. The instrument was scanned from m/z 600 to 1600, and several scans were summed to obtain the final spectrum. Mass scale calculation employed the multiple charged ions from a separate introduction of horse heart myoglobin (average molecular mass 16,952 Da).

Isolation of Spectrin, Actin, and Protein 4.1—Spectrin and protein 4.1 were purified from normal human red blood cells, and actin was purified from rabbit skeletal muscle as described previously (20). Proteins were stored at 4°C and used within 4 weeks after purification. Protein purity was assessed by 8% SDS-PAGE. Ultrafiltration with Centriprep (Amicon Inc., Beverly, MA) was used to concentrate proteins or to dialyze proteins against appropriate buffer. Protein concentrations were determined by spectrophotometry using published specific absorbivities, i.e. 1.07 cm2/mg at 280 nm for spectrin (28), 0.64 cm2/mg for F-actin at 290 nm (29), and 0.80 cm2/mg at 280 nm for protein 4.1 (7). Molar concentrations were calculated using following molecular masses, 526 kDa for spectrin dimer (30), 66 kDa for protein 4.1 (31), and 42 kDa for actin (32).

Analysis of Peptide Interaction with Spectrin by Sucrose Gradient Sedimentation—[35S]Methionine metabolically labeled GST constructs or thrombin-cleaved peptides (3–48 μM) were mixed with or without purified spectrin (6 μM) in buffer A (100 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 0.2 mM dithiothreitol, 0.1 mM DFP, 0.05 mM PMSF, 1 mM sodium azide, pH 7.5) to a final volume of 50 μl. The mixtures were incubated for 60 min at 4°C. The reaction products were then layered on top of 10–25% sucrose gradients, separated, and quantified as described previously (20) except that the ultracentrifugation was performed at 4°C, for 12 h, at 36,000 revolutions/min in a SW 50.1 rotor (Beckman Instruments Inc., Fullerton, CA). The data were analyzed using a spectrin binding model previously described (20). Unlabeled GST-21.43, GST-21.26, and a synthetic 21-aa peptide were added to the mixture of [35S]GST-21.43 (3 μM) and spectrin (6 μM) to study the ability of these peptides to inhibit the interaction of the 21.43 peptide with spectrin.

Analysis of Peptide Interaction with Spectrin by Concanavalin A-Sepharose Interaction—Purified spectrin was labeled with N-[3H]ethylmaleimide (DuPont-NEN) as described previously (5). The [3H]spectrin (2 μM) was mixed in 50-μl volume reactions with various GST fusion peptides (0–1.6 μM) in buffer A for 60 min at 4°C. The anti-GST antibody solution was subsequently added (final concentration: 6.1 or 15 μg) and the incubation continued for 30 min at 4°C. Formalin-treated insoluble protein A was then added (10% v/v), and after 15 min of incubation at 4°C, the radioactive tubes were spun for ~15 s in a microfuge at room temperature. The pellets were washed three times with cold buffer A to remove any unbound [3H]spectrin. Pellet and supernatant fractions were counted for radioactivity. For the inhibition studies, [3H]spectrin (2 μM) was preincubated during 60 min, at 4°C, in buffer A, with excess of either full-length protein 4.1, thrombin-cleaved peptides 21.43, 21.26, 0.59, or an anti-21 antibody, prior to the addition of GST-21.43 (1.6 μM) followed by the immunoprecipitation procedure.

Low Shear Viscometry—The effect of protein 41 and recombinant peptides on the gelation of spectrin-actin mixtures was assessed using a falling ball low shear viscometer (20, 33). Briefly, spectrin was preincubated at 27°C for 30 min to form spectrin tetramer. F-actin (14 μM) and spectrin tetramer (1.8 μM) were mixed first, the fusion peptides or cleaved peptides were then added to obtain a final peptide concentration ranging from 0.05 to 350 μM in either 50 or 100 μl total volume of buffer A. The mixture was quickly drawn up into a 100-μl microcapillary tube for a 60-min incubation at 4°C. Subsequently, the tubes were mounted on a ramp (60° incline), and a small steel ball was placed below the meniscus. If the ball did not move under gravity, or if the ball fell a specified distance in a time greater than 100 times the base-line control of spectrin-actin alone, then the state of the mixture was termed a gel. Experiments were repeated twice. The activity of thrombin-cleaved 21.43 and 21.37 was also measured in buffer B used for circular dichroism experiments. For the kinetic study, the time necessary to obtain a visible transition point was monitored (20) and the concentration of thrombin-cleaved peptides. This experiment was conducted in duplicate and repeated twice. Composition of the gelation
mixture was analyzed as follows. The gel was centrifuged at 80,000 revolutions/min for 7 min, at 4°C, in a TLA-100 rotor (Beckman), and the pellet and supernatant were analyzed by 12% SDS-PAGE. The amount of free and bound spectrin and actin species were determined by densitometry using a IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA).

Gel Filtration—The thrombin-cleaved peptide 21.43 preparation (containing both 21.43 and 21.37 species, see Table I) was analyzed by gel filtration on a Sephadex G-50 column (2.5 x 100 cm) equilibrated with buffer A at 4°C. The column was calibrated with the standard proteins myoglobin (M, = 16,900), cytochrome c (M, = 12,400), and aprotinin (M, = 6,500). The void volume (V,) of the column was determined with blue dextran.

Circular Dichroism Experiments—CD measurements were performed on a Jasco J-600 spectropolarimeter, in buffer B (100 mM sodium fluoride, 10 mM sodium phosphate, pH 7.4), at room temperature, in a 1-mm path length cell, at a scan rate of 10 nm/min (Jasco Inc., Easton, MD). Each spectrum was the average of five consecutive measurements. The concentrations of the HPLC-purified peptides 21.37 and 21.43 in buffer B were 4.5 and 12.5 μM, respectively. The effect of 40% (v/v) TFE, 1% (w/v) SDS, and 1% (v/v) Tween 20 on the secondary structure of the peptides was also studied on a preparation of cleaved 21.43 purified by gel filtration (with buffer B) and a preparation containing a mixture of 21.43 and 21.37. Analysis of the data was performed by the LINCOMB program after subtraction of the corresponding baseline data (34).

RESULTS

Expression of the Recombinant Protein 4.1 Peptides—Recombinant peptides corresponding to the spectrin-actin-binding domain of erythroid protein 4.1 were designed to contain the 21-aa alternatively spliced exon and various lengths of the 59-aa constitutive exon (Fig. 1). Additional peptides corresponding to non-erythroid isoforms of protein 4.1 either lacked the 21-aa exon (0.59) or had another alternatively spliced exon of 19 aa inserted along with the entire 59-aa cassette (19.59) (Fig. 1). Both SDS-PAGE and Western blotting analysis revealed one or more bands for the majority of assayed constructs (Fig. 2 and Table I). As expected from the amino acid sequence, all peptides containing the 21-aa cassette were recognized by the anti 21 antibody, while only constructs containing 31 aa or more were strongly reactive with the anti 10b antibody (Table I). Reversed-phase HPLC purification of thrombin-cleaved peptides allowed the separation and the quantification of the different species contained in each fusion protein preparation. N-terminal sequencing of HPLC-purified 21.43 indicated that the thrombin cleavage occurred at the expected site. Mass spectrometric characterization of major HPLC-purified species confirmed that expression of the constructs typically yielded a couple of major peptide species: one of the expected length and

Fig. 1. Map of the protein 4.1 recombinant peptides. A, schematic diagram of the 10-kDa domain of protein 4.1. Residues 406–472 of protein 4.1 were previously identified as the spectrin-actin-binding domain of protein 4.1 (26). The alternatively spliced 21-aa cassette and the constitutively expressed 59-aa cassette are identified. Phylogenetically conserved sequences are in gray. The indicated phosphorylation sites (at Tyr and Ser) have been previously described (41, 43). B, the different COOH-terminal truncations of the prototypical erythroid 4.1 10-kDa domain isoform termed 21.59. The construct 19.59 containing a non-erythroid alternatively spliced cassette and the construct 0.59 lacking the 21-aa cassette are also represented.

one with a C-terminal truncation ranging from 10 to 20 residues. The identification of the major peptides by mass spectrometry was precise (± 2 Da), indicating that post-translational modifications, e.g. phosphorylation or methylation, had not occurred. Note that peptides 21.43 and 21.20 generated by unidirectional deletion contained additional C-terminal aa derived from the vector sequence (Table I).

Minimal Sequence Required for Binary Interaction with Spectrin—The ability of metabolically labeled GST-4.1 fusion proteins to bind purified spectrin was first assayed using a sucrose gradient cosedimentation assay (Fig. 3). The [35S]GST-21.43 induced a 75% inhibition. One of the expected length and
Neither GST-21.26 at 45 μM induced any inhibition. These results suggest that the interaction between [35S]GST-21.43 and spectrin is specific. Both binding and inhibition data suggest that while the 21-aa cassette is necessary for binding to spectrin, it is not sufficient. Additional 43 residues of the constitutive domain along with the 21-aa cassette are required for spectrin binding in this binary assay.

To examine more labile interactions between peptides and spectrin that might not be detected by the slow sucrose gradient separation of bound and free species (≥12 h), we used a rapid coimmunoprecipitation assay (separation 15 min) (Fig. 5). An antibody directed against the GST moiety was used to immunoprecipitate GST-4.1 fusion proteins, and the amount of cosedimenting [3H]spectrin was quantified to estimate binding affinity. Consistent with above results, only peptide GST-21.43 had a moderate affinity for [3H]spectrin (K_d = 0.5 × 10^6 M⁻¹). GST-21.36 and GST-21.31 bound very weakly to [3H]spectrin while shorter peptides and GST-0.59 did not bind to [3H]spectrin (Fig. 5A). Equivalent results were obtained using 15 μM of anti-GST antibody (data not shown).

To further confirm the specific binding of 21.43 to spectrin, we performed additional inhibition studies. The cosedimentation experiment was conducted in the presence of excess of thrombin-cleaved peptides 21.43, 21.26, and a synthetic 21-aa peptide, prior to perform cosedimentation experiment in the presence of [35S]GST-21.43 (3 μM), as described under “Materials and Methods.” The control corresponded to the amount of bound [35S]GST-21.43 in the absence of inhibitor. The error bars indicate the variability of the method determined during the course of three different experiments.

| Nominal construct | Immunoreactivity | Molecular mass (Da) | Deduced species |
|-------------------|-----------------|---------------------|----------------|
|                   | Anti 21 | Anti 10b | Expected | Measured |                     |
| 21.43             | +      | ++      | 10,169   | 10,170   | 21.43,K,L,NSS (+1) |
| 21.36             | +      | ++      | 10,041   | ND       | 21.37 (−2)         |
| 21.31             | +      | ++      | 9,247    | ND       | ND                 |
| 21.26             | +      | +       | 7,637    | 7,636    | 21.26 (−1)         |
| 21.20             | +      | −       | 7,302    | 7,302    | 21.20,L,NSS (−1)   |
| 21.10             | +      | −       | 6,171    | ND       | ND                 |
| 0.59              | −      | −       | 8,882    | 8,882    | 0.59 (−1)          |

FIG. 4. Inhibition of cosedimentation of [35S]GST-21.43 binding to spectrin. The spectrin (6 μM) was incubated with excess of thrombin-cleaved peptides 21.43, 21.26, and a synthetic 21-aa peptide, prior to perform cosedimentation experiment in the presence of [35S]GST-21.43 (3 μM), as described under “Materials and Methods.” The control corresponded to the amount of bound [35S]GST-21.43 in the absence of inhibitor. The error bars indicate the variability of the method determined during the course of three different experiments.
plex Formation with Spectrin and Actin—To compare structural requirements for ternary complex formation with those for binary spectrin interactions, the ability of each recombinant peptide to form a supramolecular complex with spectrin and actin was studied using a low shear viscosity assay. Fig. 6A represents the relative ability of recombinant peptides to induce gelation of spectrin-actin mixtures. Importantly, peptides as short as GST-21.31 displayed a gelation activity similar to purified protein 4.1. GST-21.26 had a reduced activity (10-fold weaker than GST-21.43), but still led to a sol/gel phase transition, while shorter peptides GST-21.20 and GST-21.10 did not increase viscosity, even at concentrations up to 300 μM. Peptides corresponding to non-erythroid isoforms of protein 4.1 (GST-0.59 and GST-19.59) appeared to be 50-fold weaker as gelation factors than GST-21.43. Extensive control experiments showed that heat-denatured peptides (60 °C for 5 min) did not increase viscosity; thrombin-cleaved peptides had the same activity as the corresponding GST fusion proteins, and gelation activity was equivalent in buffer A versus buffer B.

The ability of peptides shorter than 21.43 to induce ternary complex formation, while being unable to bind spectrin, suggested that different mechanisms may be operative in inducing gel formation by 21.43 versus these other peptides.

To explore whether putative different ternary complex assembly pathways might exhibit different kinetics, the effect of increasing amounts of cleaved peptides on the gelation velocity of a spectrin-actin mixture was assayed. Two types of responses were noted. Increasing amounts of peptide 21.43 induced a logarithmic enhancement of the rate of gelation (Fig. 6B). In marked contrast, the rate of gelation observed with peptide 0.59 was much slower, reaching a maximum value at ~120 μM. Peptide 21.26 showed a response similar to the 0.59, albeit leveling off at 90 μM (Fig. 6B). These kinetic data support the thesis that different mechanisms of gel formation indeed exist.

To quantify more precisely the potency of various GST-recombinant peptides to incorporate spectrin into the ternary complex, the composition of the gelation mixtures was quantified by a cosedimentation assay. A mixture of 1.8 μM spectrin and 14 μM actin alone did not exhibit increased viscosity (see Fig. 5A). Under these conditions, ~35% of the total spectrin cosedimented with ~95% of the actin, indicating a weak binary interaction between spectrin and actin. In contrast, in the presence of 4.1 peptides, a correlation was observed between increase in viscosity and increased incorporation of spectrin into the ternary complex. In the presence of GST-21.43 at a concentration of ~5 μM, ~90% of the spectrin was incorporated into the ternary complex. In contrast, ~20 μM and ~50 μM of GST-21.26 and GST-0.59, respectively, were necessary to incorporate ~50% of the spectrin in a ternary complex. Peptides GST-21.20 and GST-21.10 did not increase the incorporation of spectrin with actin up to concentrations of ~55 μM. In all these experiments, 95% of actin was recovered in the pellet fraction. These results further support the idea that different mechanisms of ternary complex formation may be operative depending on the affinities of the peptides for spectrin.

Structural Characterization of the Recombinant Protein 4.1 Peptides—As shown above, GST-21.43 interacts much more strongly with spectrin in binary assays than does GST-21.36. The additional amino acid residues contained within GST-21.43 must therefore represent a part of the spectrin-binding site, or alternatively they may affect the conformation and affinity of an upstream spectrin-binding site. To explore whether alterations in conformation might account for the observed functional differences, the structure of such peptides was probed by gel filtration and circular dichroism. A thrombin-cleaved 21.43 preparation, shown by analytical HPLC and mass spectrometry to contain a mixture of the expected 21.43 peptide (55%) and a truncated 21.37 peptide (45%), was analyzed by gel filtration. In two different buffers (A and B, see “Materials and Methods”), the two peptides coeluted in a single peak. Determination of the apparent Stokes radius gave a value of ~23 Å (data not shown). Based on the molecular weight measured by mass spectrometry (see Table I), the calculated Stokes radius for the peptide 21.43 was ~17 Å. Such a difference between experimental and calculated data suggests that both the peptides 21.43 and 21.37 do not adopt a completely globular conformation.

To further characterize the secondary structure of the peptides, CD measurements were performed on the HPLC-purified peptides 21.37 and 21.43, as well as on a gel filtration-purified mixture of these two peptides. CD spectra indicated that both peptides exhibit similar secondary structures (Fig. 7). These results imply that 1) removal of 6 aa from 21.43 does not detectably alter its secondary structure and 2) these residues are directly involved in spectrin binding rather than maintenance of proper 4.1 conformation. Analysis of the curves gave a maximal α-helical content of <5% for both 21.37 and 21.43; a value significantly less than predicted from the primary structure analysis. Indeed, according to the method of Garnier et al. (35), predicted α-helical contents were 72 and 68% for 21.37 and 21.43, respectively. CD analysis of the mixture of 21.37 and 21.43, purified by an alternative gel filtration technique, also showed a low α-helical content suggesting that the HPLC purification procedure did not alter the secondary structure of the site of aspectrin-actin mixture was assayed. Two types of responses...
To determine the intrinsic propensity of the gel-filtration purified peptides to adopt a helical conformation, the CD spectra were measured in the presence of 40% TFE and 1% SDS as inducers of helicity (36–39) and of 1% Tween 20 as a negative control. Secondary structure analysis gave ~42% α-helix with TFE and ~26% with SDS while Tween 20 did not alter secondary structure (data not shown). The large increase in helicity obtained with TFE suggests that both peptides have the propensity to adopt a helical conformation.

**DISCUSSION**

We have investigated the structural requirements for protein 4.1 binary interactions with spectrin, as well as for ternary interactions with spectrin and actin. Analysis of different binding behavior observed among a nested set of truncated recombinant 4.1 peptides in several in vitro assays (sedimentation, immunoprecipitation, and viscometry) have enabled the development of a model for ternary complex assembly. Our data are consistent with a model in which a core actin binding motif is flanked by two sequence elements required for high affinity spectrin binding; details of this model and supporting evidence are discussed below.

Both binding and inhibition studies showed that the peptide 21.43 specifically bound spectrin with a moderately high affinity ($K_a = 0.2–0.5 \times 10^6 \text{M}^{-1}$). This affinity for spectrin is similar to the $0.5 \times 10^6 \text{M}^{-1}$ value previously reported using similar methods for both the prototypical SAB construct 21.59 and the full-length protein 4.1 (9, 20). Deletion of additional N- or C-terminal residues (0.59 and 21.36, respectively) led to loss of spectrin binding. Thus, residues 407–427 (the 21-aa cassette) and 464–470 (the difference between 21.36 and 21.43) are identified as two key motifs essential for a high affinity binding to spectrin. Interestingly, the physiological importance of these motifs is also supported by the observations that both are highly conserved evolutionarily, and both include phosphorylation sites previously reported to decrease 4.1-spectrin-actin interactions. Sequence comparison of 4.1 from mammalian (human, mouse, dog), amphibian (frog), and avian (chicken) sources has shown that residues 414–429 and 457–469 are
highly conserved (40). Moreover, phosphorylation of Tyr within the 21-aa cassette has been previously identified as disruptive to spectrin-actin-4.1 interactions (41). In addition, phosphorylation of protein 4.1 on Ser within the 59-aa domain also decreased the ternary complex formation and the protein 4.1-spectrin interaction (42, 43). The dramatic decrease in spectrin affinity as the 21.43 is shortened at its C terminus may thus be related to the loss of highly conserved amino acids including Ser.

Requirements to form a ternary complex with spectrin and actin were different than requirements for binary interaction with spectrin. One class of peptides, represented by 21.36 and 21.31, exhibited minimal interaction with spectrin in binary assays, but nevertheless enhanced spectrin cross-linking of actin filaments with activities similar to that of 21.43 and full-length 4.1. These peptides induced formation of a gel at physiological molar ratios (dimeric spectrin/actin/4.1 peptide 1:7:1). We interpret these results to indicate that the highly conserved residues 31–36 of the constitutive domain, while not allowing strong binary interaction with spectrin, nevertheless play a role in stabilizing spectrin binding in the presence of actin. Further truncation produced a second class of peptides (0.59 and 21.26) that was also inactive in spectrin binary assays but exhibited a qualitatively and quantitatively different ternary complex activity. These peptides were active only at concentrations 10–50-fold higher than normal 4.1 and much higher than the estimated “cellular” concentration (100,000 molecules in a cell volume of 100 femtoliters, i.e., \(3 \mu M\)). Although, we note that the red cell 4.1 can be concentrated at the membrane by additional interactions with glycoprotein C, p55 and band 3 (44–48); therefore, the effective membrane skeletal concentration of 4.1 is likely higher than 3 \(\mu M\). In addition, this class of peptides exhibited different kinetic properties of gel formation, implying a different molecular basis for ternary complex formation. We speculate that the central domain shared by peptides 0.59 and 21.26, i.e., the first 26 residues of the 59-aa domain (amino acids 428–453 of intact 4.1) may induce ternary complex formation by functioning as an actin-binding site. Hence, a weak binary interaction with F-actin involving this motif might initiate ternary complex formation in vitro, even in the absence of high affinity spectrin binding. The importance of this putative actin motif is supported by 1) the fact that the shorter peptides 21.26 and 21.10, lacking part of the putative actin binding motif, displayed no activity in any of the assays, and 2) the recent observation that a protein 4.1 motif variant with a deletion of one residue from the Lys447–448 doublet (corresponding to residues 20–21 of this actin binding motif) is unable to interact with spectrin or spectrin-actin mixtures (49).

Consistent with this model, a cooperative actin binding activity with protein 4.1 has been reported earlier using cosedimentation assays (6). Moreover, our preliminary experiments with these peptides have also shown a binary, cooperative association with F-actin (data not shown). Furthermore, cosedimentation experiments with excess of actin and limiting amounts of spectrin also provide evidence consistent with cooperative 4.1-actin binding. Peptides 21.43, 21.26, and 0.59 sedimented in molar excess of the available spectrin in this assay. All three peptides exhibited cooperative binding and reached saturation at approximately the total actin concentration, implying an additional cooperative interaction with F-actin.

The discrepancies between the calculated and experimentally determined Stokes radii and also between the predicted and measured secondary structures suggest that the peptides 21.43 and 21.37 do not require a compactly folded structure for activity. While capable of initiating gelation in buffer B, both these peptides exhibited an helicity substantially lower than predicted (this report and Ref. 31). This difference may be attributed to the fact that short polyepitopes in solution can be disordered by polar interactions between water and the peptide backbone (38). Previous studies with purified proteins, using in vitro binding assays, suggested a conformational change of one or several components of the ternary complex (3). A conformational readjustment was recently noted for recombinant peptides corresponding to the putative actin-binding site of Nebulin (50). Mechanistically, the acidic F-actin molecule might increase helicity of bound 4.1 peptide in similar fashion. Additional experiments using known inducers of helical content trifluoroethanol and SDS (37, 38, 51) indicate that peptides 21.43 and 21.37 have the propensity to adopt an \(\alpha\)-helical conformation (data not shown). Further structural studies by two-dimensional NMR will be necessary to establish whether the TF-E- and SDS-induced helicity we observed is of biological importance, and whether conformational adjustments of protein 4.1 occur upon actin and spectrin binding.

Based on the present studies as well as previous solution binding assays and visualization of protein-protein interactions by electron microscopy (3, 5, 52), we propose the following model of ternary complex assembly. Although formation of a ternary complex can arise from simultaneous interaction among three proteins, it is more likely that the ternary complex assembly begins with a binary complex. Three pathways (noted I, II, and III) schematize the possible binary interactions which initiate assembly of the ternary complex, i.e., 4.1 peptide-spectrin (I), 4.1 peptide-actin (II), and spectrin-actin (III). Our results suggest the following scenarios for the assembly of the ternary complex involving different peptides. For protein 4.1 and peptides that can strongly bind to spectrin, e.g., 21.43 and 21.59, pathway (I) would be the dominant mechanism of ternary complex assembly. This pathway would represent the physiological mechanism corresponding to the rapid binding of peptide 21.43 to spectrin followed by the rapid cross-linking of F-actin. This pathway displayed no rate limitation in the kinetic study suggesting a rapid assembly of the ternary complex. In contrast, the smaller peptide 21.26, and also the norenyltyroid peptides 0.59 and 19.59, do not have the strong affinity for spectrin necessary for initiating pathway (I) and would thus follow pathway (II). In this pathway, the peptides first bind cooperatively to F-actin, and this complex interacts slowly with spectrin. Indeed, the apparent saturation in gelation speed with increasing concentrations of 21.26 and 0.59, at fixed spectrin concentration, suggests a rate limitation most consistent with a slow spectrin cross-linking of peptide-actin complexes represented in pathway (II). For this reason, the peptides 21.31 and 21.36, while inducing ternary complex assembly with a potency similar to that of 21.43 and because of their low affinity for spectrin, would also follow pathway (II). In pathway (III), a weak interaction of spectrin with actin is followed by the binding of any functional peptide. The weak interaction of spectrin with actin is emphasized by the weak cosedimentation of spectrin with actin in the absence of peptide and also by the low viscosity of a spectrin-actin mixture.

Our data suggest the following domain map of this 64 aa sequence: two spectrin-binding sites (the 21-aa cassette and the residues 27–43 of the constitutive domain) flank an actin binding site (first 26 residues of the constitutive domain). These putative binding sites contain charged residues, consis-
ent with localization of the SAB to the surface of protein 4.1. Furthermore, we have shown that the 21-aa cassette is not a functionally autonomous structure, possessing the complete spectrin-actin binding activity of the erythroid 4.1, but does appear to be a modulator of this function.

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