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K. Weber, J. R. Glenney and D. M. Shotton

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Microfilament–membrane interaction: the brush border of intestinal epithelial cells as a model

By K. Weber and J. R. Glenney, Jr
Max Planck Institute for Biophysical Chemistry, D-3400 Göttingen, F.R.G.

The intestinal epithelium provides an excellent starting material for the isolation of natural microfilament organizations in amounts suitable for biochemical studies. The microvillus filament bundle core and the terminal web provide two distinct microfilament systems. We review the current knowledge of the filament bundle core and the attempts that have been made to reconstitute this structure from actin and its four major associated proteins. We show in addition that a high molecular mass actin-binding protein (TW-260/240) having spectrin-like properties is, next to myosin, the major associated protein of the terminal web retained in isolated brush borders. We summarize the biochemical and morphological evidence for the existence of a class of spectrin-related molecules in the cortical cytoplasm of many cell types. These findings may lead to a new understanding of membrane–microfilament interactions.

The microvillus cytoskeleton

The intestinal epithelial cell is polarized in many structural and functional aspects. Its upper part, dominated by microfilaments and various membrane specializations, can be isolated as a structurally intact unit. Such *brush borders* reveal two distinct microfilament systems. The 1000 microvilli that normally protrude into the lumen are stabilized by a filament bundle core of some twenty F-actin filaments of the same polarity as shown by myosin S_1 decoration (Begg *et al.* 1978). Below the level of the terminal web lies an anastomosing network of filaments (Hull & Staehelin 1979). This terminal web system anchors not only the rootlets of the microvillus cores but is itself anchored at the membrane by a circumferential belt of *zonula adhaerens* specializations (Farquhar & Palade 1963), and probably provides a tension within the epithelial sheet.

Microvilli are dislodged from the brush border by the application of shearing forces and they can be isolated as a homogeneous population of intact microvilli retaining the membrane. As shown in 1978 (Bretscher & Weber 1978*a*) they retain end-on attachment of the filament bundle core at the tip as well as side-on membrane attachment. In isolated microvilli slender whiskerlike protrusions span the distance between the filament bundle and the inner side of the plasma membrane covering a distance of some 30 nm. Demembranation with Triton X-100 provides a pure filament bundle preparation suitable for biochemical analysis, which reveals actin and four major associated proteins.

Immunofluorescence microscopy performed on total intestinal epithelial cells (Bretscher & Weber 1978*b*) showed that terminal web microfilaments and microvillus filament cores are distinct entities. Myosin, α-actinin and tropomyosin, known to be components of stress-fibre microfilament bundles of various cultured cells, are notably absent from microvilli and only found in the terminal web. As expected, actin antibodies decorate both microvilli and terminal web, giving rise to a much broader fluorescent zone. Since α-actinin was at that time widely

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discussed as F-actin membrane anchor (Mooseker & Tilney 1975) negative results on microvilli were startling and demanded a more detailed analysis.

Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis of isolated microvillus filament bundle cores confirmed the immunological results. The four major associated proteins present in addition to actin (see table 1) were distinct from any of the associated protein of other microfilaments known at that time. They are calmodulin (16 kDa protein), fimbrin (68 kDa protein), villin (95 kDa protein) and a 110 kDa component (Glenney & Weber 1980).

TABLE 1. MAJOR STRUCTURAL COMPONENTS OF ISOLATED MICROVILLI AND TERMINAL WEB CYTOSKELETAL ORGANIZATIONS

polypeptide	M_r/kDa	binding target	function
		microvillus	
calmodulin (C)	16	110	Ca^{2+} buffer??
actin (A)	43	F, V, 110	major structural component
fimbrin (F)	65	A	actin bundling
villin (V)	95	A	actin bundling/severing
110 kDa protein	110	A, C	membrane anchor
		terminal web	
calmodulin (C)	16	TW-240	?
actin (A)	43	F, V, (260/240), M	major structural component
fimbrin (F)	65	A	actin bundling
villin (V)	95	A	actin bundling/severing
myosin (M)	200	A	generation of tension?
TW-240	240	A, C	actin cross-linking
TW-260	260	A	actin cross-linking

Calmodulin

This protein is considered by enzymologists to be the ubiquitous Ca^{2+}-dependent modulator protein of various Ca^{2+}-regulated key enzymatic activities including various kinases such as phosphorylase kinase or myosin kinase (Cheung 1980). Although the initial biochemical experiments identifying the 16 kDa component of microvilli as calmodulin were very extensive (Howe *et al.* 1980; Glenney & Weber 1980), the question remained if calmodulin was not present due to artefacts of cell fractionation procedures. Using an antibody specific for calmodulin on total intestinal cells we showed that the microvilli region is intensely stained – a result in accord with the fractionation experiments (Glenney & Weber 1980).

The 110 kDa *protein*

Matsudaira & Burgess (1979) were the first to suggest that the 110 kDa protein is a major constituent of the side-arm structure of the filament bundle core. The exposure of bundles to ATP leads, for unexplained reasons, to smooth bundles without protrusions. When analysed by SDS–polyacrylamide gels such bundles have lost the 110 kDa protein. We subsequently showed that treatment of normal bundles with trifluoperazine, a calmodulin-specific drug, removes calmodulin without affecting the core ultrastructure, including the side arms (Glenney *et al.* 1980). Such calmodulin-depleted bundles can be recharged in a saturable fashion with exogenously supplied brain calmodulin. We then developed a new gel-overlay assay to detect

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calmodulin-binding polypeptides separated on an SDS–polyacrylamide gel (Glenney & Weber 1980). This assay revealed that the 110 kDa protein is the major calmodulin-binding protein of the microvillus cytoskeleton; later biochemical studies have upheld this proposal. Further analysis is hampered by our inability to obtain pure and native 110 kDa protein owing to its insolubility. Only when native purified 110 kDa protein becomes available can we expect to study this microfilament-associated protein as an example of a protein providing direct plasma membrane anchorage, and study its interactions with calmodulin, the filament bundle core, the plasma membrane and ATP. For the present we have to be content with ferritin immuno-electron microscopic studies (Glenney *et al.* 1982*a*), which indicate that the 110 kDa protein is present only along that part of the microvillus filament bundle core that *in situ* is in apposition to the plasma membrane. That is, the 110 kDa protein is not found in the terminal web, in agreement with the model of the 110 kDa protein as the cross-bridge.

Fimbrin

This is an F-actin bundling protein, of molecular mass 65 kDa, preferentially found in submembranous microfilaments underlying cellular extensions.

Originally, an antibody was raised against denatured fimbrin purified by SDS–polyacrylamide gel electrophoresis (Bretscher & Weber 1980*b*). This antibody revealed that fimbrin is a common component of many cell types. It is found as a component of ruffles, microvilli and microspikes. In addition we found fimbrin to be localized in the stereocilia present on the sensory hair cells of the hearing organ (Bretscher *et al.* 1982) as well as within the growth cone of cultured neurons (Shaw *et al.* 1981). Thus fimbrin was the first microfilament-associated protein with a strong preference for membrane extensions in comparison to stress fibres of cultured cells, where it is much less pronounced.

Fimbrin has now been purified in homogeneous native form (Glenney *et al.* 1981*c*; Bretscher 1981). It is a monomeric, nearly globular molecule. Although fimbrin does not seem to bind to G-actin, it has now been characterized as an F-actin-binding protein by sedimentation analysis. Under optimal conditions F-actin filaments reveal saturation at an approximate molar ratio of 1 fimbrin to 5–10 actin monomers in the F-state, a value close to the natural abundance of fimbrin in the isolated filament bundle cores. Electron microscopical analysis of such material shows reconstituted bundles of actin filaments similar to the mirovillus filament bundle core.

Villin

Purified villin is a monomeric protein of molecular mass 95 kDa (Bretscher & Weber 1980*a*), with a high-affinity Ca^{2+}-binding site, for which Mg^{2+} can only poorly compete (Glenney *et al.* 1980). In the presence of Mg^{2+} at millimolar concentrations, the Ca^{2+} dissociation constant is in the micromolar range. Villin has two morphologically and functionally distinct effects on F-actin organization governed by the presence or absence of Ca^{2+} and the molar ratio of villin to actin (Bretscher & Weber 1980*a*). This is best shown by electron microscopy.

At Ca^{2+} levels below 0.1 μM, villin added at a molar ratio of 1 villin to 5 or 10 actins, a ratio resembling its natural abundance in the microvillus, leads to long, tightly packed F-actin bundles. Such bundles are obtained by either adding villin to preformed F-actin or allowing polymerization to occur in the presence of villin. We have now analysed some of the biochemical properties of villin that one would expect for certain bundling proteins. It was found, for instance,

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that mild proteolysis *in vitro* with V_8 protease creates two fragments, a large N-terminal core of molecular mass 87 kDa and a small headpiece, which represents the carboxyterminal 76 residues of villin (Glenney & Weber 1981; Glenney *et al.* 1981 *a*). Both the core and headpiece contain an actin binding site but neither can bundle F-actin in the absence of free Ca^{2+}.

Thus both fimbrin and villin act as F-actin bundling proteins in buffers low in Ca^{2+}, in agreement with their probable function as structural regulators of the microvillus filament bundle core. We have recently been able to assemble three types of bundles *in vitro* (Glenney *et al.* 1981 *b*): fimbrin-induced, villin-induced and bundles containing both cross-linking factors. Such bundles are now undergoing detailed structural analysis and provide the first successful step towards the full reconstitution of the microvillus filament bundle core.

A totally different result is obtained in the presence of micromolar concentrations of Ca^{2+}, even at a lower villin:actin ratio. Instead of bundles or the long filaments obtained with actin alone, we observe a larger number of much shorter filament fragments (Bretscher & Weber 1980 *a*; Glenney & Weber 1981; Mooseker *et al.* 1980; Craig & Powell 1980; Matsudaira & Burgess 1982). This result is obtained by starting either with preformed F-actin or by salt-induced actin assembly. Thus in the presence of free Ca^{2+} at micromolar concentrations or above, villin is a regulator of F-actin filament length. This mechanism indicates either a Ca^{2+}-dependent severing activity or an enhanced nucleation mechanism. We have in fact found that both mechanisms seem to apply.

The nucleation activity of villin is exceedingly potent and can be easily detected at molar villin:actin ratios of as low as 1:1000. Villin can readily form low molecular mass complexes with monomeric actin that are just as potent as villin itself in nucleating actin filament assembly. Upon nucleation of actin filaments, villin remains bound to, and caps, the barbed end of the filament it has nucleated, resulting in unidirectional elongation at the free pointed ends. Thus, ideally, every villin molecule will nucleate a filament and the number of filaments will depend on the number of villin molecules provided. Because the original pool of G-actin has been kept constant, many more but correspondingly shorter filaments will be seen, as shown by electron microscopy.

Villin also shortens preformed F-actin filaments in a manner dependent on calcium and concentration. This process is not likely due to enhanced nucleation but could be explained by fragmentation of F-actin caused by viscous shear during mixing with subsequent capping at the free barbed ends, resulting in an inhibition of reannealing. There are several experiments that indicate a more direct action, i.e. F-actin 'severing'. The most direct is the immediate distintegration of microvillus filament bundle cores upon addition of calcium (Glenney *et al.* 1980; Mooseker *et al.* 1980). Since such cores are stable in EGTA and not readily amenable to weak shearing forces, and already contain villin as structural protein, the possibility of direct severing is indicated.

The function of villin's nucleation and severing activity *in vivo* has so far escaped definition. It should be remembered that for the very short lifetime of an intestinal epithelial cell (2–3 days) the microvilli and their cores seem to be stable structures and are probably not involved in dynamic processes. It is important to note, however, that relatively little is known about two important events: (1) the original morphogenesis of the brush border when the cell undergoes terminal differentiation in the crypt position of the villus, and (2) the process by which cells are sloughed off at the tip of villi. Thus, consider that millions of cells are assumed to be generated and destroyed every day in the intestine of a healthy human. It is possible that the forma-

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tion of the brush border requires a Ca^{2+} signal allowing nucleation or an F-actin shuttle mediated by villin. Additionally, the process of cell sloughing may somehow be related to an intracellular Ca^{2+} imbalance leading to a trigger of villin as a Ca^{2+}-activated 'time-bomb' resulting in the disintegration of the brush border due to F-actin severing.

For many years textbooks and reviews have propagated a report of Mooseker (1976), considered to be crucial, for active movement of microvilli due to an actomyosin-based contraction. It was reported that demembranated brush borders contract their microvilli cytoskeletons into and through the level of the terminal web when exposed both to ATP and micromolar calcium. The Ca^{2+}-activated F-actin severing activity of villin makes this highly unlikely and so does the dissolution of microvillus filament bundle cores upon addition of Ca^{2+}. A recent re-evaluation of the experiments (Burgess 1982) has now directly verified the absence of contraction and the occurrence of filament core disintegration.

Thus, we currently envisage the filament bundle cores as a stabilizer of the extended microvillus plasma membrane and we see no unambiguous experiment pointing directly towards microvillus motility. The terminal web organization of the isolated brush-border cell may well be in a state of isometric tension providing a structural scaffold to keep microvilli erect. In addition, if there were contraction within the web of the one cell a different final result is indicated for junctionally connected epithelial sheets in which the individual forces through most of the sheet will equalize each other. Thus we should be careful not always to envisage contractility and movement when we find organized actin and myosin somewhere in the neighbourhood.

The terminal web

The terminal web organization has been much less biochemically explored than the microvillus. Electron microscopy indicates an intricate network of filaments surrounding the rootlets of the microvilli and a particularly rich display of microfilaments at the *zonula adhaerens* type of membrane specializations (Hull & Staehelin 1979). Earlier immunofluorescence data (Bretscher & Weber 1978*b*) identified myosin, α-actinin and tropomyosin to be microfilament-associated proteins in the terminal web, and a later-isolated 130 kDa actin-binding protein called vinculin is also present and has been discussed as possible membrane anchor by Geiger *et al.* (1980). Isolated brush-border preparations retain less of this complexity of proteins but have still a well displayed organization in the electron microscope. SDS–polyacrylamide gels reveal, in addition to the microvillus filament core proteins, three to four high molecular mass proteins, which are structural components of the terminal web (Bretscher & Weber 1978*a*; Glenney *et al.* 1982*b*). These have molecular masses of 260, 240 and 150 kDa in addition to the myosin heavy chain (200 kDa). All these polypeptides are retained after dembranation with Triton X-100 in buffers preserving the ultrastructure and must therefore have a strong influence on the organization of the terminal web.

We have recently purified the two high molecular mass (260 and 240 kDa) polypeptides, and found that the two proteins form a stable complex abbreviated as TW-260/240, with TW standing for terminal web (Glenney *et al.* 1982*b*). Complex formation is indicated by the following experiments.

1. Both proteins co-sediment at 11 *S* and co-elute at a Stokes radius higher than 200 nm and must therefore have a highly asymmetric shape.

2. Both proteins bind F-actin and become pelletable as gelation or cross-linking factors in

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low-speed and high-speed centrifugation assays and densitometry indicates a molar ratio of 1:1 for TW-260 and TW-240. The native molecular mass of the isolated complex points to a value of close to 1 MDa, i.e. a tetramer containing two copies each of TW-260 and TW-240.

Antibodies raised against TW-260/240 reveal in immunofluorescence microscopy an intense labelling of the terminal web and also submembranous labelling along the lateral sides of the intestinal epithelial cells. A similar display is also seen in frozen sections, which indicate the presence of antigenic sites in other cell types (Glenney *et al.* 1982*b*). Thus a submembranous location is indicated in chick embryo fibroblasts and HeLa cells, as well as other cell lines. In all cases the fluorescence pattern is distinctly different from the stress-fibre organization known to occur in most of these cell types. Frozen sections also show strong staining in the cortical cytoplasm of axons in nerves and nerve bundles as well as cultured neurons. These images are strikingly similar to those seen recently by Levine & Willard (1981). They isolated two high molecular mass proteins termed fodrin, from total brain tissue, and after final purification by preparative SDS–polyacrylamide gels raised an antibody in rabbits and described a submembranous display of fodrin in many cell types. Because of the similar images obtained we purified native fodrin and compared intestinal TW-260/240, brain fodrin and red blood cell spectrin. The results can be summarized as follows.

1. All three proteins are heterodimers and contain one subunit at 240 kDa, whereas the other can be either larger as in TW-260/240 or smaller as in spectrin (220 kDa) or in fodrin (235 kDa). Direct molecular mass determination identifies fodrin as a tetramer of molecular mass 1 MDa (Glenney *et al.* 1982*d*).

2. All three proteins bind to F-actin and cross-link F-actin as assayed by low shear viscosity and high-speed or low-speed sedimentation of the resulting gel.

3. Results with various antibodies against fodrin and TW-260/240 in immuno blotting show that, although for each individual protein there is some cross-reaction between the two different chains, cross-reactivities between different proteins seem so far always to involve the so-called common 240 kDa subunits corresponding to the α-chain of spectrin (Glenney *et al.* 1982*c*). This leads to the assumption that the three 240 kDa subunits are closely related although probably not identical.

4. One-dimensional fingerprints of partial digestion with V_8 protease show that there is a strikingly common fragmentation pattern for all three 240 kDa subunits (Glenney *et al.* 1982*c*). The second subunits are much more different both between themselves and in comparison with the 240 kDa subunits. That these subunits must be somehow related is indicated by amino acid composition data and electron microscopy.

5. All three proteins bind calmodulin in a calcium-dependent manner when assayed by the ^{125}I-gel overlay assay, which indicates that this binding resides in the common 240 kDa subunit (Glenney *et al.* 1982*b*, *c*).

6. All three proteins are seen after rotary shadowing as long flexible double-stranded structures related to tetrameric human spectrin (Glenney *et al.* 1982*b*). However, we note, in spite of these similarities, reproducible morphological differences. Thus, TW-260/240 shows relatively loosely intertwined strands in which gaps between the strands are common. Fodrin, however, reveals a much tighter association of the strands with very few gaps except in fortuitous images. The contour length is about 200 nm for both fodrin and tetrameric spectrin but 260 nm for TW-260/240. In each case the population of molecules is quite homogeneous.

Why did we and others not detect spectrin-related molecules outside erythroid cells earlier,

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when antibodies to human spectrin were used on various cells in 1976? The answer seems to lie in the fact that at least some of these antibodies were strictly spectrin-specific (Glenney & Weber, unpublished), thus most probably even the common 240 kDa subunits of spectrin, fodrin and TW-260/240 are not identical.

The spectrin-related family of high molecular mass actin-cross-linking proteins is clearly distinct from smooth muscle filamin (Wang & Singer 1977) and macrophage ABP, which have been analysed in detail by Hartwig & Stossel (1981). Filamin is a homodimer of 250 kDa subunits. Filamin does not react with any of the antibodies against the spectrin family and these proteins do not react with anti-filamin antibodies (Glenney *et al.* 1982*b*). Filamin can be present in stress fibres, whereas fodrin and TW-260/240-related antigens seem to avoid stress fibres. Filamin seems not to bind calmodulin. Future experiments have to evaluate the presence and absence of filamin- and spectrin-related proteins in various cell types *in situ*.

In the brush border TW-260/240 may not only be submembranously organized but could perhaps also anchor neighbouring microvillus filament bundle cores. Electron micrographs, especially those of Hirokawa & Heuser (1981), indicate fine thin filaments in these positions that have often been considered to be solely myosin but clearly also could at least in part be TW protein, both by diameter and morphology.

Although there are now many avenues to be explored, the presence of spectrin-related molecules in non-erythroid cells may lead us to some novel aspects of submembranous actin, including possible interactions with a spectrin-binding protein, ankyrin, found in non-erythroid cells (Bennett & Davis 1981). In addition the well established red blood cell cytoskeleton may lead us to some important insights into the submembranous actin organization in non-muscle cells and perhaps to some better understanding of membrane anchorage of microfilaments and possibly the state of actin in the red blood cell.

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Discussion

D. M. SHOTTON (*Department of Zoology, University of Oxford, U.K.*). If these spectrin-like molecules are not found at the microvillus tip, what holds the ends of the actin microfilaments to the membrane at this point?

K. WEBER. Although α-actinin was previously proposed to serve this end-on anchorage role (Mooseker & Tilney 1975), immunofluorescence microscopy (Bretscher & Weber 1978*b*) as well as subcellular fractionation experiments (Bretscher & Weber 1979) have shown that α-actinin is not present in the microvillus. The 110 kDa protein, now thought to be the microfilament-membrane cross-bridge protein in the microvillus (Matsudaira & Burgess 1979), is not concentrated at the tip region as shown by immunoelectron microscopy (Glenney *et al.* 1982*a*). At present we have no further evidence to suggest what protein might anchor the microfilament at the microvillus tip; however, it should be noted that such a protein may be present in very small amounts and below the level of detection of the methods we are currently using.

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