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Calmodulin-binding domain of recombinant erythrocyte β-adducin

(cytoskeleton/proteolysis/calpain/"PEST" sequence/membranes)

DOMINICK A. SCARAMUZZINO AND JON S. MORROW*

Department of Pathology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

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ABSTRACT **Adducin is a 200-kDa heterodimeric protein of the cortical cytoskeleton of mammalian erythrocytes. Analogs are also abundant in brain and several other tissues. *In vitro*, adducin bundles F-actin and enhances the binding of spectrin to actin. Previous studies have established that the β subunit of adducin binds calmodulin (CaM) in a Ca^{2+}-dependent fashion with intermediate affinity (≈200 nM) and that this activity is destroyed by proteolysis. We have confirmed the trypsin sensitivity of CaM binding by β-adducin and the existence of a 38- to 39-kDa protease-resistant core. Calpain I digestion generates a larger core fragment (49 kDa) that is also devoid of CaM-binding activity. Use of recombinant β-adducin peptides generated from partial cDNA clones identified strong CaM-binding activity within the protease-sensitive domain in residues 425–461: KQQKEKTRWLNTPNTYLRVNVADEVQRNMGSPRPKTT in single-letter amino acid codes. This region of the molecule is highly conserved between mouse, rat, and human and shares structural features with CaM-binding sequences in other proteins. Multiple flanking PEST sequences (sequences rich in proline, glutamic acid, serine, and threonine residues that enhance proteolytic sensitivity) may contribute to the protease sensitivity of this region. Consensus sequences for phosphorylation by cAMP-dependent kinases and by protein kinase C (or CaM-dependent kinase) are also found within or near this CaM-binding domain. Collectively, these data suggest a structural basis for the regulation of adducin by Ca^{2+}-dependent CaM binding and possibly by covalent phosphorylation and calpain I-mediated proteolysis as well.**

Erythrocyte adducin is a heterodimeric protein of the cortical cytoskeleton. It is composed of related but nonidentical α and β subunits of 103 and 97 kDa. Recently, the complete primary structures for both rat spleen adducin (1) and human erythrocyte adducin (2) have been deduced by cDNA cloning. These studies establish adducin and its multiple isoforms as a new family of proteins in a variety of tissues and cultured cell lines, including those from brain, kidney, liver, etc. (3–7). Characteristically, adducins bind calmodulin (CaM) and are phosphorylated by cAMP-dependent and -independent kinases and by protein kinase C (3, 8–10).

In vitro adducin stimulates the binding of spectrin to F-actin and tightly bundles F-actin (11, 12). Both activities are diminished in the presence of Ca^{2+} and CaM, suggesting one pathway by which adducin's function may be regulated. Adducin is also associated at unit stoichiometry with high molecular mass spectrin–actin–protein 4.1 complexes extracted from fresh erythrocyte membranes (11), an observation consistent with estimates of 30,000 copies of adducin per cell (13).

The *in vivo* role of adducin remains uncertain. Its capacity to mediate actin–actin and spectrin–actin associations suggests a role in cytoskeletal assembly and stabilization. During erythropoiesis, its stabilization at the membrane precedes that of spectrin (7), and this process may be phosphorylation dependent (8, 9). In other tissues, adducin has been identified at the lateral margin of epithelial cells and particularly in regions of cell–cell contact, where it is one of the first proteins to be recruited (4). Thus, adducin may play a role in establishing topographic polarity and lateral organization in the spectrin-based cytoskeleton.

Little is known concerning the functional organization of the adducin molecule. Sequence comparisons suggest the presence of an actin-binding domain near the amino terminus of both subunits (2). The CaM-binding activity of the protein resides in the β subunit, which is also phosphorylated by both protein kinase C and cAMP-dependent kinase (9). Previous attempts to isolate CaM-binding activity in adducin peptides generated by protease digestion have failed, although the existence of a protease-resistant 39-kDa "core" has been demonstrated (14).

The current study confirms the protease sensitivity of CaM binding by adducin, demonstrates Ca^{2+}-dependent CaM binding in recombinant peptides generated from cDNA clones encoding the protease-sensitive regions of mouse β-erythrocyte adducin, and defines by deletional analysis the sequence necessary for this activity. The CaM-binding site in β-adducin displays several interesting features, including a sensitivity to calpain I digestion. These results have been previously reported in abstract form (15).

MATERIALS AND METHODS

Preparation and ^{125}I-Labeling of Proteins. Erythrocyte adducin was prepared and purified from Triton X-100-extracted cells (11). ^{125}I-labeled CaM (bovine brain) was prepared as before (16). Calpain I from bovine heart was a gift from Dorothy Croall (University of Maine, Farmington, ME).

Protein Digestions. Unless otherwise indicated, adducin (0.1 mg/ml) was digested at room temperature (RT) with trypsin (Worthington) at 1 μg/ml in 20 mM borate/30 mM NaCl, pH 8.5, for up to 3 hr. Digestion with calpain I (4–9 μg/ml) was in 50 mM Tris/0.5 mM EDTA/0.5 mM EGTA/2 mM $CaCl_2$/5 mM 2-mercaptoethanol, pH 7.5, at RT.

Preparation and Purification of Recombinant Peptides. Primers to β-adducin cDNA were prepared from published sequence information (5). Primers 1811 (5′-TGGGGTGAGCGGGCCTGAGGACTC-3′) and 1838 (5′-TACCGCCACCCCTTTGTCCAAGAGAA-3′) were used with PCR to generate a 540-base-pair (bp) clone from a mouse spleen λgt11 cDNA library (provided by Sherman Weissman, Yale University). This clone was confirmed as p3A1.1 (5) by restriction mapping with *Sau*3AI and *Hae* III. By using established methods (17), p3A1.1 was cut with *Sau*3AI and *Rsa* I, inserted into the *Bam*HI–*Sma* I cloning site of pUC-19

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Abbreviations: CaM, calmodulin; GST, glutathione *S*-transferase; PEST sequence, sequence rich in proline, glutamic acid, serine, and threonine residues that enhance proteolytic sensitivity; RT, room temperature.

*To whom reprint requests should be addressed.

by T4 DNA ligase, and cloned in HB101 *Escherichia coli* (clone p3A1.1-A1, nucleotides 51–449). Five additional subclones were obtained by PCR by using primers with 5′ *Bam*HI and *Eco*RI restriction sites as follows: subclone A2 [base pairs 51–201: primers 3893 (5′-CGGGATCCCAGCCACAGTCAC-3′) and 5384 (5′-CGGAATTCCTCACACCTCGTCAGCCACGTT-3′)]; subclone A3 [base pairs 124–449: primers 5385 (5′-CGCGGATCCCAGAAGCAGCAGAAGGA-3′) and 3844 (5′-CGGAATTCCCGGACTCCGGCTCTTCT-3′)]; subclone A4 [base pairs 51–246: primers 3893 and 4124 (5′-CGGAATTCCTTCATCCACGTGGTCT-3′)]; subclone A5 [base pairs 217–294: primers 4451 (5′-CGCGGATCCAGTCCCCGACCAAAGACCA-3′) and 4452 (5′-CGGAATTCCTCATTCAATCCGTATGGGCAT-3′)]; subclone A7 [base pairs 172–449: primers 4450 (5′-CGATGGATCCTACCTGCGGGTGAAC-3′) and 3844]; and subclone A8 [base pairs 51–171 bp: primers 3893 and 3894 (5′-CGAATTCGGTGTTGGGAGTGTT-3′)]. In addition, subclone A3 was digested with *Bam*HI and *Bsa*AI, creating subclone A6 (base pairs 124–236). All clones and amplified products were verified by cDNA sequencing. Subclones A1–A8 in the appropriate pGEX expression vector (Pharmacia LKB) were introduced into *E. coli* (HB101 or W3110) and screened for β-adducin expression, and the peptide was purified as before (18).

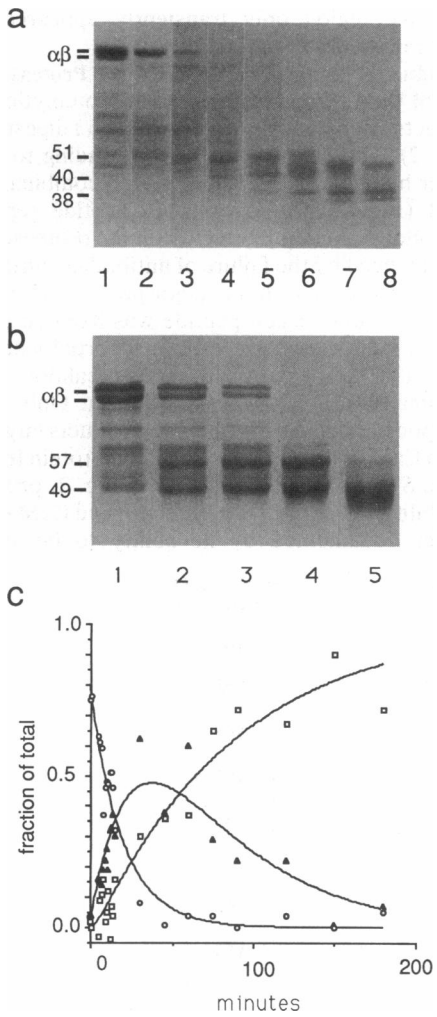

FIG. 1. Adducin is rapidly degraded by trypsin to a 40- to 38-kDa protease-resistant domain and by calpain I to a 49-kDa resistant domain. (*a*) Digestion with trypsin at 1 μg/ml. Lanes: 1, starting material; 2–5, digestion for 5, 15, 45, and 120 min, respectively; 6–8, 30 min of digestion with increasing concentrations of trypsin (approximately 20, 50, and 100 μg/ml, respectively). Molecular mass (kDa) is indicated to the left. (*b*) Digestion with calpain I at 4.5 μg/ml. Lanes: 1, starting material; 2–4, digestion for 30, 60, and 120 min, respectively (note that both subunits of adducin were degraded with a half-life of about 60 min); 5, digestion with calpain I at 9 μg/ml for 120 min. The 49-kDa degradation product was resistant to further breakdown by the protease. (*c*) Densitometric measurement of trypsin digestion products at 30°C: their abundance as a function of time was fit to a three-step kinetic model ($A \geq B \geq C$) by nonlinear regression analysis. The half-life ($t_{1/2}$) of intact adducin under these conditions was 14 min. No differences were noted between α- or β-adducin. The prominent intermediate (51 kDa) was degraded with a $t_{1/2}$ of 20 min. The 38- to 40-kDa products were extremely stable ($t_{1/2} >> 180$ min) and resisted further digestion. The sequential appearance of the 51- and 40-kDa peptides indicates that one is derived from the other. The increasing half-lives of the smaller fragments indicate that adducin must contain at least two regions—one stable to protease and the other unstable.

CaM-Binding Assays. CaM-binding activity was assessed by the retention of peptides on immobilized CaM or by their specific crosslinking to CaM in solution. Two-milliliter columns of CaM-Sepharose 4B (Pharmacia) were equilibrated at

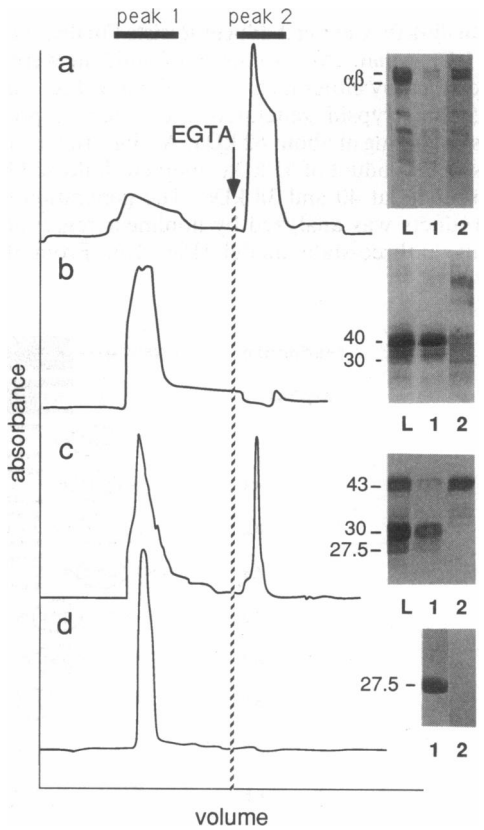

FIG. 2. Recombinant peptides from the protease-sensitive domain of adducin bind CaM. Intact α,β-adducin or recombinant β-adducin peptides were assayed for their ability to bind CaM-Sepharose. Elution profiles a–d and SDS/PAGE analysis (*Insets*) are shown. Lanes in SDS/PAGE *Insets*: L, material loaded onto the column; 1, flow-through; 2, material eluted with 5 mM EGTA. All gels were Coomassie blue-stained except for lane 2 in profile b *Inset*, which is silver-stained and contains no bands that were not in control silver-stained lanes. Profiles: a, intact adducin; b, adducin digested with trypsin (the predominate component in this mixture is the 38- to 40-kDa core domain, which is not retained by the affinity column); c, recombinant β-adducin from the β-p3A1.1-A1 clone; d, GST alone. In profile c and *Inset*, note the ability of the 43-kDa β-adducin–GST fusion protein to bind CaM. Also present in these preparations was a variable amount of proteolytic products between 44 and 30 kDa. This latter product represents a small portion of β-adducin sequence joined to GST and does not bind CaM. The extreme protease sensitivity of this region of adducin defied attempts to prepare peptides free of all proteolysis.

RT with 10 mM Tris/0.15 mM NaCl/1 mM $CaCl_2$/5 mM 2-mercaptoethanol/0.15 mM phenylmethylsulfonyl fluoride (PMSF). Peptides in this buffer were eluted with 5 mM EGTA. Alternatively, 2 μM adducin peptides were crosslinked at RT to ^{125}I-labeled CaM by using 1 mM 3,3'-dithio-bis(sulfosuccinimidylpropionate) (Pierce) in 10 mM Hepes/0.13 M KCl/20 mM NaCl/0.5 mM EGTA/0.15 mM PMSF with or without 1 mM $CaCl_2$.

Other Procedures. DNA sequencing was performed by using either Sequenase (United States Biochemical) or TaqTrack (Promega) kits. Immunoblotting, SDS/PAGE, and protein determinations were by established methods (19–21). Antibodies to α,β-adducin were prepared from adducin electroeluted from SDS/PAGE gels (22) and were affinity-purified. PEST sequence analysis was done with computer program PEST-FIND provided by Martin Rechsteiner (23); PEST sequences enhance proteolytic sensitivity and are rich in proline, glutamic acid, serine, and threonine.

RESULTS

A 38- to 49-kDa Core of Adducin Resists Further Trypsin or Calpain I Digestion. Both subunits of adducin were rapidly degraded at RT by either trypsin or calpain I (Fig. 1 *a* and *b*). Within 5 min trypsin generated a cascade of proteolytic products beginning at about 62 kDa. As digestion proceeded, a quasi-stable product of 51 kDa emerged, followed by more stable products at 40 and 38 kDa. The generation of these major products was analyzed by nonlinear regression analysis using a three-state model (Fig. 1*c*). From this, the half-life of intact adducin was estimated to be 14 ± 5 min; the 51-kDa product, 20 ± 6 min; and the 40-kDa product, >>180 min. A similar analysis using calpain I (Fig. 1*b*) identified the simultaneous disappearance of both α- and β-adducin with a half-life of approximately 60 min and the coincident appearance of peptides at 57 and 49 kDa that resisted further digestion. With higher levels (9 μg/ml for 120 min) of calpain I, the 57-kDa peptide was degraded to 49 kDa, and there was a suggestion of some slight reduction in the size of the 49-kDa peptide. In all digestions, fragments derived from the protease-sensitive region only transiently appeared, as evidenced by immunoblotting.

Only Adducin Peptides Derived from the Protease-Sensitive Region Bind CaM. None of the major (proteolytically resistant) products after either trypsin or calpain I digestion bound CaM (Fig. 2). The sensitivity of CaM binding to proteases was further highlighted by results with recombinant peptide p3A1.1-A1 (Fig. 2, profile c). This peptide represents a portion of adducin that is distinct from the protease-resistant core, as evidenced by the failure of antibodies purified to this peptide to cross-react with the major proteolytic products of trypsin. This 43-kDa fusion peptide was itself so proteolytically sensitive that during preparation it partially broke down to a major 30-kDa fragment and minor breakdown products of approximately 41, 37, 36, and 33 kDa. Only the intact 43-kDa peptide and minor breakdown products larger than 33 kDa bound CaM, while the 30-kDa fusion protein fragment or glutathione *S*-transferase (GST) alone (Fig. 2, profile d) did not. The ability of the 43-kDa peptide to bind CaM in solution was further documented by its ability to be specifically

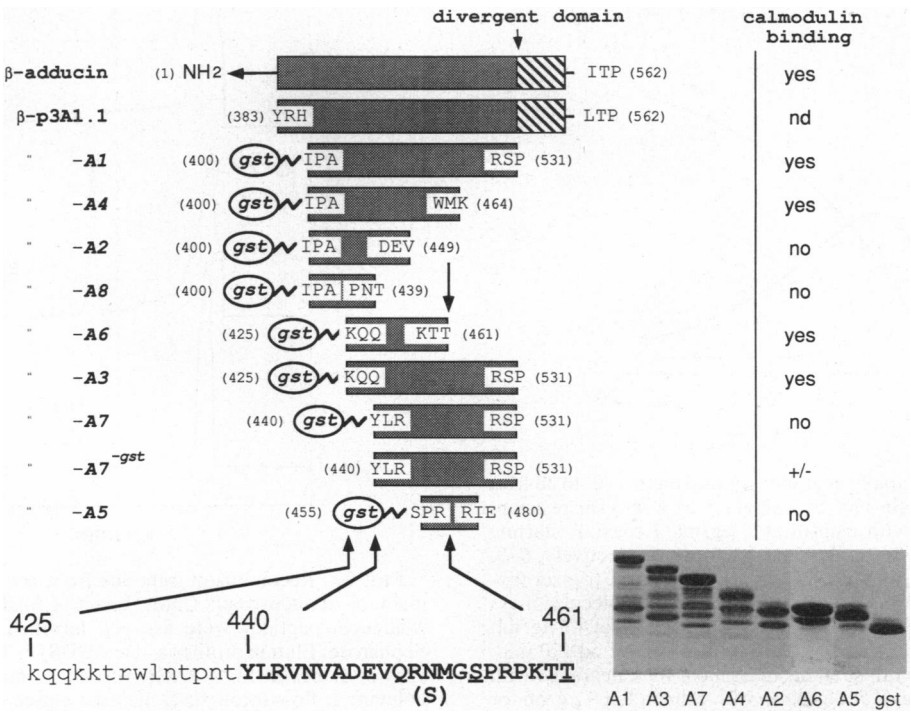

FIG. 3. Deletional analysis identifies the CaM-binding domain of β-adducin. Recombinant peptides representing deletional mutants of β-adducin were tested for their ability to bind immobilized CaM in a Ca^{2+}-dependent manner. While these assays are inherently nonquantitative, the molar concentration of recombinant peptide (or native adducin) that was loaded onto the affinity column was uniform within a factor of 10. Therefore, binding in this assay comparable to native adducin (which was used as a rough criterion for "positive binding") indicates that the binding affinity of the peptide relative to native adducin is probably well within a factor of 10. In every instance, these peptides demonstrated significant sensitivity to protease damage, and no proteolytic GST-containing fusion peptide fragments of <32 kDa bound CaM. Except for peptides p3A1.1-A2, -A5, and -A6, the carboxyl termini of all recombinant peptides were protected by a five-residue extension (-EFIVTD in single-letter code). Peptides p3A1.1-A2 and -A5 contained no extension, while -A6 ended in -GIHRD. Separate experiments established that these extension sequences alone did not bind CaM. Peptide p3A1.1-A6 bound CaM, defining a minimal size for this site (residues 425–461). Peptide p3A1.1-A7 also demonstrated some binding activity after the removal of GST with thrombin, indicating that the minimal CaM-binding sequence may include only residues 440–461 (shown in boldface). (*Inset*) Coomassie blue-stained gel of recombinant proteins. All peptides suffered a variable amount of protease degradation, which could not be eliminated despite vigorous attempts.

crosslinked to CaM by 3,3′-dithio-bis(sulfosuccinimidylproprionate) in a Ca^{2+}-specific manner (data not shown).

Deletional Analysis Identifies the Site of CaM Binding as Residues 425–461 of β-Adducin. Since the 43-kDa GST–adducin peptide bound CaM and its 30-kDa proteolytic product did not, a series of recombinant peptides with sequence deletions were prepared from clone p3A1.1-A1 (Fig. 3). Peptides p3A1.1-A1, -A3, -A4, and -A6 bound CaM. The smallest of these was p3A1.1-A6 containing residues 425–461, indicating the presence of a CaM-binding domain within this region. Further deletions at the 3′ end eliminated activity, as in peptides p3A1.1-A2 and -A8. To exclude steric interference, the GST sequence at the amino terminus was removed by thrombin cleavage of peptides p3A1.1-A3 and -A7. In some experiments, weak CaM-binding activity was detected in peptide p3A1.1-A7 after GST removal but this finding was difficult to consistently demonstrate because of the sensitivity of this peptide to proteases. However, the presence of some activity in p3A1.1-A7 suggested that a minimal calmodulin-binding domain may reside in residues 440–461. Within this region are consensus sequences for phosphorylation by cAMP-dependent kinases (residue 461) and by protein kinase C or CaM-dependent kinases (residue 455). Preliminary experiments indicate that both protein kinases A and C phosphorylate the p3A1.1-A1 recombinant peptide (data not shown).

The CaM-Binding Domain Is Flanked by PEST Sequences. The extreme sensitivity of the CaM-binding site to proteases was reminiscent of proteins characterized by the presence of PEST sequences (23–25). In many of these [e.g., brain spectrin (16)], there is either an inhibitory or synergistic effect of CaM on the rate of calpain I proteolysis. In the present experiments, CaM did not significantly inhibit the digestion of adducin by calpain I (data not shown). An analysis of rat spleen and human erythrocyte β-adducin sequence revealed the presence of PEST or PEST-like sequences flanking the CaM-binding site in the protease-sensitive region of the molecule (Fig. 4). These are most frequent in the carboxyl-terminal third distal to the CaM-binding site, but sites approaching PEST sequence criteria flank the CaM site closely. It is unknown whether these sequences actually account for the extreme sensitivity of this part of the molecule to proteolysis.

DISCUSSION

These results establish that (*i*) residues 425–461 of β-adducin interact with CaM; (*ii*) this region is highly sensitive to a number of proteases including calpain I; (*iii*) this region contains multiple sequence motifs rich in the amino acids proline, glutamic acid, serine, and threonine, all known to enhance proteolytic sensitivity (PEST sequences); and (*iv*) this region contains two putative consensus sequences for covalent phosphorylation by protein kinase A, by the multifunctional CaM kinase, or by protein kinase C (27). A comparison of the sequences of human (2), rat (1), and mouse (5) adducin also indicates almost perfect sequence conservation of this region among these β-adducins. Conversely, β-adducin sequence diverges markedly from α-adducin (which does not bind CaM) beyond residue 435, further confirming the critical importance of this region for CaM binding. Collectively, these data suggest a tripartite domain organization of β-adducin (Fig. 4*b*).

The binding site identified here may demonstrate an unusual variation of the basic–amphipathic helix motif that characterizes many CaM-binding proteins and supports the notion that CaM can interact with proteins through a variety of seemingly unrelated sequences (28). With use of an algorithm established for the identification of putative CaM-binding sites (29), only a 12-residue sequence beginning at position 445 and truncated by a proline at position 456 was found to approach the stringent criteria of mean hydropho-

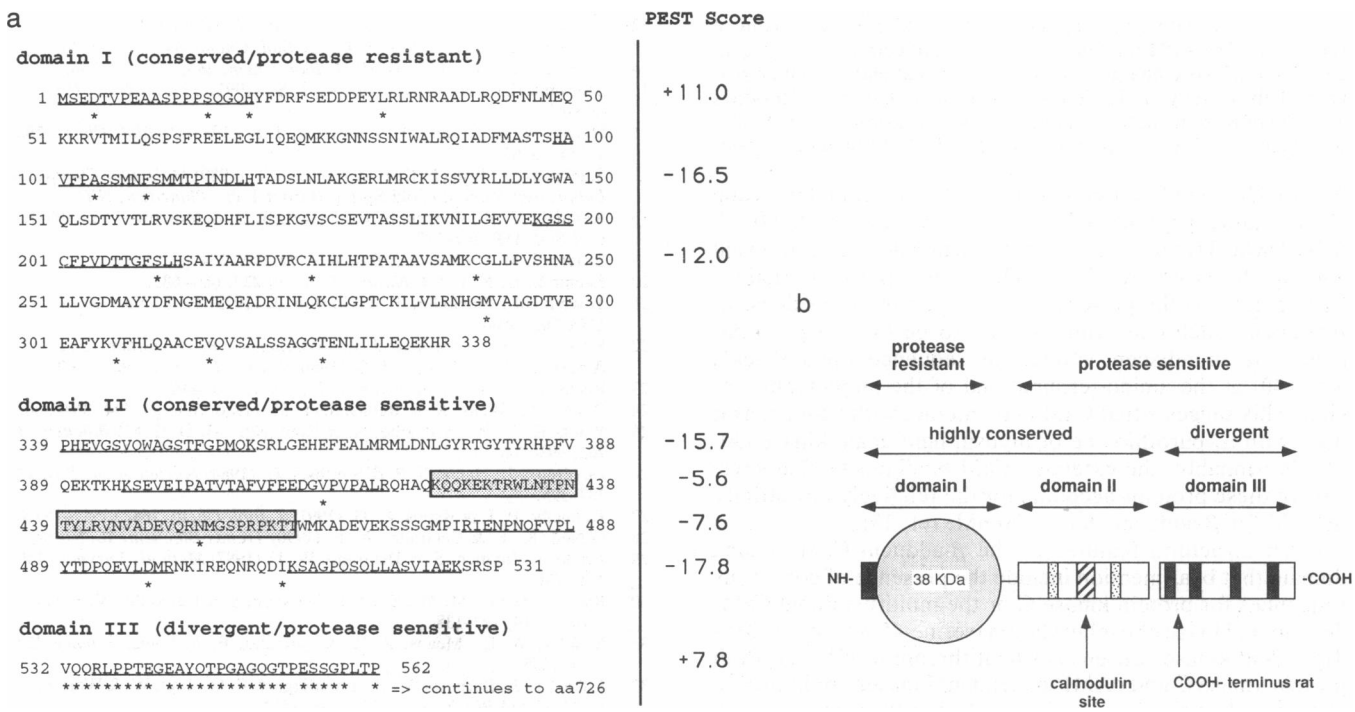

FIG. 4. CaM-binding domain of β-adducin and its relationship to PEST sequence motifs and putative sites of phosphorylation. (*a*) The complete amino acid sequence of β-adducin from rat (1) was aligned with human (2) using the program BESTFIT (26). Nonidentical residues in human are marked with an asterisk. The CaM-binding domain is shaded. PEST sequence candidates are underlined. PEST sequences bestow enhanced susceptibility to intracellular proteolysis and are often found in CaM-binding proteins (25). Note the regions with PEST scores approaching significance (≥0) that flank the CaM-binding domain and the high degree of sequence conservation in domains I and II. (*b*) Domain model of β-adducin, demonstrating the relationship of the CaM-binding domain to other structural features.

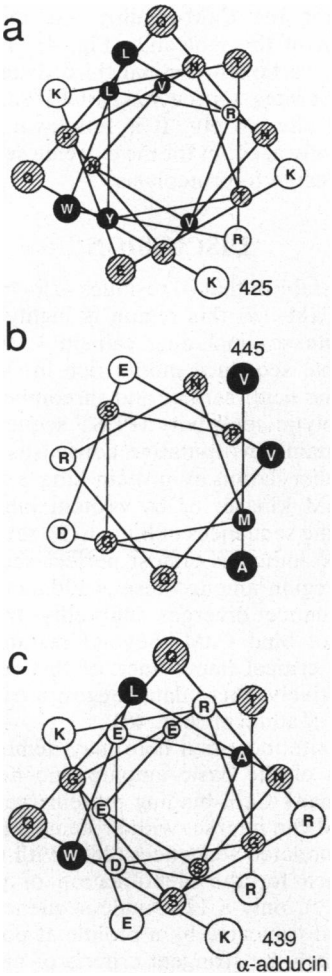

FIG. 5. Helical-wheel diagrams of the CaM-binding domain of β-adducin. The CaM-binding domain of β-adducin contains a concentration of basic charge at its amino-terminal end and an amphipathic helix distally. (*a*) Residues 425–445 of β-adducin. (*b*) Residues 445–456 of β-adducin. (*c*) Residues 439–459 of α-adducin. Note that α-adducin is highly divergent over most of the CaM-binding domain.

bicity (⟨Hb⟩) and hydrophobic moment (⟨μ_H⟩) found in strong CaM-binding peptides (⟨Hb⟩ = −0.16; ⟨μ_H⟩ = 0.49). A helical-wheel representation of this region does indeed reveal amphipathic structure (Fig. 5). However, a third criterion for CaM binding is the presence of clustered basic residues. In β-adducin, such clustering appears to be located predominantly in an adjacent stretch of sequence (amino acids 425–440) at the amino-terminal end of the amphipathic region. This suggests that CaM may interact with adducin as it does with α_1-purothionine or myosin light chain kinase (30, 31). Presumably, the extended CaM-binding site characteristic of these proteins accounts for the relatively low affinity of CaM for β-adducin ($K_d \approx 230$ nM; ref. 13).

Other structural features of the β-adducin CaM-binding domain that bear mention include the presence of consensus sequences for protein kinase C or the multifunctional CaM-dependent kinase phosphorylation (serine-455), and a cAMP-dependent kinase consensus site at threonine-461 (27). Both protein kinase C and cAMP-dependent kinases are known to phosphorylate native adducin (8–10). In other proteins such as myosin light chain kinase (32) and MARCKS (myristoylated alanine-rich C kinase substrate; ref. 33), CaM and phosphorylation act competitively to regulate function. The close proximity of putative phosphorylation sites to the locus of CaM binding in β-adducin lends support to an earlier speculation that the action of CaM and protein kinase C phosphorylation on adducin may be coupled (10) and to our own observations that cAMP-dependent phosphorylation inhibits CaM binding to native adducin (data not shown). If adducin's actin and spectrin–actin binding activities are coordinately regulated by CaM and protein kinase C (or A), then like the MARCKS protein (34), adducin would represent a point of convergence of the Ca^{2+}/CaM and protein kinase C signal-transduction pathways that control the cortical cytoskeleton.

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