Primary Structure and Domain Organization of Human Alpha and Beta Adducin

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Abstract. Adducin is a membrane-skeletal protein which is a candidate to promote assembly of a spectrin-actin network in erythrocytes and at sites of cell-cell contact in epithelial tissues. The complete sequence of both subunits of human adducin, alpha (737 amino acids), and beta (726 amino acids) has been deduced by analysis of the cDNAs. The two subunits have strikingly conserved amino acid sequences with 49% identity and 66% similarity, suggesting evolution by gene duplication. Each adducin subunit has three distinct domains: a 39-kD NH_2-terminal globular protease-resistant domain, connected by a 9-kD domain to a 33-kD COOH-terminal protease-sensitive tail comprised almost entirely of hydrophilic amino acids. The tail is responsible for the high frictional ratio of adducin noted previously, and was visualized by EM. The head domains of both adducin subunits exhibit a limited sequence similarity with the NH_2-terminal actin-binding motif present in members of the spectrin superfamily and actin gelation proteins. The COOH-termini of both subunits contain an identical, highly basic stretch of 22 amino acids with sequence similarity to the MARCKS protein. Predicted sites of phosphorylation by protein kinase C include the COOH-terminus and sites at the junction of the head and tail. Northern blot analysis of mRNA from rat tissues, K562 erythroleukemia cells and reticulocytes has shown that alpha adducin is expressed in all the tissues tested as a single message size of 4 kb. In contrast, beta adducin shows tissue specific variability in size of mRNA and level of expression. A striking divergence between alpha and beta mRNAs was noted in reticulocytes, where alpha adducin mRNA is present in at least 20-fold higher levels than that of beta adducin. The beta subunit thus is a candidate to perform a limiting role in assembly of functional adducin molecules.

PLASMA membranes of eukaryotic cells contain a system of structural proteins known as the spectrin-based membrane skeleton that has a general role in organization of certain integral membrane proteins and of coupling these proteins to cytoplasmic proteins (2, 16, 29). Spectrin, the principal component of the membrane skeleton, is a rod-shaped protein that associates with F-actin at each end. Spectrin and actin together with accessory proteins form a regular geodesic domelike structure in erythrocytes where short actin filaments are associated with five and seven spectrin molecules to form a polygonal network (5, 28). The striking images of the spectrin skeleton raise the issue of how such a structure could be assembled and has focused attention on the accessory proteins that interact with spectrin and actin at spectrin-actin junctions (1).

Adducin is a membrane-skeletal protein that promotes association of spectrin with actin, and this interaction is regulated by calcium/calmodulin (14, 31). Adducin is comprised of two subunits with M_r 103,000 (alpha) and 97,000 (beta) and was originally purified from human erythrocytes based on its calmodulin-binding activity (13). Adducin also is a substrate for protein kinase C in intact cells as well as under in vitro conditions (6, 27, 33, 39). A protein closely related to erythrocyte adducin has been purified from brain membranes (3) and immunologically related isoforms are present in other tissues and cell types including liver, kidney, lung and testes, and various cultured cells (3, 24, 40). Adducin is localized at sites of cell-cell contact in epithelial tissues and in cultured cells such as keratinocytes and MDCK cells (24). Co-localization of adducin with spectrin in a detergent-stable complex at sites of cell-cell contact and the ability to promote formation of spectrin-actin complexes have suggested that adducin has a role in assembly of a spectrin-actin network (14, 24).

Limited proteolytic digestion of adducin has revealed that each subunit is comprised of two distinct domains (23). The NH_2-terminal 39-kD region is protease-resistant and globular in shape while the COOH-terminal domains are extremely protease sensitive. These properties suggested that adducin is shaped like a ball with a tail on one end, although the tail was not noted previously by rotary shadowing EM (13). The COOH-terminal domains contain phosphorylation sites, the binding site for calmodulin, and are required for association with spectrin and actin (23).

In this study, we have determined the amino acid sequence for both subunits of adducin from analysis of their cDNAs.

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The subunits are remarkably similar throughout their sequences, with 49% identity and 66% similarity. Both subunits contain a hydrophilic COOH-terminal region corresponding to the protease-sensitive domain noted previously. A single alpha adducin mRNA is expressed in all tissues while beta adducin mRNA has tissue-specific variability.

Materials and Methods

Protein Isolation and Sequencing

Adducin and its NH_2-terminal protease resistant domains were purified from human erythrocytes essentially as described (23). Small peptides, which were present in tryptic digests of adducin, were separated from the protease-resistant domain by passage through a Mono Q column equilibrated with 10 mM sodium phosphate, pH 7.5, 1 mM NaEDTA, 1 mM NaN_3, 0.05% Tween 20, 0.5 mM DTT, pH 7.5 and loaded on a Poly F reverse phase column. The column was eluted using a linear gradient of 0.2% TFA in water to 0.1% TFA in acetonitrile. Fractions containing a single band on SDS-polyacrylamide gels were sequenced directly. Impure fractions were pooled in small batches, diluted with 0.2% (vol/vol) TFA, loaded on a Vydac C18 column and eluted as described above. Fractions containing a single band were sequenced. Some fractions were sequenced after transfer to Immobilon paper (Millipore Corp., Bedford, MA) as described (9, 30). Smaller bands obtained after chymotryptic digestion of the protease resistant domains were also sequenced after transfer to Immobilon. Peptides were sequenced on an Applied Biosystems Protein Sequencer by Judy Phelps in the Howard Hughes Biopolymer facility (Duke University Medical Center).

Cloning and Sequencing of Adducin cDNAs

Unless specified, molecular cloning methods were performed essentially as described by Sambrook et al. (1989). cDNA clones for adducin were initially isolated by screening a lambda gt 11 human reticulocyte library (7) with an affinity-purified antibody against adducin. Clone R8, which encodes alpha adducin (see Results), was obtained on antibody screening, subcloned into pBluescript SK II (+) (Stratagene, La Jolla, CA) and sequenced in both directions using the dideoxy chain termination method (36). Subsequent alpha adducin clones were isolated from either the human reticulocyte or a K562 (human erythroleukemic cell line) 5′ stretch lambda gt 11 library (Clontech, Palo Alto, CA) using ^{32}P-labeled cDNA fragments prepared by randomly primed DNA synthesis (Multiprime System; Amersham Corp., Arlington Heights, IL). Clones for beta adducin were isolated by screening the K562 library with a 300-bp cDNA probe. The probe was synthesized by reverse transcriptase-polymerase chain reaction of K562 cell RNA using primers derived from a partial mouse spleen cDNA sequence (35). The inserts were subcloned into pBluescript KS II (+) (Stratagene) and sequenced in both directions using the dideoxy chain-termination method.

Expression of Partial Alpha Adducin in Escherichia coli

Clone R8 was ligated in a unique EcoRI site in pGEMEX-1 (Promega Biotec, Madison, WI) which contains a T7 RNA polymerase promoter. The recombinant plasmid was used to transform JM109 (DE 3), a non-expressor strain of *E. coli*, and plasmids were identified containing the correct orientation of the clone in the plasmid. An expressor strain of *E. coli* (BL 21) was subsequently transformed with the selected recombinant plasmid (38). Bacteria were induced with IPTG to express a fusion protein which contained 30 kD of NH_2-terminal viral protein Gene 10 sequence followed by the polypeptide encoded by clone R8.

Antibodies

Antibodies to individual subunits of adducin were raised by immunizing rabbits with bands cut from SDS-polyacrylamide gels and affinity purified using either intact adducin or the expressed fusion protein coupled to CNBr-activated sepharose as an immunoadsorbant (8).

Northern and Southern Blot Analyses

Total RNA from rat tissues and K562 cells (3×10^8 cultured cells) was extracted using RNAzol (Cinna/Biotex) according to instructions provided by the manufacturer. Human reticulocyte RNA was prepared from blood obtained from patients with polycythemia or undergoing exchange transfusions (17) with the modification that the acid precipitate was extracted with four volumes of RNAzol and RNA purified as for rat tissues. Poly A^+ RNA was isolated from total RNA by adsorption to oligo dT-cellulose. Chromosomal localization of alpha and beta adducin was evaluated using blots of DNA from human/hamster cell hybrids (Bios Corp., New Haven, CT).

Electron Microscopy

200 μl of 53 μg/ml adducin and 57 μg/ml NH_2-terminal domains in 10 mM sodium phosphate, pH 7.5, 1 mM EDTA, 1 mM NaN_3, 0.5 mM DTT, were loaded on a 6.0-ml gradient of 15–40% glycerol in 0.1 M ammonium bicarbonate, 5 mM DTT. The gradients were centrifuged at 50,000 rpm for 15 h in SW-50.1. Fractions were collected (250 μl) and run on a SDS-polyacrylamide gel. Fractions containing protein were used for low angle rotary shadowing with platinum and carbon (12). Negative staining was performed by applying 2–5 μg/ml adducin or the NH_2-terminal domains to carbon coated grids (Ted Pella Inc., Irvine, CA), which were washed with 10 mM sodium phosphate, 1 mM Na EDTA, 0.5 mM DTT, pH 7.5, and coated with 1% uranyl formate.

Computer Analysis of Sequence Data

The nucleotide and amino acid sequences of adducin subunits were analyzed using the MacVector Program (International Biotechnology Inc., New Haven, CT). Comparison of sequences of adducin subunits was performed with the BESTFIT Program of University of Wisconsin Genetics Computer Group (UWGCG, Madison, WI). Database searches were performed using the WORDSEARCH program and sequence alignments were made using LINEUP and PROFILE programs of UWGCG. Potential phosphorylation sites were identified by the PC Gene program, Prosite (Intelligenetics, Mountain View, CA).

Results

Isolation and Characterization of Human Adducin cDNAs

A human reticulocyte lambda gt11 library (7) was initially screened with an antibody that recognized both subunits of adducin with equal affinity. Two clones were isolated which hybridized with one another. The deduced amino acid sequence from clone R8 revealed an open reading frame which contained an identical match with the sequences of four peptides isolated from a tryptic digest of adducin (corresponding to 62 residues), confirming that clone R8 encoded a portion of one of the adducin subunits. The presence of a stop codon in clone R8 (followed by several other stops in all reading frames) indicated that the sequence represented the COOH-terminal coding and 3′ untranslated region of one adducin subunit. Several overlapping clones encoding the complete subunit were isolated by subsequent screening of the reticulocyte and K562 libraries (Fig. 1 *a*).

Antibodies raised against the individual subunits of adducin were not completely specific to one subunit and could therefore not be used to determine the subunit encoded by clone R8. Therefore, clone R8 was expressed in bacteria, and the expressed fusion protein was used to isolate antibodies specific for this polypeptide. Antibody isolated from antisera raised against intact adducin reacted selectively with the alpha subunit, indicating that clone R8 encoded the alpha subunit of adducin (data not shown).

Clones for beta adducin could not be isolated from the reticulocyte library despite repeated antibody screens, but were obtained from a K562 human erythroleukemia cell library using the following approach. Four peptides (corre-

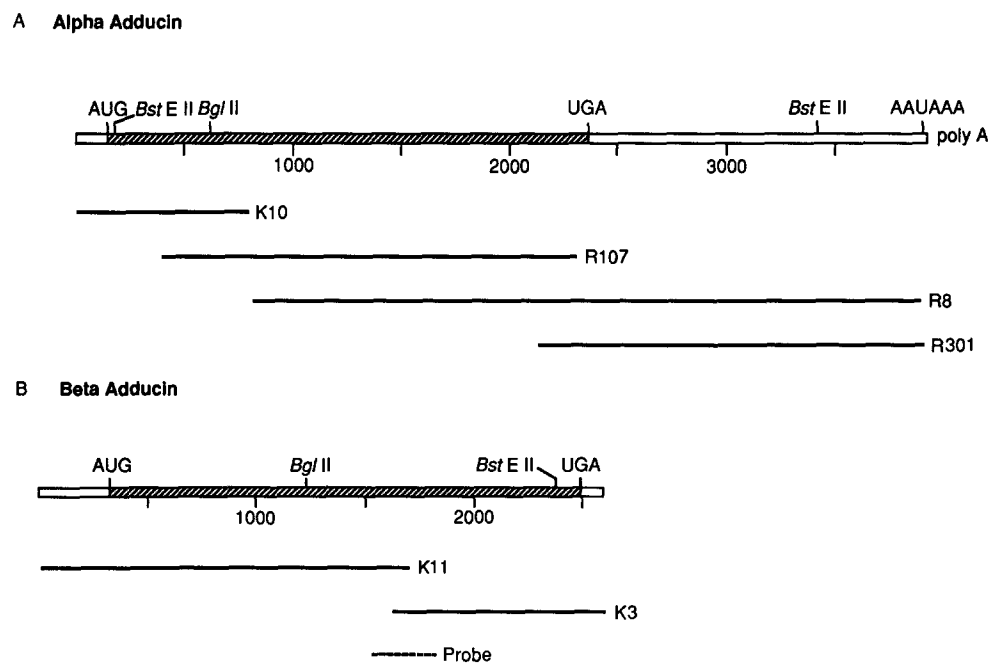


Figure 1. Restriction maps and alignment of cDNA clones for alpha (*a*) and beta (*b*) adducin. The composite cDNAs were created from overlapping clones. The prefix K or R designates the library (K562 or reticulocyte) from which the clone was isolated. The open reading frame is indicated by a cross-hatched box. AUG and UGA represent the start and stop codons. A polyadenylation signal (AAUAAA) is present 13 bp upstream of a poly A tail on clone R301.

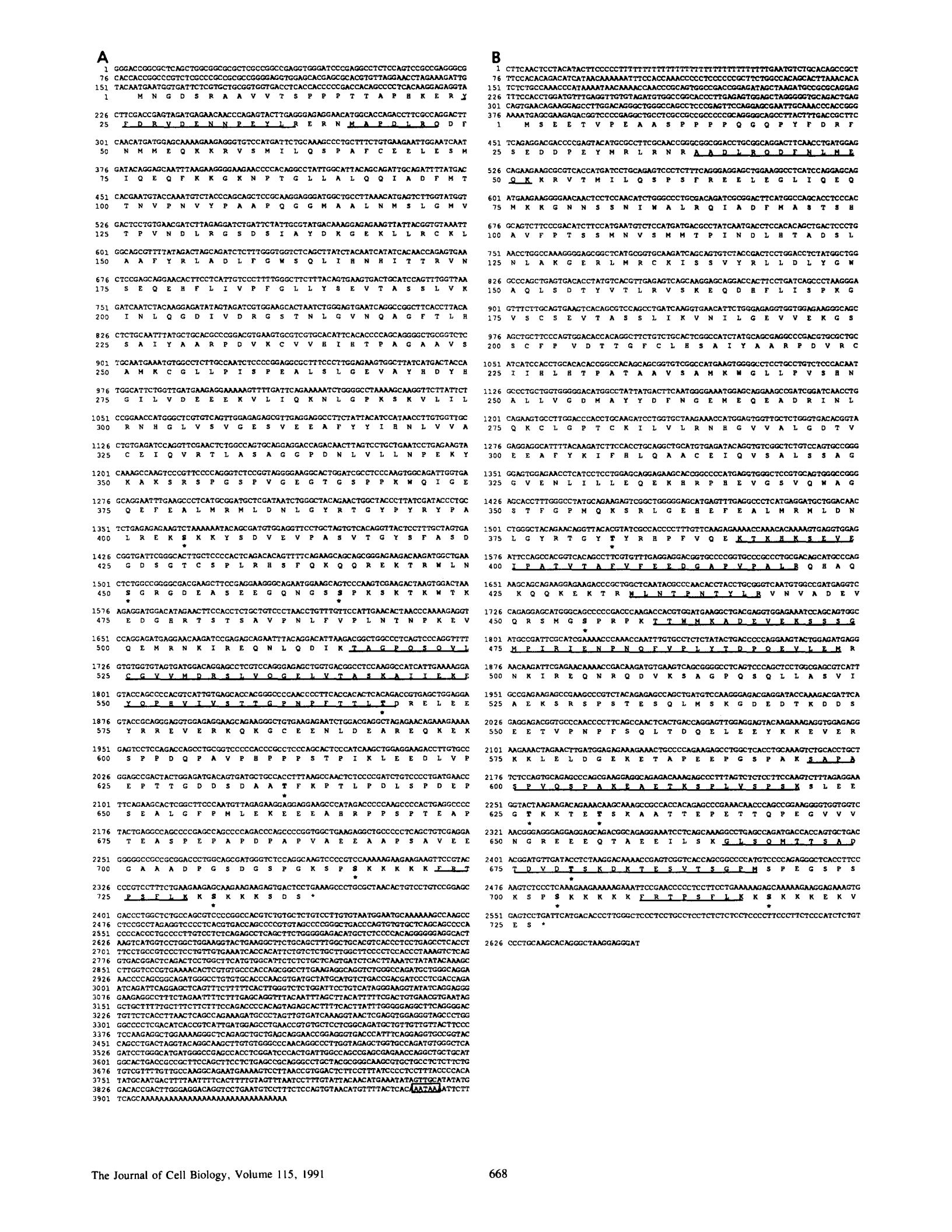
sponding to 79 residues) matched identically with portions of amino acid sequence derived from a 540-bp partial mouse spleen adducin cDNA sequence (35). These peptides also matched the deduced amino acid sequence from alpha adducin clone R8 in small segments, suggesting that the peptides were related but not identical to alpha adducin and represented beta adducin. Oligonucleotide primers were synthesized based on mouse adducin cDNA sequence in the portions that matched with peptide sequence. These primers were used to synthesize a 300-bp cDNA fragment from K562 mRNA reacted with reverse transcriptase to synthesize cDNA followed by amplification of cDNA with the polymerase chain reaction (Gilligan, D. M., R. Joshi, E. Otto, T. McLaughlin, and V. Bennett. 1990. *J. Cell Biol.* 111:46*a*.) The 300-bp cDNA was subcloned and sequenced and showed 87% nucleotide identity with the mouse spleen adducin sequence. Two clones (K11 and K3), obtained by screening the K562 library with the 300-bp probe, overlapped by 60 bp and together encoded a protein with an open reading frame of 726 residues (Fig. 1 *b*). The predicted amino acid sequence obtained from the composite cDNA sequence of beta adducin clones was clearly distinct from that of alpha adducin confirming that the clones indeed encoded the beta subunit of adducin.

Analysis of Nucleotide and Amino Acid Sequence

The nucleotide and predicted amino acid sequences for alpha and beta adducin are shown in Fig. 2. It was not possible to directly ascertain the NH_2-terminal sequence by protein sequence since the NH_2-terminus of both adducin subunits is blocked (23). However, the presence of stop codons in all reading frames upstream of the proposed start site suggests that translation starts at the methionine indicated in this figure. The predicted initiation codon is followed by an open reading frame of 2211 nucleotides (737 residues) in alpha adducin and 2,178 nucleotides (726 residues) in beta adducin. Alpha adducin has a long 3′ untranslated region of 1,540 bp, which contains a polyadenylation signal 13 nucleotides 5′ to a poly A tail.

The molecular weight of adducin calculated from the amino acid sequence (alpha = 81 kD; beta = 80 kD) is smaller than that predicted by SDS-PAGE (M_r alpha = 103 kD; beta = 97 kD). The discrepancy between predicted molecular weight and the relative molecular weight is due to anomalous migration of adducin polypeptides on SDS electrophoresis. Plots of migration as a function of polyacrylamide concentration provide a way of extrapolating migration rates to zero polyacrylamide where proteins binding equivalent amounts of SDS per gram of protein will have equal migration rates. Migration of adducin polypeptides extrapolates to a lower rate than standard proteins (data not shown) indicating that these polypeptides either bind less SDS per gram or that they have a net basic charge. Since the measured pI of adducin is less than seven (23), the most likely explanation is reduced binding of SDS. The COOH-terminal domain of adducin polypeptides is hydrophilic and is likely to be responsible for reduced electrophoretic migration. In support of this interpretation, the expressed fusion protein comprising the COOH-terminal domain of alpha adducin migrated at 105 kD instead of the actual molecular weight of 85 kD (not shown). Finally, further evidence that the deduced sequence is complete is that the total number of nucleotides sequenced for alpha adducin (3,936 bp) is very close to the observed mRNA of 4 kb (Fig. 6).

The deduced amino acid sequences for alpha and beta adducin are strikingly conserved, with an overall identity of 49% and a similarity of 66% (Fig. 3). The similarity is greater towards the NH_2-terminal half of adducin (56% identity, 76% similarity) consistent with conclusions from peptide map analysis (23). However, stretches of closely related sequences are distributed along the entire length of these polypeptides. The COOH-terminal end of both subunits contains an identical 22 amino acid highly basic domain (11 lysine residues). This domain is similar to a domain



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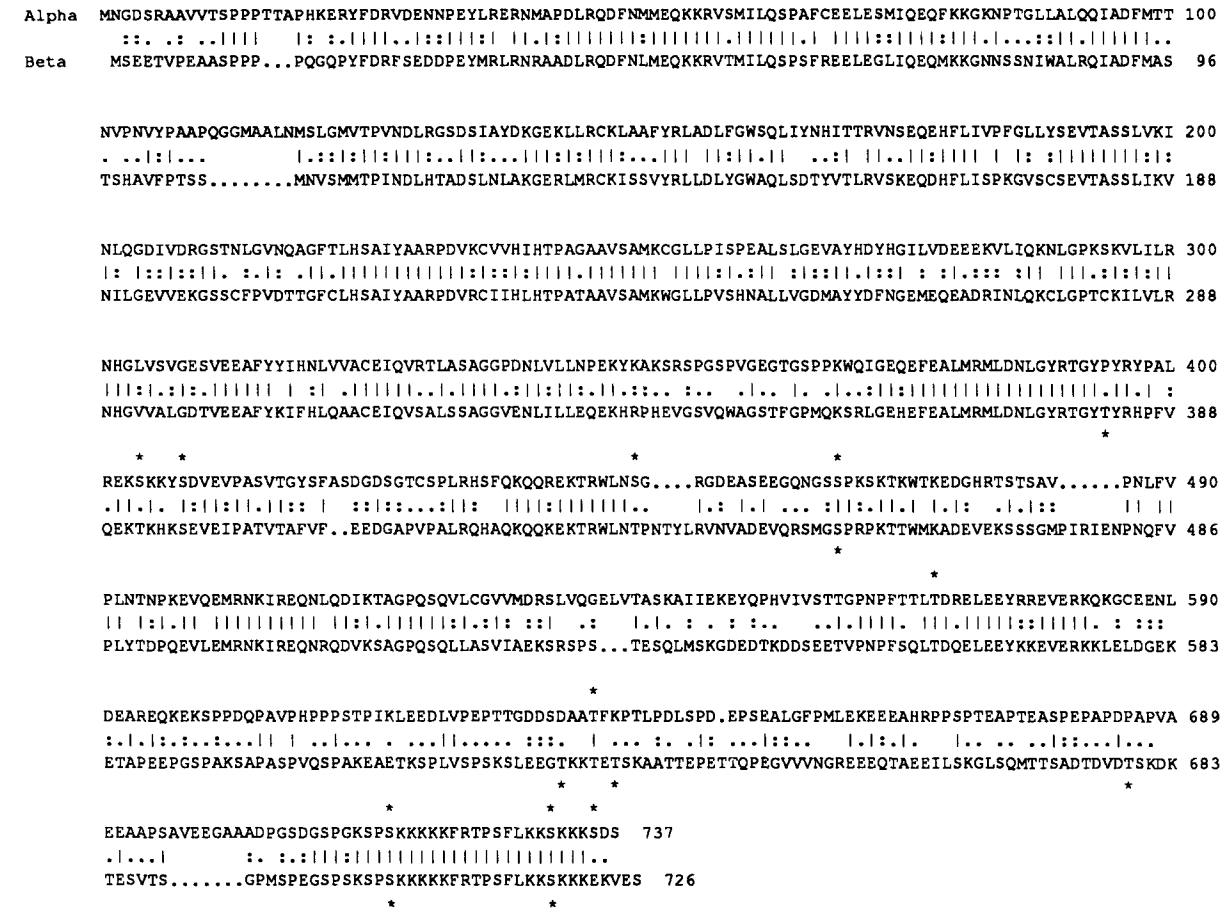

Figure 3. Bestfit analysis of amino acid sequences for alpha and beta adducin. Sequences were aligned using the Wisconsin Genetics Computer Group program. Lines indicate identical residues, two dots indicate conservative substitutions. Sequences have 49% identity and 66% similarity. Asterisks indicate potential phosphorylation sites.

in the MARCKS protein which contains protein kinase C phosphorylation sites and exhibits calmodulin binding (19).

Adducin is a major substrate for protein kinase C and contains multiple potential protein kinase C phosphorylation sites (Fig. 2). Several of these predicted protein kinase C sites are in a strategic location at the junction between head and tail domains (see below). Additional potential sites are in the COOH-terminal basic stretch noted above. The predicted sites are most likely not all utilized by protein kinase C since adducin incorporates only 2–3 mols of phosphate per mol (3, 27, 39). At least one of the sites at the junction between head and tail domains is phosphorylated under in vitro conditions, since progressive proteolysis of adducin phosphorylated by protein kinase C yields a labeled polypeptide of M_r = 48,000, while polypeptides of lower relative molecular weight are not labeled (Joshi, R., and V. Bennett, unpublished data). Two potential protein kinase A sites were detected in alpha adducin at residues 407 and 734. Additional predicted sites are present in the head domains, but are not phosphorylated in vitro (23) and therefore not included in this analysis. Consensus sequences for phosphorylation by cdc2 kinase and cGMP-dependent protein kinase also are present, although it is not known whether adducin is a substrate for these kinases.

Potential calmodulin binding sites are residues 425–444 and 705–721 in beta adducin, based upon an aromatic residue in a region exhibiting some segregation of polar and hydrophobic residues in a helical wheel diagram (32). Residues 705–721 of beta adducin are identical to residues 718–734 in alpha adducin and may be a calmodulin binding site in both subunits. Calmodulin binds preferentially to the beta subunit of erythrocyte adducin (13), although a lower affinity interaction with the alpha subunit could also occur.

Domain Structure of Adducin

Previous mapping of domain structure of adducin by controlled proteolysis has revealed two major subdivisions

Figure 2. Nucleotide and deduced amino acid sequences for human erythrocyte alpha (*a*) and beta (*b*) adducin. Underlined amino acids were identical to sequences of peptides isolated from proteolytic digestion of adducin. Potential protein kinase C phosphorylation sites in the COOH-terminal domains of adducin are indicated by asterisks (PC Gene Prosite Program, Intelligenetics). The polyadenylation signal for alpha adducin is boxed. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X 58141 for alpha and X 58199 for beta adducin.

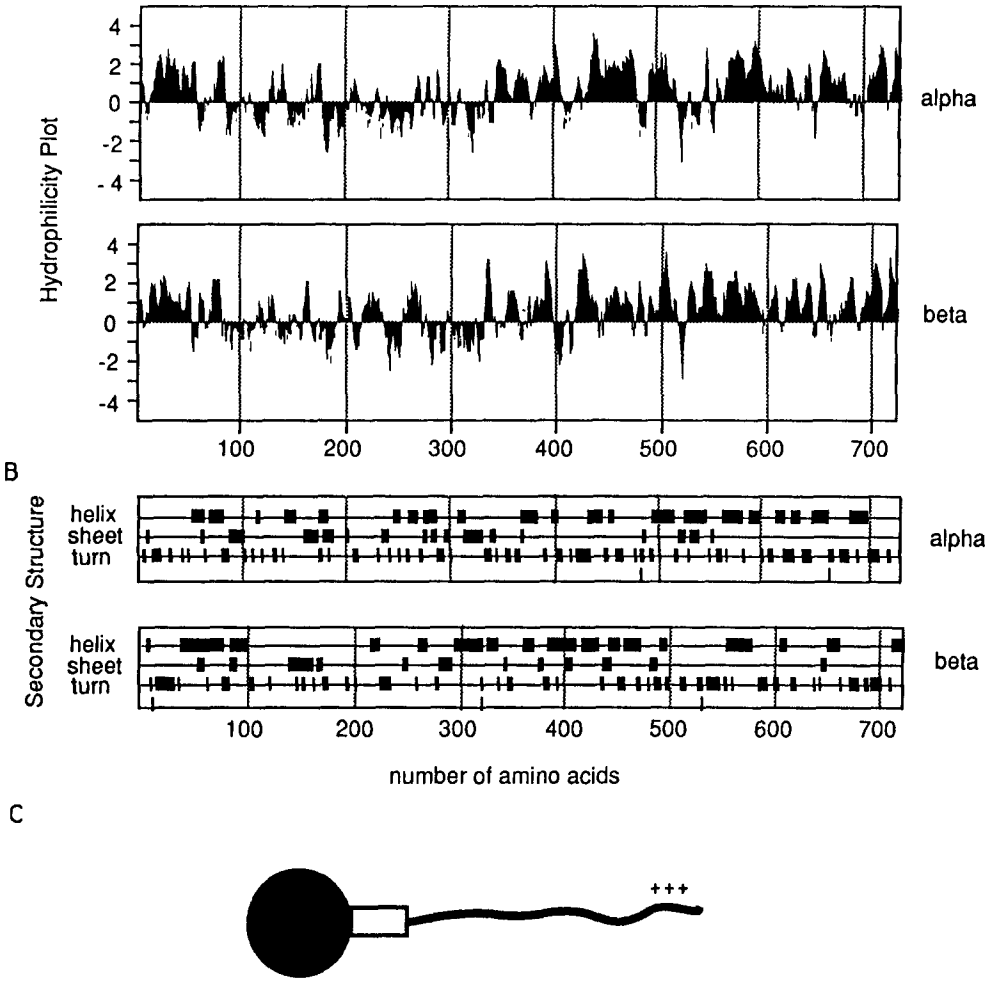

Figure 4. Analysis of alpha and beta adducin amino acid sequences. (*A*) Hydrophilicity profiles of alpha and beta adducins. Plots were determined using the MacVector program (International Biotechnologies Inc.) and a window length of 7 residues. (*B*) Secondary structure analysis of alpha and beta adducin according to the method of Chou and Fasman showing predicted regions of alpha helix, beta sheet, and turns. (*C*) Schematic model of folding of an adducin subunit (equivalent for alpha and beta subunits).

within each polypeptide: a protease-resistant NH_2 terminal domain of M_r = 39,000 and a protease-sensitive COOH-terminal region (23). A stable chymotryptic fragment of M_r = 48,000 also has been noted (3, 23), suggesting a subdomain of M_r = 9,000 between the protease-sensitive and protease-resistant domains. The hydrophilicity profile of adducin subunits is consistent with results from protease digestion, and resolves distinct regions within the primary sequence. The COOH-terminal regions from residues 350–737 (alpha) and 338–726 (beta) are highly enriched in hydrophilic residues, while the NH_2-terminal segments contain a mixture of hydrophobic and hydrophilic residues (Fig. 4). The hydrophilic domain does contain a few regions with less polar residues, and one of these occurs between residues 410–430 (alpha) and 398–420 (beta). The residues from 350 to 430 (alpha) and 338–418 (beta) would correspond to the M_r = 9,000 subdomain noted above. This profile is consistent with three distinct domains in adducin: a NH_2-terminal protease-resistant domain of 350 residues followed by a 80 residue subdomain and ending in a protease-sensitive 300 residue COOH-terminal domain. Secondary structure predictions shown in Fig. 4 suggest that the COOH-terminal domains have multiple stretches of predicted alpha helices interrupted by regions rich in proline residues.

Physical properties of adducin as compared to those of the NH_2-terminal domains suggested that while adducin is relatively asymmetric with a frictional ratio of 1.5-1.8 (3, 13, 39), the NH_2-terminal domains are approximately spherical in shape (23). One interpretation based on this information is that the COOH-terminal domain forms a tail which is the primary contributor to the asymmetry observed in intact adducin. The maximum length of the tail would be 45 nm if it is organized as an extended alpha helix (0.15 nm/residue), although the presence of multiple proline-rich turns suggests a more contorted folding. Two predictions that could be tested by EM are that a tail should be observed and that the isolated NH_2-terminal domain would have the same dimensions as the globular portion of intact adducin. Extended tails have not been noticed previously in adducin by rotary shadowing and the molecule was predicted to be an oblate ellipse (13). However, a negative result is difficult to interpret since tails have been difficult to visualize in other proteins such as synapsin (21, 37).

A careful reexamination of rotary shadowed images of adducin (Fig. 5) demonstrates the presence of tails on some molecules (~10 per cent in this experiment). In some examples, the tails appear folded into globular structures at their ends. The maximum lengths of these folded tails were ~5–6

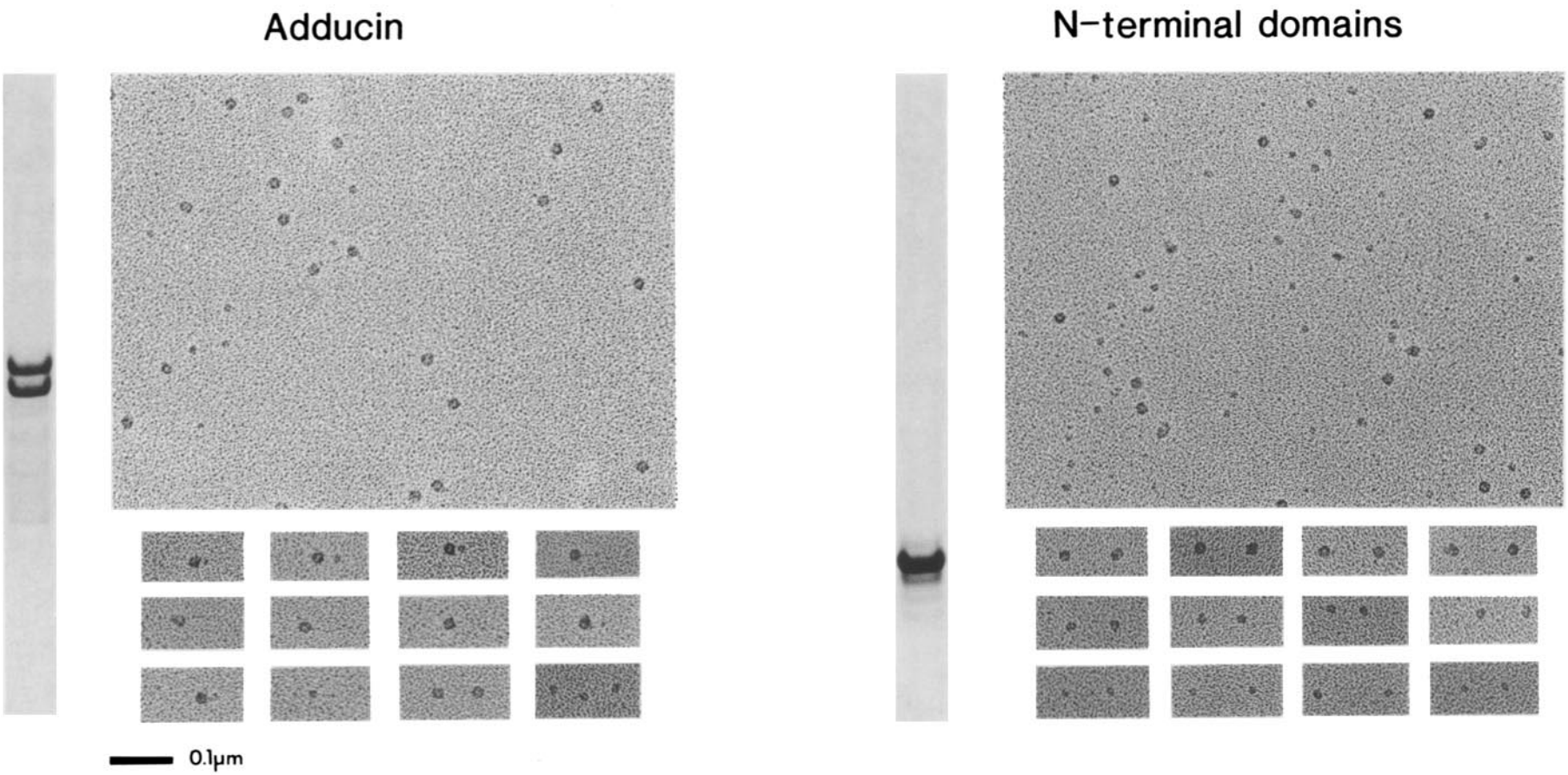

Figure 5. Visualization of intact adducin (*left*) and adducin NH_2-terminal head domain (*right*) by EM. Platinum/carbon replicas were prepared by low angle rotary shadowing (See Materials and Methods). The NH_2-terminal domain was isolated by anion exchange chromatography following digestion of adducin with trypsin as described (23). Coomassie blue-stained SDS-gels of proteins are shown on the left of each panel. Bar, 100 nm.

nm. The diameters of tails are difficult to estimate since these values are close to the size of the platinum grains. Sizes of the globular structures observed in adducin and the NH_2-terminal domains were very similar in rotary shadowed images (Fig. 5). The major structure visualized in these electron micrographs therefore is comprised of the NH_2-terminal domain. These results support the prediction from analysis of amino acid sequence that adducin contains a head domain and a flexible tail.

The diameter of the most prevalent structure in intact adducin and NH_2-terminal domains is 20 nm in shadowed images, although smaller structures of 12 and 8 nm also are evident (Fig. 5). The 20-nm forms comprised ~70% of intact adducin structures (157 out of 218) and 50% of NH_2-terminal domains (130 out of 267).

The heterogeneity in size of adducin could result from a mixture of monomers and dimers or of dimers and tetramers (see Discussion). Some insight into whether the large images represent dimers or tetramers can be gained by comparison of the actual size with values predicted from calculations based upon the approximately spherical shape indicated by physical properties of the NH_2-terminal domain (23). The size of the 20-nm structure visualized by platinum shadowing includes the contribution of metal in addition to protein. A more accurate estimate of the size of the protein is 10–12-nm based on negative staining of adducin (13). Minimum sizes of dimers and tetramers of the NH_2-terminal domain are 5.6 and 7.1 nm, respectively, if these are folded into a smooth sphere (from the relationships V = mol wt (partial specific volume)/Avogadros number) and $V = 4/3\ R^3$). More realistic values taking into account hydration would be 7.1 nm, and 8.9 nm for dimers and tetramers, respectively (calculated assuming only 50 per cent of the volume is protein and the rest is water). These calculations indicate that the 10–12-nm structures are probably tetramers of NH_2-terminal domains and that smaller structures are dimers.

Expression of Alpha and Beta Adducin mRNA

Alpha adducin clone R107 hybridized to a single band of 4.0 kb in a Northern blot of RNA from various rat tissues (liver, brain, kidney, and spleen), human reticulocytes, and K562 cells (Fig. 6, *left panel*). The amount of alpha adducin mRNA in liver (lane *A*) is much less than in kidney (lane *B*), brain (lane *C*), or spleen (lane *D*). The level of alpha adducin mRNA in 10 μg of poly A RNA from kidney, brain, or spleen is similar to the amount of alpha adducin mRNA detected in 10 μg of total RNA from K562 cells (lane *E*) or reticulocytes (lane *F*), suggesting greater expression in K562 cells and reticulocytes.

On a duplicate blot, beta adducin clone K11 hybridized to mRNAs of several different sizes. Liver and kidney (lanes *A* and *B*) have no detectable beta adducin mRNA, in contrast to alpha adducin (lanes *A* and *B* in the left panel). Beta adducin clone K11 hybridized to an 8.1-kb mRNA in brain (lane *C*) and, on longer exposure, to 4.0- and 3.8-kb mRNAs (data not shown). In spleen (lane *D*), three mRNAs are evident of 3.7, 3.3, and 3.0 kb. In total RNA from K562 cells (lane *E*), two major transcripts were detected of 4.0 and 3.8 kb. In contrast to alpha adducin (lane *F* in the left panel), much less beta adducin mRNA was present in reticulocytes (lane *F*) and required poly A selection and longer exposure for detection (lane *F'*).

The major differences in levels of alpha and beta adducin mRNAs in kidney, liver, and reticulocytes indicate unequal stability of alpha and beta adducin mRNAs and/or distinct transcriptional regulation. Another striking contrast between the Northern blots for alpha and beta adducin is provided by the observation that a single-sized mRNA from spleen, K562 cells, and brain hybridizes to the alpha adducin probe while mRNAs of several different sizes hybridize to the beta adducin probe.

Sequence Similarity of Adducin Subunits with the Actin-binding Domains of the Spectrin/Actin-binding Protein Super Family

Database searches with alpha and beta adducin amino acid sequences did not show any extended homology with known proteins. However, an interesting similarity was noted be-

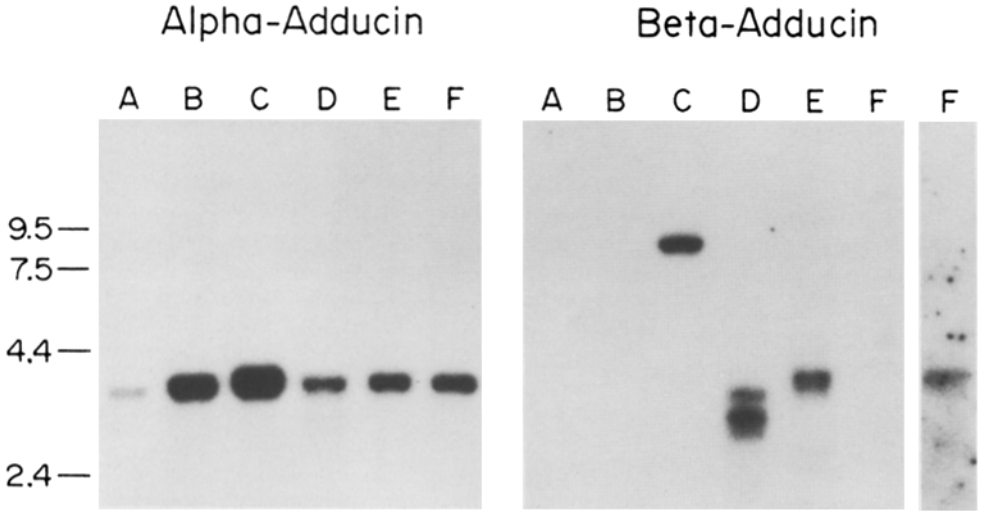

Figure 6. Detection of alpha and beta adducin mRNAs in various tissues by Northern blot analysis. Duplicate blots were hybridized to ^{32}P-labeled probes prepared by random priming from alpha (*R107*) or beta (*K11*) cDNAs. Lanes contained 10 μg of either poly A^+ RNA from adult rat liver (*A*), kidney (*B*), brain (*C*), spleen (*D*), or total RNA from human K562 (*E*) or reticulocytes (*F*). Lane *F'* contained 5 μg of poly A^+ RNA from human reticulocytes and was hybridized to the PCR-derived beta probe shown in Fig. 1 *b*. Sizes from an RNA ladder (obtained from Bethesda Research Laboratories, Gaithersburg, MD) are indicated in kb on the left.

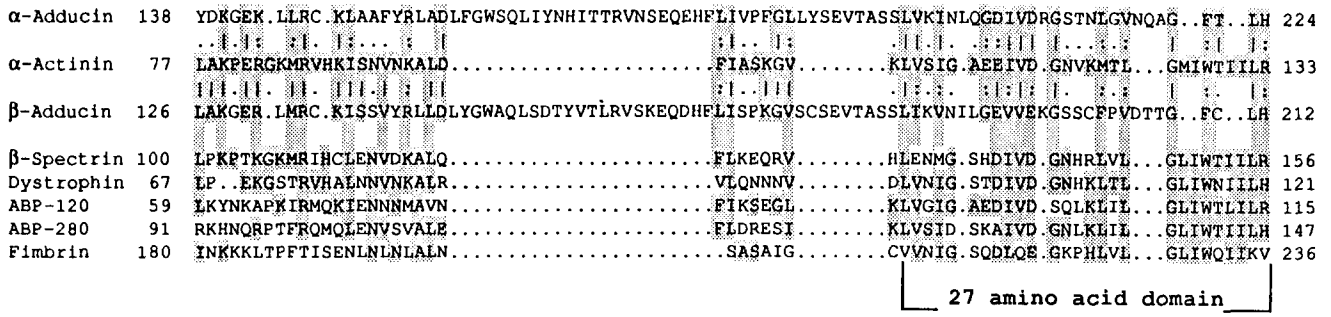

Figure 7. Comparison of alpha and beta adducin sequence with human alpha-actinin (42), human erythrocyte beta spectrin (41), human dystrophin (25), *Dictyostelium* (*D.d.*) ABP-120 (4), human endothelial ABP-280 (18), and the first NH_2-terminal repeat of chick fimbrin (10). Amino acid positions are shown on either side of the aligned sequences. Shaded areas include identical residues and conservative substitutions in comparison to the alpha-actinin sequence. Alignments between alpha-actinin and beta adducin (first two domains) and between alpha-actinin and alpha adducin (third domain) were determined using the Bestfit program from the Wisconsin Genetics Computer Group. The 27 amino acid domain essential for actin binding in *Dictyostelium* ABP-120 is indicated.

tween adducin and the highly conserved NH_2-terminal F-actin binding domains of the spectrin superfamily (spectrin, alpha-actinin, dystrophin) and certain actin gelation proteins (ABP-120, ABP-280, and fimbrin). A 27 amino acid segment is 60–80% conserved among these proteins and has recently been shown to be essential for actin binding by ABP-120 (4). A computer assisted search of adducin sequence with this 27 amino acid domain revealed a related sequence in the NH_2 terminal globular domains of both subunits (Fig. 7). Alpha adducin has nine identical residues and six conservative substitutions out of the 27 amino acid domain while beta adducin has four identical residues and 10 conservative substitutions. Two additional segments of similarity between adducin and other actin-binding domains are revealed by insertion of two gaps of 23 and 8 residues (Fig. 7). Interestingly, human alpha-actinin is more closely related to beta adducin than to other members of the family in this region. No apparent similarities were found between adducin and members of the actin capping/severing/depolymerization family of proteins such as gelsolin, villin, etc.

Chromosomal Localization of Alpha and Beta Adducin Genes

Southern blots of genomic DNA from a panel of hybrid cell lines with deletions of various human chromosomes suggests that the alpha subunit is encoded by a gene located on chromosome 4. Clone R107 (representing 2-kb-coding sequence from alpha adducin) hybridized to cell lines containing chromosome 4 in a panel of DNA from several human/hamster hybrid cell lines (data not shown). Unfortunately, clone K11 (representing beta adducin) did not hybridize to any cell line suggesting that the chromosome carrying beta adducin was absent in this panel. A careful analysis of the chromosomal representation in these hybrid lines showed that chromosome 2 was present only in a single cell line. Controls rerun by Bios Corp. suggested that chromosome 2 in the panel cell line did indeed have a deletion. It is possible that beta adducin is present on chromosome 2 although further experiments are required to verify this localization.

Discussion

This report describes the complete primary sequence for alpha and beta subunits of human adducin, presents evidence that the genes encoding these subunits are expressed in multiple tissues, and implicates the beta subunit as the most variable and potentially limiting subunit in assembly of intact adducin. The primary sequences of the subunits provide several interesting insights into the structure and evolution of adducin. Alpha and beta adducin are closely related with an overall identity in amino acid sequence of 49%, suggesting evolution through gene duplication. The sequence data combined with earlier analysis of domain structure (23) support folding of adducin subunits into a globular NH_2-terminal head domain linked by a connecting domain to an extended tail. The tail of adducin would contribute to the high frictional ratio noted previously (3, 13, 39), and has been visualized in this report by EM (Fig. 5).

Separation of adducin into distinct domains or modules suggests how this protein could associate selectively with spectrin–actin complexes in preference to actin or spectrin alone in in vitro assays (3, 14). Separate binding sites of low affinity for spectrin and F-actin, one in the head and the other in the tail, would result in weak association with individual spectrin or F-actin molecules. However, providing adducin and actin associate closely on spectrin, a spectrin–actin complex would allow adducin to bind simultaneously with both spectrin and actin. Participation of adducin in a ternary complex as opposed to binary interactions with spectrin or actin alone would be greatly favored due to the increase in the effective concentration of interacting sites. In thermodynamic terms, ternary complexes are favored because of a reduction in entropy related to rotation and diffusion of the interacting proteins (for a discussion of quantitative aspects of multivalent protein–protein associations see Jencks [22] and Erickson [11]). A flexible tail would be ideally suited for a role requiring simultaneous association with two large molecules such as spectrin and actin filaments. The head domain exhibited no detectable binding to F-actin in co-sedimentation assays (23). However, direct evaluation of activity of isolated tails in binding to actin was not feasible since the tail was destroyed by even mild proteolysis. It will be important in future experiments to evaluate the roles of heads and tails of adducin using recombinant polypeptides.

Physical properties and cross-linking experiments with adducin indicate that adducin subunits are assembled into sta-

ble dimers and that dimers participate in a dimer-tetramer equilibrium (3, 13, 23) (see Results). Consideration of available information suggests a model where the first level of organization involves association of subunits into stable parallel dimers involving tail-tail and head-head contacts. The observation that removal of the tail by proteolysis results in a major reduction in efficiency of cross-linking of adducin to dimers and tetramers (23) may be interpreted as evidence for extensive tail-tail contacts. An anti-parallel arrangement of subunits is unlikely based on the size of the heads, which are 10–12 nm based on EM of intact adducin (13). A single head of 39 kD folded into a sphere would have an expected diameter of 6 nm (see Results). Evidence for head-head contacts within dimers is based on cross-linking of isolated heads to dimers, although this occurred with reduced yield compared to intact adducin.

Several observations suggest that adducin dimers participate in a dimer-tetramer equilibrium that involves head-head contacts. As discussed in Results, the 10–12-nm structures visualized by negative staining (13) are approximately the expected size for a tetramer comprised of 39-kD heads. Isolated heads have a predicted molecular weight of 120 kD, and are either trimers or a mixture of dimers and tetramers (23). In support of a tetramer is the observation that the heads can be cross-linked as tetramers (23). A possible model incorporating these observations would predict adducin dimers associated by head-head contacts to form tetramers. Tetramers with actin-binding domains located in their tails would explain ability of adducin to bundle actin filaments (31). Such a configuration also suggests how adducin can recruit additional spectrin molecules to adducin-spectrin-actin ternary complexes (14).

Adducin sequence, while unique in the data bases examined, exhibits localized similarities to the MARCKS protein at the COOH-terminus and to the actin-binding domains of members of the spectrin/alpha actinin/dystrophin super family in the NH_2 terminal head domain. The relationship of adducin to MARCKS could result from the large number of basic residues in both sequences and may not represent an evolutionary relationship between these proteins. However, the shared basic sequence could be of functional significance. For example, MARCKS has recently been reported to associate with F-actin, and the basic sequence which also contains sites of phosphorylation by protein kinase C was implicated in this interaction (Hartwig, J. H., P. A. Jammey, A. Rosen, M. Thelem, A. C. Nairn, and A. Aderem. 1990. *J. Cell Biol.* 111:8*a*). The similarity between adducin and members of the spectrin superfamily is more extensive and may reflect a distant relationship between adducin and other members of the superfamily. The functional significance of the sequence similarities between adducin and actin-binding proteins is not clear, since the related sequence is located in the head domain of adducin which does not associate with F-actin (23). The actin-binding domains of spectrin and other proteins have additional functions such as binding to accessory proteins or to an adjacent subunit. The sequence shared with adducin thus may be involved in an activity unrelated to actin-binding. In any event, the regions of sequence similarity to MARCKS and actin-binding proteins are candidates for site-directed mutagenesis and deletions in future exploration of active sites of adducin.

An unanticipated implication of data presented in this study is that the same genes encoding alpha and beta adducin are expressed in other tissues. Alpha adducin probes hybridize under conditions of high stringency with a mRNA of identical size in multiple tissues. While alpha adducin has not been characterized by sequencing of cDNA, it is likely that the mRNAs are very similar or identical. A portion of the beta adducin gene is expressed in brain based upon the sequence of a partial cDNA clone isolated from a human brain cDNA library which is identical to erythrocyte beta adducin (Gilligan, D. M., T. McLaughlin, and V. Bennett, unpublished observations). Other erythrocyte membrane-skeletal proteins encoded by identical genes expressed in other tissues include ankyrin (referred to as ankyrin$_R$ to distinguish this isoform from other members of the family) (26, 34), and the beta subunit of spectrin (41). These considerations indicate that detailed analysis of proteins such as erythrocyte adducin will be relevant in the context of other tissues.

Expression of mRNAs encoding alpha and beta adducin are subject to distinct regulation. A striking divergence between message levels occurs in reticulocytes which have at least 20-fold higher levels of alpha adducin mRNA relative to beta adducin mRNA (Fig. 6) even though the polypeptide subunit stoichiometry of alpha and beta adducin is 1:1 in mature erythrocytes. Further studies will be required to resolve whether alpha and beta adducin mRNAs have differences in stability or translation efficiency. One possibility is that rapid turnover of beta adducin mRNA may limit the synthesis of beta adducin subunits and thereby regulate the assembly of adducin heterodimers. Analysis of adducin mRNA in other tissues reveals different levels of expression of alpha and beta subunits as well as several size mRNAs related to beta adducin. Alpha adducin mRNA is detected in liver and kidney while beta adducin mRNA is not, suggesting that alpha adducin may be expressed without beta adducin. However, it is also possible that in liver and kidney beta adducin mRNA is degraded rapidly while alpha adducin mRNA is relatively stable.

Alpha adducin expression has been noted in the absence of beta adducin in human fibroblasts and keratinocytes based on detection of immunoreactive polypeptides (24, 40). These observations suggest either that beta adducin variants are present that do not react with antibodies or that homodimers of alpha adducin are functional. Evidence for self association between alpha subunits is provided by cross-linking with *O*-phenanthroline/copper which forms disulfide bonds between adjacent cysteine residues. Alpha adducin was completely cross-linked to higher forms by this reagent leaving a portion of the beta subunit in an uncomplexed form (13). It will be of interest to evaluate properties of recombinant alpha and beta adducin to determine if these polypeptides can assemble into homo-dimers and tetramers and to evaluate functional activities.

The observation of several size mRNAs hybridizing to the beta adducin probe suggests possibilities of splicing or other posttranscriptional modifications of pre-mRNA. A partial cDNA sequence for mouse spleen beta adducin (35) exhibits 98% identity with human beta adducin for 149 amino acids, followed by 31 residues that abruptly diverge and may represent a splice site. The 8.1-kb beta adducin mRNA may also

result from alternative splicing of pre-RNA, although the possibility that this mRNA is encoded by a closely related gene has not been excluded.

Mechanisms of site directed as opposed to random assembly of structural proteins are crucial to understanding formation of a cell. Adducin has features that suggest such a role as an assembly factor that has the potential to promote localized organization of spectrin into a spectrin–actin lattice. Adducin interacts with spectrin and actin according to an ordered pathway beginning with association of spectrin and actin to form a spectrin–actin complex followed by association of adducin, and finally by recruitment of a second spectrin to the adducin-spectrin–actin ternary complex (3, 14). One consequence of such an ordered reaction is that assembly of a spectrin–actin network could be directed to particular regions of cell membranes where either spectrin or adducin are concentrated. Sites of cell–cell contact in epithelial tissues contain a stable complex of spectrin and adducin (24) and are an example of a domain that may result by such a directed assembly reaction. The structure of adducin suggests a testable hypothesis for how this protein could participate in assembly of a spectrin–actin network. The availability of adducin cDNAs will allow evaluation of these ideas in in vitro assays and in the context of cells.

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