Studies on the Spectrin-like Protein from the Intestinal Brush Border, TW 260/240, and Characterization of Its Interaction with the Cytoskeleton and Actin

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ABSTRACT The terminal web of the intestinal brush border contains a spectrin-like protein, TW 260/240 (Glenney, J. R., Jr., P. Glenney, M. Osborne, and K. Weber, 1982, *Cell*, 28:843–854.) that interconnects the "rootlet" ends of microvillar filament bundles in the terminal web (Hirokawa, N., R. E. Cheng, and M. Willard, 1983, *Cell*, 32:953–965; Glenney J. R., P. Glenney, and K. Weber, 1983, *J. Cell Biol.*, 96:1491–1496). We have investigated further the structural properties of TW 260/240 and the interaction of this protein with actin. Salt extraction of TW 260/240 from isolated brush borders results in a loss of terminal web cross-linkers primarily from the apical zone directly beneath the plasma membrane. Morphological studies on purified TW 260/240 using the rotary shadowing technique confirm earlier results that this protein is spectrin-like and is in the tetrameric state in buffers of low ionic strength. However, examination of TW 260/240 tetramers by negative staining revealed a molecule much straighter and more uniform in diameter than rotary-shadowed molecules. At salt concentrations at (150 mM KCl) and above (300 mM KCl) the physiological range, we observed a partial dissociation of tetramers into dimers that occurred at both 0° and 37°C. We also observed (in the presence of 75 mM KCl) a concentration-dependent self-association of TW 260/240 into sedimentable aggregates.

We have studied the interaction of TW 260/240 with actin using techniques of co-sedimentation, viscometry, and both light and electron microscopy. We observed that TW 260/240 can bind and cross-link actin filaments and that this interaction is salt- and pH-dependent. Under optimum conditions (25–75 mM KCl, at pH 7.0) TW 260/240 cross-linked F-actin into long, large-diameter bundles. The filaments within these bundles were tightly packed but loosely ordered. At higher pH (7.5) such bundles were not observed, although binding and cross-linking were detectable by co-sedimentation and viscometry. At higher salt (>150 mM KCl), the binding of TW 260/240 to actin was inhibited. The presence of skeletal muscle tropomyosin had no significant effect on the salt-dependent binding of TW 260/240 to F-actin.

The ubiquitous presence of spectrin-like proteins in the "cortical" cytoplasm of cells has been recently established by numerous biochemical and immunological studies on a wide variety of cell types and tissues (reviewed in references 1 and 2). By analogy with erythrocyte spectrin (for review see reference 3), it is likely that these spectrin-like proteins play important roles in the cytostructural matrix associated with the plasma membrane and perhaps with certain intracellular membranes as well. Among the best characterized of the nonerythroid "spectrins" with respect to biochemical properties are proteins purified from intestinal epithelial cells (4) and from brain tissues by several laboratories variously referred to as fodrin (5, 6), calspectin (7), or brain spectrin (8, 9).

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Taken together, these biochemical and structural studies on spectrin-like proteins indicate that like bona fide spectrin these proteins have diverse functional properties. They bind to and cross-link actin filaments (4, 6–9). They have the potential, at least, to bind specifically to membranes, as determined by binding to inside-out membrane vesicles from erythrocytes (8, 9). Finally, they bind to, in a calcium-dependent fashion, the ubiquitous regulatory protein, calmodulin (7, 9–14).

With respect to structure-function relationships, among the best understood of these spectrin-like proteins is that found in the brush border of chicken intestinal epithelial cells, termed TW 260/240 by Glenney et al. (4) who first purified and characterized this protein. The apical brush border surface of intestinal epithelial cells consists of a tightly-packed array of microvilli, each of which contains a supporting bundle of actin filaments, cross-linked by at least two bundling proteins, fimbrin (15–17) and villin (18–22). The basal or "rootlet" ends of these bundles descend below the plasma membrane into the apical cytoplasm of the cell. This region is called the terminal web because of the dense meshwork of filamentous material that interdigitates with and presumably cross-links adjacent core rootlets to each other (for studies on the structure of the terminal web see references 23–28). Results of both biochemical (4) and immunological studies (see below) indicate that TW 260/240 is exclusively localized in the terminal web region. Electron microscopy-immunolocalization studies using antibodies to either TW 260/240 (29) or brain fodrin (30) indicate that TW 260/240 (or its equivalent in mammalian brush borders) comprises at least one type of "interdigitating" filaments that cross-link core rootlets. A second class of cross-linking filaments that appear to be greater in diameter than the TW. 260/240 filaments when visualized by the quick-freeze, deep-etch rotary replication (QFDERR)[1] technique (27) may be comprised of brush border myosin that, like TW 260/240, is localized exclusively in the terminal web (31–34). The studies of Hirokawa et al. (30) raise the possibility that the spectrin-like protein of the brush border, in addition to cross-linking core rootlets, may be involved in cross-linking core rootlets to intermediate filaments, to the plasma membrane, and perhaps to coated and smooth surface vesicles, which are found in large numbers in the terminal web, at least in isolated brush borders (35, 30; for details see Discussion).

In this report we will present results of experiments that extend the elegant studies of Glenney et al. (4) and further elucidate the functional properties of the TW 260/240 molecule and the interaction of this protein with actin filaments. Some of the experiments reported here have appeared elsewhere in preliminary form (36).

MATERIALS AND METHODS

Brush Border Isolation: Brush borders were isolated from intestinal epithelium of chickens according to the method of Mooseker et al. (31) using modifications described in Keller and Mooseker (37). In this method, brush borders, once isolated by homogenization of epithelial cells in low ionic strength medium containing divalent cation chelators, are transferred to a buffer termed brush border stabilization solution (BBSB) containing 75 mM KCl, 5.0 mM $MgSO_4$, 1.0 mM EGTA, 0.2 mM dithiothreitrol. 10 mM imidazole-Cl, pH 7.2 with 0.2 mM phenylmethylsulfonyl fluoride and 20 trypsin inhibitor U/liter aprotinin (Sigma Chemical Co., St. Louis, MO) to inhibit proteolysis. In later experiments, we switched to a solution identical to BBSB, but containing only 0.1 mM $MgSO_4$ (15), because we found that the salt extraction of TW 260/240 was far more efficient using brush borders prepared with the BBSB buffer containing the lower Mg^{++} concentration. For some experiments brush borders were demembranated with BBSB containing 1% Triton X-100 (38) prior to salt extraction for isolation of TW 260/240.

Isolation of TW 260/240, Actin, and Tropomyosin: For most of the experiments reported here, TW 260/240 was purified by the methods of Glenney et al. (4). In brief, this procedure involved extraction of brush borders in elevated salt (0.3 M KCl) and purification of TW 260/240 from the salt extract by gel filtration on an agarose column (we used Bio-Rad A15 M, 200-400-mesh; Bio-Rad Laboratories, Richmond, CA). In our initial experiments (conducted before the publication of reference 4) we employed essentially the same method except that Triton-treated brush borders were used and higher salt (0.6 M KCl) was used for extraction of TW 260/240. We switched to the method of Glenney et al. (4) because this method can also be used for obtaining highly enriched fractions of the microvillus bundling protein, fimbrin. Fractions of purified TW 260/240 from the gel filtration column were concentrated either by dialysis against Aquacide (Calbiochem-Behring Corp., San Diego, CA) as in (6) or by chromatography on hydroxyl apatite (HAP). We found the latter method preferable because this technique also reduced the extent of proteolysis that occurred in the TW 260/240 fractions upon storage. In preparation for HAP chromatography the TW 260/240 fractions from the gel filtration column were made 0.5 mM potassium phosphate, their pH was adjusted to 7.2 with HCl, and they were applied to a 5-ml HAP column equilibrated with 0.6 M KCl, 0.5 mM EDTA, 10 mM Tris, 0.2 mM dithiothreitol and 5.0 mM potassium phosphate at pH 7.2 (HAP starting buffer). The TW 260/240 was eluted by application of a linear gradient of 5–300 mM phosphate in HAP starting buffer. The TW 260/240 elution peak was at ~110 mM phosphate. Further concentration was achieved by dialysis of the TW 260/240 fractions back into HAP starting buffer, and application to a small (~1 ml) HAP column and elution by a "step" application of 0.5 M potassium phosphate in HAP starting buffer. The concentration of TW 260/240 was estimated according to the method of Lowry et al. (39) using BSA as a standard.

Actin was isolated from chicken skeletal muscle by the method of Spudich and Watt (40). For certain experiments (see Results), G-actin was further purified by gel filtration on Sephadex G-150 (41). Tropomyosin was isolated from chicken skeletal muscle by the method of Bailey (42) as modified by Eisenberg and Kielly (43).

Actin-TW 260/240-binding Studies: For co-sedimentation studies, aliquots (175 μl) containing 4.65 μM F-actin polymerized in the presence or absence of TW 260/240, various concentrations of TW 260/240 (0.1–0.93 μM; based on dimer mol wt 500,000), 0.025–0.3 M KCl, 2 mM $MgSO_4$, 1 mM EGTA, and 10 mM imidazole-Cl (pH 7.0–7.5). In one series of experiments, tropomyosin (1.65 μM) was included (1:7 molar ratio with actin). After 30–60 min of incubation at room temperature, the samples were spun in an Airfuge (Beckman Instruments, Palo Alto, CA) for 30 min at an air pressure setting of 24 psi. Supernates were removed, the pellets gently washed (without suspension) with identical buffer, and then both supernates and pellets were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS PAGE) (44, 45). The relative amounts of actin and TW 260/240 in the supernate and pellet fractions were determined by densitometry of tube gels using a Gilford spectrophotometer (Gilford Instruments, Oberlin, OH) measuring absorbance at 660 nm. Protein peaks were quantitated by cutting and weighing.

The effects of TW 260/240 on the high-shear viscosity of actin solutions (both on assembly and on the steady-state viscosity of F-actin) was assayed by Ostwald viscometry using viscometers (Cannon Instruments, State College, PA) with flow rates of ~60 s.

Electron Microscopy: The morphology of individual TW 260/240 molecules was assayed by both conventional negative-staining techniques and low-angle rotary shadowing (46, 47). To test the effects of salt and temperature on the presumed tetrameric form (4) of the TW 260/240 molecule, samples of TW 260/240 (0.7 μM) were dialyzed (4°C) overnight against either 50, 150, or 300 mM KCl with 2 mM $MgCl_2$, 0.2 mM dithiothreitol, 1 mM EGTA, and 10 mM Tris (pH 7.5). At 0 time, the samples were split and either stored on ice or incubated at 37°C for 30 min. Aliquots (90 μl) of the various samples were rapidly diluted with 50, 150, or 300 mM ammonium acetate (210 μl) and 700 μl of glycerol, then sprayed onto freshly cleaved mica, and rotary shadowed with platinum-carbon in an Edwards high-vacuum evaporator (Sussex, England) by the method of Tyler and Branton (47). To quantitate the salt- and temperature-dependent dissociation of TW 260/240 tetramers into dimers, measurements of molecule lengths from random survey micrographs (210 molecules per condition were measured) were made using an electronic planimeter (Numonics, Landsdale, PA). Samples of TW 260/240 were also visualized by conventional negative-staining procedures by the application of TW

[1] *Abbreviations used in this paper:* BBSB, brush border stabilization solution; HAP, hydroxyl apatite; QFDERR, quick-freeze, deep-etch rotary replication.

260/240 solutions (0.03 mg/ml) onto carbon-parlodian-coated grids and staining with 1% uranyl acetate.

Electron microscopy of TW 260/240-actin filament mixtures was performed by examination of conventional negatively-stained preparations. Preparations of salt-extracted and control brush borders were fixed and embedded for thin-section microscopy according to the method of Begg et al. (28)

RESULTS

Effects of Salt Extraction on Terminal Web Structure

As first demonstrated by Glenney et al. (4), treatment of membrane-intact or demembranated brush borders (results not shown) with elevated salt (0.2–0.5 M KCl) results in extraction of 70–90% of the TW 260/240 without extraction of myosin, the other main constituent protein of the terminal web not associated with the microvillar core rootlets (Fig. 1 *b*). Salt extraction released into the supernatant a considerable amount of fimbrin, villin, and some actin, in addition to TW 260/240. At the level of the light microscope, salt extraction has little detectable effect on brush border morphology (results not shown). However, examination of these preparations by electron microscopy revealed a dramatic difference in the terminal web organization of brush borders depleted of TW 260/240 (Fig. 1). In control preparations (Fig. 1 *a*) we have observed interdigitating filaments at all levels of the core rootlet, from the point of emergence from the microvillar membrane to their basal ends. On the basis of examination of numerous micrographs, we have observed, however, that the density of interdigitating filaments is non-uniform along the length of the core rootlets, with the minimum density at the apical and basal ends of the core rootlets and the maximum in the central region of the terminal web. In salt-extracted brush borders (Fig. 1 *c*), there were still numerous cross-linking filaments, presumably consisting of myosin molecules of an as yet undefined aggregation state (27), but they were restricted primarily to the "central zone" defined above. The apical zone of the terminal web directly beneath the plasma membrane was virtually devoid of cross-linking filaments, a morphology that was observed in essentially all the brush borders in which the section plane was favorable. We also observed a reduction in the density of cross-linking filaments associated with the basal ends of the core rootlets. In addition, the circumferential, "contracting" ring (37, 35, 48, 49) of actin filaments associated with the junctional margin of the brush border at the level of the zonula adherens was intact in the salt-extracted brush borders, but it appeared somewhat less densely packed than in control preparations (results not shown, but see reference 37). Numerous cross-linking filaments resembling myosin filaments (Fig. 1 *c*) connected the zonula adherens contracting ring to the adjacent core rootlets. This observation is consistent with the greater concentration of myosin detected at the lateral margin of the terminal web by immunofluorescence techniques (32).

Structure of TW 260/240

As first demonstrated by Glenney et al. (4), rotary shadowed preparations of purified TW 260/240 sprayed onto mica from buffers of relatively low ionic strength (10–100 ml KCl) reveal a uniform population of highly elongated, "flexible appearing" molecules ~240–260 nm in length (Fig. 2 *a*). These molecules are strikingly similar to the tetrameric form of erythrocyte spectrin (3, 46, 47, 50), although considerably longer (~250 vs. 200 nm). Like erythrocyte spectrin, TW 260/240 is a double-stranded molecule with the strands loosely coiled along their length and tightly associated at the two ends. Assuming that the molecules visualized by this technique are indeed the tetrameric form of TW 260/240 (see Discussion), one can readily discern the characteristic "head to head" association of the two presumed heterodimers at the center of the molecule, particularly in those molecules in which this association appears to be partially disrupted (Fig. 2 *a*).

The morphology of the TW 260/240 molecule determined by the technique of negative staining (Fig. 2 *b*) was markedly different from that discerned by the rotary shadowing method. Although the TW 260/240 molecule visualized by negative staining had approximately the same length (~240 nm) as those visualized by rotary shadowing, they appeared much straighter and stiffer, and the coiled configuration of subunits was not readily discernible. As expected, negatively-stained molecules were also considerably thinner in diameter (2–3 nm), and were more uniform in diameter than rotary shadowed molecules.

In addition to the presumed tetrameric form of TW 260/240, preparations of this protein also contained variable amounts of aggregated material, particularly at low ionic strength (Fig. 2 *c*). Most of these aggregates (when visualized by the rotary shadowing technique) appeared to be comprised of disordered arrays of individual tetrameric molecules.

Effects of Salt and Temperature on the Organization of the TW 260/240 Molecule

In studies on the dimer-tetramer association of human erythrocyte spectrin, Ungewickell and Gratzer (51) demonstrated that dimer-tetramer association is governed by a simple equilibrium that is salt dependent (dimer favored at low salt) and temperature dependent (low temperature inhibits the conversion between dimer and tetramer). Moreover, these investigators found that at physiological ionic strength and temperature (150 mM salt at 37°C), erythrocyte spectrin is present as a roughly equal mixture of dimers and tetramers. The results summarized above and those of Glenney et al. (4) suggest that the stable form of TW 260/240 at low ionic strength is the tetramer, rather than the dimer. However, in our studies, the preparations of TW 260/240 were always maintained at 0–4°C prior to application to the mica or electron microscopy grid. Thus, conversion into the dimer form could have been inhibited by the low temperature of these preparations. Moreover, initial studies, on the examination of the effects of high ionic strength on the TW 260/240 molecule (>150 mM salt) we noted the presence of two distinct populations, one with molecules 260 nm long and the other with molecules approximately half that length (Fig. 2, *d* and *e*), indicating that at least partial dissociation of the presumed tetrameric form of TW 260/240 could occur at elevated salt concentrations. To determine the effects of both temperature and salt on the association state of the TW 260/240 molecule, experiments similar to those of Ungewickell and Gratzer (51) were performed as described in Materials and Methods. Results of these quantitative studies (Fig. 3) indicate that the association state of TW 260/240 is salt dependent and temperature independent. At 50 mM KCl, TW 260/240 was present as the 250-260-nm (presumably) tetrameric form before and after warming to 37°C. At 150 mM salt, there was a shift to approximately equal numbers of the 260- and 130-nm forms, both before and after warming,

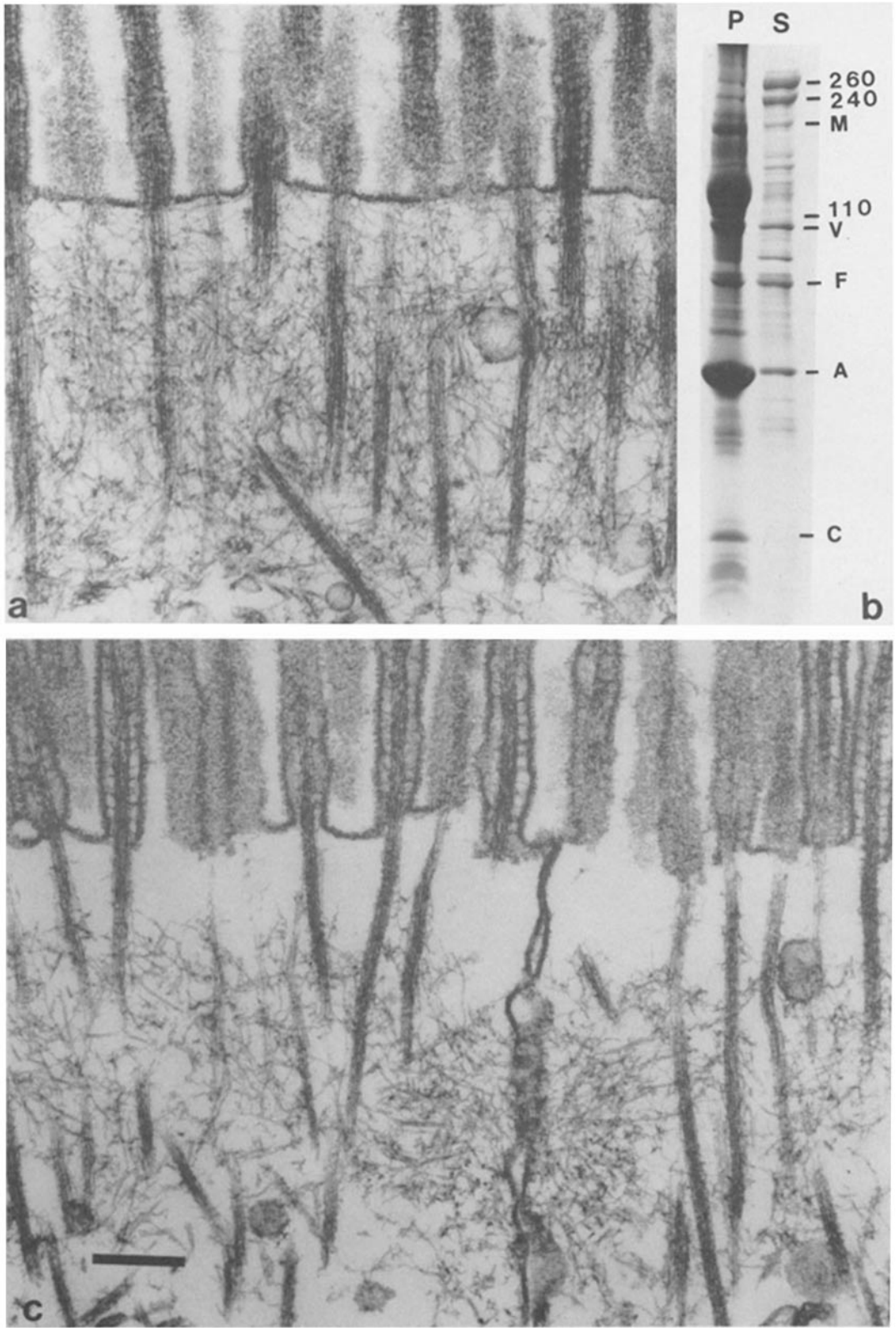

FIGURE 1 Salt extraction of TW 260/240 from the isolated brush border. (*a*) Terminal web region of a brush border in a preparation before extraction. Filamentous material interdigitating between the "rootlet" ends of microvillus core is present at all levels of the rootlets. The density of cross-linkers is somewhat lower within the apical zone of the terminal web, directly beneath the plasma membrane. (*b*) SDS PAGE of a brush border preparation extracted with 0.3 M KCl, following TW 260/240 isolation procedures (4). Pellet (*P*) and supernate (*S*) fractions, loaded stoichiometrically, after a 10,000-*g* spin are shown. Most of the TW 260/240 has been extracted, together with lesser amounts of villin (*V*), fimbrin (*F*), and actin (*A*). Myosin (*M*) and the lateral-bridge protein 110-kdalton protein (*110*)-calmodulin (*C*) have remained in the pellet fraction. (c) Terminal web region of a brush border in the salt-extracted pellet fraction depicted in *b*. Note the loss of interdigitating filaments (compared with *a*) from the apical zone of the terminal web directly beneath the plasma membrane. There is also a lower density of filamentous material associated with the basal ends of the rootlets. Numerous filaments interdigitating between rootlets still remain in the central zone of the terminal web. The junctional complex is intact, including the "contracting ring" of actin filaments (37, 35, 48, 49) associated with each side of the zonula adherens. Bar, 0.2 μm. × 75,000.

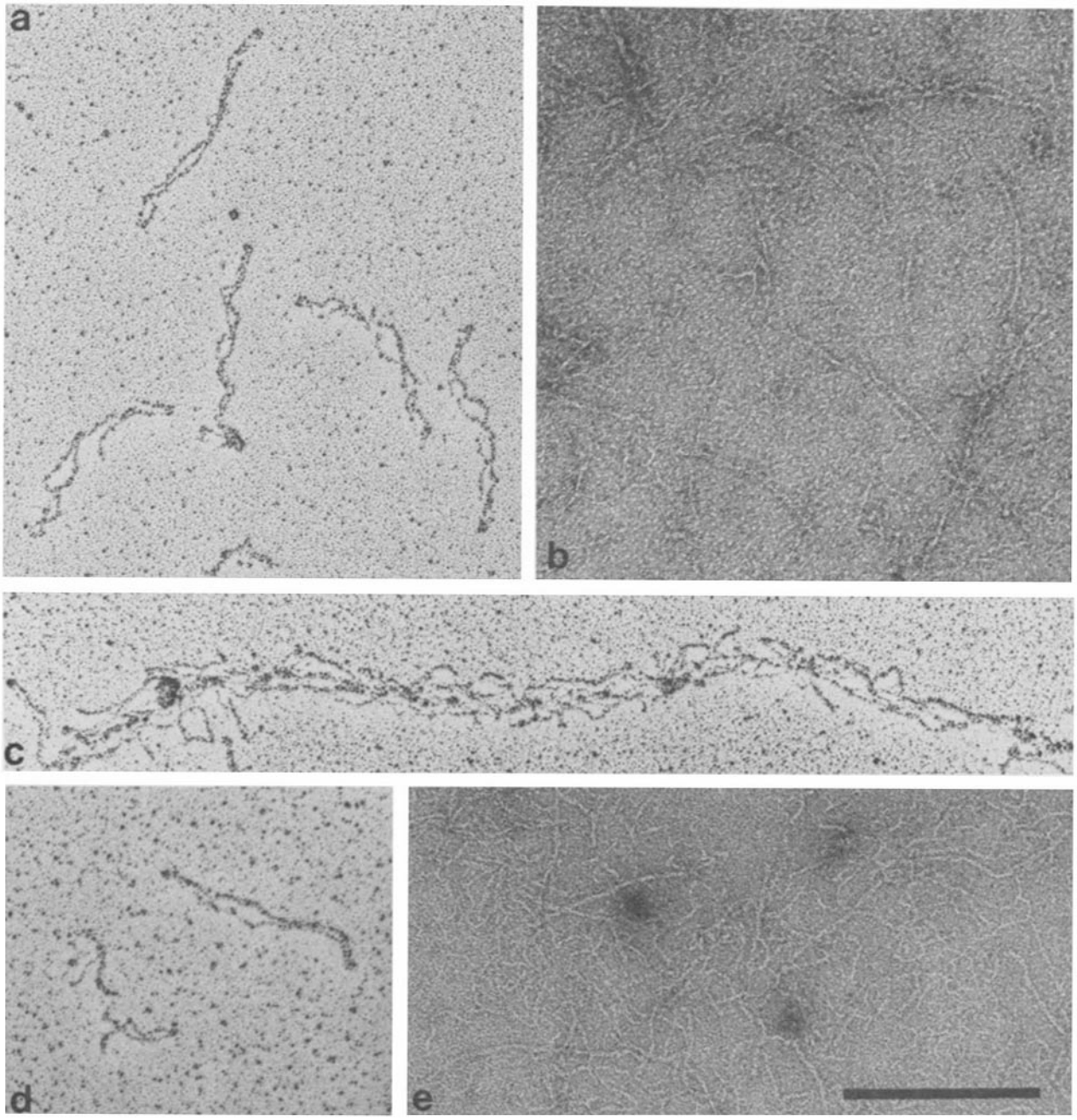

FIGURE 2 Morphology of TW 260/240 tetramers and dimers. (a) Rotary shadowed molecules of TW 260/240 from a preparation of purified protein in 50 mM KCl. Under these conditions, only the presumed (see text) tetrameric form is present. (*b*) The same preparation as in *a* visualized by negative staining. Note that the TW 260/240 molecules appear straighter and more uniform in diameter than in *a*. (*c*) Rotary shadowed replica of aggregates of laterally-associated TW 260/240 tetramers present at low ionic strength (25 mM KCl). (*d*) Rotary shadowed TW 260/240 molecules in the presence of 150 mM KCl. Both (presumed) tetramer and dimer forms are present. (*e*) Negatively-stained preparation of TW 260/240 in the presence of 150 mM KCl. Bar, 0.2 μm. × 150,000.

and the proportion of dimers present was only slightly increased when the salt was elevated to 0.3 M KCl. Finally, this salt-dependent dissociation of TW 260/240 is a reversible process, since TW 260/240 preparations dialyzed from high salt into low salt contain only the tetrameric form (as in Fig. 2*a*).

Interaction of TW 260/240 with Actin

CO-SEDIMENTATION STUDIES: Glenney et al. (4) reported that TW 260/240 in the presence of 25 mM KCl, 1 mM MgCl, at pH 7.3, will co-sediment with actin filaments, demonstrating that this protein will bind to F-actin under these conditions. We have conducted similar co-sedimentation assays to investigate a number of parameters with respect to the binding of F-actin by TW 260/240 including (*a*) concentration dependence of TW 260/240 on binding, (*b*) ionic strength, (*c*) pH, and (*d*) presence of tropomyosin.

The analysis of the concentration dependence of TW 260/240 on actin binding was conducted by addition of various amounts of TW 260/240 (0.11–0.93 μM; these concentrations

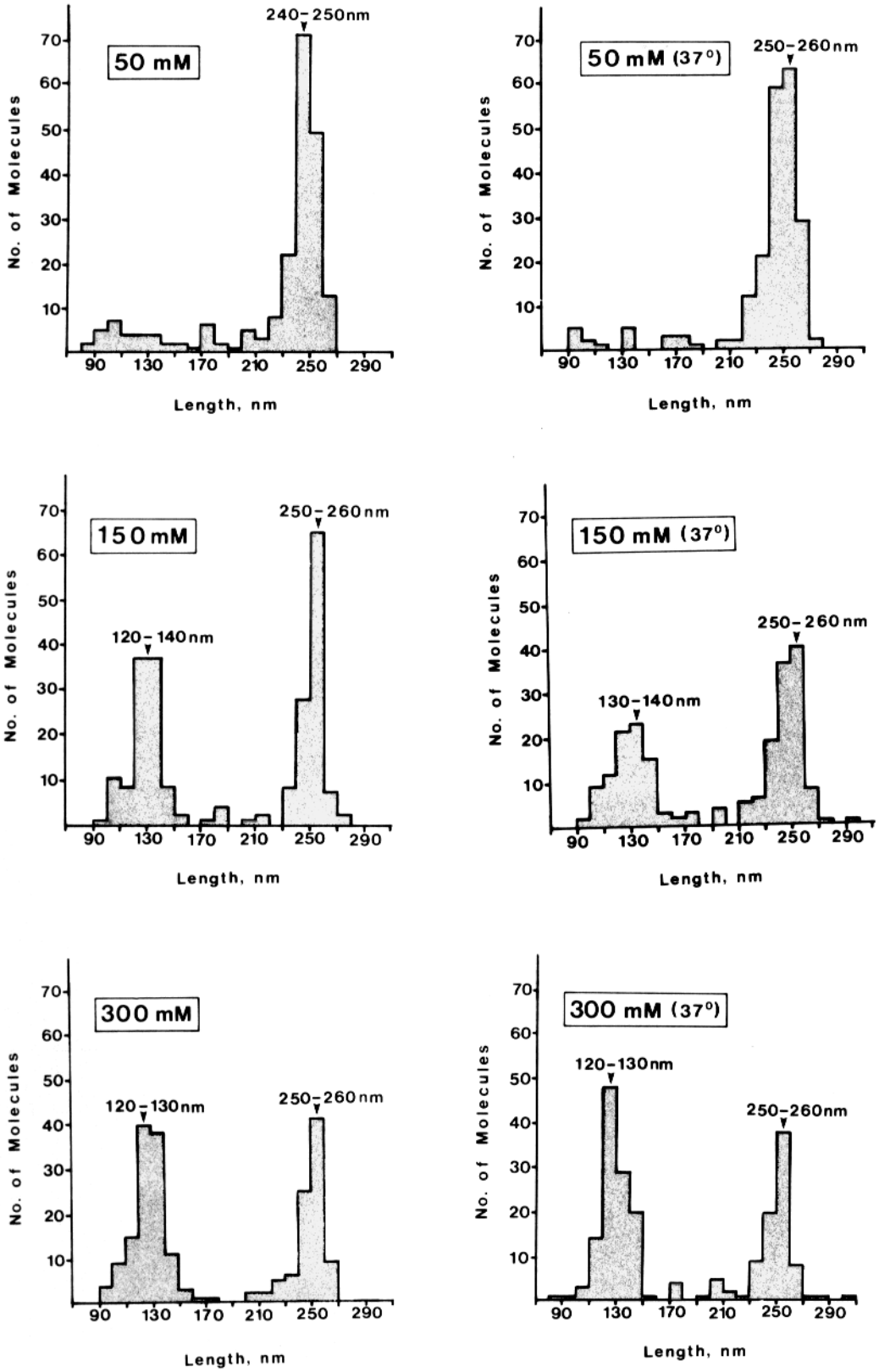

FIGURE 3 Effect of ionic strength and temperature on the association state of TW 260/240. Each histogram depicts the distribution of molecule lengths measured from rotary-shadowed preparations in the presence of 50, 150, and 300 mM KCl. The histograms on the left are measurements of molecules at 0°C, and those on the right are from preparations warmed to 37°C for 30 min.

based on 500,000-moi-wt dimer) to 4.65 μM F-actin in a solution containing 75 mM KCl, 2.0 mM $MgSO_4$, 1.0 mM EGTA, and 10 mM imidazole-Cl, pH 7.0. These conditions reflect those that have been determined to be optimum for retaining the biochemical and structural integrity of the brush border cytoskeletal apparatus in vitro (23). Under these conditions, TW 260/240 co-sediments with actin over the range of TW 260/240 concentrations tested (Fig. 4, *a* and *b*). We did observe, however, a reproducible increase in the percent of nonpelletable TW 260/240 above ratios of TW 260/240 to actin of 1:10 (i.e., there is an increase in the relative amount of TW 260/240 in the supernate fraction). We were unable to determine whether TW 260/240 binding is saturable because we also observed a concentration-dependent increase in

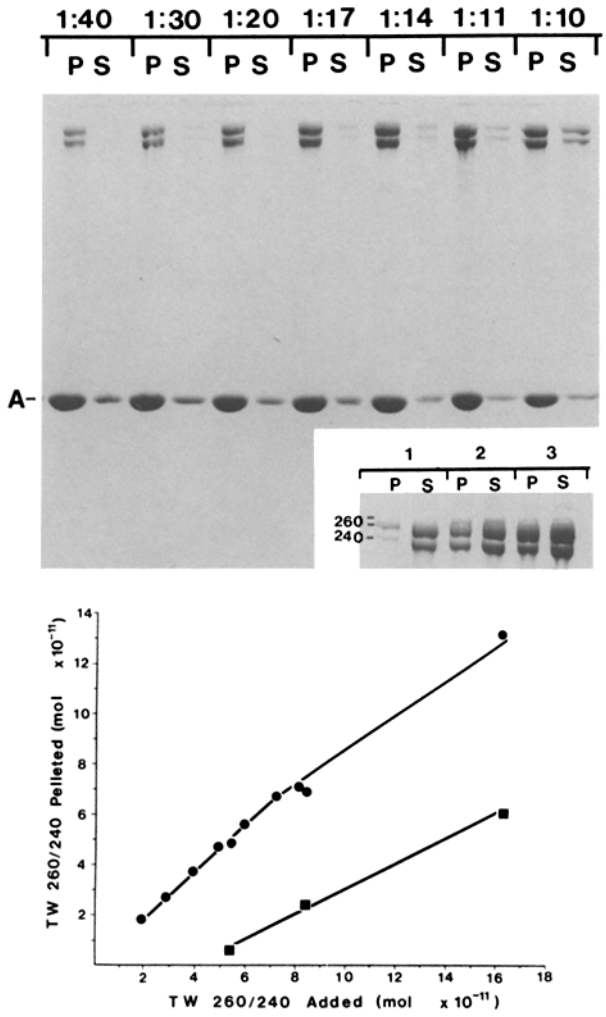

FIGURE 4 Co-sedimentation of various concentrations of TW 260/240 with F-actin. (a) SDS PAGE of mixtures of TW 260/240 and F-actin (*A*) at molar ratios of TW 260/240:actin from *1:40–1:10* in the presence of 75 mM KCl, 2 mM $MgCl_2$ at pH 7.0. Pellet (*P*) and supernate (*S*) samples after high-speed sedimentation are shown. The *P* and *S* lanes for *1:11* and *1:10* are ratios slightly underloaded relative to the other samples. (*inset*) Pellet (*P*) and supernate (*S*) of TW 260/240 samples sedimented in the absence of F-actin. The concentrations of samples *1*, *2*, and *3* were 0.31, 0.46, and 0.93 μM, respectively. These concentrations are equivalent to those used for sedimentation in the presence of actin for ratios for TW 260/240:actin of 1:15, 1:10, and 1:5. Note that the 260-kdalton-subunit actually is resolvable into 2–3 sub-bands. (*b*) Quantitation of co-sedimentation experiments comparable with those in *a* by densitometry of SDS PAGE, using tube gels to eliminate loading and band dimension problems. The amount of TW 260/240 that pellets in the presence (●) and absence (■) of F-actin is plotted against the total amount of TW 260/240 added. Note the concentration-dependent increase in TW 260/240 that pellets in the absence of actin.

the amount of TW 260/240 that sedimented in the absence of actin at concentrations >0.3 μM. These sedimentable aggregates formed in concentrated samples of TW 260/240 were dispersed by dilution of the protein (results not shown).

The effect of salt on TW 260/240 actin binding was analyzed by co-sedimentation of 0.2 μM TW 260/240 in the presence of 4.65 M actin at 25, 75, and 150 mM KCl at pH 7.5 and 7.0 (Fig. 5, *a* and *b*). As has been demonstrated for brain fodrin (6), TW 260/240 binds most avidly to actin at low ionic strength. In the presence of 150 mM KCl at pH 7.0 the amount of binding as measured by co-sedimentation was greatly reduced, and at pH 7.5 this binding was reduced to the levels of TW 260/240 which sediment in the absence of actin. The effects of salt on TW 260/240 binding to purified actin from skeletal muscle was mirrored by the salt-dependent solubilization of TW 260/240 from the isolated brush border cytoskeletal apparatus in which release of this protein from the terminal web occurred at salt concentrations >~150 mM KCl (Fig. 5*c*). However, only ~50% of the TW 260/240 was solubilized at 150 mM. Higher salt (>300 mM) was required to achieve 80–90% extraction.

We also examined the effects of tropomyosin on the binding of TW 260/240 to F-actin. The rationale for these experiments was that tropomyosin is present on the actin filaments of the microvillus core rootlets (33, 20) to which TW 260/240 molecules are presumably bound. Results of these experiments (Fig. 5*b*) indicate that skeletal muscle tropomyosin has little if any significant effect on interaction of TW 260/240 with actin over the range of salt concentrations analyzed.

VISCOMETRY STUDIES: The effects of TW 260/240 on the salt-dependent polymerization and steady-state viscosity of actin were examined by high-shear viscometry. In initial studies, we examined the effects of low ratios (~1:80) of TW 260/240 on the polymerization of actin (7 μM) induced by the addition of 2 mM $MgCl_2$ and 20 mM (Fig. 6*a*) or 75 mM (Fig. 6*b*) KCl at pH 7.5. At even these low concentrations of TW 260/240, we observed a marked increase in the final plateau viscosity reached at steady state in both 20 and 75 mM KCl. There was no significant effect on the rate of viscosity increase, however. We observed the same increase in plateau viscosity in the absence (1 mM EGTA) and presence of various concentrations of Ca^{++} from 0.1 to 100 μM, at both 20 and 75 mM KCl. (Fig. 6*a* shows the effect of 100 μM Ca^{++} at 20 mM KCl. The slight reduction in plateau viscosity compared with that in the absence of Ca^{++} was not reproducibly observed.) It is important to note that the increase in plateau viscosity induced by TW 260/240 cannot be due primarily to an increase in polymer present. In the same viscometers, this plateau viscosity, in the absence of TW 260/240, can only be matched by increasing the actin concentration from 7 to almost 12 μM; thus, TW 260/240 must affect the high-shear viscosity of actin by cross-linking filaments and/or greatly increasing average filament length.

We examined the concentration-dependence of TW 260/240 on actin polymerization and plateau viscosities of F-actin at steady state using 4.65 μM actin at various ratios of TW/260/240 to actin from 1:160 to 1:20. In these experiments gel-filtered G-actin was used, and polymerization was initiated by addition of 75 mM KCl, 2 mM $MgCl_2$ at pH 7.0, the same conditions used for analysis of concentration-dependent binding to actin (Fig. 4). As reported above, we observed an increase in plateau viscosity at ratios of TW 260/240 to actin as low as 1:160, with this effect reaching a maximum in the range of 1:80 (Fig. 6*c* and results not shown). Surprisingly, at higher concentrations of TW 260/240 (e.g., 1:40 ratio) this effect was reduced, and at a ratio of 1:20 the final plateau viscosity was significantly lower than that of control preparations in the absence of TW 260/240. This reduction in viscosity at high concentrations of TW 260/240 is most likely due to the formation of macroscopic aggregates (which were readily visible with the naked eye) presumably consisting of gelled networks of actin filaments (see below and Fig. 7). In

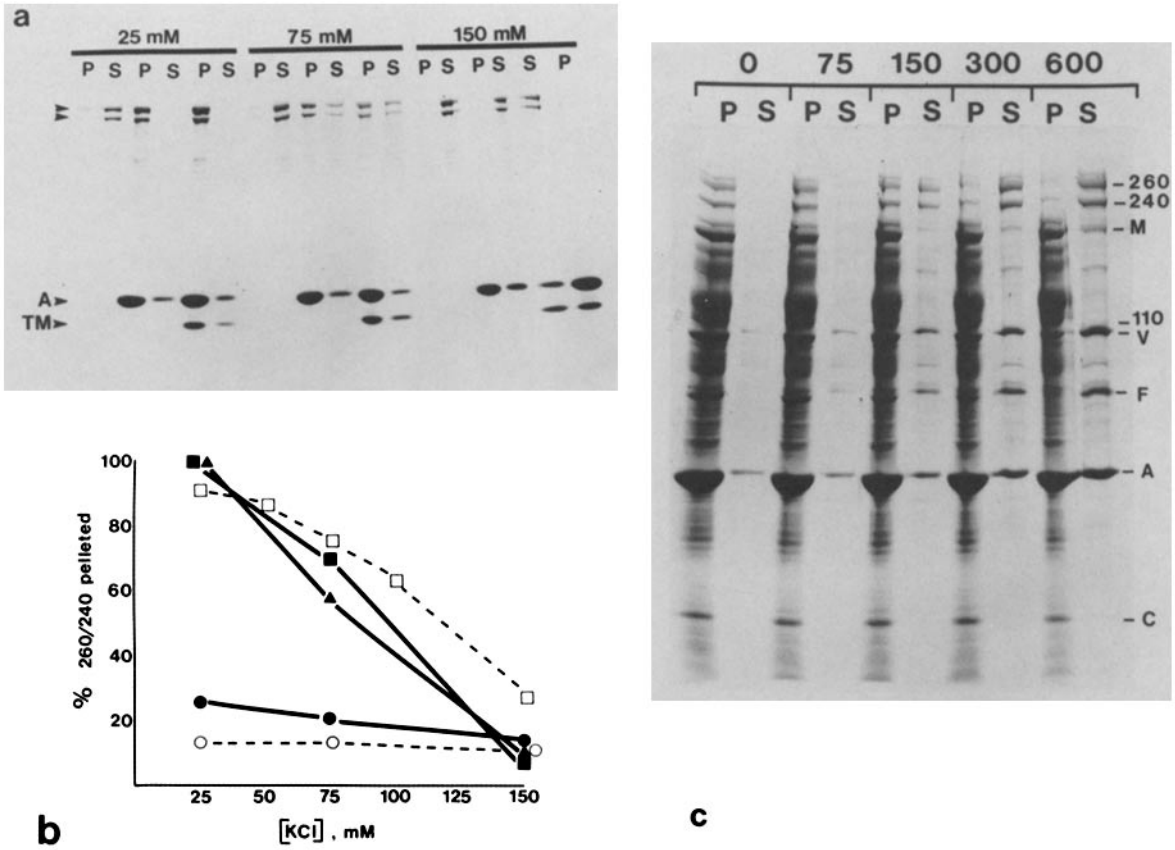

FIGURE 5 Salt- and pH-dependence of TW 260/240-actin interaction. (*a*) SDS PAGE experiments in which 0.2 μM TW 260/240 was added to 4.65 μM F-actin (*A*) in the abscence or presence of tropomyosin (*TM*). Salt conditions were either 25, 75, or 150 mM KCl with 2 mM $MgCl_2$ at pH 7.5. Pellet (*P*) and supernate (*S*) fractions are shown. Arrowheads indicate the TW 260/240 subunits. (*b*) The results of the experiments in *a* are plotted as the percent of TW 260/240 that pelleted in the absence (●) and in the presence of F-actin without (■) and with (▲) tropomyosin. The dashed lines show results of a similar experiment conducted with a different preparation of TW 260/240 at pH 7.0. ○, TW 260/240 alone; □, TW 260/240 in the presence of actin. (*c*) Salt-dependent extraction of TW 260/240 from the isolated brush border. Using solution conditions identical to those in *a*, membrane-intact brush borders were extracted with various concentrations of KCl from 0 to 600 mM. Pellet (*P*) and supernate (*S*) after a 10,000-*g* spin were analyzed by SDS PAGE. Abbreviations as in Fig. 1.

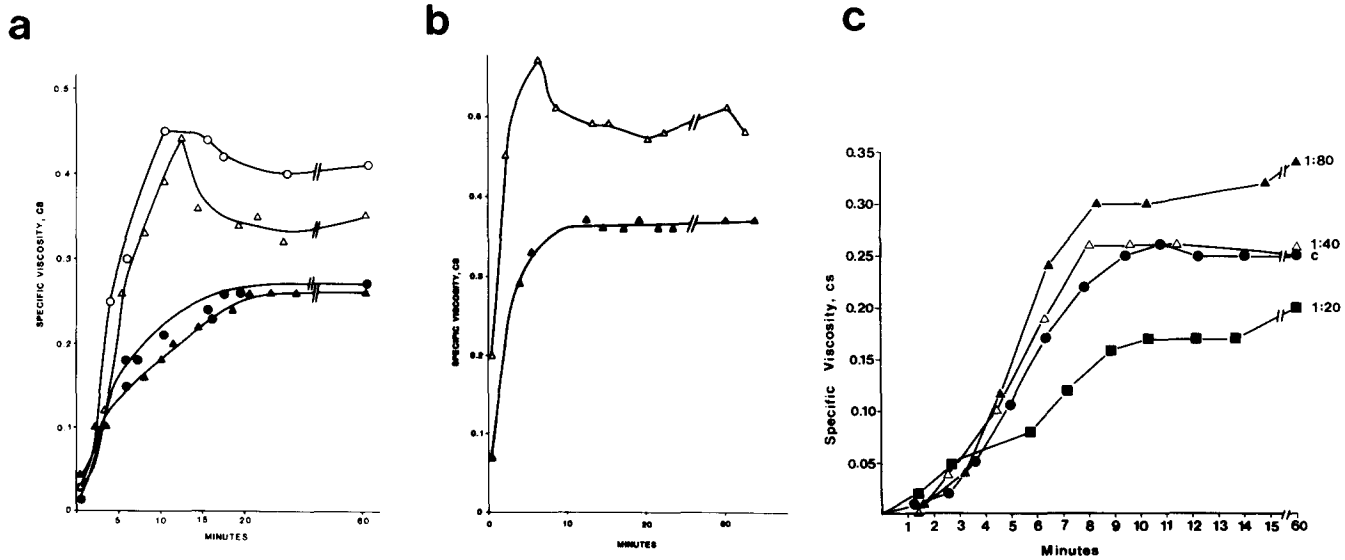

FIGURE 6 High-shear viscometry of actin polymerization in the presence of TW 260/240. (*a*) Effects of 90 nM TW 260/240 on the polymerization of 7 μM actin in the presence of 25 mM KCl, 2 mM $MgCl_2$ at pH 7.5. The specific viscosities of actin alone (●▲) and with TW 260/240 (○△) in the presence of 1 mM EGTA (●○) or 0.2 mM $CaCl_2$ (▲△) are plotted as a function of time after addition of salt. (*b*) As in *a*, except that the KCl concentration was 75 mM. The viscosities for actin (▲) and actin plus TW 260/240 (△) in the presence of 1 mM EGTA are plotted. (*c*) Concentration dependence of TW 260/240 on the assembly and plateau viscosity of actin. Polymerization of gel-filtered G-actin at 4.65 μM was initiated by addition of 75 mM KCl, 2 mM $MgCl_2$ at pH 7.0 in the absence of TW 260/240 (C, ●), and in the presence of various ratios of TW 260/240:actin of 1:20 (■), 1:40 (△), and 1:80 (▲).

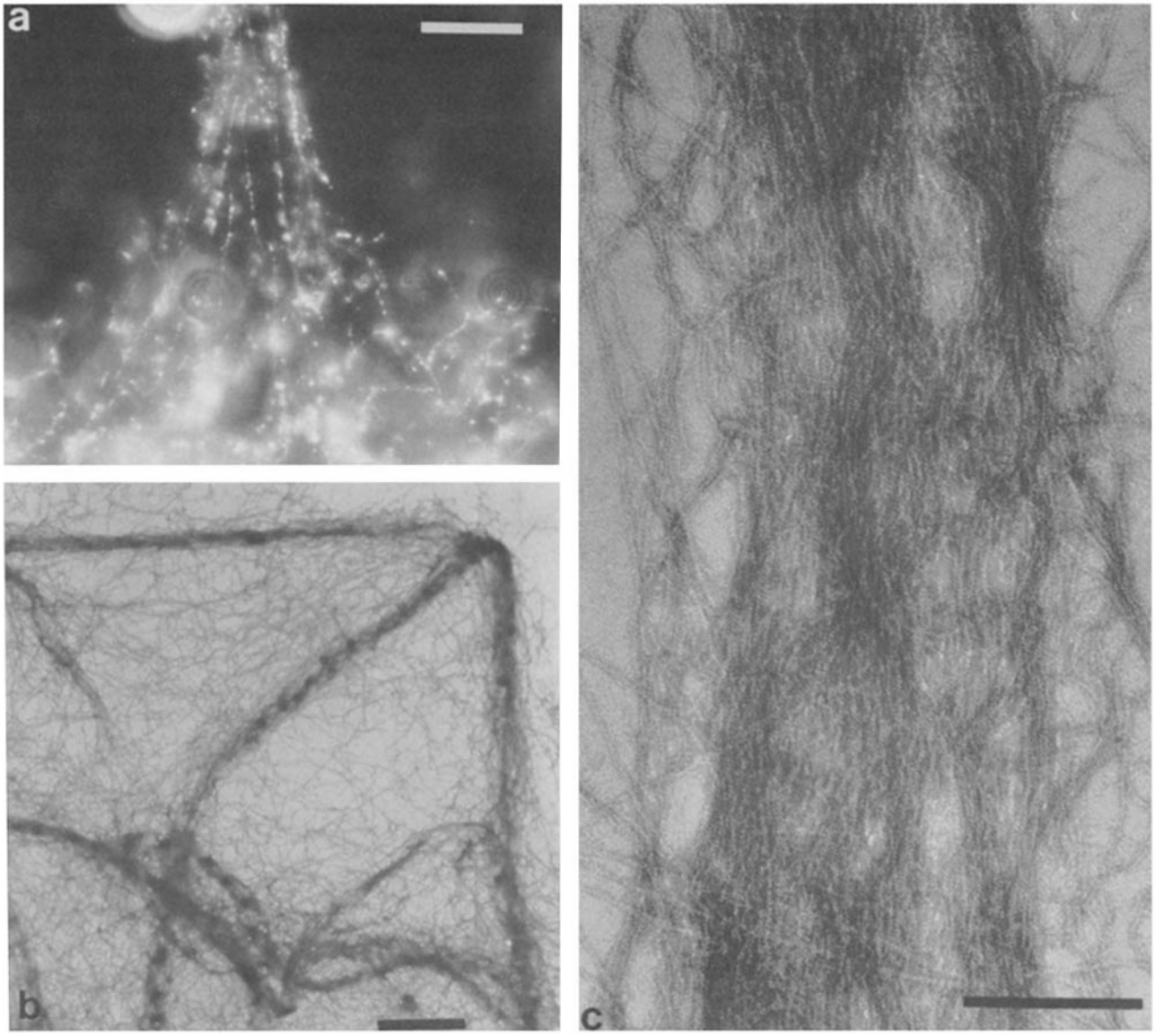

FIGURE 7 The cross-linking of F-actin by TW 260/240. (a) Dark-field light micrograph of a mixture of F-actin (4.65 μM) "gelled" by the addition of 0.3 μM TW 260/240 in the presence of 75 mM KCl, 2 mM $MgCl_2$ at pH 7.0. Bar, 10 μm. × 1,500. (*b*) Low magnification, negative-stain preparation of the electron micrograph of the preparation in *a*. Bar, 1 μm. × 13,500. (*c*) Higher magnification of a bundle of actin filaments cross-linked by TW 260/240. Bar, 0.2 μm. × 135,000.

these experiments, which utilized nuclei-free, gel-filtered actin (41), we observed no detectable effect of TW 260/240 on the nucleation phase of actin polymerization.

MORPHOLOGICAL STUDIES: The results of the viscometry studies above (Fig. 6) indicate that TW 260/240 can cross-link actin filaments even at very low ratios of spectrin to actin, and that this cross-linking is of sufficient strength to be at least partially stable to the high-shear forces applied within the Ostwald viscometer. That TW 260/240 is a potent cross-linker of actin filaments is dramatically confirmed by visual and microscopic examination of TW 260/240-actin mixture. As long as the salt concentration is <150 mM, addition of TW 260/240 to actin filaments will induce the formation of macroscopic "chunks" of material that, when viewed by dark field light microscopy, consist of fine fibrillar networks, presumably comprised of cross-linked aggregates of actin filaments (Fig. 7*a*). Such networks are not found at higher salt concentrations (>150 mM KCl).

Ultrastructural examination (Fig. 7, *b* and *c*) of these gelled networks of actin-TW 260/240 by the negative-staining technique indicates that they are comprised of extremely long, interconnected bundles of actin filaments. These bundles were quite large in diameter and, although the filaments within them were tightly packed, there was no detectable axial order such as that seen in Mg^{++} paracrystals or filament bundles cross-linked by fimbrin (15–17), or fascin (see reference 52 for discussion and references).

At higher pH (pH 7.5), addition of TW 260/240 (1:15 molar ratio) to actin at the same salt concentration (75 mM) as that for experiment described above (Fig. 7) did not induce the formation of extensive fibrillar networks (detectable by either light or electron microscopy), even though TW 260/240 did bind to, and cross-link, actin filaments under these conditions, as determined by co-sedimentation (Fig. 5) and viscometry (Fig. 6*b*). Examination of this preparation by negative staining revealed that no extensive aggregation of

filaments occurs and that individual molecules of TW 260/240 are readily detectable both in the "background" and in apparent association with actin filaments through T-like connections to the sides of actin filaments (Fig. 8).

To better visualize the interaction of TW 260/240 with actin filaments, we attempted to use the rotary-shadowing technique, the method that has been routinely used for the visualization of the interaction of spectrin-like molecules with actin (for review, see reference 3). Unfortunately, in our hands, we found this method useless, because the atomization technique used for application of samples to mica induced disruption of actin filament structure into short oligomers and small molecules identical in dimension to rotary-shadow G-actin molecules (results not shown at request of reviewers). This same effect was observed by Glenney et al. (6) who observed that extensive glutaraldehyde fixation was required to maintain filament structure. Even then, only short fragments of filaments were observed.

DISCUSSION

Properties of TW 260/240

Morphological studies first conducted by Glenney et al. (4) on purified TW 260/240 using the technique of rotary shadowing clearly established the spectrin-like morphology of this molecule, and showed that in buffers of low ionic strength this protein is most likely in the tetrameric state. In favorable replicas (e.g., Fig. 2, *a* and *d*), four individual strands within the molecule can be readily resolved. Moreover, the marked similarity in structural organization to the tetrameric forms of erythrocyte spectrin and brain fodrin, molecules for which molecular weight determinations have been conducted (3, 6), further supports the validity of this assumption.

It is clear that the rotary shadowing technique has been an excellent method for deciphering the subunit organization of TW 260/240. However, the apparent "floppiness" and, in particular, the loosely coiled association of subunits within the molecule may be exaggerated by the technique. This notion is supported by the morphology of TW 260/240 tetramers in negatively-stained preparations (Fig. 2*b*). By this technique, the molecule is straight, not "floppy," and is uniform in diameter along its length, indicating that its subunits may be more tightly associated than in molecules visualized by rotary shadowing technique. Our attempts to visualize TW 260/240 interaction with actin filaments using this technique supply further evidence that considerable disruption of "weak" subunit interactions could occur during specimen preparation for rotary shadowing. It is apparent that the shear forces applied to actin filaments in the atomization step used for applying samples to mica chips are of sufficient magnitude to disrupt these filaments into short oligomers and monomers. It is also possible that the "stiffer" appearance of TW 260/240 by negative staining is an artifact of that technique, as suggested by one of our reviewers. However, it is important to note that the in situ morphology of TW 260/240, visualized either in standard thin section (Fig. 1) or by QFDERR (30), is most consistent with the negatively-stained image of the TW 260/240 molecule.

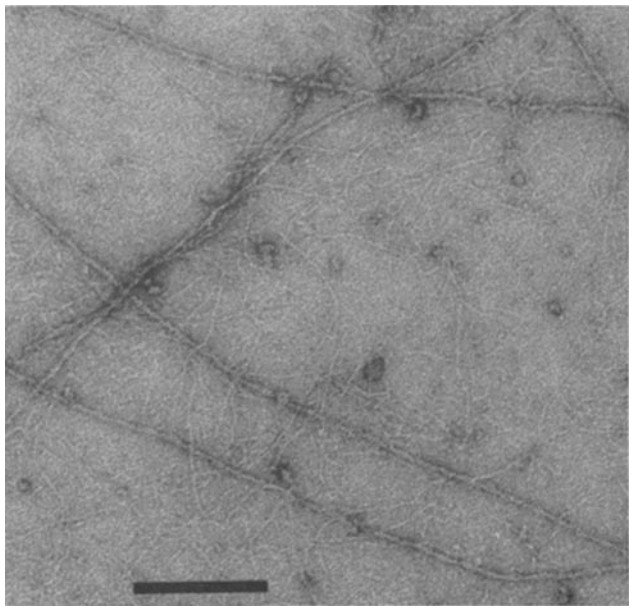

FIGURE 8 Effect of pH on the structural interaction by TW 260/240 with F-actin. Negatively stained preparation. Electron micrograph of a mixture of F-actin and TW 260/240 under conditions identical to those in Fig. 7, except at higher pH (pH 7.5). Note that the filaments are not bundled, and that numerous TW 260/240 molecules are evident in apparent lateral association with filaments and in the "background." Bar, 0.2 μm. × 85,000.

Although TW 260/240 is a tetramer at relatively low ionic strength (4; Fig. 2), we have observed that at salt concentrations at (150 mM KCl) or above (300 mM KCl) the physiological range, partial dissociation of tetramers into dimers occurs (Figs. 2 and 3). In these experiments we observed approximately equal numbers of dimers and tetramers, indicating that ~20–30% of the total tetramer population had dissociated into dimers, at both 150 and 300 mM KCl (Fig. 3). One explanation for the effect of salt on the tetramer is that there is a shift in equilibrium toward the dimer state. Another possibility is that these preparations of TW 260/240 contain two populations of tetramers, one of which is more sensitive to salt-dependent dissociation. A factor that could contribute to such an effect is the phosphorylation state of TW 260/240. We have observed that the 260 kdalton subunit can be phosphorylated in situ by addition of [γ^{32}P]ATP to isolated brush borders (see Fig. 6, reference 37), or by addition of exogenous, cAMP-dependent kinase (Keller, T., and D. Fishkind, unpublished observations). Perhaps the extent of phosphorylation of the 260-kdalton subunit somehow affects the association state of TW 260/240 at physiological ionic strength. It is important to note, however, that phosphorylation of erythrocyte spectrin has no effect on the dimer-tetramer equilibrium (51). On the other hand, our results demonstrate that TW 260/240 is quite distinct from spectrin in that the tetramer, not the dimer of TW 260/240, is stable at low ionic strength, and that the salt-dependent dissociation of tetramers is temperature independent. Clearly, further experiments are required to decipher the nature of dimer-tetramer association and to reveal what, if any, effect phosphorylation has on this process. Despite the fact that we do not adequately understand the parameters that govern the association/dissociation of TW 260/240 dimers, our results indicate that reversible dimer-tetramer association could be an important factor in regulating the function of this protein in vivo (possible functions of TW 260/240 are discussed in more detail below).

Another property of TW 260/240 that could contribute to its function is raised by the observation that this protein self-associates, in a concentration-dependent manner, to form

sedimentable aggregates (Fig. 4). We have presented preliminary evidence that at low ionic strength these aggregates are comprised of side-to-side arrays of tetramers (Fig. 2*c*). However, at higher ionic strength (75–150 mM) we have also observred rosette-shaped arrays (results not shown) similar in morphology to the oligomeric forms of spectrin observed by Morrow and Marchesi (50). More experiments using native gel electrophoresis (50) will be required to determine whether TW 260/240 can self-associate into discretely-sized oligomeric forms in a spectrin-like, concentration-dependent manner. Either type of self-association could contribute to the formation of extensive TW 260/240 networks in the terminal web, which could surround, and presumably interact with, the core rootlets.

One final aspect of the TW 260/240 molecule revealed in our studies, for which we have, as yet, no explanation, is that the 260-kdalton subunit actually was resolvable on SDS PAGE into 2–3 "sub-bands" (see Figs. 1*b*, 4*a*, and 5). The significance of this micro-heterogeneity within the 260-kdalton subunit is not known. It is highly reproducible with respect to number and relative intensity of bands, when different preparations were run on the same gel, indicating that proteolysis is an unlikely explanation. Moreover, all of the subunit bands were phosphorylatable, and phosphorylation has no effect on the number or relative intensity of these bands (unpublished observations). Thus, the nature of this micro-heterogeneity remains a mystery, although it is quite clear that all of these 260-kdalton bands are bona fide subunits of the molecule, as all remain inseparable through gel filtration, HAP chromatography, and actin-co-sedimentation.

Interaction of TW 260/240 with Actin

We have investigated various parameters regarding the binding of TW 260/240 to actin filaments. The conditions that optimize this interaction are physiological with respect to pH (pH 7.0 is better than pH 7.5) but not ionic strength. TW 260/240 binds to, and cross-links actin filaments most avidly at low ionic strength. At physiological ionic strength, this interaction was inhibited with respect to amount of TW 260/240 bound (Fig. 5), and extent of cross-binding, as determined by both light (dark field) and electron microscopy. Similar salt and pH dependence has been recently reported for the interaction of brain fodrin (spectrin) with actin (6).

The marked inihibitory effect of physiological concentrations of monovalent salt (150 mM KCl) on the in vitro binding of TW 260/240 to actin is disturbing (Fig. 5), given its presumed function in situ as a cross-linker of core rootlets in the terminal web. One consideration is that our in vitro studies were conducted at much lower concentrations of actin and TW 260/240 than must be present locally within the terminal web in vivo. Alternatively, our test tube experiments may simply lack some factor(s) or ionic conditions that are required for association of TW 260/240 with the cytoskeletal apparatus. For example, binding to the actin filaments of core rootlets could be facilitated by the presence of the other proteins present on those filaments, such as tropomyosin, fimbrin, or villin. Thus far we have investigated the effect of only one of these proteins, tropomyosin (albeit using skeletal, not brush-border, tropomyosin), and found no effect (Fig. 5, *a* and *b*). However, this notion should be examined further. Extraction of isolated brush borders with 150 mM KCl at pH 7.5 resulted in solubilization of only 30–50% of the TW 260/240 (Fig. 5*c*), not the 90–100% expected from the in vitro binding studies (Fig. 5, *a* and *b*). These results suggest that the interaction of TW 260/240 with the cytoskeletal apparatus cannot be explained completely by the characteristics of its binding to pure actin filaments. A simple explanation is that the purification procedure somehow alters the actin-binding sites on TW 260/240. A more interesting possibility is that the brush border contains a class of TW 260/240 binding sites that are salt-insensitive, for example, sites on the brush border membrane. This explanation is unlikely, since we have observed similar levels (~50%) of TW 260/240 extraction at 150 mM KCl using brush borders demembranated with Triton X-100 (results not shown).

Despite the questions we have raised above, the observations regarding the effects of physiological salt on TW 260/240 dimer-tetramer association, and on its interaction with actin, could be telling us something important about the behavior of this protein in vivo. From chemical and structural studies on the isolated brush border, it has been demonstrated that the terminal web is a highly stable structure. However, it is important to note that brush borders are prepared by homogenizing epithelial cells in low ionic strength buffer. Thus, rapid dilution of intracellular salt could "freeze in place" TW 260/240 molecules that, prior to lysis, may have been in a highly dynamic state with respect to dimer-tetramer (and perhaps oligomer) equilibrium, and filament interaction. It is reasonable to assume that an image of the terminal web as cytoplasmic "concrete" may be a misleading one, and one that is, in fact, inconsistent with the absorptive functions of the intestinal epithelial cell.

The combined results of viscometry (Fig. 6), light, and electron microscopy (Fig. 7) demonstrated that TW 260/240 is a potent cross-linker of actin filaments, even at relatively low ratios of TW 260/240 to actin. The exact structural nature of this cross-linking remains unclear, however. The localization studies of Glenney et al. (29) and Hirokawa et al. (30) indicate that TW 260/240 appears to form lateral, bivalent connections between adjacent core rootlets. In our in vitro studies, at salt concentrations (20–75 mM KCl) where detectable TW 260/240 binding and cross-linking occur, we have observed two types of binding of TW 260/240 to actin. At pH 7.5, we observed lateral T-like connections of TW 260/240 to actin filaments, but no apparent aggregation of filaments (Fig. 8), although cross-linking was detectable by viscometry (Fig. 6, *a* and *b*). At pH 7.0, in the presence of either 25 (results not shown) or 75 mM KCl, TW 260/240 induced the formation of long, large-diameter, interconnected actin bundles. Given the close packing of filaments within these bundles, it is unlikely that cross-linking by TW 260/240 is mediated by simple T-connections between filaments; if so, the spacing between filaments would be considerably greater, up to a maximum of the tetramer length of ~250 nm. Extensive examination of negatively-stained preparations of these bundles such as those in Fig. 7 failed to reveal the presence of obvious cross-linkers between filaments within the bundle, and only occasionally were cross-linkers visible at the edges of bundles where filaments tend to splay off from the main mass of the bundle. Obviously, super-positioning of filaments would tend to obscure such cross-linkers. Another explanation for the apparent disappearance of TW 260/240 in these bundles is that the TW 260/240 molecule cross-links filaments at acute angles, so that the long axis of the tetramer would lie almost parallel to the filaments in the bundle. This would require considerable flexibility in the actin-binding

domains at the two ends of the tetramer, a property consistent with the apparent flexibility of spectrin-like molecules. A more interesting possibility is that the actin-binding domain of each dimer within the TW 260/240 tetramer actually interacts with more than one actin monomer within the filament in a fashion analogous to the binding of tropomyosin to actin. If so, a certain portion of each end of the tetramer might either wrap around, or perhaps lie "in the groove" of the actin filament. As a result, the portion of the tetramer spanning the gap between two cross-linked filaments could be considerably shorter than the extended length of the tetramer. In this regard, it is also important to note that the spacing between the outer surface of adjacent microvillar core rootlets is considerably less (~100–200 nm) than the length of the TW 260/240 tetramer (these unpublished measurements were made on quick-freeze, deep-etch preparations of isolated chicken intestinal brush borders, done in collaboration with N. Hirokawa, Department of Biophysics, Washington University, St. Louis, MO).

Possible Functions for TW 260/240

The short answer to questions regarding the function of TW 260/240 or any of the other cytoskeletal proteins in the brush border is "we don't know." Nevertheless, one can make some reasonable guesses based on the characterization of this spectrin-like protein, reported here, and by Glenney et al. (4), and on the results of recent immunolocalization studies (29, 30).

It is clear that much of the TW 260/240 in the terminal web is involved in cross-linking of core rootlets to one another, a function that is consistent with the actin-binding properties of purified TW 260/240. However, it is important to note that there are still questions regarding the exact location of the TW 260/240 cross-links in the terminal web. The results of salt extraction experiments (Fig. 1) suggest that much of the TW 260/240 is localized primarily in the apical zone of the terminal web, directly beneath the plasma membrane. We have also observed a depletion of cross-links associated with the very basal ends of the core rootlets. We cannot determine from these morphological observations whether the central zone of the terminal web, which presumably contains the myosin cross-links, also contained TW 260/240 before salt extraction. The immunological studies of Hirokawa et al. (30) do provide some evidence for the mutually exclusive distribution of TW 260/240 (or its mammalian equivalent) in the terminal web of mouse brush borders. On the other hand, Glenney et al. (29) have reported a uniform distribution of TW 260/240 at all levels of the core rootlets, although at least some of the data they present are consistent with a somewhat reduced concentration of TW 260/240 in the "central zone" of the terminal web (see Fig. 3, *a* and *b*, and reference 29). It will be important to study the possible competitive interactions of TW 260/240 and myosin with actin.

In addition to questions regarding the exact location of TW 260/240, we are still in the dark regarding the functional significance of its interaction with core rootlets. As we have already discussed above, the impression derived from electron micrographs that the terminal web is a rigid, cross-linked array of cytoskeletal elements may be a misconception. Given our observations of the salt dependence of TW 260/240-actin binding, it is possible that the TW 260/240 cross-links may not be tightly associated with the microvillar core rootlets. Our results also indicate that TW 260/240 may not be required for maintaining the spatial and structural integrity of the terminal web. Extraction of isolated brush borders with high salt solubilized most of the TW 260/240 from the terminal web (Fig. 1 *b*). Even though this results in a substantial loss of TW 260/240 cross-linkers, the terminal web did not collapse, round up, or fall apart. The spatial order of the core rootlets was unaffected, the microvilli remained upright, and the plasma membrane remained tightly associated with the cytoskeletal apparatus (Fig. 1 *c*).

A logical extension of the above discussion is that the function of TW 260/240 may not be solely architectural, in a static sense. One exciting possibility is that TW 260/240 is involved in "membrane traffic" through the terminal web. For example, TW 260/240 may participate in endocytosis of apical membrane, which is mediated, at least in part, by coated vesicles (53). In addition, apical membrane may be replaced by Golgi-derived vesicles (54) that we have speculated are actively transported to the membrane along core rootlets, perhaps by vesicle-associated myosin (35, 37). This speculation was based on the observation that in QFDERR preparations of isolated brush borders there are numerous vesicles attached to the core rootlets. More recently, Hirokawa et al. (30) have demonstrated that the connections between these vesicles and the core rootlet may be comprised, at least in part, of TW 260/240 (or its equivalent in mammalian brush borders). Thus, TW 260/240 might facilitate the attachment of endocytotic and/or Golgi-derived vesicles to the core rootlets. Given the possible "loose" association of TW 260/240 with the core rootlets, this spectrin-like protein, pehaps in concert with myosin, might also facilitate the movement of these vesicles up and/or down the core rootlets. We readily admit, however, that the possible involvement of TW 260/240 and myosin in membrane traffic in the brush border is, at this point, strictly fantasy. The composition of the rootlet-associated vesicles will have to be examined, and their presence in intact cells will have to be demonstrated. In addition, we will have to determine whether TW 260/240, like other spectrin-like proteins, can interact specifically with membranes, and whether there are membrane binding sites (e.g., ankyrin-like; 55, 3, 8, 9) on these vesicles or on the plasma membrane.

One final property of TW 260/240 that should be discussed with respect to the function of this protein is its interaction with the Ca^{++}-dependent regulatory protein, calmodulin. It has been shown by acrylamide gel overlay techniques that the 240-kdalton subunit binds to calmodulin in the presence but not absence of calcium (11, 12, 14). Little else is known about this interaction, however. Future studies will hopefully address a number of questions, including (*a*) the nature of TW 260/240-calmodulin interaction in solution, under physiological conditions, (*b*) the effect of calmodulin on the interaction of TW 260/240 with actin and other cytoskeletal proteins of the terminal web, and (*c*) the possible role of calmodulin in modulating the interaction of TW 260/240 with the plasma membrane, vesicles, and intermediate filaments (30).

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