Calcium-sensitive, Lipid-binding Cytoskeletal Proteins of the Human Placental Microvillar Region

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Abstract. In this study we describe a group of Ca^{2+}-sensitive proteins located in the microvillar region of the human placental syncytiotrophoblast. By following the distribution of proteins between the particulate and supernatant phases of detergent-solubilized microvilli in the presence of defined concentrations of free Ca^{2+}, we demonstrate a class of proteins of subunit molecular weights 72,000, 69,000, 38,000, 36,000, and 32,000 that associate with both the cytoskeleton and lipid at high concentrations of free Ca^{2+}.

These proteins can be released from microvilli using EGTA-containing buffers. Although they do not bind to phenyl–Sepharose, they will bind to phospholipids immobilized on phenyl–Sepharose columns in a Ca^{2+}-dependent manner and show a marked preference for phospholipids with negatively charged headgroups.

The results provide evidence for a sequence of events which may occur within the microvillus as the localized concentration of intracellular free Ca^{2+} rises.

MANY of the events occurring at the maternal surface of the human placenta such as receptor-mediated endocytosis and control of the cytoskeletal structure are thought to be regulated by the levels of intracellular free Ca^{2+}.

The placental microvillus has recently been shown to contain two distinct groups of calcium-sensitive proteins. The first is a group of proteins that is released from microvillar cytoskeletons at micromolar levels of free Ca^{2+} (Ca^{2+}-soluble proteins). These include α-actinin, an 80,000-mol-wt protein, and actin (38). The 80,000-mol-wt protein is recognized by antibodies raised to the 80,000-mol-wt protein found as a minor component of chicken intestinal brush border cytoskeletons (Edwards, H. C., and A. G. Booth, unpublished result). The latter protein is now called ezrin (4).

The second group comprises polypeptides that are associated with the cytoskeleton at millimolar levels of free Ca^{2+} (EGTA-soluble proteins). These have subunit molecular weights of 72,000, 69,000, 38,000, 36,000, 32,000, and 10,000. They are confined almost exclusively to the microvillar region of the placental chorionic villus.[1] Several of these proteins show immunological cross-reactivity with the *Torpedo* protein calelectrin (36).[1] Furthermore, the 36,000-mol-wt polypeptide is recognized by antibodies raised to a similar protein, P36, from intestinal brush borders (13).[1] The 72,000 and 69,000-mol-wt polypeptides cross-react with antibodies to a protein (protein III) from bovine liver (33).[1]

Similar groups of proteins which bind to actin filaments or cytoskeletons with a range of affinities for Ca^{2+} have been identified in the intestinal brush border, liver mem-

brane vesicles, brain cortex, adrenal medulla, and blood cells (13, 15, 32, 33, 37, 39). At least one of these proteins binds to actin filaments at concentrations significantly greater than the range of intracellular free calcium ion concentrations. The P36 protein of the intestinal brush border requires concentrations of Ca^{2+} of 10^{-4}–10^{-3} M for binding to actin (15). However, this requirement for Ca^{2+} is markedly reduced in the presence of phosphatidylserine (14, 22) with which it has been shown to bind directly.

In this study we have examined the effect of Ca^{2+} on the association of proteins with the cytoskeleton in the placental microvillus. By solubilizing whole microvilli in media containing defined concentrations of Ca^{2+}, rather than studying the association or dissociation of proteins from cytoskeletons, we hoped to avoid any artifacts arising from the preparation of cytoskeletons.

We have also compared the abilities of the Ca^{2+}-released proteins and the Ca^{2+}-associating proteins to bind to microvillar lipid and the effect of Ca^{2+} on this. Finally, we examined the specificity of binding to lipid to establish whether this is achieved through increased hydrophobicity as is suggested by Shadle et al. (33) or by ionic interactions as proposed by Rhoads et al. (32). The physiological implications of our results are discussed.

Materials and Methods

Preparation of Microvilli from Human Placenta

Microvilli were prepared essentially as described by Booth et al. (3). Human placentae were processed within 15 min of delivery. All procedures were carried out at 4°C. The cord, membranes, and major blood vessels were removed and the placenta was minced. An equal volume of 0.15 M

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1. Walker, J. H., C. Boustead, H. C. Edwards, E. Fawell, and A. G. Booth, manuscript submitted for publication.
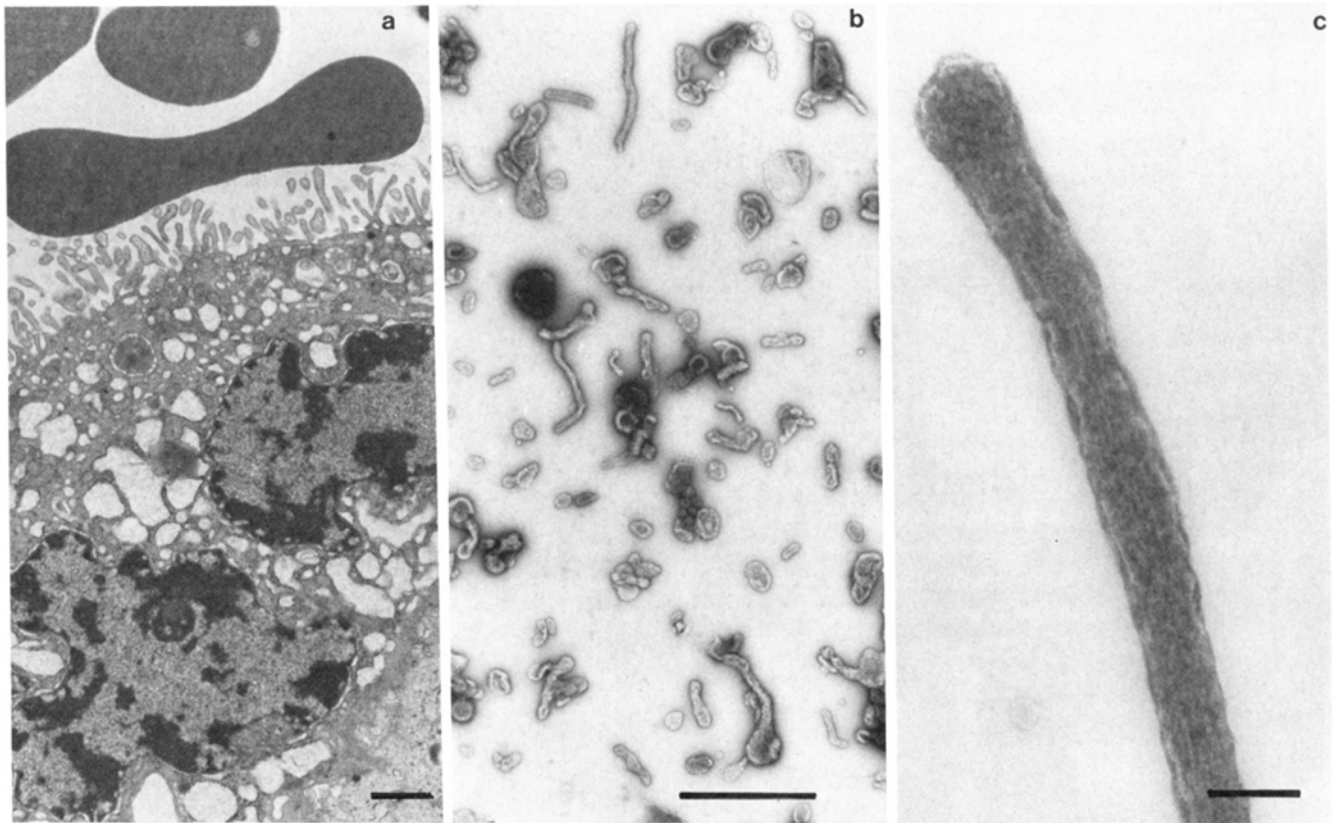
Figure 1. The human placental microvillus. (*a*) Ultrathin section of human placenta showing the maternal surface. (*b*) Microvilli were fixed immediately after their preparation, by resuspension to a concentration of 1 mg of protein/ml in 0.15 M NaCl, 10 mM Hepes, pH 7.4, containing 0.25% glutaraldehyde. After incubation for 10 min at 4°C, 1 M NH₄Cl was added to a final concentration of 20 mM and the microvilli were dialyzed against 0.15 M NaCl, 10 mM Hepes, pH 7.4, for 18 h at 4°C. Microvilli were visualized for electron microscopy by negative staining with 2% uranyl acetate. (*c*) Fixed microvilli were solubilized in 0.15 M NaCl, 10 mM Hepes, pH 7.4 containing 5% Triton X-100 for 30 min at 4°C. After centrifugation for 10 min at 20,000 *g*, the pellet was resuspended and washed twice in detergent-free buffer. The detergent-solubilized microvilli were visualized as above. Bars, 0.1 μm.
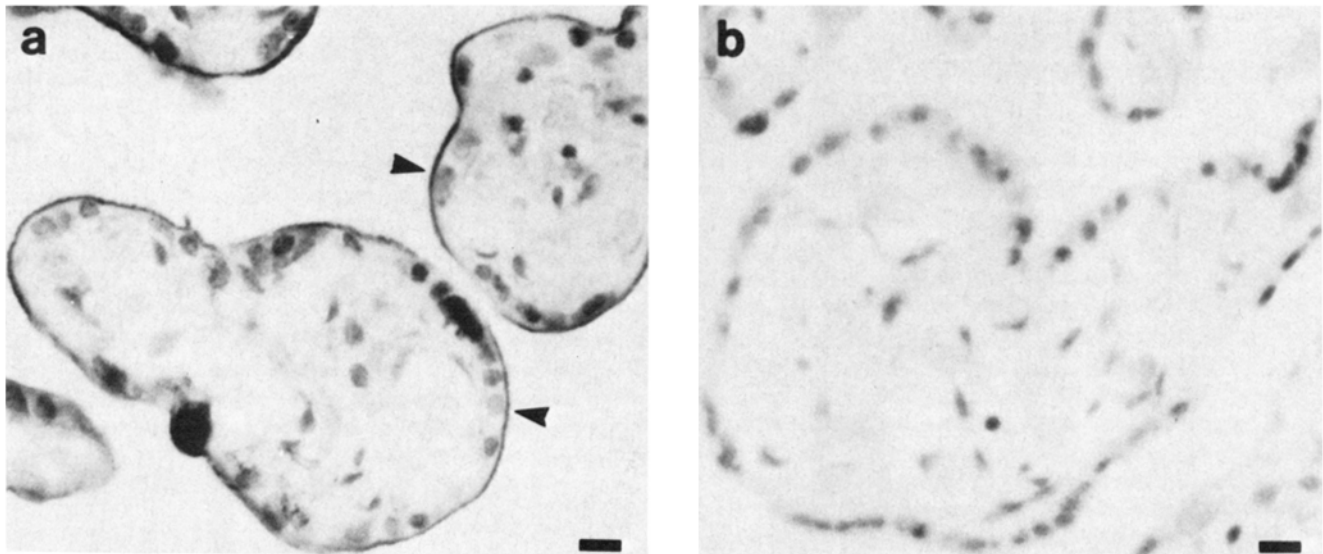

Figure 2. Immunolocalization of 69,000- and 72,000-mol-wt proteins. Small pieces of human placenta were fixed in 3.7% formaldehyde and embedded in paraffin essentially as described (40). Sections (3 μm) were cut and after removal of paraffin were incubated with normal swine serum for 20 min; anti-p68 serum or preimmune serum diluted in 150 mM NaCl, 50 mM Tris, pH 7.4 containing 4% bovine serum albumin (4% TBSA) for 30 min; peroxidase-labeled anti–rabbit IgG (Miles Laboratories Inc., Elkhart, IN) diluted 1:200 in 4% TBSA for 30 min. Peroxidase activity was visualized with 0.05% (wt/vol) 3,3'-diaminobenzidine and 0.01% (vol/vol) H₂O₂ in 50 mM Tris, pH 7.4. Sections were counterstained with Mayer's haemotoxylin. (*a*) Anti-68 (36). (*b*) Preimmune serum. Bars, 10 μm.

NaCl was added and the mixture stirred for 1 h. The homogenate was passed through a nylon sieve, the filtrate collected, and then centrifuged at 2,000 g for 10 min (Sorvall SS34 rotor; Beckman Instruments, Inc., Palo Alto, CA). The supernatant was then centrifuged for a further 10,000 g for 10 min in the SS34 rotor. After careful removal, the supernatant was centrifuged at 90,000 g for 30 min in an A130 rotor (Beckman Instruments, Inc.). Pelleted material was resuspended to a final volume of 50 ml with 10 mM mannitol, 2 mM Tris, pH 7.0, using a loose fitting Dounce homogenizer and solid MgCl$_2$·6H$_2$O added to a concentration of 10 mM. After stirring for 10 min the aggregated material was removed by centrifugation at 5,000 g for 10 min (SS34 rotor) and the supernatant centrifuged for 20,000 g for 30 min. The resultant pelleted material (microvilli) was resuspended with the required buffer to a final concentration of 5 mg of protein/ml.

Preparation of Microvillar Cytoskeletons

Cytoskeletons were prepared from the microvilli by resuspension to a protein concentration of 5 mg/ml in Buffer A (75 mM NaCl, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM Hepes, pH 7.4) containing 5% Triton X-100 and either 1 mM EGTA or defined concentrations of free Ca^{2+} (38). After incubation at 25°C for 15 min cytoskeletons were collected by centrifugation at 20,000 g for 15 min at 25°C (SS34 rotor). Pelleted material was resuspended and washed twice in the appropriate buffer containing 0.1% Triton X-100. Finally, the cytoskeletons were resuspended to a protein concentration of 3 mg/ml in detergent-free buffer.

Preparation of Ca^{2+}-sensitive, Cytoskeletal Proteins

(a) Preparation of EGTA-soluble Proteins. Cytoskeletons were prepared from microvilli in the presence of 1 mM CaCl$_2$. After centrifugation at 20,000 g for 15 min at 4°C (SS34 rotor), the pelleted material was resuspended in Buffer A containing 1 mM EGTA and dialyzed exhaustively against the same buffer for 18 h. The supernatant containing the EGTA-soluble proteins was collected by centrifugation at 20,000 g for 15 min at 4°C.

(b) Preparation of Ca^{2+}-soluble proteins. Cytoskeletons were prepared from microvilli using buffers containing 1 mM EGTA. After centrifugation at 20,000 g for 15 min at 4°C (SS34 rotor), the pelleted material was resuspended in Buffer A containing 1 mM CaCl$_2$ and dialyzed exhaustively against the same buffer for 18 h. The supernatant containing the Ca^{2+}-soluble proteins was collected after centrifugation at 20,000 g for 15 min at 4°C.

Preparation of Liposomes

Microvilli were resuspended and washed in Buffer A containing 1 mM EGTA for 15 min at 25°C and then pelleted by centrifugation at 20,000 g for 15 min. Lipid was then prepared by chloroform/methanol extraction (10). Liposomes were prepared from lipid extracted from placental microvilli or commercially prepared phospholipids (Sigma Chemical Co., Poole, Dorset, UK), by sonication in the required buffer under N$_2$.

Preparation of Buffers with Defined Concentrations of Free Ca^{2+}

Buffers contained CaCl$_2$ and EGTA in ratios appropriate to produce the required final concentration of free Ca^{2+} as previously described (38). Care was taken to monitor and maintain constant pH. Buffers contained no MgCl$_2$.

Electrophoresis and Scanning of Gels

Electrophoresis in 7–17% polyacrylamide gradient gels was performed by the method of Laemmli (24). Gels were stained as previously described (2). Gel tracks were scanned and integrated on an LKB UltraScan XL gel scanner.

Electron Microscopy

Samples were fixed with glutaraldehyde and osmium tetroxide, dehydrated through graded alcohol solutions, and embedded in Taab 812. Ultrathin sections were stained with lead citrate and uranyl acetate.

Results

The Human Placental Microvillus

Extensive areas of the apical surface of the human placental syncytiotrophoblast are covered by microvilli (Fig. 1 *a*). These do not exhibit the characteristic uniformity of the intestinal brush border microvilli and there is a lack of any obvious terminal web in the underlying region (29). This may be explained by the absence of lateral membranes in the syncytium, on which a structure such as the terminal web might be attached.

Examination of the microvillus preparation under the electron microscope (Fig. 1, *b* and *c*) showed that the integrity of the microvillus structure is maintained and that contaminating membranes have been removed. This is largely achieved by the selective aggregation of membranous material by MgCl$_2$ (3).

An enrichment of the plasma membrane marker protein alkaline phosphatase of 35-fold from the original homogenate is typical, giving a final specific activity of ∼5.5 µmol/min per mg of protein. Activities of other marker enzymes were all substantially depleted (3).

Immunolocalization of the proteins of the 69,000- and 70,000-mol-wt proteins with an antibody raised to a similar protein isolated from bovine liver and designated p68 (36)[1] showed that these proteins were concentrated in the microvillar region at the surface of the placental syncytium (Fig. 2).

Ca^{2+}-sensitive Interaction of Proteins with the Cytoskeleton

To examine the effect of Ca^{2+} on the interaction of proteins with the cytoskeleton, samples of microvilli were exposed to defined concentrations of Ca^{2+} and treated with Triton X-100. The insoluble material (cytoskeletons) and detergent-soluble material were examined by SDS-PAGE. Polypeptides of molecular weights 36,000, 69,000, and 72,000 were almost completely associated with the cytoskeleton when the concentration of free Ca^{2+} was >3 µM (Fig. 3). Two polypeptides of 38,000-mol-wt and 32,000-mol-wt also bound to the cytoskeleton as the Ca^{2+} concentration increased. However, the transition of these proteins from soluble to bound was not as clearly delineated as that of the first three proteins. The difference between these two groups of Ca^{2+}-associating proteins was evident when the amount of each protein present in the detergent-soluble material was quantified (Fig. 4). The 36,000-, 69,000-, and 72,000-mol-wt polypeptides each had a $K_{0.5}$ value for binding of 4–5 µM, whereas the $K_{0.5}$ value for the 32,000- and 38,000-mol-wt polypeptides was 10^{-4}–10^{-3} M.

Binding of Ca^{2+}-sensitive Proteins to Microvillar Membrane Lipid

Since the EGTA-soluble proteins share many characteristics with other Ca^{2+}-sensitive membrane-associated proteins,[1] some of which have been shown to have lipid-binding properties (14, 22, 33), we examined the effect of Ca^{2+} on the binding of both the EGTA-soluble proteins and the Ca^{2+}-soluble proteins to liposomes of microvillar lipids. We found that all the EGTA-soluble proteins (those with molecular weights of 72,000, 69,000, 38,000, 36,000, and 32,000) bound directly to the liposomes in the presence of millimolar Ca^{2+}
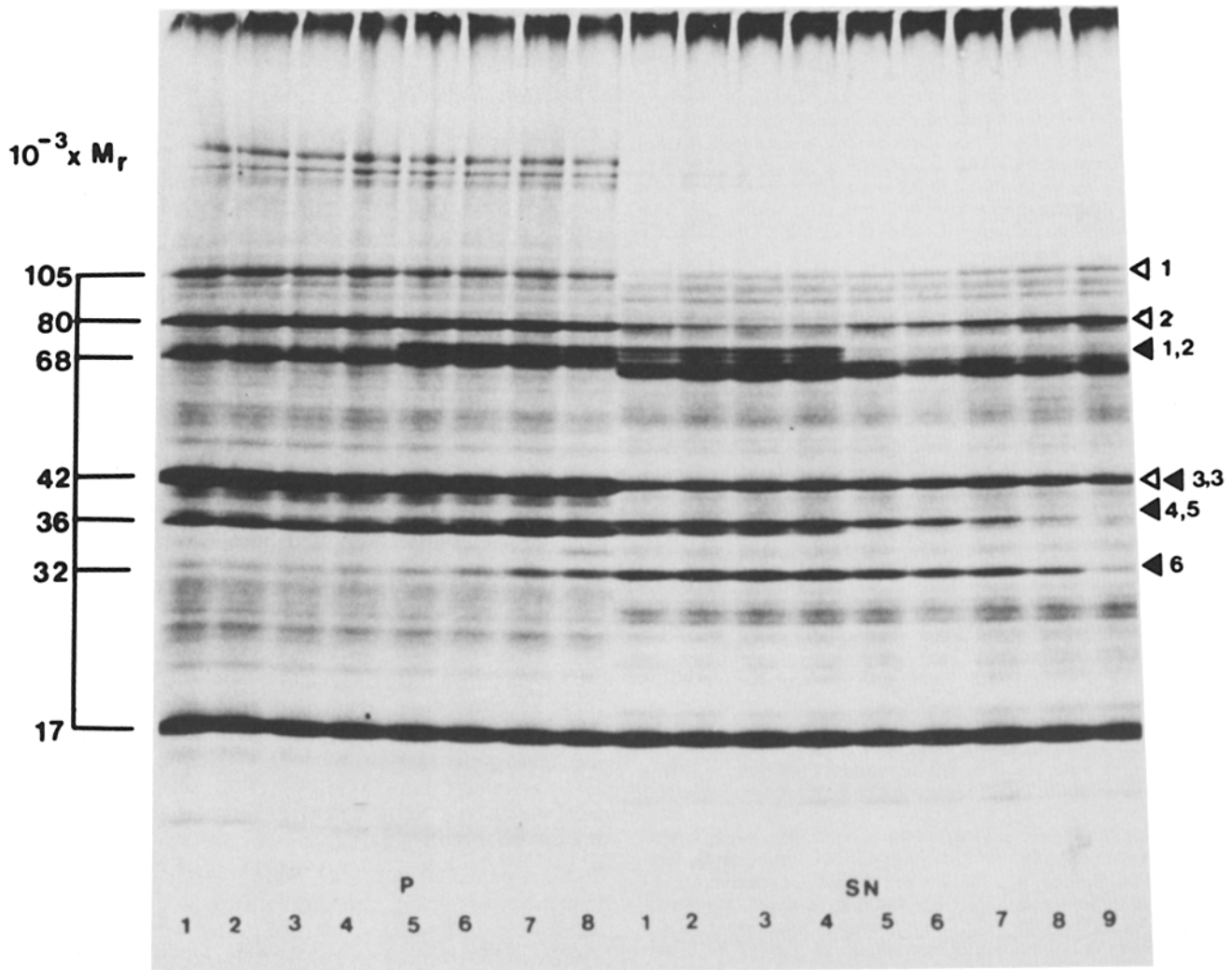
Figure 3. The effect of Ca^{2+} on the interactions of microvillar proteins with the cytoskeleton. Samples of microvilli (5 mg of protein), after centrifugation at 20,000 g for 15 min at 4°C (Sorvall SS34 rotor), were resuspended in 1 ml of Buffer A containing 5% Triton X-100 and one of a range of defined concentrations of free Ca^{2+}. Each sample was then dialyzed for 18 h at 4°C against Buffer A containing 0.1% Triton X-100 and the same concentration of free Ca^{2+}. After centrifugation at 20,000 g for 15 min at 4°C, the pellets were resuspended in 1 ml of Buffer A. Equal volumes of resuspended pellets (P) and detergent supernatants (SN) were examined by SDS-PAGE. 5 μg of hemoglobin was added to each sample as an internal standard for gel scanning. The concentrations of free Ca^{2+} were as follows: (lanes *1*) 0.1 μM; (lanes *2*) 0.3 μM; (lanes *3*) 1 μM; (lanes *4*) 3 μM; (lanes *5*) 8.6 μM; (lanes *6*) 22.5 μM; (lanes *7*) 100 μM; (lanes *8*) 300 μM; and (lane *9*) 1 mM. The Ca^{2+}-soluble proteins are indicated (*1*, α-actinin; *2*, 80,000-mol-wt protein; *3*, actin). The EGTA-soluble proteins are indicated (*1*, 72,000; *2*, 69,000; *3*, actin; *4*, 38,000; *5*, 36,000; *6*, 32,000).

(Fig. 5, lane *3*). The Ca^{2+}-soluble proteins (α-actinin, the 80,000-mol-wt-protein, and actin) did not bind in the presence or absence of Ca^{2+} (Fig. 5, lanes *5–8*).

The Ca^{2+}-dependent binding of the EGTA-soluble proteins to membrane lipid was quantified. Liposomes and proteins preequilibrated with buffers containing defined concentrations of free Ca^{2+} were mixed. After incubation at 25°C for 15 min and centrifugation, liposomes and supernatants were subjected to SDS-PAGE (Fig. 6) and scans of the gels were integrated (Fig. 7). Again we found coordinated binding of the 72,000-, 69,000-, and 36,000-mol-wt polypeptides to the lipid, with a $K_{0.5}$ value of ~50 μM, somewhat higher than the value for the binding of these proteins to the cytoskeleton. The 32,000- and 38,000-mol-wt proteins also bound to the microvillar lipid liposomes, but a $K_{0.5}$ value could not be obtained even though almost 100% of these pro-

teins was lipid bound at the highest Ca^{2+} concentration used.

The EGTA-soluble Proteins Interact with the Head Groups of Polar Lipids

Several proteins similar to the EGTA-soluble placental proteins have been shown to interact with hydrophobic matrices in a Ca^{2+}-dependent manner (18, 28, 33, 36). The p36 protein of intestinal brush borders has also been shown to interact directly with phosphatidylserine but not phosphatidylcholine in the presence of Ca^{2+} (14). In the light of this we have attempted to establish whether the proteins are binding to membrane lipid by a Ca^{2+}-dependent hydrophobic interaction or via the lipid headgroups.

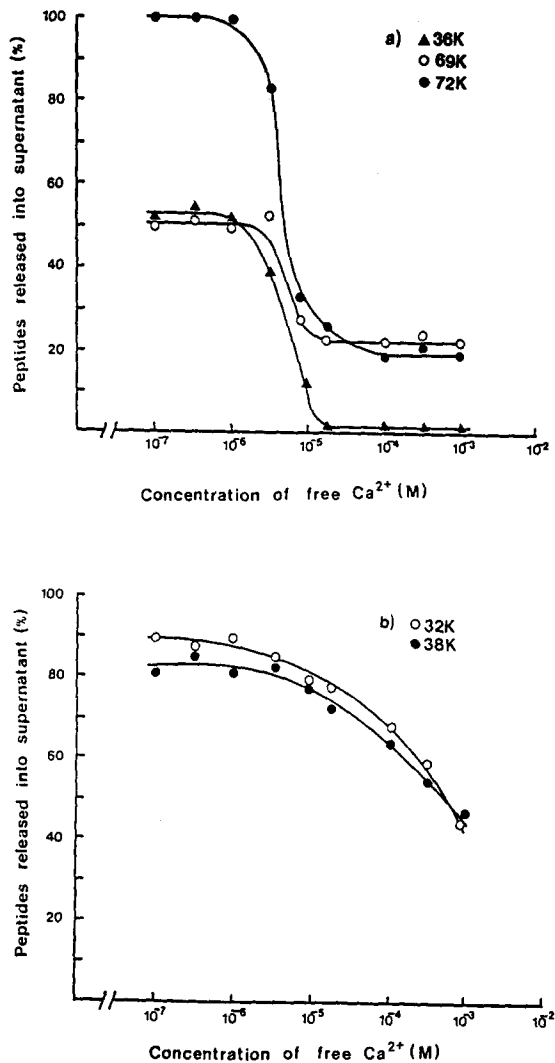When the EGTA-soluble proteins were examined by chro-

Figure 5. The interaction of Ca^{2+}-sensitive proteins of the cytoskeleton with microvillar membrane lipid. Samples of EGTA-soluble proteins (lanes *1-4*) and Ca^{2+}-soluble proteins (lanes *5-8*) equilibrated with Buffer A containing either 1 mM EGTA ($-C$) or 1 mM $CaCl_2$ ($+C$) were incubated with microvillar liposomes prepared in the same buffers, for 15 min, at 25°C. After centrifugation at 20,000 g, for 15 min, proteins in the liposomal pellets (P) and supernatants (S) were precipitated with ice-cold acetone and resuspended to an equal volume of Buffer A before examination by SDS-PAGE. Lane M contains molecular weight markers.

Figure 4. The effect of Ca^{2+} on the interactions of microvillar proteins with the cytoskeleton. Samples of cytoskeletons and detergent-soluble proteins prepared from microvilli in the presence of defined concentrations of Ca^{2+} were subjected to SDS-PAGE, as described in Fig. 3. The gels were scanned after staining and destaining and the amount of each peptide present in the supernatant was calculated as a percentage of the total using the peak areas. These results represent the means of six separate experiments. $K_{0.5}$ for the interaction of the 36,000-, 69,000-, and 72,000-mol-wt polypeptides (*a*) with the cytoskeleton was between 4 and 5 μM. The corresponding $K_{0.5}$ for the 38,000- and 32,000-mol-wt polypeptides (*b*) was between 0.1 and 1 mM.

matography on phenyl-Sepharose columns, only a small proportion of the 72,000-, 69,000-, and 32,000-mol-wt polypeptides bound in the presence of 1 mM Ca^{2+} (Fig. 8, lane *4*). In contrast, when columns were first coated with microvillar lipid almost all the proteins bound in a Ca^{2+}-dependent manner. (Almost all the actin present bound to the phenyl-Sepharose in a Ca^{2+}-independent manner. This is evident in Fig. 8.)

The interaction of the EGTA-soluble proteins and individual phospholipids was studied by using commercial preparations of phospholipids oriented on phenyl-Sepharose columns. The results are shown in Fig. 9.
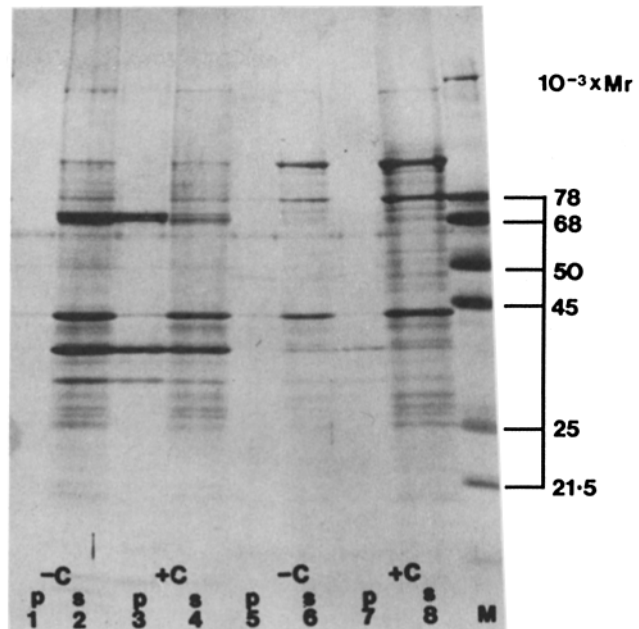
The proteins showed a marked affinity for the negatively charged phospholipids; i.e., cardiolipin, phosphatidylinositol, and phosphatidylserine, but also bound to phosphatidylethanolamine. They bind to cardiolipin, a lipid characteristically associated with the inner mitochondrial membrane, sufficiently strongly to resist elution by 2 mM EGTA. No binding was detected to phosphatidylcholine or sphingomyelin. Similar results were obtained when the immobilized lipids were replaced, where possible, by liposomes (result not shown).

Discussion

A Different Class of Microvillus?

Previous studies on the structure of placental microvillar cytoskeleton have revealed several basic differences with that of the intestinal and renal brush borders. Placental microvilli are more dynamic structures, actively involved in the processes of receptor-mediated endocytosis. This is reflected in an apparent lack of organization and uniformity and also the absence of an extensive terminal web in the underlying syncytioplasm (29). The microfilaments are much closer to the plasma membrane than those in brush borders (11, 17). Large amounts of actin are always released into the membrane supernatant on solubilization of microvilli. It is noticeable that placental actin displays an affinity for hydrophobic matrices (see Fig. 8). Similar findings have been reported elsewhere (1).
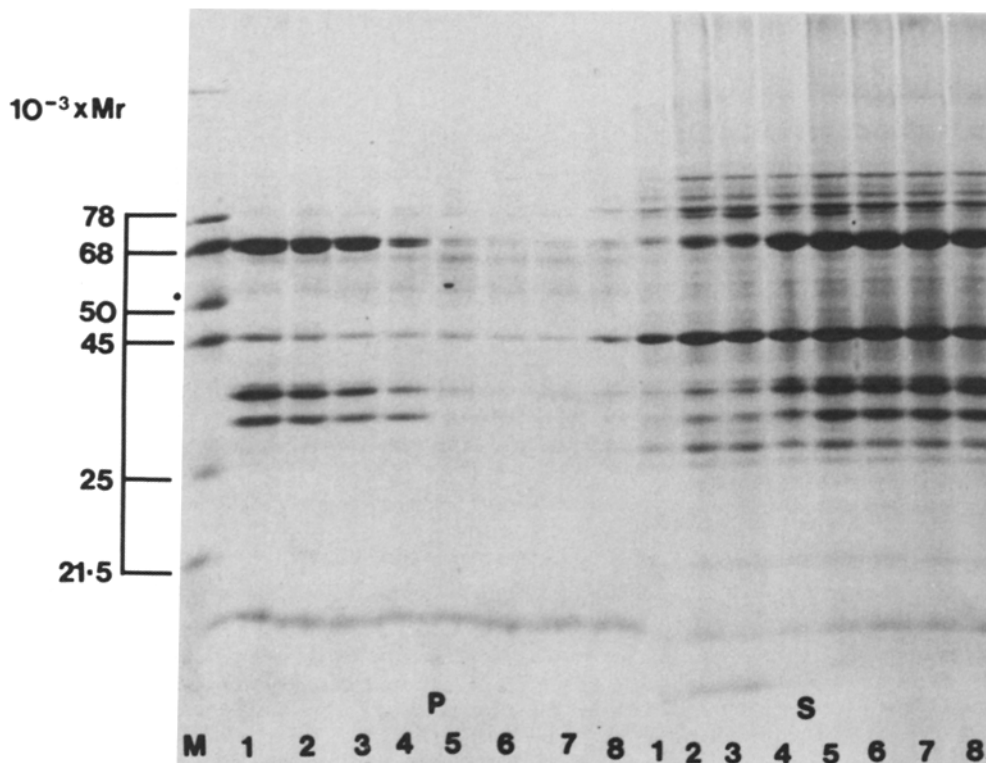
$10^{-3} \times Mr$

78
68
50
45
25
21·5

P   S

M 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Figure 6. The effect of free Ca^{2+} concentration on the interaction of the EGTA-soluble proteins with microvillar liposomes. Samples of EGTA-soluble proteins and microvillar liposomes, equilibrated with Buffer A containing defined concentrations of free Ca^{2+} were incubated together for 15 min at 25°C. After centrifugation at 20,000 *g* for 15 min, samples of the liposomal pellets (*P*) and supernatants (*S*) were examined by SDS-PAGE. The Ca^{2+} concentrations used were as follows: (lanes *1*) 1 mM; (lanes *2*) 0.3 mM; (lanes *3*) 0.1 mM; (lanes *4*) 22.5 μM; (lanes *5*) 8.6 μM; (lanes *6*) 3 μM; (lanes *7*) 1 μM; (lanes *8*) 0.3 μM. Lane *M* contains molecular weight markers.

Because of the lack of uniformity of placental microvilli, biochemical analysis has proved to be the most successful means for studying its cytoskeletal structure. We have already shown placental microvilli contain no fimbrin, villin, or the 110K protein of brush borders (1) but contain α-actinin (38), a common component of many plasma membrane–associated microfilamentous structures (7, 8, 20, 27). More recently we have shown placental microvilli to contain a group of Ca^{2+}-sensitive, membrane-binding proteins,[1] which are similar to a group of proteins described as mammalian cal-electrins (36). The placental microvillar 36,000- and 32,000-mol-wt proteins also show similarities to protein I and protein II of the intestinal epithelial cell (where they are localized in the terminal web region [13]). From this and other evidence we suggest placental microvilli represent a different class, perhaps more closely aligned to the α-actinin-containing microvilli of ascites cells (8).

Protein–Lipid Interaction

Several of the proteins that show similarities to the placental microvillar EGTA-soluble proteins are purified using hydrophobic adsorptive techniques in the presence of Ca^{2+} (12, 33, 36). One reason for this may be an increase in the hydrophobic nature of the proteins on binding Ca^{2+} (33), in a similar manner to that reported for calmodulin (18, 23, 28). Alternatively, it has been suggested that this effect is caused by contaminating membrane lipids (12). Our results would seem to support the latter, since the placental EGTA-soluble proteins require the presence of lipid in order to bind to phenyl–Sepharose columns and do not become intrinsically hydrophobic in the presence of Ca^{2+}.

We suggest that the proteins interact with the lipid headgroups for two reasons. First, the lipids immobilized on the phenyl–Sepharose will be oriented with their headgroups

facing the medium. It is unlikely that the proteins interact with the hydrophobic portions of the immobilized lipids, since they do not bind to the phenyl–Sepharose matrix. Secondly, the proteins showed no binding with the positively charged phospholipids, phosphatidylcholine and sphingomyelin, clearly indicating interaction with the headgroups. It is of interest that phosphatidylcholine and sphingomyelin are more commonly associated with the outer layer of the plasma membrane and the other lipids studied, with the inner layer. The actual mechanism of binding is unclear; it might involve direct protein–headgroup interaction or it might involve Ca^{2+} cross-links between the lipid and the protein. The former mechanism is certainly possible, since two of the proteins (the 36,000- and the 38,000-mol-wt proteins) are basic[1] and would carry net positive charge at physiological pH values. The latter mechanism has been demonstrated for the binding of blood-clotting factor V to acidic phospholipids (30, 32).

Little is presently known about the individual lipid-binding characteristics of the EGTA-soluble proteins. The p36 protein of intestinal brush borders has been shown to bind to phosphatidylserine (14, 22). It is possible that one or more proteins acts as an anchor by binding to lipid in the presence of Ca^{2+}, with the remainder binding on to this, perhaps in a cooperative manner.

Regulation of Cytoskeletal Structure by Ca^{2+}

Various events affecting cytoskeletal structure are regulated by Ca^{2+}. Although many of these events are thought to be mediated by calmodulin we have shown that at least in the case of the Ca^{2+}-sensitive release of α-actinin from cytoskeletons this is not the case (38). There is now increasing evidence for the direct involvement of a wide range of Ca^{2+}-sensitive proteins in the structure of the cytoskeleton and its
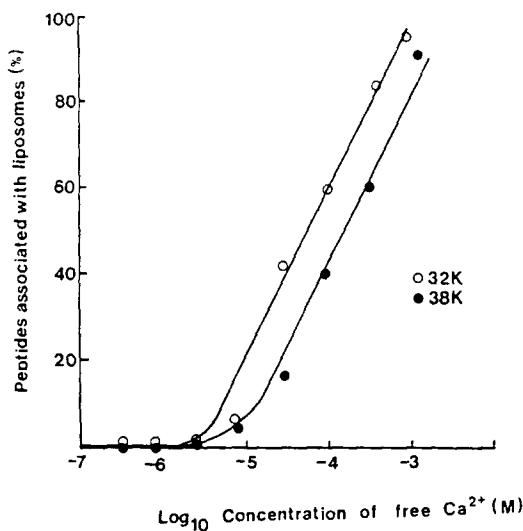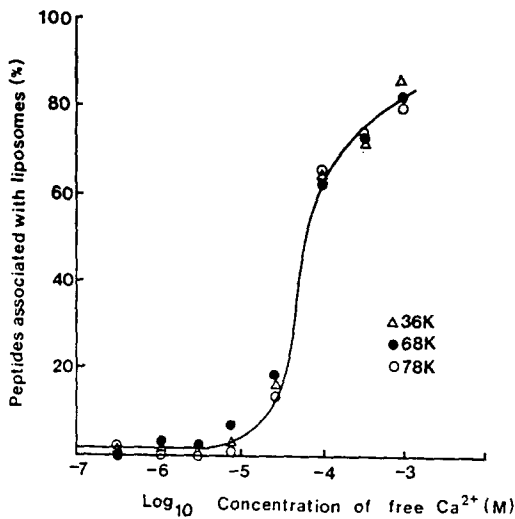
Figure 8. Phenyl–Sepharose chromatography of EGTA-soluble proteins. Samples of EGTA-soluble proteins (1 mg/ml) (prepared as described in Materials and Methods), equilibrated with Buffer A containing either 1 mM EGTA (−C) or 1 mM CaCl₂ (+C), were applied to phenyl–Sepharose columns (2 ml) equilibrated with the same buffer. After applying the samples, the columns were further washed with 10 ml of the same buffer. Samples in EGTA were eluted with 10 ml Buffer A containing 2% Triton X-100. Samples in Ca²⁺ were eluted with 10 ml Buffer A containing 2 mM EGTA and then with 10 ml Buffer A containing 2% Triton X-100. The experiment was repeated using columns coated with microvillar lipid. Suspensions of microvillar lipid (1 mg/ml) in Buffer A, were mixed with phenyl–Sepharose for 5 min at 25°C. After columns were poured, they were washed extensively with the appropriate starting buffer (lanes 6–10), before samples were applied. Samples of protein not adsorbed to the column in starting buffer (lanes 1, 3, 6, and 8), proteins eluted by EGTA (lanes 4 and 9), and proteins eluted with detergent (lanes 2, 5, 7, and 10) were examined by SDS-PAGE. Lane M contains molecular weight markers.

Figure 7. The effect of free Ca²⁺ concentration on the interaction of the EGTA-soluble proteins with microvillar liposomes. Samples were subjected to SDS-PAGE as described in the legend to Fig. 4. After scanning, the percentage of individual EGTA-soluble proteins associating with the liposomes (pellets) was quantified. These results represent the means of three experiments.

regulation, with calmodulin relegated to the role of Ca²⁺ buffer (15, 16).

We have previously suggested that α-actinin and the proteins now identified as the EGTA-soluble proteins were behaving in a manner similar to the flip-flop exchange of proteins described by Sobue et al. (34, 35). In the light of the results described in this paper, we now propose a series of events which might take place if the localized intracellular concentration of free Ca²⁺ was to increase. The values for half-maximal binding of α-actinin to cytoskeletons (38), EGTA-soluble proteins to cytoskeletons, and EGTA-soluble proteins to membrane lipid are 0.3, 4, and 50 μM, respectively. We suggest that if the local concentration of Ca²⁺ was to increase, the first cytoskeletal event would be the release of α-actinin from the actin microfilaments. Nonmuscle α-actinin has been shown to act as an actin cross-linking protein (6, 9, 25, 26). There is some evidence for it forming a
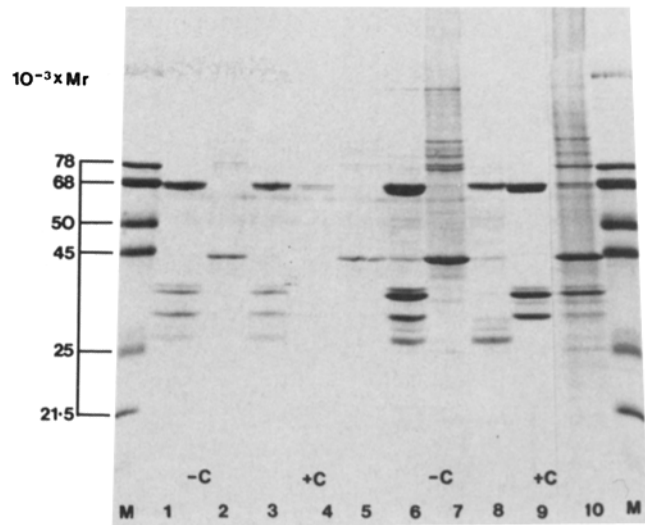
direct link between actin and the membrane (5); however the placental protein showed no ability to bind to membrane lipid (Fig. 5). If the local Ca²⁺ concentration was to rise further, the EGTA-soluble proteins would bind to the cytoskeleton. At local Ca²⁺ concentrations of 50 μM and above, the actin filaments would become linked to the membrane. This would provide a mechanism for the controlled interaction of microfilaments with plasma membrane components. It would also provide a mechanism for the rapid assembly and disassembly of microvilli in response to the events of endo- and exocytosis occurring at the plasma membrane.

Do These Events Happen under Physiological Conditions?

At least the first two events, the release of α-actinin and the association of the EGTA-soluble proteins, occur at micromolar levels of Ca²⁺, a level of Ca²⁺ which might be achieved intracellularly. However, the binding to lipid seems to require rather high concentrations, ∼50 μM, and it is not clear whether this concentration would ever be attained in any intact cell. Since this study has only examined the sensitivity of these proteins to Ca²⁺, we cannot rule out other forms of regulation. For instance, in the case of the P36 protein of

$10^{-3} \times M_r$

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a b c a b c a b c a b c a b c a b c

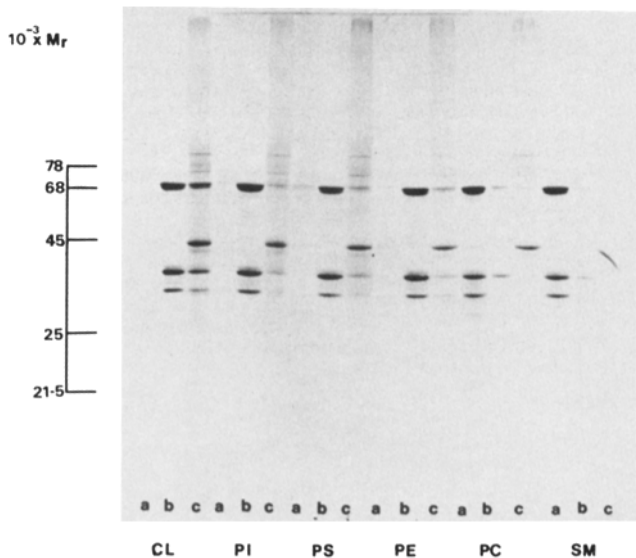CL   PI   PS   PE   PC   SM

Figure 9. Binding of EGTA-soluble proteins to individual phospholipids. Samples of EGTA-soluble proteins equilibrated with Buffer A containing 1 mM CaCl₂, were applied to phenyl–Sepharose columns (2 ml), which had been coated with 2.5 mg of phospholipid (as described in the legend to Fig. 8), equilibrated with the same buffer. After washing with 10 ml of starting buffer, adsorbed proteins were eluted, first with 10 ml of Buffer A containing 2 mM EGTA and then with 10 ml of Buffer A containing 2% Triton X-100. Peak samples of unadsorbed protein (lanes *a*), proteins eluted by EGTA (lanes *b*) and proteins eluted by 2% Triton X-100 (lanes *c*), were examined by SDS-PAGE. Phospholipids were cardiolipin (*CL*), phosphatidylinositol (*PI*), phosphatidylserine (*PS*), phosphatidylethanolamine (*PE*), phosphatidylcholine (*PC*), and sphingomyelin (*SM*).

brush borders it has been demonstrated that the presence of phosphatidylserine increased the affinity of this protein for Ca^{2+} by two orders of magnitude (14). There is also evidence for regulation by phosphorylation and dephosphorylation. The placental microvillar proteins with molecular weights of 80,000 and 36,000 have been shown to cross-react immunologically with ezrin (Edwards, H. C., and A. G. Booth, unpublished result) and P36[1] of the intestinal brush border, respectively. Both these proteins identify with cellular substrates for tyrosine kinases (13, 19), often associated with the receptors of growth factors, several of which are present in the placenta microvillar plasma membrane. Moreover, recent studies report similarities between the P36 protein and lipocortins, phospholipase A2 inhibitors (21) with possible involvement in membrane breakdown and fusion.

We do not as yet know whether factors such as phosphorylation or lipid-binding would affect the sensitivities of the placental proteins to Ca^{2+}. However, it is clear that Ca^{2+} at least plays a major role in the regulation of placental microvillar structure and function.

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