Journal of Cellular Biochemistry 30:271-279 (1986)
Membrane Skeletons and Cytoskeletal-Membrane Associations 157-165

Reevaluation of the Hydrophobic Nature of the 110-kD Calmodulin-, Actin-, and Membrane-Binding Protein of the Intestinal Microvillus

Karen A. Conzelman and Mark S. Mooseker

Department of Biology, Yale University, New Haven, Connecticut 06511-8112

A complex of calmodulin (CM) and the 110-kD (110K) subunit composes the helical array of cross-bridges linking the microvillus actin filament bundle with the membrane. The hydrophobic properties of the 110K protein, assessed by the detergent phase partitioning assay [Bordier C: J Biol Chem 256:1604, 1981], are highly dependent on the solution conditions used in its isolation. The ATP-dissociable 110K-CM complex [Howe and Mooseker: J Cell Biol 97:974, 1983] exhibits hydrophilic characteristics in this assay. In contrast, the 110K subunit extracted from brush borders by Triton X-100, sodium dodecyl sulfate, and sodium pyrophosphate (detergent-treated 110K) [Glenney JR, Glenney P: Cell 37:743, 1984] behaves as a hydrophobic protein. However, because the soluble hydrophilic 110K-CM can be rendered hydrophobic by treating the complex with the same detergent and salt conditions used in the preparation of detergent-treated 110K, the properties of detergent-treated 110K seem likely to be an effect of the solution conditions on its native conformation, sedimentability, or exposure of binding domains. In addition, the detergent-treated 110K is devoid of calmodulin and no longer exhibits the actin-binding activity characteristic of the ATP-dissociable 110K-CM and of the intact complex in situ. With two partially purified preparations of the 110K subunit exhibiting such dramatically distinct properties, it seems premature to define the nature of the 110K subunit's association with the membrane at this time.

Key words: brush border, actin binding, actin-membrane interaction, 110-kD protein, calmodulin, 110kD-calmodulin complex, hydrophobic properties

The interaction of actin filaments with membranes is a universal aspect of cytoskeletal organization in eucaryotic cells. One of the best characterized examples of this interaction is in the microvilli of the intestinal epithelial brush border [for review, see 1,2]. The actin filament bundle within the microvillus is attached laterally to the membrane by a helical [3] array of bridges [4,5]. These filament-membrane

Received May 10, 1985; accepted October 18, 1985.

cross links are composed, at least in part, of a complex of the Ca^{++} binding protein calmodulin (CM) and a 110-kD subunit (110K) [for review, see 6]. Recently, several laboratories have purified the 110-kD-calmodulin complex (110K-CM) [7–9]. Studies by these three groups indicate that, like the lateral bridge [10], the purified 110K-CM complex binds to actin in the absence but not the presence of ATP. Not surprisingly, the 110K subunit is an ATP-binding protein [11]. Collins and Borysenko [8] have shown recently that the 110K-CM has ATPase activity in the presence of EDTA, an activity considered diagnostic for myosin. This observation, together with the ATP-dependent interaction of 110K-CM with actin, has given rise to exciting speculation that 110K-CM could be a membrane-associated mechanoenzyme.

Although there is good agreement regarding the ATP-dependent binding interaction of the 110K-CM with the core filaments, the nature of its association with the membrane remains controversial. Several investigators have observed that 110K-CM is extracted as a soluble complex from either membrane intact brush borders [7,8] or microvilli [12] by ATP. Howe and Mooseker [7] have noted that the ATP-dependent extraction of 110K-CM is potentiated by slightly elevated salt (0.2 M KCl or greater). These relatively mild extraction conditions are characteristic of peripheral membrane proteins [13]. In addition, Coudrier et al [14] have identified a 200-kD membrane protein in porcine microvilli (generally present as a 140-kD proteolytic fragment), which binds to the 110K subunit in vitro. Taken together, these results suggest that 110K-CM provides a peripheral link to the membrane potentially mediated by an integral membrane protein.

In contrast, Glenney and Glenney [15] have suggested that the 110K subunit is an integral membrane protein. They found that extraction of 110K from brush border required detergent, and that detergent was necessary for 110K solubility in aqueous buffers, properties characteristic of integral membrane proteins [13]. Further evidence for the hydrophobic nature of the 110K subunit includes 1) detergent phase partitioning as assayed by the technique of Bordier [16], 2) reconstitution of 110K into artificial liposomes, and 3) phenylisothiocyanate labeling of hydrophobic domains of 110K in isolated microvilli. Moreover, they have suggested that the isolated 110K-CM might lack the hydrophobic domain(s) involved in membrane insertion either as a result of proteolysis or loss of boundary lipids.

The results of Glenney and Glenney [15] provide a clear demonstration of the presence of hydrophobic domain(s) on the 110K subunit isolated by their method (refered to here as *detergent-treated 110K*). However, we believe that it is premature to identify the 110K as an integral membrane protein based on the evidence presented in their study. The procedures used for purification of detergent-treated 110K include the addition of the detergents Triton X-100 and sodium dodecyl sulfate (SDS) followed by ammonium sulfate precipitation. Such procedures might either artifactually expose or, in the case of SDS binding, actually generate hydrophobic domains not present in the native 110K-CM. To address these issues, we have compared the hydrophobic properties of the 110K subunit prepared by the method of Glenney and Glenney [15] with those of the 110K-CM under several solution conditions. For these studies, the Triton X-114 detergent phase separation assay of Border [16] was used.

MATERIALS AND METHODS

Brush Border Isolation

Brush borders were fractionated from chicken intestinal epithelial cells in solution I (75 mM KCl, 10 mM imidazole Cl (pH 7.2), 0.1 mM $MgCl_2$, 1 mM

EGTA, 0.02% sodium azide, 0.2 mM dithiothreitol) [17] by the method of Mooseker and Howe [18] with the modifications of Keller and Mooseker [19]. To help control proteolysis, 10–20 trypsin inhibitor units of Aprotinin (Sigma Chemical Co., St. Louis, MO) per liter and 0.2 mM phenylmethylsulfonylfluoride (Eastman Kodak Co., Rochester, NY) were added to all solutions.

110-kD Protein Extraction and Partial Purification

110K-CM was extracted from membrane intact brush borders by addition of 10 vol of solution I containing 4 mM ATP. The ATP extract was then loaded onto a hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) column (10 ml bed volume) preequilibrated in solution I with an additional 0.225 M KCl and 75 mM KPO_4 (pH 7.0) and eluted with 150 ml linear 75–200 mM KPO_4 gradient (pH 7.0). Peak fractions were pooled and dialyzed into solution I with 2.5 mM $MgCl_2$ (solution IM). This 110K-CM-enriched preparation containing contaminating polypeptides of approximately 240-, 220-, 150-, 95- and 80-kD molecular weights was used for subsequent analysis without further purification in order to minimize the loss of calmodulin [see 7,8].

Alternatively, 110K was prepared according to the method of Glenney and Glenney [15]. In brief, this procedure involves extraction of demembranated brush borders with 0.25% Triton X-100, 0.05% SDS, and 10 mM sodium pyrophosphate in solution I and subsequent ammonium sulfate fractionation. The resulting 110K-enriched precipitate, which in our hands contains variable amounts of a 43-kD polypeptide and other minor contaminants, is dialyzed into distilled water.

Triton X-114 Phase Separation Assay

The hydrophobic nature of the proteins in brush border extracts and in various 110K-enriched preparations was assessed by their relative partitioning into the detergent and aqueous phases of a 1% solution of Triton X-114. These assays were done according to the method of Bordier [16] using either distilled water (for detergent-treated 110K), solution IM (for hydroxylapatite-enriched 110K-CM and detergent-treated 110K), or solution IM with an additional 0.125 M KCl (for unfractionated brush border extracts) as the buffer. In addition to the 110K-CM prepared by hydroxylapatite fractionation and the detergent-treated 110K, the phase partitioning characteristics of the 110K-CM treated with 0.25% Triton X-100, 0.05% SDS, and 15% ammonium sulfate and of the detergent-treated 110K incubated with exogenous brain calmodulin (0.3 mg/ml) in solution IM were also determined.

Actin-Binding Assay

110K prepared by the two methods described above was incubated in solution IM with 0.3 mg/ml F-actin at room temperature for 20 min in the presence or absence of 4 mM ATP. Aliquots were spun in an airfuge (Beckman Instruments Inc., Palo Alto, CA) for 30 min at 25 psi. The composition of the resulting pellets and supernatants was assessed by SDS-gel electrophoresis.

Preparation of Other Proteins

Calmodulin was prepared from bovine brain by the method of Burgess et al [20] (graciously provided by Dr. C.L. Howe). G-actin was prepared from chicken breast muscle by the method of Spudich and Watt [21]. For actin-binding assays, the actin

was prepolymerized in solution IM by a 20-min incubation at 20°C with periodic pipetting.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [22] was performed using 6–16% linear gradient gels or minigels [23].

RESULTS

Interaction of 110K-CM and Detergent-Treated 110K Subunit With Actin

The native state of the 110K subunit can best be assessed by its ATP-dependent interaction with actin; this activity mirrors the behavior of the lateral bridge in isolated brush borders. In contrast to the 110K-CM (Fig. 1a) [7], the detergent-treated 110K subunit does not bind to actin (Fig. 1b). Moreover, addition of exogenous calmodulin to the calmodulin-stripped detergent-treated 110K does not restore ATP-dependent actin-binding activity (data not shown).

Hydrophobicity of 110K-CM and Detergent-Treated 110K Subunit

The hydrophobicity of 110K-CM and detergent-treated 110K subunit was evaluated using the detergent-phase partitioning assay of Bordier [16]. This assay is based on the temperature-dependent behavior of the detergent Triton X-114. When heated above its cloud point, Triton X-114 will partition into a viscous, pelletable, detergent-rich phase and an aqueous (detergent-poor) supernatant phase. Many proteins with hydrophobic domains, such as the major glycoproteins of the brush border membrane

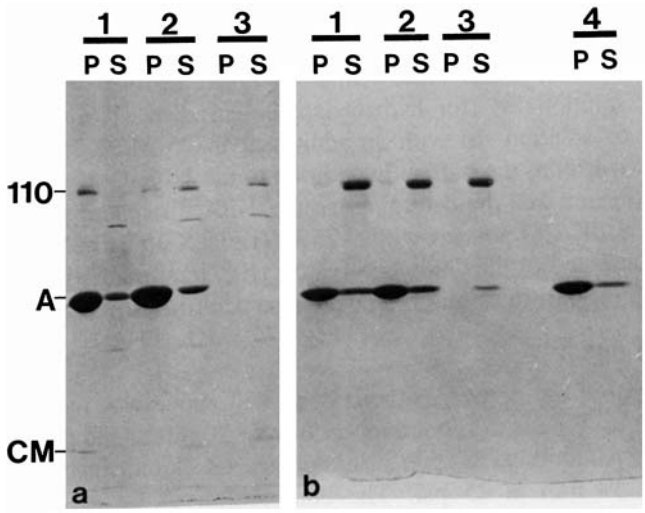

Fig. 1. Cosedimentation analysis of the actin-binding activities of 110K preparations; 110K prepared either by hydroxylapatite fractionation (a) or by the method of Glenney and Glenney [15] (b) was incubated with F-actin in the absence (lanes 1) or presence (lanes 2) of ATP before being subjected to airfuge centrifugation. SDS-PAGE analysis of the resulting pellets (P) and supernatants (S) is shown here. Lanes 3 in a and b and lane 4 in b represent, respectively, the sedimentation profiles of each of the 110K preparations and of F-actin alone.

[24] (Fig. 2a), partition quantitatively into the detergent phase, whereas most hydrophilic proteins partition into the aqueous phase. With this technique, soluble cytoskeletal proteins present in an ATP extract of either membrane-intact or demembranated brush borders exclusively partition into the aqueous phase. This includes myosin, actin, villin, the nonerythroid spectrin, TW 260/240, the 110K subunit and calmodulin (Fig. 2b,c).

Consistent with the results of Glenney and Glenney [15], we found that the detergent-treated 110K partitions into the detergent phase (as does a 43-kD polypeptide that contaminates this preparation; Fig. 3, lane 1). In contrast, the 110K-CM in the hydroxylapatite-enriched preparation (Fig. 3, lane 2), like the complex in the unfractionated ATP extract (Fig. 2b,c), partitions exclusively into the aqueous phase. However, the phase partitioning behavior of both the detergent-treated 110K and the 110K-CM is highly dependent on the solution conditions used. Treating the hydrophilic 110K-CM with the detergent and ammonium sulfate conditions used by Glenney and Glenney [15] reverses the phase partitioning behavior of the 110K subunit (Fig.

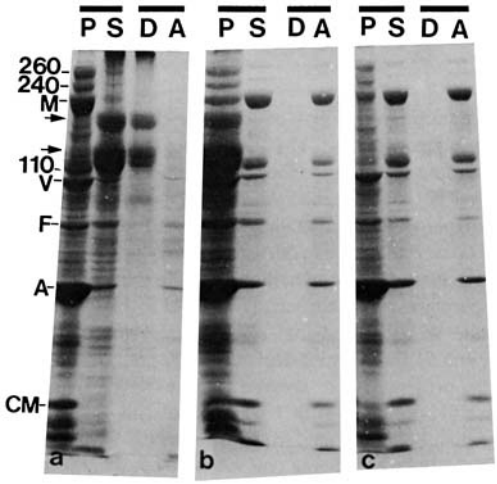

Fig. 2. Detergent phase partitioning assay of Triton X-114 and ATP extracts. Brush borders were treated either with 1% precondensed Triton X-114 [16] in solution IM or with solution IM with an additional 4 mM ATP and 0.125 M KCl at 4°C for 10 min. They were centrifuged at 15,600g for 5 min, and the resulting pellets (P) and supernatants (S) are shown. The proteins in each of the supernatant fractions were assayed for their relative partitioning into the detergent (D) and aqueous (A) phases of Triton X-114. The mobility of the proteins TW 260/240 (260 and 240), myosin heavy chain (M), 110-kD subunit (110), villin (V), fimbrin (F), actin (A), and calmodulin (CM) are indicated. a) Triton X-114 extraction of brush borders. Nonionic detergent solubilizes the major glycoproteins (arrows) of the brush border (S) [21] from the cytoskeleton (P). These integral membrane glycoproteins partition into the detergent phase (D), whereas the small amount of extracted cytoskeletal proteins present is found exclusively in the aqueous phase (A). b) ATP extraction of membrane-intact brush borders. The cytoskeletal proteins solubilized (S) from the brush border cytoskeleton by ATP and 0.2 M KCl, including the 110K subunit and CM, partition exclusively into the aqueous phase. c) ATP extraction of dembranated brush borders. Brush borders demembranated with Triton X-114 (as in Fig. 2a-P) were subsequently extracted with ATP in solution IM with an additional 0.125 M KCl. The extraction of 110K-CM is more complete under these conditions than from membrane-intact brush borders (compare Figs. 2b-S with 2c-S); nevertheless, all 110K and CM partition into the aqueous phase (A). Residual amounts of the integral membrane glycoproteins are found in the detergent phase (D).

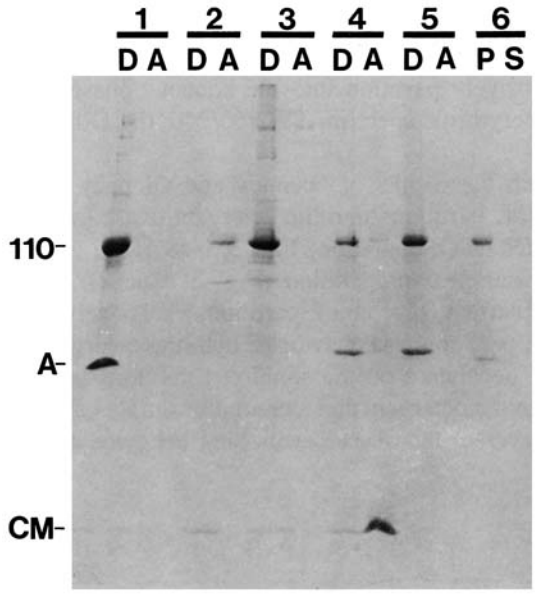

Fig. 3. Triton X-114 phase partitioning assay of 110K-enriched fractions. D is the detergent-rich phase and A is the detergent-poor (aqueous) phase. 1) 110K prepared by detergent and pyrophosphate extraction of demembranated brush borders according to the method of Glenney and Glenney [15]. This preparation is depleted in calmodulin. The band marked A comigrates with actin on SDS-PAGE. 2) 110K-CM partially purified by hydroxylapatite fractionation of an ATP extract of brush borders. 3) Detergent (0.25% Triton X-100 and 0.05% SDS) was added to hydroxylapatite enriched 110K-CM (as in 2 but a different column fraction) to duplicate the conditions used in the Glenney and Glenney procedure (15). This detergent-treated preparation was subsequently brought to 15% saturation with ammonium sulfate, and the resulting precipitate was dialyzed into solution IM. Note again that this preparation has no associated calmodulin. 4) and 5) Calmodulin-depleted 110K prepared as in 1 was incubated in solution IM for 90 min at 4°C in the presence (4) or absence (5) of excess exogenous brain calmodulin (0.3 mg/ml). 6) Detergent-treated 110K prepared as in 1 was mixed with Triton X-114 as for the Bordier assay but was kept on ice rather than being warmed to the detergent cloud point. The fractions here represent the distribution of 110K in the pelleted volume equivalent to the detergent phase (P) and the supernatant volume equivalent to the aqueous phase (S). Although there is no phase separation under these conditions, most of the detergent-treated 110K is found in the pellet.

3b, lane 3). These conditions also remove much of the calmodulin from the complex. Conversely, the phase partitioning behavior of at least some of the detergent-treated 110K is reversed by incubation with exogenous calmodulin (Fig. 3, lane 4). The effect of calmodulin does not seem to be simply nonspecific competition with the 110K subunit for SDS or Triton X-100 binding; other added protein (bovine serum albumin) does not alter the separation of detergent-treated 110K into the detergent phase (data not shown). Finally, we have noticed that the detergent-treated 110K tends to aggregate under the conditions of the Bordier assay [16]. Therefore, separation of the 110K into the detergent phase could be a consequence of sedimentability rather than hydrophobicity. In fact, in samples that were never warmed to the Triton X-114 cloud point, most of the detergent-treated 110K was still found in a volume equivalent to that of the detergent phase "pellet" (Fig. 3, lane 6).

DISCUSSION

The nature of a protein's association with a biomembrane has been operationally defined by its extractibility and solubility in neutral, aqueous buffers. Classically, peripheral membrane proteins and nonmembrane proteins are defined by the relatively mild conditions required for their extraction and by their solubility in aqueous solutions. In contrast, integral membrane proteins require detergents, chaotropic agents, or organic solvents to be dissociated from membranes and once solubilized are usually associated with lipids. Furthermore, in aqueous solution, integral membrane proteins are generally insoluble or aggregated [13]. Based on these criteria and on the results of the detergent phase partitioning assay, the 110K-CM extracted from brush borders by ATP treatment is a peripheral membrane protein, whereas the detergent-treated 110K subunit has properties consistent with classification as an integral membrane component. However, the results presented above suggest that the differences in the hydrophobic properties of these two preparations of 110K are most likely a consequence of the differences in the treatments used in their partial purification from brush border cytoskeletons.

The hydrophobic nature of the detergent-treated 110K subunit might be a result of the loss of calmodulin during its isolation. This is consistent with the shift into the aqueous phase of at least a small proportion of the detergent-treated 110K reconstituted with exogenous calmodulin and of the shift into the detergent phase of the 110K subunit derived from the 110K-CM by detergent and ammonium sulfate treatment. Calmodulin may help to maintain the native conformation of the 110K, minimizing the exposure of internalized hydrophobic peptide regions. Exposure of the calmodulin-binding domain of 110K itself might account for its phase partitioning, in that the binding sites of other calmodulin binding proteins have been characterized as amphipatic [25]. A more provocative notion is that calmodulin binding somehow regulates the exposure of a hydrophobic insertion sequence involved in membrane binding. Finally, calmodulin binding, either by affecting the exposure of hydrophobic regions or by some other conformational change, might simply minimize the aggregation and sedimentation of 110K. As described above, we have noted the aggregation of the detergent-treated 110K during the Bordier assay. Howe and Mooseker [7] and Collins and Borysenko [8] have also reported an increase in the aggregation of 110K coincident with the loss of calmodulin. Since the phase partitioning assay is valid only for soluble proteins, it is important to be cautious in interpreting these data.

Although the effect of calmodulin on the partitioning of 110K appears to be specific, the treatment of the protein with detergent and ammonium sulfate might also account for its behavior in the Bordier assay. All the other polypeptides in the detergent-treated 110K preparation, including one comigrating with actin on SDS-PAGE, partition into the detergent phase; only the partitioning of the 110K subunit is shifted by incubation with calmodulin.

Clearly the ATP-dissociable 110K-CM behaves as a hydrophilic protein in the detergent phase partitioning assay. Although not all the 110K-CM is solubilized by ATP from membrane-intact brush borders, nearly complete extraction of the complex is achieved by ATP treatment of demembranated brush borders (Fig. 2c). The 110K-CM in both of these extracts partitions exclusively into the aqueous phase (Fig. 2b,c). This result argues against the existence of two populations of 110K, one peripherally and one integrally associated with the membrane. Moreover, in that the 110K subunit

of the isolated complex can be rendered hydrophobic by the same detergent and ammonium sulfate conditions used in the preparation of detergent-treated 110K, the soluble 110K-CM cannot simply be a hydrophilic proteolytic fragment of a larger integral membrane protein.

On the basis of these results, we feel that it is premature to classify 110K as an integral membrane protein. Such an assignment has implications not only for its role in brush border structure and function but also for its synthesis, transport, and compartmentalization within the cell. In fact, Cowell and Danielsen [26,27] have recently provided biosynthetic data to suggest that 110K, like villin and actin, probably is not synthesized on the rough ER, as is typical for integral membrane proteins, but rather is synthesized on free ribosomes. This would suggest that the 110K either is a peripheral membrane protein or is inserted into the membrane posttranslationally, as has been demonstrated for the microsomal membrane proteins cytochrome b_5 and NADH:cytochrome b_5 oxidoreductase [see 26,27 for discussion].

The results presented here do not rule out the possibility that the 110K-CM directly links the microvillus core to the membrane. The detergent phase partitioning technique is by no means a definitive assay for a protein's interaction with the membrane. However, the results from this assay do suggest that the hydrophobic properties of the isolated, detergent-treated 110K probably reflect the effects of the purification procedure on the 110K subunit rather than its native properties. In that three of the four lines of evidence presented by Glenney and Glenney [15] for the integral association of the 110K subunit with the membrane used protein purified by this detergent treatment, it is important to be cautious in interpreting these data. Nevertheless, the specific phenylisothiocyanate labeling of 110K in membrane-intact microvilli also reported by Glenney and Glenney [15] does suggest that 110K has some hydrophobic domains potentially responsible for either membrane insertion or hydrophobic interactions with other proteins such as calmodulin. Clearly, more work is necessary to define the molecular basis of the interaction of the 110K-CM with the microvillus membrane.

ACKNOWLEDGMENTS

We acknowledge the work of Dr. C.L. Howe, whose preliminary experiments laid the foundation for the study reported here. We thank Dr. T.C.S. Keller for several insightful discussions during the course of this work. We also acknowledge the excellent technical support of Michael Boyd, John Santarelli, and Anna Goglia and the assistance of Kris Mooseker in manuscript preparation. This work was supported by predoctoral fellowships from NSF and Miles Corporation to K.A.C. and by March of Dimes grant I-924 and NIH grant AM25387 to M.S.M.

REFERENCES

1. Bretscher A: In Dowben R, Shay J (eds): "Cell and Muscle Motility." New York: Plenum Press, 1983, pp 239–268.
2. Mooseker MS: Cell 35:11, 1983.
3. Matsudaira P, Burgess D: Cold Spring Harbor Symp Quant Biol 46:845, 1982.
4. Mukherjee TM, Staehelin LA: J Cell Sci 8:573, 1971.
5. Mooseker MS, Tilney LG: J Cell Biol 67:725, 1975.

6. Mooseker MS, Bonder EM, Conzelman KA, Fishkind DJ, Howe CL, Keller TCS: J Cell Biol 99:104s, 1984.
7. Howe CL, Mooseker MS: J Cell Biol 97:974, 1983.
8. Collins JH, Borysenko CW: J Biol Chem 259:14128, 1984.
9. Verner K, Bretscher A: J Cell Biol 99:351a, 1984.
10. Matsudaira P, Burgess D: J Cell Biol 83:667, 1982.
11. Mooseker MS, Bonder EM, Conzelman KA, Fishkind DJ, Howe CL, Keller TCS: In Donowitz M, Sharp GWG (eds): "Mechanisms of Intestinal Electrolyte Transport and Regulation by Calcium (Kroc Foundation Series Vol 17)." New York: Alan R. Liss, Inc., 1984, pp 287–307.
12. Verner K, Bretscher A: Eur J Cell Biol 29:187, 1983.
13. Singer SJ: Annu Rev Biochem 43:805, 1974.
14. Coudrier E, Reggio H, Louvard D: EMBO J 2:469, 1983.
15. Glenney JR, Glenney P: Cell 37:743, 1984.
16. Bordier C: J Biol Chem 256:1604, 1981.
17. Bretscher A: Proc Natl Acad Sci USA 78:6849, 1981.
18. Mooseker MS, Howe CL,: In Wilson L (ed): "Methods in Cell Biology." Vol 25, New York: Academic Press, 1982, pp 144–175.
19. Keller TCS, Mooseker MS: J Cell Biol 95:943, 1982.
20. Burgess WH, Jemiolo DK, Kretsinger RH: Biochim Biophys Acta 623:257, 1980.
21. Spudich JA, Watt S: J Biol Chem 246:4866, 1971.
22. Laemmli V: Nature 227:680, 1970.
23. Matsudaira P, Burgess D: J Cell Biol 83:667, 1982.
24. Mooseker MS, Stephens RE: J Cell Biol 86:466, 1980.
25. Cox JA: Fed Proc 23:3000, 1984.
26. Cowell GM, Danielsen EM: FEBS Lett 172:309, 1984.
27. Cowell GM, Danielsen EM: Biochem J 225:275, 1985.