# Binding Sites of Calmodulin and Actin on the Brain Spectrin, Calspectin

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ABSTRACT We used rotary-shadowing electron microscopy to map the calmodulin- and actin-binding sites on the brain spectrin, calspectin (or fodrin). Calspectin dimers appeared as rods 110 nm long and joined in a head-to-head manner to form tetramers 220 nm long. We determined calmodulin-binding sites by a ferritin-labeling method combined with biotin-avidin complex formation. Ferritin particles were found to attach to the head parts of calspectin dimers at a position 10–20 nm from the top of the head. The number of the calmodulin-binding sites seemed to be only one for each dimer and two for each tetramer. In contrast, the actin-binding sites were localized at the tail ends of the calspectin molecules. The tetramers attached to muscle F-actin with their tail ends and often cross-linked adjacent filaments. The results are discussed in view of the analogy to the erythrocyte spectrin.

Spectrin, together with actin, has been shown to be the major protein of the cytoskeletal network underlying the human erythrocyte membrane (for reviews, see references 1, 2). The network is generally thought to control cell shape and to regulate the lateral mobility of integral membrane proteins. In thin-section electron microscopy, the network shows a layered construction underneath the membrane; a filamentous (spectrin-actin) meshwork is horizontally disposed, being connected to the membrane through vertical components (3, 4). Similar layered structures underlying the plasmalemma are frequent in various cell types (5, 6). Thus we have been led to propose a concept of the "plasmalemmal undercoat" (5). Recently, spectrinlike proteins have been demonstrated to occur in a variety of nonerythroid cells and tissues (for reviews, see references 7–9). These proteins have been purified to homogeneity and characterized (10, 11). They share certain characteristic properties with the erythrocyte spectrin: (*a*) they are high-molecular-weight proteins, comprising heterodimers that join each other to form tetramers; (*b*) they can bind actin and calmodulin; and (*c*) their molecular shapes are alike, looking like elongated rods. Furthermore, they have been shown by immunofluorescence to occur in the cell periphery, possibly associated with the plasmalemma (7, 12). Since these proteins are closely related, if not identical, to erythrocyte spectrin, they may be categorized as a family of "spectrins." Interestingly, these spectrins appear to vary in subunit composition in different sources (7). Such variable compositions of spectrins may reflect the different functions that are required by respective cell types. In this sense, an emphasis should also be placed on the differences in molecular nature and subunit composition.

Calspectin (or fodrin) has been isolated as a spectrinlike, calmodulin- and actin-binding protein from a membrane fraction of brain (11, 13). In the course of establishing the similarity and difference in function among spectrins, we attempted to ultrastructurally determine the calmodulin- and actin-binding sites on the calspectin molecule. We believe that this approach may lead to better understanding of the functional differentiation of spectrins in various cell types.

## MATERIALS AND METHODS

*Materials:* Calspectin was purified from fresh bovine brain (obtained from a local slaughterhouse) according to the method of Kakiuchi et al. (14), with a modification (15). Calmodulin was prepared from bovine brain by the procedure of Kakiuchi et al. (16). Rabbit skeletal muscle G-actin was prepared by the method of Spudich and Watt (17).

*Ferritin Labeling of Calmodulin-binding Sites:* To determine electron microscopically the calmodulin-binding sites on calspectin molecules, we used a ferritin-labeling method combined with a high-affinity interaction of biotin and avidin (18), according to the method used by Tyler et al. (19) for determining band 4.1 binding sites on spectrin. Biotinyl-*N*-hydroxysuccinimide ester was synthesized from biotin and *N*-hydroxy-succinimide. 15 μl of biotinyl-*N*-hydroxysuccinimide ester dissolved in dimethyl formamide (12 mg/ml) was mixed and incubated with 190 μl of purified calmodulin (400–500 μg/ml in 100 mM KCl, 3 mM $NaN_3$, 0.4 mM phenylmethylsulfonyl fluoride, 100 mM borate buffer, pH 8.5) at 23°C for 3 h. Biotin-conjugated calmodulin (85 μg/ml) was obtained by eluting in a Sephadex G-25 column with 5 mM sodium phosphate buffer containing 130 mM KCl, 20 mM NaCl, 0.2 mM dithiothreitol,



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2 mM $NaN_3$, pH 7.5. Biotin-conjugated calmodulin (40 μl) was then mixed with 6 μl of calspectin (800 μg/ml in 40 mM KCl, 0.1 mM EGTA, 0.1 mM dithiothreitol, 5 mM Tris-HCl buffer, pH 7.5) and the mixture was incubated in the presence of 0.1 mM $CaCl_2$ for 12 h at 4°C. Finally, 6 μl of avidin-ferritin complexes (5 mg/ml in 0.9% NaCl) was added to label the biotin-conjugated calmodulin that had been bound to the calspectin. Activation of the calmodulin-deficient brain phosphodiesterase by calmodulin and biotin-labeled calmodulin was determined as described by Kakiuchi et al. (16).

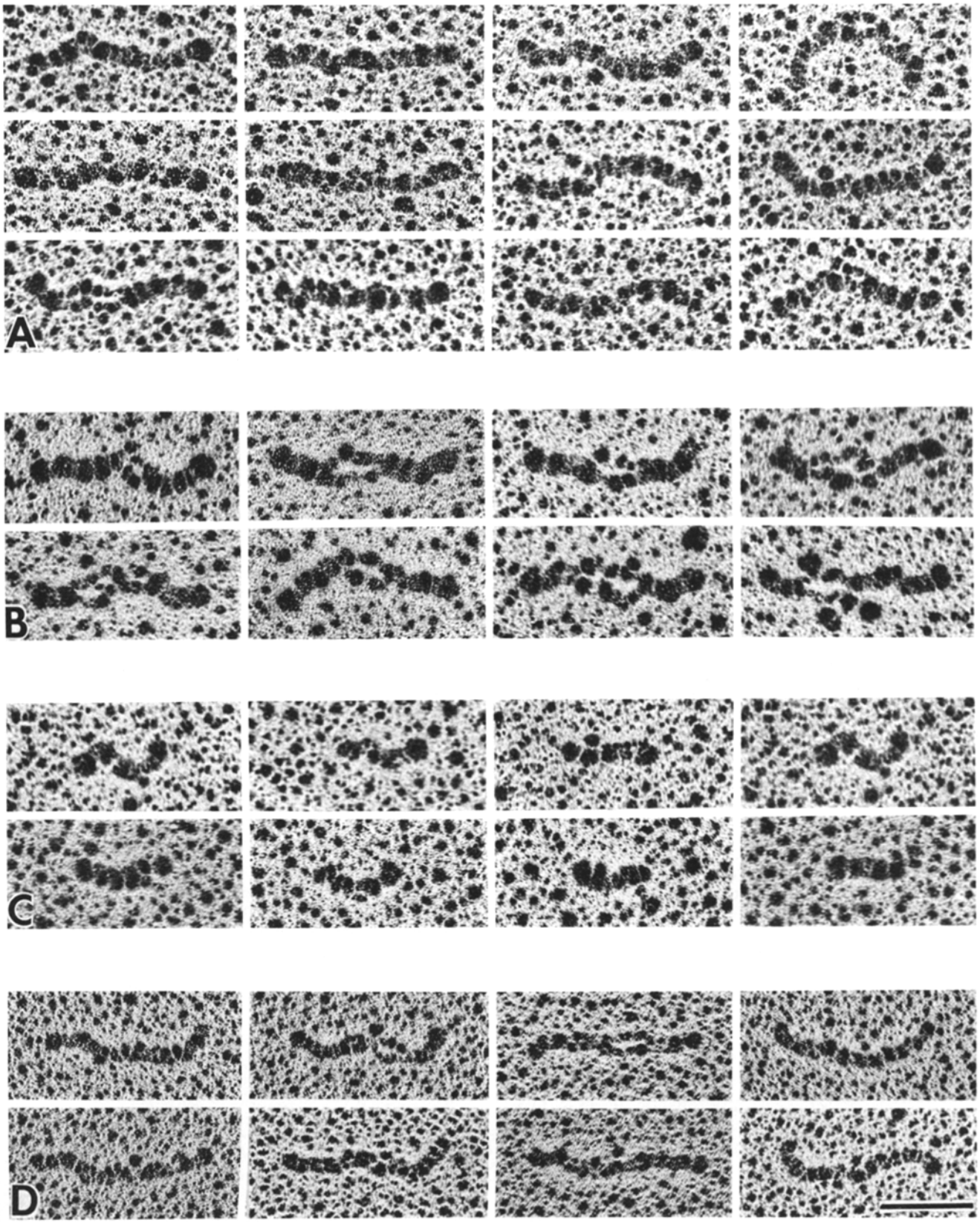


FIGURE 1 Morphology of calspectin molecules in rotary-shadowed preparations. (*A*) Calspectin tetramers. (*B*) Calspectin tetramers after incubation in 0.1 M KCl: The separation of two twisted strands in the middle portion is enhanced. (*C*) Calspectin dimers. (*D*) Calspectin tetramers reconstituted from dimers. Bar, 0.1 μm. × 168,000.

*Calspectin Binding with F-actin:* G-actin (200 μg/ml) was polymerized in 5 mM Tris-HCl buffer (pH 7.5) containing 20 mM KCl, 2 mM $MgCl_2$ and 1 mM ATP, at 25°C for 30 min. F-actin thus prepared was diluted to half-protein concentration by adding the same buffer and then incubated with the equal volume of calspectin solution (800 μg/ml in 40 mM KCl, 0.1 mM EGTA, 0.1 mM dithiothreitol, 5 m Tris-HCl, pH 7.5) at 24°C for 30 min.

*Electron Microscopy:* Calmodulin- or actin-bound calspectin solution was mixed with an equal volume of glycerol and sprayed on freshly cleaved pieces of mica according to the method described by Tyler and Branton (20). After drying under $10^{-6}$ Torr for 10 min, the samples were rotary-shadowed with platinum-carbon approximately at a shadowing angle of 5°. Shadowed films were floated on water and picked up onto Formvar-coated grids and observed in a Hitachi 11-DS electron microscope at an accelerating voltage of 75 kV.

## RESULTS

### Morphology of Calspectin Molecules

In rotary-shadowing electron microscopy, calspectin molecules look like elongated rods of ~220 nm in length for the tetramers and 110 nm for the dimers as previously described (11). Each rod showed two twisted strands, with the middle portion of the tetrameric rod more or less separated (Fig. 1, *A* and *C*). Such separation was enhanced when the tetramers were incubated in 0.1 M KCl at 37°C for 30 min (Fig. 1*B*). Biochemical and electron microscopic analyses of the tetramer-dimer interconversion indicated that the tetrameric rods were formed by the head-to-head association of two dimers (Fig. 1*D*).

### Calmodulin-binding Sites on Calspectin

The biotin-conjugated calmodulin was mixed with calspectin to obtain biotin-calmodulin-calspectin complexes, to which ferritin-avidin complexes were then added to detect the calmodulin-binding sites on calspectin molecules. We expected the ferritin-labeling method for calmodulin through biotin-avidin interaction to be better than a direct method using ferritin-calmodulin conjugates because of a specific, high-affinity interaction of biotin and avidin with relatively less steric hindrance. Moreover, calmodulin was not severely affected by biotin conjugation. Biotin-conjugated calmodulin was capable of activating the calmodulin-deficient brain phosphodiesterase (Fig. 2), though its potency slightly decreased from that of the native form of calmodulin. The amounts of calmodulin and biotin-conjugated calmodulin required for half-maximum activation of enzyme were 50 and 80 ng, respectively. The present preparations contained mainly tetrameric forms of calspectin with some fractions of dimeric forms. The calmodulin-calspectin complexes thus labeled showed that one or two ferritin particles were attached to individual tetrameric rods near the midpoints (Fig. 3, *A* and *B*). It should be recognized that the ferritin particles represent single ferritin molecules and aggregates of two ferritin molecules, the latter of which were formed with glutaraldehyde during avidin-ferritin conjugation. The maximum number of the ferritin-attached sites was two for a tetramer. Ferritin particles were situated 10–20 nm apart from the exact midpoint of the tetramer. When two particles attached to one tetrameric rod, they were found to be separated ~35 nm from each other in the center of the rod. There was no instance in which two particles were found on the same side of the midpoint. Furthermore, when two strands were separated at the middle portion of the tetramer, ferritin particles labeled only one strand for each dimer. Ferritin particles attached to the head portions of the dimers; this was well consistent with the observation on the tetramers (Fig. 3*C*). From these observations, the calmodulin-binding sites appeared to be two for a tetramer and one for a dimer. We then attempted to directly visualize the calmodulin molecules bound on calspectin. In rotary-shadowed preparations of calmodulin-calspectin complexes, small spherical dots were found near the midpoints of calspectin tetramers, similar in position to that labeled by ferritin (Fig. 3*D*). Such dots may represent calmodulin molecules, but further study is needed to unequivocally identify the calmodulin molecules in rotary-shadowed preparations.

### Actin Binding to Calspectin

In rotary-shadowing electron microscopy, muscle F-actin–calspectin complexes showed that calspectin rods were attached to actin filaments with their tail ends in an end-on fashion (Fig. 4). The tetrameric rods were often seen to cross-link adjacent actin filaments. These results indicate that the actin-binding sites are localized at the tail ends of calspectin molecules, as such at both ends of the tetramer.

## DISCUSSION

Our study has successfully mapped calmodulin- and actin-binding sites on the brain spectrin, calspectin (or fodrin), by low-angle rotary-shadowing electron microscopy. Calspectin molecules were seen as elongated rods 110 nm in length for dimers and 220 nm for tetramers, which are similar to human erythrocyte spectrin except that calspectin rods appear more rigid. The calmodulin- and actin-binding sites were found to be distributed symmetrically against the midpoint of the tetrameric rod at the head parts and at the tail ends of the calspectin, respectively. The results unambiguously confirm that the calspectin tetramer is formed by a head-to-head association of two heterodimers each of which comprises α- and β-subunits.

Our paper is the first ultrastructural demonstration of the calmodulin-binding sites on one of many calmodulin-binding proteins using a novel ferritin-labeling method (18, 19). This method may be very useful in demonstrating that there are calmodulin-binding sites not only on isolated proteins but also in situ in cells. Our results show that the number of calmodulin-binding sites is two for a tetramer and one for a dimer. These results, together with our earlier finding that the

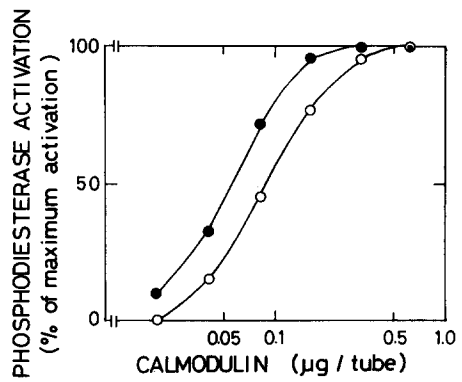


FIGURE 2 Activation of the calmodulin-deficient brain phosphodiesterase by calmodulin and biotin-labeled calmodulin. The assay was carried out as previously described in Kakiuchi et al. (16) in the presence of 0.1 mM CaCl. Protein concentration was determined as previously described in (16). ●, native calmodulin. ○, biotin-conjugated calmodulin.

isolated $\alpha$-subunit of calspectin is a calmodulin-binding polypeptide (13), establishes unequivocally that the calmodulin binding of calspectin is attributed solely to its $\alpha$-subunit. Since the isolated $\alpha$-subunit of erythrocyte spectrin was found to be a calmodulin-binding protein (11), a similar conclusion may be drawn for spectrin. Earlier observations indicating the inability of the $\beta$-subunit polypeptide of calspectin to bind to calmodulin could not be interpreted unambiguously (11, 21), because in those experiments calmodulin binding was examined by the gel overlay technique on SDS gels, and the possibility was not excluded that the binding ability of the peptide may have been destroyed during treatment in SDS.

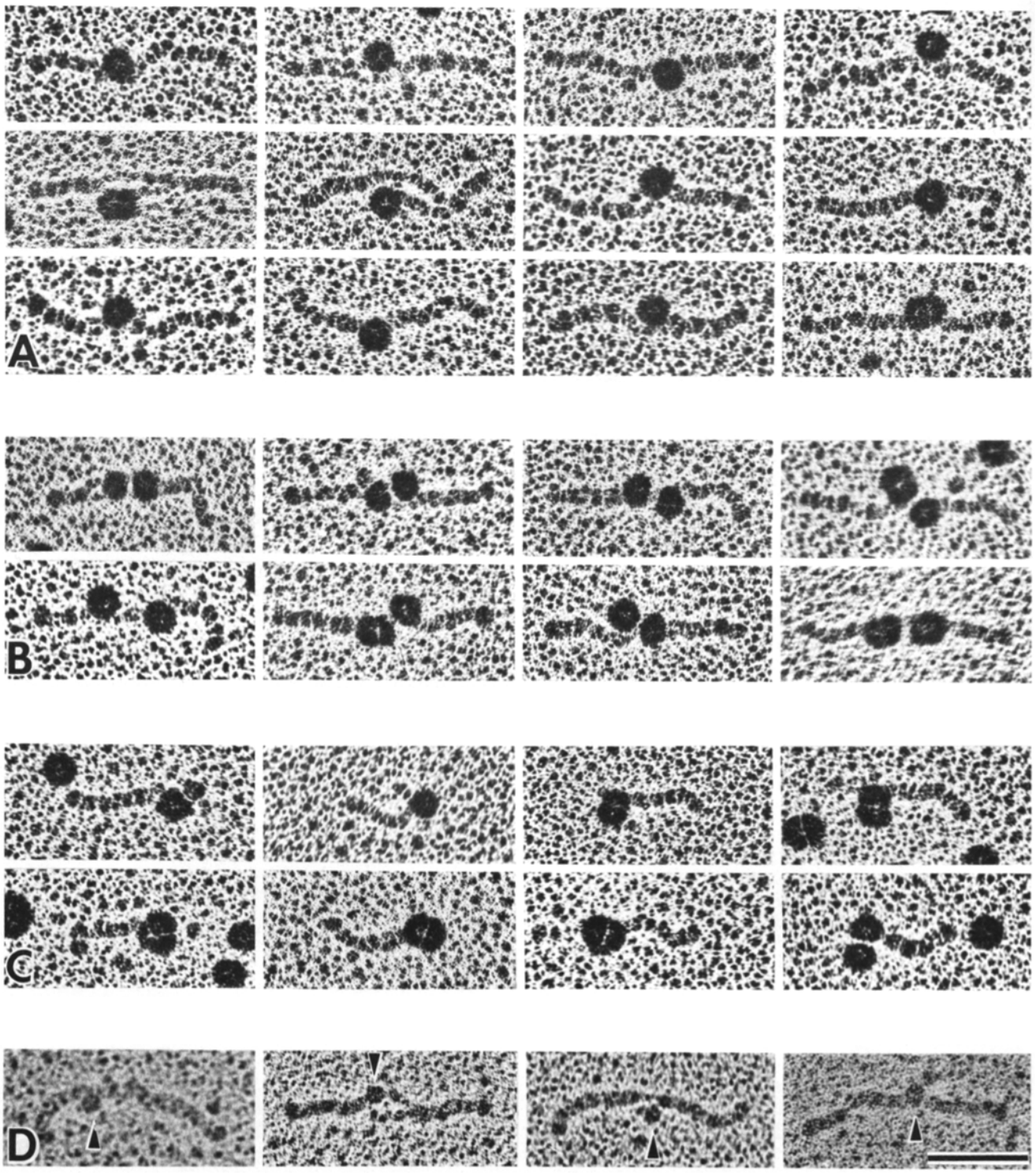


FIGURE 3 Mapping of calmodulin-binding sites on calspectin molecules. (*A–C*) Calspectin molecules in which calmodulin-binding sites are labeled with ferritin particles. (*A*) Individual calspectin tetramer labeled with a single ferritin particle. (*B*) Individual calspectin tetramer labeled with two ferritin particles. (*C*) Individual calspectin dimer labeled with a ferritin particle. (*D*) Calspectin tetramers incubated with biotin-conjugated calmodulin. Small spherical dots are found near the midpoints of the calspectin tetramers (see arrowheads). Bar, 0.1 $\mu$m. × 168,000.

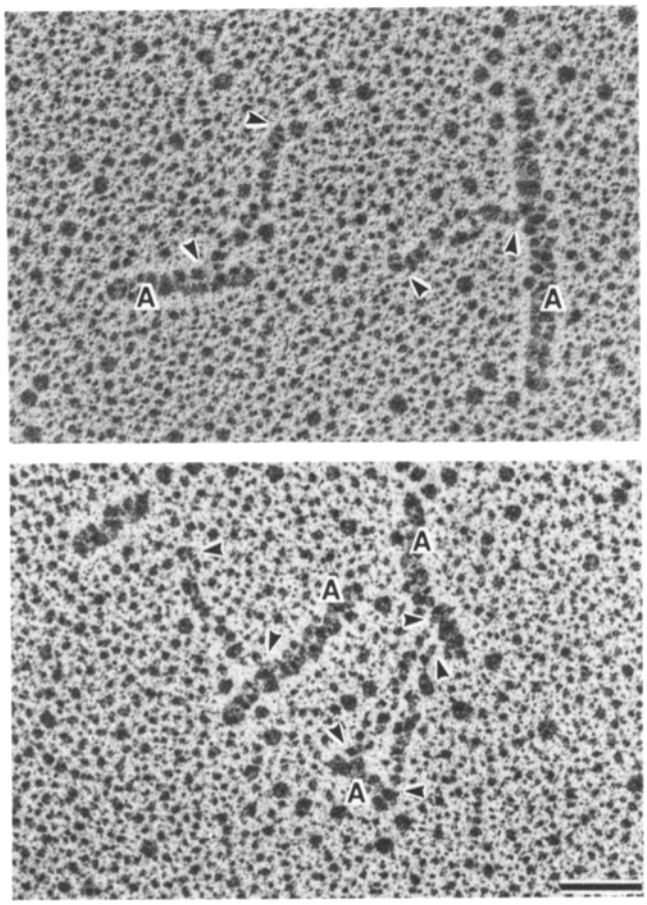


FIGURE 4 Rotary-shadowing electron micrographs of actin-calspectin complexes. Calspectin tetramers were attached to actin filaments (*A*) with their tail ends (arrowheads) in an end-on fashion. The tetrameric rods are often seen to cross-link adjacent actin filaments (see the *lower* panel). Bar 0.1 μm. × 110,000.

Our results also establish that calmodulin attaches to calspectin polypeptides at their head part (near the midpoint of the tetramer).

The significance of calmodulin binding to calspectin is not clear. Calmodulin can also bind to erythrocyte spectrin but only in the presence of urea (22). The calmodulin-binding sites on the erythrocyte spectrin may somehow be masked in the native form. The difference in calmodulin-binding abilities between these two spectrin species may reflect their functional difference. Interestingly, the erythrocyte spectrin possesses the binding site for band 2.1 protein, ankyrin, in the head part of spectrin (19). The β-subunit of the erythrocyte spectrin is responsible for ankyrin binding (23), whereas in calspectin the calmodulin binding is attributed to the α-subunit. The possible relation between calmodulin and ankyrin also remains to be elucidated.

The actin-binding sites are localized at the tail ends of calspectin molecules, similar to those of the erythrocyte spectrin (24). This suggests the functional similarity between these two spectrins. Through this mode of association, "spectrins" and actin can form some supramolecular organizations inside various cells. In the erythrocyte membrane, actin exists as short filaments that appear to play a key role in formation of the continuous network of spectrin (1, 4). Calspectin, with actin, may also form the cytoskeletal network underlying the plasmalemma in cells of brain tissues, though some other types of organization cannot be ruled out. Moreover, biochemical and immunofluorescence studies indicate that the spectrins differ in their subunit composition from one another, suggesting that this family of proteins might have been evolved to meet the different functional requirements in respective cells. Further detailed studies of the similarity and difference in chemical properties among the spectrins are needed to better understand their functions inside the cells.

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