Identification of the Spectrin Subunit and Domains Required for Formation of Spectrin/Adducin/Actin Complexes

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Adducin is an actin-binding protein that has been proposed to function as a regulated assembly factor for the spectrin/actin network. This study has addressed the question of the subunit and domains of spectrin required for formation of spectrin/adducin/actin complexes in vitro. Quantitative evidence is presented that the β-spectrin N-terminal domain plus the first two α-helical domains are required for optimal participation of spectrin in spectrin/adducin/actin complexes. The α subunit exhibited no detectable activity either alone or following association with β-spectrin. The critical domains of β-spectrin involved in complex formation were determined using recombinant proteins expressed in bacteria. The N-terminal domain (residues 1-313) of β-spectrin associated with F-actin with a K_d of 26 μM, and promoted adducin binding to F-actin with half-maximal activation at 110 nM. Addition of the first α-helical domain (residues 1-422) lowered the K_d for F-actin by 4-fold to 6 μM, but also reduced the capacity by 3-fold and had no effect on interaction with adducin. Further addition of the second α-helical domain (residues 1-528) did not alter binding to F-actin but resulted in a 2-fold increased activity in promoting adducin binding with half-maximal activation at 50 nM. Addition of up to eight additional α-helical domains (residues 1-1388) resulted in no further change in F-actin binding or association with adducin. These results demonstrate an unanticipated role of the first repeat of β-spectrin in actin binding activity and of the second repeat in association with adducin/actin, and imply the possibility of an extended contact between adducin, spectrin, and actin involving several actin subunits.

The spectrin-based membrane skeleton was first visualized as a detergent-insoluble assembly of proteins in erythrocytes (Yu et al., 1973). Spectrin and its associated proteins are now known to be expressed in cells of most metazoan organisms (reviewed by Bennett and Gilligan (1993)). Proposed functions of spectrin-based membrane skeletons include maintenance of mechanical stability of erythrocyte membranes, organization of integral proteins in specialized membrane domains, and regulation of vesicle-membrane interactions. Electron microscopy has resolved the erythrocyte membrane skeleton as a two-dimensional network with 5-6 spectrin molecules linked to short actin filaments to form a sheet of 5-6-sided polygons (Byers and Branton, 1985; Liu et al., 1987; Shen et al., 1986). These striking images raise the issue of how such a regular network can be assembled and have focused attention on the accessory proteins localized at spectrin-actin junctions (reviewed by Bennett (1990)).

Adducin is localized at spectrin-actin junctions (Derick et al., 1992) and has been proposed to contribute to assembly of the spectrin-actin network (Gardner and Bennett, 1987). Adducin was initially discovered as a calmodulin-binding protein in erythrocytes (Gardner and Bennett, 1986) and now is known to be expressed in brain as well as most other tissues (Bennett et al., 1988; Joshi et al., 1991). Adducin binds to spectrin-actin complexes with high affinity but binds to either spectrin or actin alone with low affinity. Adducin also promotes addition of a second spectrin molecule to spectrin-actin complexes in a calcium-calmodulin regulated manner (Gardner and Bennett, 1987, 1988). In epithelial tissues and cultured cells, adducin is co-localized with spectrin and actin at lateral domains of cell-cell contact (Kaiser et al., 1989). Adducin is phosphorylated by protein kinase A and protein kinase C (Waseem and Palfrey, 1988; Ling et al., 1986) and is a candidate to participate in dynamic behavior of the spectrin skeleton.

Understanding the structural basis for the spectrin/adducin/actin complex promises to provide insights into assembly and regulation of the membrane skeleton network. The domain structure of adducin includes an N-terminal 39-kDa globular domain connected by a 9-kDa neck domain to a 33-kDa protease-sensitive tail domain (Joshi and Bennett, 1990; Joshi et al., 1991). Tail domains of both α and β adducin are responsible for binding of adducin to spectrin-actin complexes (Hughes and Bennett, 1995). Spectrin is composed of rod-shaped α and β subunits laterally associated in an anti-parallel orientation. The region of spectrin involved in association with actin contains the C terminus of the α subunit and the N terminus of β-spectrin. The N terminus of β-spectrin contains a conserved actin-binding domain (Karinch et al., 1990), which is also found in a large family of actin-binding proteins (reviewed by Matsudaira (1991)). β-Spectrin also contains 17 repeats of a 106-residue motif folded into triple α-helical units (Yan et al., 1993) followed by a C-terminal variable region (Hu et al., 1992; Byers et al., 1992; Winkelmann et al., 1990; 1991). The 106-residue motif is also found in other actin-cross-linking proteins including α-actinin and dystrophin, but a role of repeat domains in actin binding has not been addressed.

In this study, we report that the β-spectrin N-terminal domain plus the first two α-helical repeats are required for full activity in formation of a ternary complex with adducin and actin. We also present evidence that the first α-helical repeat of β-spectrin contributes to association with actin filaments. These findings support a model for the adducin/spectrin/actin complex with lateral association of spectrin and adducin tail domains along the actin filament and involving several actin subunits.

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EXPERIMENTAL PROCEDURES

Materials

2β-H Bolton-Hunter reagent was from ICN Radiochemicals. Disopropyl fluorophosphosphate, leupeptin, pepstatin A, dithiothreitol, phenylmethylsulfonyl fluoride, benzamidines, NaF, NaBr, NaEDTA, NaEGTA, HEPES, Tween 20, Triton X-100 were from Sigma. Dnase, bovine serum albumin, urea, and sucrose were from U. S. Biochemical Corp. 4-(2-Aminoethyl)benzenesulfonyl fluoride was from Boehringer Mannheim. The pGEMEX expression vector was from Promega. S-Sepharose resin, Mono Q, and Mono S columns were obtained from Pharmacia Biotech Inc. Electrophoresis's reagents were from Bio-Rad.

Methods

Procedures—Determination of protein concentration was performed by the procedure of Bradford (1976) using bovine serum albumin as a standard. Alternatively, in the case of purified proteins, absorbance at 280 nm was measured and the concentration of protein was calculated using an extinction coefficient estimated from amino acid sequence by the methods of Gill and von Hippel (1989). Polyacrylamide gel electrophoresis was performed using 0.2% SDS in the buffers of Fairbanks et al. (1971) and 1.5-mm-thick 3.5–17% exponential gradient slab gels. Protein iodination was performed with Bolton-Hunter reagent (Bennet, 1983).

Purification of Proteins—Actin was isolated from acetic powder of rabbit skeletal muscle (Pardee and Spudich, 1982) from a 10-ml sample of HPLC fractionation (3.9 mg/ml starting solution) in a 1.5-liter HPLC-Superose 6 column equilibrated with the same buffer. Fractionation was monitored by SDS-polyacrylamide gel electrophoresis.

Brain spectrin subunits were purified as essentially as described (Davis and Bennett, 1983). All procedures were performed at 4°C. Briefly, spectrin (3 mg) was dialyzed against 7 M urea, 10 mM sodium phosphate buffer, pH 6.3, and to a 6-ml hydroxylapatite column. α-Spectrin was eluted with 10 M urea, 10 mM sodium phosphate buffer, pH 6.3, and the column was washed with the same buffer for 6 h. β-Spectrin was eluted with 250 mM phosphate buffer, 7 M urea, 0.05% Tween 20, 10 mM DTT, pH 7.1. Spectrin subunits were dialyzed against 10 mM phosphate buffer, pH 7.4, 1 M NaBr, 6 mM urea, 10 mM glycine, 1 mM NaH2PO4, 0.05% Tween 20, 1 M NaCl, 1 mM DTT for 6 h, followed by dialysis against the same buffer without urea for another 6 h to renature the proteins. β-Spectrin was further fractionated by gel filtration on Superose 6 to remove aggregated material. Finally, the non-aggregated β-spectrin was concentrated on a 0.5-mL hydroxylapatite column.

Association of α-Spectrin and β-Spectrin (residue 1-1388)—65 μg of α-spectrin and 110 μg of β-spectrin 1-1388 were combined in a buffer containing 6 M urea, 1 M NaCl, 10 mM sodium phosphate, pH 7.4, 1 M NaEDTA, 1 M NaN3, 0.05% Tween 20, 0.5 M DTT, 10% sucrose and were dialyzed for 6 h against the same buffer without urea. Then the dimer αβ12,1388 was purified on a 25-ml HPLC-Superose 6 column with a flow rate of 0.25 ml/min. The column buffer was the same as the dialysis buffer. A 200-μl sample was loaded, and fractions (0.25 ml each) were monitored by SDS-polyacrylamide gel electrophoresis.

Expression of β-Spectrin Constructs in E. coli—Human βo spectrin cDNA was used as a template in subcloning (Hu et al., 1992). An Ndel site followed by an ATG codon was included at the beginning of the PCR product. An EcoRI site following a stop codon was placed at the end of the PCR product. These restriction sites were used to clone the PCR products into the pGEMEX vector, a Pet7-plasmid with a Tet promoter. The recombinant plasmids were transformed into BL21 (DE3/pLysS) bacterial strain. Overexpression of recombinant polypeptides in this E. coli host was induced by 0.5 mM isopropyl-1-β-D-galactopyranoside (Studier et al., 1990). After 3 h of induction with isopropyl-1-β-D-galactopyranoside, 3 liters of bacterial culture were centrifuged at 5,000 × g for 10 min at 4°C.

All purification steps were performed at 4°C and were monitored by SDS-polyacrylamide gel electrophoresis. Each overexpressed construct had the expression level of over 50% of total proteins and the mobility corresponding to the predicted molecular mass. Precise procedures for purification varied for different constructs as well as different preparations of the same construct. In general, the bacterial pellet was lysed by freezing/thawing once and resuspended in 100–200 ml of 50 mM sodium phosphate, pH 7.4, 1 mM NaEGTA, 25% sucrose, with protease inhibitors (1 mM 4-(2-Aminoethyl)benzenesulfonyl, 10 mM benzamidines, 10 μg/ml pepstatin A, 200 μg/ml phenylmethylsulfonyl fluoride as final concentrations), and incubated on ice for 30 min. Dnase was added to a final concentration of 40 μg/ml to 30 μg/ml in order to stabilize the preparation. Then protein was extracted by addition of 2 volumes of Triton containing buffer (200 mM NaCl, 20 mM sodium phosphate, pH 7.4, 2 mM NaEDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors as mentioned above). The suspension was filtered through a 18-gauge needle once and centrifuged for 20 min at 5,000 × g. Usually 50–90% of construct proteins were in the Triton-soluble fraction, which was collected as described. The supernatant of this fractionate was precipitated by addition of solid (NH4)2SO4 to achieve 60% saturation (3.9 mg/ml starting solution). After incubation for 30 min on ice, the precipitated proteins were pelleted by centrifugation for 30 min at 5,000 × g. The protein pellet was resuspended in 20–30 ml of 1 M NaBr, 10 mM sodium phosphate, pH 7.4, 1 mM NaEDTA, 1 mM NaN3. 0.05% Tween 20, 0.5 mM DTT at a protein concentration of 0.5–3 mg of protein/ml and was dialyzed against the same buffer for 6 h before it was loaded into a 15-liter Superose 12 column equilibrated with the same buffer. Fractions containing the construct protein were pooled and dialyzed against 50 mM NaBr, 10 mM sodium phosphate, pH 7.4, 1 mM NaEDTA, 1 mM NaN3, 0.05% Tween 20, 0.5 mM DTT for 6 h. Then the protein was loaded into a 1-ml Mono Q and other Mono S ion exchange column and eluted by a linear 0.05–0.5 M NaBr gradient. Typically a 3-liter culture can produce 2–20 mg of protein with over 95% purity.

RESULTS

Identification of the Spectrin Subunit Required for Association with Adducin/Actin—The first step in mapping the spectrin contribution to spectrin/adducin/actin complexes was to determine which subunit(s) of spectrin are involved. The approach involved determining the activity of native spectrin and isolated spectrin subunits (see “Methods”) in promoting adducin binding to actin (Fig. 1). Native spectrin tetramer increased binding of 125I-labeled adducin to F-actin in a saturable manner with half-maximal activation at approximately 10 nM spectrin. Adducin can associate with actin independently from spectrin, and this is a high capacity but low affinity binding with a Kd of 28 μM (Gardner and Bennett, 1987). Assays in this study were performed with 5 nM adducin and 1.5–2 μM F-actin. Under these conditions, the extent of binding of adducin to actin is 2–3-fold higher in the presence of spectrin. The concentration required for half-maximal activation of adducin binding is used in this study as a measure of activity of spectrin in stabilizing spectrin/adducin/actin complexes. Double-reciprocal plots of this data are linear (Fig. 1B) and allow estimates of concentrations required for half-maximal activation.

α-Spectrin exhibited essentially no activity in this assay. The lack of activity of the α subunit is not due to aggregation since a gel filtration column profile of α-spectrin showed that over 90% of this subunit was in the non-aggregated fractions (data not shown). β-Spectrin, in contrast to the inactive α subunit, increased binding of 125I-labeled adducin to F-actin in a saturable manner with half-maximal activation at 22 nM. This apparent affinity is lower than that of native spectrin (10 nM), but still displays high affinity binding. In other experiments, the concentration required for half-maximal activation for the α subunit varied from 20–40 nM. β-Spectrin exhibited only half the extent of activation of the native spectrin tetramer. The basis of reduced extent and reduced affinity of β-spectrin compared to spectrin tetramer is not known. However, β-spectrin differs from native spectrin in several respects that could be relevant: (a) β-spectrin is a monomer while native spectrin is an α2β2 tetramer, and β-spectrin therefore only has a single-
binding site compared to two sites in the native tetramer; (b) monomeric \( \beta \)-spectrin lacks lateral contacts with an \( \alpha \) subunit. The question of whether \( \alpha \)-spectrin contributes to \( \beta \)-spectrin activity in the complex will be addressed below (see Fig. 8). The conclusion from the data in Fig. 1 is that, to a first approximation, ability to associate with adducin/actin complexes resides in \( \beta \)-spectrin.

Mapping Adducin and Actin Binding Domains in \( \beta \)-Spectrin—\( \beta \)-Spectrin constructs were evaluated to determine the minimal region sufficient for full activity of \( \beta \)-spectrin in forming spectrin/adducin/actin complexes (Fig. 2). The N-terminal domain (residues 1–313) includes the actin-binding domain (residues 47–186) (Karinch et al., 1990) as well as all the N-terminal sequence before the first \( \alpha \)-helical domain. Increasing numbers of \( \alpha \)-helical domains were added to the N-terminal domain of \( \beta \)-spectrin based on definition of Drosophila \( \beta \)-spectrin segments (Byers et al., 1992). The \( \alpha \)-helical domains can be folded properly and individually if their boundaries correspond to folding units (Winograd et al., 1991). Constructs were expressed in bacteria and purified by ion exchange chromatography and gel filtration. Proper folding was evaluated by circular dichroism spectroscopy, which provides a sensitive measure of \( \alpha \)-helical secondary structure. The constructs exhibited spectra corresponding to 60–70% \( \alpha \)-helix (results not shown), which is consistent with the helix composition of native spectrin (Calvert et al., 1980; Yan et al., 1993). For some constructs, protease resistance also was evaluated. The results of circular dichroism spectra and protease resistance combined with behavior on gel filtration indicated that the bacterially expressed \( \beta \)-spectrin constructs were properly folded and unaggregated.

\( \beta \)-Spectrin constructs were evaluated for activity in promoting binding of adducin to F-actin (Figs. 1, 3, and 4). A construct containing the N-terminal domain plus 10 \( \alpha \)-helical domains (residues 1–1388) was equivalent to the intact native \( \beta \) subunit in terms of the concentration required for half-maximal activation, although residues 1–1388 exhibited a greater extent of activation (Fig. 1). \( \beta \)-Spectrin constructs also were equivalently active that contained the N-terminal domain plus either 2, 3, 4, or 10 \( \alpha \)-helical domains (Fig. 3). Therefore, the N-terminal domain plus the first two triple helical domains is sufficient for full activity of \( \beta \)-spectrin. However, deletion of the second \( \alpha \)-helical domain reduced the apparent affinity 2-fold resulting in half-maximal activation at 120–140 nM (Fig. 4). Additional deletion of the first \( \alpha \)-helical domain resulted in no further
change in activity. A construct containing the first and second 
α-helical domains but lacking the N-terminal domain exhibited 
no detectable activity in promoting binding of adducin to 
F-actin.

These results indicate that the β-spectrin N-terminal do-
main plus the first two α-helical domains are required for full 
activity in forming a spectrin/adducin/actin complex, but the 
N-terminal domain alone or combined with the first α-helical 
domain still is about 50% as active (Fig. 5). The differences in 
adducin-dependent activities of β-spectrin constructs could 
be dependent on association with F-actin and/or on direct inter-
action with adducin. To determine which possibility is the basis 
for the 2-fold difference, it was necessary to evaluate actin 
binding affinity of these constructs.

Effect of α-Helical Domains on Actin Binding Activity of the 
β-spectrin N-terminal Domain—The N-terminal domain alone 
or combined with the first α-helical domain were labeled and 
assayed for activity in co-sedimenting with F-actin (Fig. 6A).

Since spectrin and actin alone associate with a relatively 
low $K_d$ in the micromolar range, 10–20 μM concentrations of spect-
rin polypeptides were required to approach saturation. Such 
concentrations of spectrin are not obtainable with native spec-
trin due to solubility problems and due to formation of bundles 
of actin filaments, but could be achieved with recombinant 
spectrin polypeptides. Double-reciprocal plots of the binding 
data for the two constructs revealed differences in capacity and 
affinity for actin (Fig. 6). The N-terminal domain alone associ-
ated with F-actin with a $K_d$ of 26 μM, and a capacity of 1 
molecule/1–2 F-actin subunits. The fact that the capacity for 
spectrin domain was less than the theoretical 1:1 ratio could be 
due to negative cooperativity at high occupancy and/or partial 
denaturation of F-actin. The possibility that recombinant spe-
trin polypeptides altered the actual amount of polymerized actin 
is ruled out by a parallel assay using $^{125}$I-labeled actin 
icubated with different concentrations of spectrin constructs. 
The results showed no effect of spectrin construct on the actual 
amount of F-actin recovered in the pellets under assay condi-
tions (data not shown). The N-terminal domain plus the first 
α-helical domain exhibited a 4-fold higher affinity but a 3-fold 
lower capacity. The difference in affinity and capacity for this 
construct were confirmed in four separate experiments, includ-
ing different preparations of F-actin and of spectrin polypep-
tides. Thus, while the absolute values for capacity were not

![Image](http://www.jbc.org/)

**Fig. 3.** β-Spectrin constructs 1–1388, 1–777, 1–643, and 1–528 have the same effect on promoting $^{125}$I-labeled adducin binding to F-actin. $^{125}$I-labeled adducin (5 nM, 127,000 cpm/pmol) was incubated with F-actin (2.0 μM) in the presence of increasing concentration of β-spectrin 1–1388 ( ), 1–777 ( ), 1–643 ( ), or 1–528 ( ). The co-sedimentation assay is described in Fig. 1. The data are expressed as the means of duplicate determinations. The difference between each pair of duplicate determinations is less than 5%. The adducin co-sedimented with actin alone (0.38 nmol/μmol actin) and adducin sedi-
mented without actin (0.11 nmol/μmol actin) have been subtracted.

**Fig. 4.** Activity of β-spectrin residues 1–313, 1–422, 1–528, 284– 
528 in promoting binding of adducin to F-actin. A, $^{125}$I-labeled adducin (5 nM, 215,000 cpm/pmol) was incubated with F-actin (1.5 μM) and increasing concentrations of β-spectrin constructs encompassing residues 1–313 ( ), 1–422 ( ), 1–528 ( ), or 284–528 ( ). Co-sedimentation assay and data processing are as described in Fig. 3. The data are expressed as the means of duplicate determinations. The difference between each pair of duplicate determinations is less than 5%. The adducin co-sedimented with actin alone (0.63 nmol/μmol actin) and adducin sedimented without actin (0.15 nmol/μmol actin) have been subtracted. B, the data of A are expressed as a double-reciprocal plot. The concentration of spectrin required for half-maximal activation equals the reciprocal of the x axis intercept.

**Fig. 5.** Concentrations of β-spectrin constructs required for half-maximal activation of binding of $^{125}$I-labeled adducin to F-actin. Values for the concentrations required for half-maximal activation were determined by the x axis intercept of the double-reciprocal plot of 1/spectrin construct-dependent binding of $^{125}$I-labeled adducin to F-actin versus 1/spectrin construct. The data were collected from the same assays as noted in Figs. 3 and 4. Each value reported is the result of one independent binding assay.
rigorously determined, the difference in capacity between constructs was a consistent finding.

Although addition of the second α-helical domain to the N-terminal domain plus the first α-helical domain increased the requirement for half-maximal activation of adducin binding 2-fold, this addition had no measurable effect on affinity for actin (Fig. 7). Moreover, further addition of up to 3-fold with little change in the concentration required for half-maximal activation of adducin binding. Increased affinity due to spectrin dimerization rather than interaction with actin is unlikely based on the finding of reduced capacity, as well as lack of positive cooperativity and lack of evidence for β-spectrin self-association. The second α-helical domain contributes to interaction of β-spectrin with adducin independent of actin, since addition of the second domain adds little to actin affinity, but does increase stability of adducin/spectrin/actin complexes.

Effect of α-Spectrin on Association of β-Spectrin with Adducin/Actin Complexes—The N-terminal domain of β-spectrin is closely aligned with the C-terminal domain of α-spectrin to form lateral interchain binding between spectrin subunits (Speicher et al., 1992; Viel and Branton, 1994). α-Spectrin could therefore affect the association of β-spectrin with adducin and actin. β-Spectrin resides 1–1388 (N-terminal domain plus 10 α-helical domains) is equivalent to native β-spectrin in terms of forming adducin/actin complexes (Fig. 1) and contains the sites for association with α-spectrin (Speicher et al., 1992; Viel and Branton, 1994). β-Spectrin 1–1388 lacks the site required for end-end association with the N-terminal domain of α-spectrin, and therefore will form laterally associated heterodimers with α-spectrin but will not form end-end tetramers. β-Spectrin 1–1388 was therefore used to determine effects of α-spectrin on adducin/actin interactions (Fig. 8).

The α-spectrin/β-spectrin 1–1388 heterodimers were formed by association of α-spectrin with excess β-spectrin 1–1388 and purified by gel filtration to remove free β-spectrin 1–1388 (see "Methods"). The final dimer fraction contained α-spectrin and β-spectrin 1–1388 in a molar ratio of approximately 1:1 (not shown). The heterodimer has the same activity in promoting binding of adducin to F-actin as β-spectrin 1–1388 alone (Fig. 8). Thus α-spectrin assembled with β-spectrin 1–1388 under these experimental conditions has no detectable role in modulating stability of spectrin/actin/adducin complexes.

Effect of Adducin on Binding of α-Actinin to F-Actin—The N-terminal and first two α-helical domains of β-spectrin exhibit sequence similarity to α-actinin (Byers et al., 1989). It was of interest to determine if α-actinin also shared ability to interact with adducin. The effect of adducin on binding of chicken smooth muscle α-actinin and spectrin to F-actin were compared in Fig. 9. Labeled α-actinin associated with F-actin in the absence of adducin, indicating that α-actinin was active (data not shown). However, adducin had no effect on binding of α-actinin to F-actin even though adducin did promote binding of spectrin with half-maximal stimulation at 40 nM adducin.
Adducin therefore is selective for spectrin/adducin complexes and is not likely to stabilize actin interactions of α-actinin. Other proteins with an actin-binding domain share even less similarity with β-spectrin residues 1–528 than α-actinin. Thus dystrophin and other members of the family are not likely to have actin binding activity promoted by adducin either, although further experiments are required to demonstrate this issue.

**DISCUSSION**

This study presents quantitative evidence that the β-spectrin N-terminal domain plus the first two α-helical domains are responsible for participation of spectrin/adducin/actin complexes formed in vitro assays. α-Spectrin exhibited no detectable activity either alone or following association with β-spectrin 1–1388. The primary activity of β-spectrin in association with actin and adducin/actin complexes resides in the N-terminal domain. However, the first α-helical domain also is involved in association with actin and increases the $K_d$ of the N-terminal domain for F-actin by 4-fold. The second α-helical domain of β-spectrin contributed little to actin-binding but did increase by 2-fold the activity of the N-terminal domain plus first α-helical domain in stabilizing spectrin/adducin/actin complexes. Although the region of β-spectrin required for adducin/actin binding activity is homologous to the N terminus of α-actinin, α-actinin is not affected by adducin in actin-binding assay. Thus the interactions between spectrin and adducin are specific for spectrin and are not likely shared by other members of the spectrin/α-actinin/dystrophin family of actin-binding proteins.

Involvement of β-spectrin and its actin-binding N-terminal domain in an actin-related complex was anticipated in view of studies with spectrin (Karinch et al., 1990) and other members of related actin-binding proteins (Mimura and Asano, 1987; Fabbrizio et al., 1992; Hemmings et al., 1992; Lebart et al., 1993; Levine et al., 1992; McGough et al., 1994). However, a role for the first two α-helical domains of β-spectrin in interactions with actin and adducin has not been reported previously, and has interesting implications for arrangement of components of spectrin/actin and spectrin/adducin/actin complexes (see below).

**Fig. 8.** Effect of α-spectrin on activity of β-spectrin (1–1388) in promoting binding of $^{125}$I-labeled adducin to F-actin. The dimer of α-spectrin/β-spectrin 1–1388 (■) was formed and purified as described under “Experimental Procedures.” α-Spectrin (65 μg, ▲), β-spectrin residues 1–1388 (40 μg, ▼) were both denatured and then renatured to establish parallel conditions with dimer preparation. The adducin promoting activity of α-spectrin, β-spectrin residues 1–1388, or dimer α/β–1,388 was assayed as noted in Fig. 1. The adducin binding to actin alone (0.40 nmol/μmol actin) has been subtracted from spectrin-dependent adducin binding to F-actin.

The use of monomeric forms of β-spectrin permits measurement of the actin binding affinity without complication of actin cross-linking activities. Our result shows that β-spectrin N-terminal residue 1–422 has full actin binding affinity with a $K_d = 6 \mu M$, which is the first documented affinity for β-spectrin binding to actin. Consistent with this value are the actin binding affinities measured for filamin N-terminal 190-kDa proteolytic fragment ($K_d = 3 \mu M$) (Goelin et al., 1990), α-actinin N-terminal residue 1–269 ($K_d = 4 \mu M$) (Way et al., 1992b), and dystrophin N-terminal residues 1–431 or smaller peptides ($K_d = 1–5 \mu M$) (Fabbrizio et al., 1993). Different binding assay systems might cause variations, such as the dystrophin actin binding affinity ranging from 0.1 μM (Corrado et al., 1994) to 44 μM (Way et al., 1992a). β-Spectrin residues 1–313 have a low actin binding affinity $K_d = 26 \mu M$, comparable to the dystrophin-related protein, utrophin, which has a N-terminal 281-amino acid actin-binding domain that associates with actin with a $K_d = 19 \mu M$ (Winder et al., 1995). These results combined with the present study indicate that the full actin binding affinity of this family of proteins is in the micromolar range and the domains outside the homologous actin-binding domain may be required to attain maximal activity.

The finding that addition of the first α-helical domain both enhances affinity of the N-terminal domain and reduces the capacity of actin filaments suggests a model for spectrin/actin complexes shown in Fig. 10. The dimensions of most of the protein subunits and segments have been measured by a variety of approaches and these dimensions are drawn in scale. According to this scheme, the N-terminal domain of spectrin binds to the interface between two actin subunits, as described for the homologous domain of α-actinin (McGough et al., 1994). In addition, optimal binding also involves contact between the first α-helical domain and at least one additional actin subunit. The α-helical domain thus stabilizes a spectrin-actin complex but also inhibits binding of immediately adjacent spectrin molecules through steric hindrance. The first α-helical domain has no detectable independent actin-binding site, suggesting two possibilities. Either the interaction with actin is too weak to be measured experimentally (i.e. a $K_d$ greater than 100 μM), or a
Adducin has a diameter of 89 Å for tetramer based on hydrodynamic second spectrin molecule to the ternary complex (Hughes and Bennett, 1987). The adducin C-terminal tail domains, with unstructured random coil conformation, bind spectrin/actin to form ternary complex and recruit a secondary actin binding site is created after the N-terminal adducin tail and involvement of the spectrin/actin-binding tail domain (Kuhlman et al., 1994). The predicted coil regions between helix A and B in both the first and second α-helical domains (both are 9–10 amino acid stretches without any similarities) as well as the end of the helix A of the second domain. These differences in primary structure are candidates to explain the functional differences between spectrin and adducin with respect to adducin. It will be of interest in future experiments to design mutations targeting these potential ternary binding sites to study their functional consequence in the formation of spectrin/actin/adducin complexes in cells.

β-Spectrin residues 1-528 can be used in future experiments as a monomeric form of native spectrin with full activity in association with actin and adducin, but which avoids complications due to actin filament cross-linking activity and limiting solubility. This new reagent makes it possible to fully saturate actin filaments with spectrin and adducin presumably in a homogeneous orientation. Therefore a high resolution structure of spectrin/adducin/actin complex can, in principle, be solved by image analysis of decorated actin filaments as has been reported for α-actinin (McGough et al., 1994).

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Figure 10. Schematic model of a spectrin/adducin/actin complex. The actin filament appears as two top helices twisting slowly round each other with maximal diameter 90–95 Å, and the dimension of one subunit along the filament is 55 Å (Holmes et al., 1990). A single spectrin repeat domain is a three-helix bundle about 50 Å long and 20 Å in diameter (Yan et al., 1993). The N-terminal construct 1-313 (homologous to α-actinin residue 1-290) is assumed to have a size similar to α-actinin N terminus (McGough et al., 1994). The head domain of adducin has a diameter of 89 Å for tetramer based on hydrodynamic measurement (Ioshi et al., 1991). The adducin head domain caps the fast-growing end of the actin filament (Kuhlman et al., 1996). The orientation of actin filament end/adducin versus spectrin is arbitrary. Adducin C-terminal tail domains, with unstructured random coil configuration, bind spectrin/actin to form ternary complex and recruit a second spectrin molecule to the ternary complex (Hughes and Bennett, 1995). β-Spectrin 1-422, is proposed to bind to more than one subunit of F-actin. Several mapped binding sites are marked as follows: A, β-spectrin 1-313 binding to actin; B, β-spectrin first α-helical domain binding to actin; C, adducin binding to β-spectrin 1-313; D, adducin binding to β-spectrin second α-helical domain. The scale bar (only for the ternary complex) stands for 50 Å.
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