Mapping the Domain Structure of Human Erythrocyte Adducin*

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Adducin is a 200-kDa heterodimeric protein associated with the erythrocyte membrane skeleton which binds to Ca\(^{2+}\)/calmodulin, promotes binding of spectrin to actin, and is a substrate for protein kinases C and A. Adducin polypeptides can be structurally and functionally divided into two distinct regions. The amineterminal 39-kDa domain of each subunit is more basic and resistant to proteases than the C-terminal 60-64-kDa domain, which is very sensitive to proteolytic degradation. Two-dimensional peptide map analysis revealed that the 39-kDa protease-resistant domains represent a portion of adducin which is highly conserved between the \(\alpha\) and \(\beta\) subunits whereas the protease-sensitive regions are different in each subunit. Comparison of the structural and functional properties of purified 39-kDa domains with intact adducin showed that the 39-kDa domains were not phosphorylated by protein kinases C or A and did not bind to Ca\(^{2+}\)/calmodulin or interact with spectrin and actin. This suggests that the protease-sensitive domains may perform the various functions of adducin since these activities were all lacking from the protease-resistant domains. It is also possible that the conserved and variable domains are both required for one or more activities of adducin or that the 39-kDa domains play a role in maintaining the oligomeric state of adducin necessary for interaction of the variable domains with spectrin-actin complexes.

A well defined network of peripheral proteins referred to as the membrane skeleton lies on the cytoplasmic surface of erythrocyte plasma membranes. The major membrane peripheral proteins are spectrin and actin, which form the two-dimensional structural lattice of the membrane skeleton. Other proteins have been isolated from the membrane skeleton and are likely to participate in the assembly and maintenance of the membrane skeleton. These accessory proteins include protein 4.1, protein 4.9, adducin, tropomyosin, and tropomodulin, a tropomyosin-binding protein. The skeleton is linked to the bilayer through association of spectrin with ankyrin and protein 4.1, which in turn are attached to integral membrane proteins (for reviews, see Bennett, 1989, 1990; Mische and Morrow, 1988; Marchesi, 1985). Isoforms of erythrocyte membrane skeletal proteins have been isolated from other cell types and have closely related activities in \textit{in vitro} assays (Bennett, 1990; Coleman \textit{et al.}, 1989; Goodman \textit{et al.}, 1988).

Adducin is a recently identified membrane skeletal protein that was first purified from human erythrocytes (Gardner and Bennett, 1986) and subsequently isolated from bovine brain membranes (Bennett \textit{et al.}, 1988). Isoforms of this protein have been detected in lung, kidney, testes, and liver (Bennett \textit{et al.}, 1988). Erythrocyte adducin is a 200-kDa heterodimeric protein present at about 30,000 copies/cell. Adducin binds with high affinity to Ca\(^{2+}\)/calmodulin (Gardner and Bennett, 1986) and is a substrate for protein kinases A and C (Ling \textit{et al.}, 1986; Cohen and Foley, 1986; Wassem and Falpey, 1988). Adducin associates selectively with spectrin-actin complexes with higher affinity than with either protein alone (Gardner and Bennett, 1987; Bennett \textit{et al.}, 1988). Adducin also recruits additional spectrin molecules to the spectrin-actin complex (Mische \textit{et al.}, 1987; Gardner and Bennett, 1987). The interactions of adducin with spectrin and actin are distinct from those of protein 4.1, which interacts with spectrin alone but not with actin and binds equivalently to spectrin and complexes of spectrin and actin (Cohen and Foley, 1984). The ability of adducin to promote spectrin-actin interactions could be important for formation of the polygonal lattice of the spectrin-actin network which has been visualized by electron microscopy (Byers and Branton, 1985; Shen \textit{et al.}, 1986; Liu \textit{et al.}, 1987). Recruitment of additional spectrin molecules to actin filaments by adducin is inhibited by calmodulin (Mische \textit{et al.}, 1987; Gardner and Bennett, 1987), suggesting that this activity is subject to regulation in the cell.

The goal of this study was to define the structural and functional domains of human erythrocyte adducin using limited proteolytic digestion of adducin as an initial approach. Each adducin subunit was found to contain a 39-kDa amineterminal protease-resistant domain that is highly conserved between the \(\alpha\) and \(\beta\) subunits. Adducin subunits also contain 60-64 kDa protease sensitive domains that are distinct from each other based on peptide maps. The protease-sensitive domains of adducin may perform functions of binding to spectrin-actin complexes, actin, and calmodulin as well as contain substrate sites for protein kinases \(\alpha\) and \(\beta\) since these activities were all lacking from the protease-resistant core domains.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free Na\(^{22}\)I was purchased from Amersham Corp. \(^{3}H\)-Labeled Bolton-Hunter reagent and \([\gamma-^{32}\text{P}]\)ATP were from ICN. Diisopropyl fluorophosphate, leupeptin, pepstatin, dithiothreitol, phenylmethylsulfonyl fluoride, EGTA, catalytic subunit of protein kinase A, sodium bromide, Tween 20, Triton X-100, trypsin, and \(\alpha\)-chymotrypsin were purchased from Sigma. Mono S and Mono Q columns, cyanogen bromide-activated Sepharose CL-4B, Superose 12, protein A, and ampholines were obtained from Pharmacia LKB Biotecnology Inc. Ethylene glycol bis(succinimidyl succinate) was from Pierce

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Chemical Company. Nitrocellulose paper and electrophoresis reagents were bought from Bio-Rad. Calmodulin was purified from bovine brain as described earlier (Gopalakrishna and Anderson, 1982). Actin was isolated from acetone powder of rabbit muscle (Pardee and Spudich, 1982) and further purified by gel filtration on Superose 12. Bovine brain spectrin was purified essentially as described earlier (Davis and Bennett, 1983; Bennett et al., 1986). Bovine brain protein kinase C was a generous gift from Dr. Robert Bell, Dept. of Biochemistry, Duke University Medical Center.

Methods

Protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was performed using 0.2% SDS with buffers of Fairbanks bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was performed as described (Davis and Bennett, 1983). Protein A was labeled with Bolton-Hunter reagent as described (Bennett, 1983). Actin was isolated from acetone powder of rabbit muscle as described earlier (Gopalakrishna and Anderson, 1982). Adducin and the core polypeptides were purified as described earlier (Pardee and Spudich, 1982) and further purified by gel filtration on hydroxylapatite, Mono Q, and Mono S columns, and eluted at low flow rates to obtain maximal separation. The two adducin subunits could only be partially separated even under the most optimal conditions. Because the two subunits of adducin were not easily separated we decided to subject total adducin to proteolytic degradation, study the various proteolytic fragments, and subsequently map these fragments to the two subunits.

Restricted Proteolytic Digestion of Adducin—Adducin (Fig. 1A, lanes 2 and 6, approximately 0.5 mg/ml) was digested using two different proteases, trypsin and chymotrypsin, for 30 min at 4 °C as described in Fig. 1. Adducin was extremely sensitive to trypsin and chymotrypsin under these conditions. Enzyme to substrate ratios of 1:2500 were sufficient for significant degradation of adducin. The major proteolytic product at 0.2 μg/ml trypsin was about 45-49 kDa in size (lane 3). A similar molecular mass polypeptide was seen with 0.2 μg/ml chymotrypsin (lanes 7-9). Two discrete bands of 43 and 39 kDa were seen upon increasing the trypsin concentration to 0.8 μg/ml (lane 4). There was an increase in the amount of the 39-kDa band and a decrease in the 43-kDa band at 3.2 μg/ml trypsin as compared with 0.8 μg/ml trypsin (lane 5). The 43-kDa polypeptide can also be visualized at 3.2 μg/ml chymotrypsin (lane 9).

The relationship between the 43- and 39-kDa bands and adducin subunits could result from one of three possibilities. 1) Both the 43- and 39-kDa bands arise from only one subunit of adducin. 2) The 43-kDa band originates from one adducin subunit and the 39-kDa band from the other. 3) The 43- and 39-kDa bands each contain portions from both subunits of adducin. Our data support the third possibility for several reasons. Densitometry of SDS-polyacrylamide gels indicated that the relative areas of 43- and 39-kDa polypeptides combined were 40-46% of the areas of both subunits. These polypeptides thus cannot both be derived from a single subunit of adducin since in this case only 20-25% of the protein would be recovered in the smaller polypeptide. The 43- and 39-kDa bands are not derived from individual adducin subunits since the amounts of the 43- and 39-kDa bands are different at different concentrations of trypsin even though both subunits of adducin are degraded simultaneously, as seen at 0.2 μg/ml trypsin and chymotrypsin. Moreover, a careful examination of the digestion pattern with increasing amounts of trypsin suggests that 39-kDa polypeptides were derived from the initial 43-kDa proteolytic fragment. This progressive degradation of polypeptides from 43 to 39 kDa is apparent

\* S. Salardi and V. Bennett, unpublished observations.
from lanes 4 and 5 in Fig. 1A. A higher concentration of the 43-kDa band as compared with the 39-kDa band is evident with 0.8 µg/ml protease whereas the 43-kDa bands are reduced compared with the 39-kDa band at 3.2 µg/ml trypsin. The total amount of staining in the combined 39- and 43-kDa bands remained approximately constant at both protease concentrations. Although both the 43- and 39-kDa bands appear as a single polypeptide in Fig. 1, they are actually composed of two very similar sized polypeptides, as visualized upon a longer run and lighter loading of the gel (data not shown). The presence of two polypeptides in at least the 39-kDa band can be easily seen in the two-dimensional SDS/isoelectric focusing gel in Fig. 3 (bottom panel).

These results suggest that each adducin subunit has a protease-sensitive region of 60–64 kDa and a more protease-resistant domain of 39 kDa. Following even mild proteolysis of adducin no discrete lower molecular mass bands could be observed, suggesting that the protease-sensitive region of adducin is reduced to small peptides. The 39-kDa domain is only relatively insensitive to proteases since treatment of adducin with higher concentrations of chymotrypsin at higher temperatures does degrade this domain further (data not shown).

Purification of the 39-kDa Protease-resistant Domain—The 39-kDa tryptic fragments were purified in mg amounts as an initial step toward the characterization of their functions. Adducin, when proteolyzed with trypsin (3 µg/mg adducin) (Fig. 1, panel B), generated equal amounts of the 43- and 39-kDa polypeptides. This digest was passed over a Mono Q column and eluted with a linear salt gradient. The 39-kDa domain represented the major product obtained on elution. The 43-kDa domain presumably was degraded to the 39-kDa polypeptides during purification. Small molecular mass peptides that represent the protease-sensitive domains did not bind to the Mono Q column and migrated with the dye front on SDS-polyacrylamide gels. The 39-kDa protease-resistant domains referred to here as the "core" were used for further structural and functional characterization.

Comparison of Adducin Subunits and the Protease-resistant Domains by Peptide Mapping—Two-dimensional peptide mapping of radioiodinated polypeptides was used to analyze the region of adducin present in the 39- and 43-kDa domains. Fig. 2 shows the peptide maps of α and β adducin subunits and the 39- and 43-kDa domains. Panel G is a composite of the map of α and β adducin analyzing the spots common between adducin and the 43- and 39-kDa domains. Adducin subunits share about 35–40% of the radioiodinated peptides, as noted previously for erythrocyte (Gardner and Bennett, 1986) and brain adducin (Bennett et al., 1988). 92 and 85% of the spots common to both subunits of adducin are present in the 43- and 39-kDa domains, respectively, indicating that the 43- and 39-kDa domains form most of the conserved region of adducin. The fact that not all the common spots are present in the protease-resistant domains suggests that the conserved region of adducin extends a little beyond the 43-kDa domain. Conversely, about 90–95% of the spots present in the 43- and 39-kDa domains were present in total adducin. Some (two to four) faint spots were present in maps of the 43- and 39-kDa polypeptides which were absent in maps of α or β adducin. These additional spots may be due to the fact that the 43- and 39-kDa domains were generated after trypptic digestion of adducin and later digested with chymotrypsin for peptide mapping. The data presented here clearly indicate that the 43- and 39-kDa polypeptides represent most of the region of adducin which is conserved between the α and β subunits.

Physical Properties of the Core Domain—The core domain in dilute solution has a Stokes radius of 4.3 nm, a sedimentation coefficient of 6.8 S, a partial specific volume of 0.73 cm³/g estimated from the amino acid composition, and a frictional ratio of 1.15 (Table I). A molecular mass of 120 kDa was calculated for the core based on these values. The value of 120 kDa is significantly larger than the molecular mass of 78–80 kDa expected for a dimer comprised of 39–40-kDa
TABLE I

Physical properties of the core

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_e$ (nm)</td>
<td>4.3</td>
</tr>
<tr>
<td>Sedimentation coefficient, $s_{20,w}$</td>
<td>6.8</td>
</tr>
<tr>
<td>Partial specific volume</td>
<td>0.73</td>
</tr>
<tr>
<td>Molecular mass, calculated</td>
<td>118,606</td>
</tr>
<tr>
<td>Frictional coefficient, $f/f_0$</td>
<td>1.15</td>
</tr>
<tr>
<td>Monomer molecular mass by SDS-polyacrylamide gel electrophoresis</td>
<td>39,000</td>
</tr>
</tbody>
</table>

*Gardner and Bennett (1986).

The Stokes radius was determined from gel filtration on a Superose 12 column (see "Methods").

The sedimentation coefficient was estimated from sedimentation on 5–20% sucrose gradients (Martin and Ames, 1961).

The value was estimated from the amino acid composition (Cohn and Edsall, 1943).

The molecular mass and frictional ratio were calculated according to the following equations (Tanford, 1961):

$\text{molecular weight} = 6\pi N R_e s_{20,w} / (1 - e^{s_{20,w}})$, and $f/f_0 = R_e \left( \frac{4\pi N}{3M_r(\bar{\rho} + \delta\rho)} \right)^{1/3}$, with an assumed hydration $\delta$ of 0.4 g/g of protein (Kuntz and Kauzmann, 1974).

In order to compare the isoelectric point of adducin with the core, the proteins were first resolved by isoelectric focusing (IEF) in the first dimension. 10 μg of adducin or core was focused on 1.5-mm tube gels (4% acrylamide, 1.25% bisacrylamide) in the presence of urea (9.2 M) and ampholines (1%, pH 3.5/10; 4%, pH 5–8). The gels were run as described by O’Farrell (1975). After equilibration for 30 min in 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 mM DTT, the focused gels were laid over a 7–17% exponential gradient slab gel and fixed in position with 2% agarose. After electrophoresis the gel was transferred to nitrocellulose and the bands visualized by immunoblotting.

The top panel shows adducin, and the bottom panel shows the core.

FIG. 3. Immunoblot of a two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) of adducin and core after isoelectric focusing (IEF) in the first dimension. 10 μg of adducin or core was focused on 11-cm × 1.5-mm tube gels (4% acrylamide, 1.25% bisacrylamide) in the presence of urea (9.2 M) and ampholines (1%, pH 3.5/10; 4%, pH 5–8). The gels were run as described by O’Farrell (1975). After equilibration for 30 min in 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5 mM DTT, the focused gels were laid over a 7–17% exponential gradient slab gel and fixed in position with 2% agarose. After electrophoresis the gel was transferred to nitrocellulose and the bands visualized by immunoblotting. The top panel shows adducin, and the bottom panel shows the core.

FIG. 4. Chemical cross-linking of adducin and core to oligomeric forms. $^{131}$I-Labeled adducin or core (30 μg/ml, 2.2 × 10$^6$ cpm/μg) (lanes 1 and 5) in 10 mM sodium phosphate, 100 mM NaCl, 1 mM NaEDTA, 0.5 mM DTT, pH 7.3, was incubated with 1 μg/ml ethylene glycol bis(succinimidy1 succinate) in the presence of 0 (lanes 2 and 6), 4 (lanes 3 and 7), and 8 M urea (lanes 4 and 8) for 30 min at 4 °C. The reaction was terminated by the addition of glycine to 10 mM, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The state of cross-linking was determined by comparison with known molecular weight markers.

The presence of two polypeptides of different molecular masses in the core is evident from this figure since the lower molecular mass polypeptide is slightly more basic and can be seen as distinct lower spots.

Chemical Cross-linking of Adducin and Core Domains—Earlier studies of erythrocyte and brain adducin have shown that adducin can be cross-linked to higher oligomers (Gardner and Bennett, 1986; Bennett et al., 1988) using chemical cross-linking agents. These studies suggested that adducin had the potential to interact with itself to form tetramers as well as larger oligomers. The ability of the core and adducin to cross-link to higher oligomers was compared using ethylene glycol bis(succinimidy1 succinate) at a concentration of 1 mg/ml as the cross-linking agent. Most of the adducin polypeptides are cross-linked to very high oligomeric form(s) that barely penetrate the gel (Fig. 4, lane 2). Dimers, trimers, and tetramers of adducin are also present although in lower amounts. The core also is cross-linked to a dimer and tetramer although higher oligomers are not evident (lane 6). The predominant cross-linked form of the core was a dimer. Cross-linking is significantly reduced in the presence of 4 and 8 M urea.
Comparison of the Binding of Adducin and the Core Domains with Ca\(^{2+}\)/Calmodulin—To determine whether the core domain binds calmodulin in a Ca\(^{2+}\)-dependent interaction, \(^{125}\)I-labeled adducin and core were applied to a calmodulin affinity column. Fig. 5 shows that adducin (top panel) bound to the column in the presence of Ca\(^{2+}\) and could be subsequently eluted by replacing Ca\(^{2+}\) with EGTA. The core (bottom panel), however, did not bind the calmodulin affinity column in the presence of Ca\(^{2+}\), suggesting that this portion of the molecule lacks the site that interacts with calmodulin. This result implies that the variable portion of the $\beta$ subunit contains the calmodulin binding site, as the $\beta$ subunit has been shown earlier to bind calmodulin (Gardner and Bennett, 1986).

Comparison of Interaction of Adducin and Core Domains with Spectrin and Actin—\(^{125}\)I-Labeled adducin and core were incubated with spectrin alone, actin filaments alone, or with spectrin and actin filaments followed by sedimentation of actin filaments through a sucrose barrier gradient (Fig. 6). Both adducin (lanes 1) and the core (lanes 2) remained entirely in the supernatant in the absence of spectrin and actin. Adducin did not sediment with spectrin alone (lanes 2), associated weakly to actin alone (lanes 3), but did sediment with spectrin and actin together (lanes 4). These findings were consistent with earlier results (Gardner and Bennett, 1987). The core, however, does not sediment with either spectrin alone (lanes 6), actin alone (lanes 7), or with spectrin and actin (lanes 8) under similar conditions. These results demonstrate that the core by itself cannot interact with spectrin and actin. We do not know as yet if the core domain contributes in binding of adducin to spectrin and actin by maintaining the proper conformation of the variable domain, contains only part of the active binding site, or plays no role in this interaction.

Comparison of the Phosphorylation of Adducin and Core Domains—Adducin is a substrate of both protein kinases C and A (Ling et al., 1986, Waseem and Palfrey, 1988). The results shown in Fig. 7, panel A, confirm that adducin is a good substrate for protein kinase C in the presence of Ca\(^{2+}\) and phosphatidylserine (lane 4). No phosphorylation is seen in the absence of either of these cofactors (lanes 2 and 3). A comparison of the phosphorylation of adducin and core with protein kinase C (panel B) and the catalytic subunit of protein kinase A (panel C) clearly shows that the core domain cannot function as a substrate for either protein kinase A or C. If adducin is first phosphorylated and then digested with protease, the resulting core domain still does not contain phosphate (not shown). The phosphorylation sites are therefore limited to the variable region of adducin. Limited proteolytic digestion of adducin after phosphorylation with protein kinase C showed that there is at least one phosphorylation site close to the proteolytic cleavage site of the core on adducin (data not shown).

DISCUSSION

This study represents an initial step in the structural and functional characterization of erythrocyte adducin. Each adducin subunit contains two domains: a relatively protease-resistant region of 39 kDa which is nearly identical between subunits based on peptide maps, and a highly protease-sensitive region of 60-64 kDa which is unique to each subunit. Earlier studies had shown that $\alpha$ and $\beta$ subunits of adducin share a considerable degree of homology although the distribution of the conserved peptides on adducin was not known (Gardner and Bennett, 1986). Similarity of protein sequence in some portions of the variable domains cannot be excluded since these domains share some similar properties such as size, protease sensitivity, and the presence of phosphorylation sites. The 39-kDa conserved domains have blocked amino termini based on results with automated Edman degradation as do both subunits of adducin (not shown). The conserved
domains therefore are located at the amino-terminal region of adducin.

Physical properties of the core suggest that it is globular in shape in contrast to adducin, which is relatively asymmetric. The extreme protease sensitivity of the variable domains implies a nonglobular folding for this region of adducin. These features suggest the possibility that adducin is shaped like a ball with a tail on one end, with the globular amino-terminal domain forming the head and the protease-sensitive variable domains extending out in the form of a tail. An extended tail domain has not been resolved by electron microscopy of adducin (Gardner and Bennett, 1986), although a tail wrapped around a head domain would be consistent with available features suggesting the possibility that adducin is shaped like a cursor by gene duplication. The variable portions could then be responsible for forming part of the active binding site(s). None of these functions of adducin could be localized by direct experiments to the variable domains, as these regions were completely destroyed upon proteolysis. Lack of phosphorylation sites on the core suggests that these sites are present exclusively on the variable region of α and β subunits of adducin. These results imply that the variable portion serves as the regulatory domain that modulates activities of phosphorylation and possibly binding of calmodulin.

This study has provided important information about the structure of adducin and the functions of a conserved 39-kDa amino-terminal domain. The high degree of similarity between the conserved domains of adducin subunits strongly suggests that the two subunits originated from a single precursor by gene duplication. The variable portions could then have diverged to perform separate functions while the core remained conserved, reminiscent of the original gene product. The variable domains of adducin could not be isolated from proteolytic digests and characterized directly due to their profound sensitivity to proteases. We are presently engaged in cloning human erythrocyte adducin and hope to identify more completely the various functional sites of adducin by studying the functions of the expressed normal and mutated proteins.

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REFERENCES

Bennett, V. (1990) Physiol. Rev. in press
Davis, J., and Bennett, V. (1983) J. Biol. Chem. 258, 7757-7766
Goodman, S. R., Kieth, K. E., Whitfield, C. F., Riedler, B. M., and...
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