

# Tropomyosin Distinguishes between the Two Actin-binding Sites of Villin and Affects Actin-binding Properties of Other Brush Border Proteins

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**Abstract.** The intestinal epithelial cell brush border exhibits distinct localizations of the actin-binding protein components of its cytoskeleton. The protein interactions that dictate this subcellular organization are as yet unknown. We report here that tropomyosin, which is found in the rootlet but not in the microvillus core, can bind to and saturate the actin of isolated cores, and can cause the dissociation of up to 30% of the villin and fimbrin from the cores but does not affect actin binding by 110-kD calmodulin. Low speed sedimentation assays and ultrastructural analysis show that the tropomyosin-containing cores remain bundled, and that 110-kD calmodulin remains attached to the core filaments.

The effects of tropomyosin on the binding and bundling activities of villin were subsequently determined by sedimentation assays. Villin binds to F-actin with an apparent  $K_a$  of  $7 \times 10^5 \text{ M}^{-1}$  at approximate physiological ionic strength, which is an order of magnitude

lower than that of intestinal epithelial cell tropomyosin. Binding of villin to F-actin presaturated with tropomyosin is inhibited relative to that to pure F-actin, although full saturation can be obtained by increasing the villin concentration. Villin also inhibits the binding of tropomyosin to F-actin, although not to the same extent. However, tropomyosin strongly inhibits bundling of F-actin by villin, and bundling is not recovered even at a saturating villin concentration. Since villin has two actin-binding sites, both of which are required for bundling, the fact that tropomyosin inhibits bundling of F-actin under conditions where actin is fully saturated with villin strongly suggests that tropomyosin's and one of villin's F-actin-binding sites overlap. These results indicate that villin and tropomyosin could compete for actin filaments in the intestinal epithelial cell, and that tropomyosin may play a major role in the regulation of microfilament structure in these and other cells.

**A**CTIN filaments in cells are organized into bundled linear arrays that are used for structural support or for cellular movement. The assembly of actin filaments into bundles is mediated by a variety of actin-binding proteins that have been grouped into various categories based on their functional properties (Pollard, 1984). Interestingly, actin-binding proteins are not evenly distributed along cellular actin bundles. For instance, the well-studied stress fibers of tissue culture cells possess periodic arrays of specific actin-binding proteins; regions of the stress fiber containing tropomyosin (TM),<sup>1</sup> caldesmon, myosin, and myosin light chain kinase alternate with regions containing  $\alpha$ -actinin (Bretscher and Lynch, 1985).

Microvillar actin bundles from intestinal epithelial cell brush borders contain different actin-binding proteins in each of the two major domains, the microvillus core and the rootlet (Mooseker, 1985). The microvillar actin bundles possess two actin-bundling proteins, villin and fimbrin, as well as the 110-kD protein and calmodulin complex that form the

cross-filaments that link the core bundle to the overlying membrane. Once the actin bundle enters the apical cytoplasm as the rootlet, additional actin-binding proteins are associated. These include: TM (Bretscher and Weber, 1978a; Geiger et al., 1981; Drenckhahn and Groschel-Stewart, 1980), which is likely associated along the length of the actin filaments; TW 260/240 (Glenney and Glenney, 1983), and myosin (Bretscher and Weber, 1978a; Mooseker et al., 1978), which are inter rootlet linkers; and caldesmon (Bretscher and Lynch, 1985), whose precise localization is unknown. One actin-binding protein, villin, is associated with the entire microvillar bundle, including the rootlet (Drenckhahn et al., 1983).

Villin is the best characterized microvillar actin filament-binding protein. In the absence of  $\text{Ca}^{++}$  it serves as an effective actin filament-bundling protein in that it has two distinct actin-binding sites (Glenney et al., 1981b). In the presence of micromolar free  $\text{Ca}^{++}$ , villin becomes an F-actin-severing protein. The amino terminal third of villin contains the  $\text{Ca}^{++}$ -regulated severing activity, whereas the carboxy-terminal headpiece ( $M_r$  8,500) retains a  $\text{Ca}^{++}$ -independent

1. Abbreviation used in this paper: TM, tropomyosin.

actin-binding domain (Glenny et al., 1981b; Matsudaira et al., 1985a, b; Hesterberg and Weber, 1986). While this series of domain mapping studies has revealed actin-binding regions of the villin molecule, nothing is known about the site(s) on the actin molecule or filament where villin binds.

Two basic approaches have been taken for localizing protein-binding domains on actin or on actin filaments. Optical and x-ray diffraction methods have been used to determine the binding domains of several thin filament proteins, including TM (Egelman and DeRosier, 1983) and fascin, a sea urchin egg actin-bundling protein that has two actin-binding sites (DeRosier et al., 1977; Spudich and Amos, 1979). Attempts to reveal actin-binding domains for villin by such methods have been unsuccessful (Matsudaira et al., 1983). Biochemical approaches, including the use of spectroscopic probes with cleavage and cross-linking studies, have yielded significant information about the protein-binding domains on actin. These kinds of studies have revealed, on a linear protein sequence map, the binding domains on actin for actin, TM, myosin, and several nonmuscle actin-binding proteins including DNase I, profilin, and depactin (Hambly et al., 1986).

In the present study we analyzed differential localization of actin-binding proteins along the filament so as to comment on the sites on actin where villin binds. At present, there is little information about the mechanism whereby actin-binding proteins are differentially bound along the length of an actin filament bundle or how different actin-binding proteins enhance or inhibit the interactions of other actin-binding proteins. For example, of the microvillus core proteins, it is known that the bundling abilities of villin and fimbrin are synergistic (Glenny et al., 1981a), and yet it is not known how TM is restricted to the rootlet portion of the core.

We have recently characterized the low  $M_r$  TM isoforms of chicken intestinal epithelial cells and found, surprisingly, that their measured association constant for F-actin is 10-fold greater than that of brain and much closer to that of gizzard smooth muscle TM (Broschat and Burgess, 1986). Since TM binds along the length of the actin filament at known domains on actin, and serves to stabilize the filament (Fujime and Ishiwata, 1971; Wegner, 1982; Fattoum et al., 1983; Bonder and Mooseker, 1983), it is likely that it will affect the interaction of various actin-binding proteins. In fact, both nonmuscle and muscle TMs, which have been shown to have remarkable sequence homologies, have been shown to affect the roles of several actin-binding proteins (Cote, 1983). For instance, muscle TM interferes with the binding of  $\alpha$ -actinin, spectrin, and filamin to actin filaments (Zeece et al., 1979; Endo and Masaki, 1982; Koteliensky et al., 1983). Muscle and nonmuscle TMs inhibit the actin-severing abilities of villin (Mooseker et al., 1982; Bonder and Mooseker, 1983) and other calcium-regulated actin-severing proteins (Bernstein and Bamberg, 1982; Fattoum et al., 1983). Two recent reports show that while muscle TM interferes with the actin-bundling ability of a 55-kD tissue culture cell actin filament-bundling protein, the nonmuscle TM isoforms from rat tissue culture cells do not inhibit 55-kD actin bundling (Yamashiro-Matsumura and Matsumura, 1985; Matsumura and Yamashiro-Matsumura, 1986). In fact, the low  $M_r$  TM isoforms were found to be competed off the actin filament by 55-kD, again suggesting common actin binding domains between TM and an actin-binding protein. Therefore, TM is a

good candidate for modulation of the binding properties of actin-binding proteins.

Since intestinal epithelial cell TM binds strongly to actin and yet is restricted to the rootlet (Drenckhahn and Groschel-Stewart, 1980), we investigated the ability of intestinal epithelial TM to bind to isolated microvillar cores and the resultant effects on core bundling in an attempt to determine why it is not found in the core in vivo. We also analyzed the effects of TM on the actin-binding and bundling properties of villin. We find that TM is capable of full saturation binding to microvillar cores, can displace villin and fimbrin from cores, has a higher affinity for actin filaments than does villin, can prevent villin from complete bundling of actin, and can displace villin from actin bundles. However, villin only weakly alters the binding properties of TM. Moreover, our results suggest that villin binds to two distinct sites on the actin filament and that one of these sites overlaps with TM's actin-binding site.

## Materials and Methods

### Microvillus Isolation

Adult chicken intestinal epithelial cell brush border microvilli were isolated according to the procedure of Bretscher and Weber (1978b) except that the protease inhibitors, phenylmethylsulfonyl fluoride (0.1 mM) and aprotinin (0.3 mg/liter) were included at each step during brush border and microvillus preparation. Microvillar cores were demembrated by a 30-min-1-h incubation in 1% Triton X-100 in Solution I (75 mM KCl, 0.1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM imidazole, pH 6.9). Cores were washed 2-3 times in Solution I and were then transferred into 150 mM KCl, 0.1 mM MgCl<sub>2</sub>, 1 mM EGTA, 10 mM imidazole, pH 6.9, for TM competition studies.

### Proteins

Intestinal epithelial cell TM was purified according to established methods (Broschat and Burgess, 1986). Briefly, TM was isolated from the EDTA/EGTA supernatant of disrupted cells (Matsudaira and Burgess, 1979) by heat denaturation and isoelectric precipitation at pH 4.6, followed by DEAE-cellulose and phenylsepharose chromatography. Thus isolated, epithelial cell TM is composed of four isoforms with  $M_s$ 's of 32-34 kD on Laemmli gels.

Villin was purified according to the methods of Bretscher and Weber (1980) from calcium-extracted brush borders. Briefly, this purification used ammonium sulfate fractionation of the calcium extract of brush borders followed by DNase-affinity chromatography. As purified, villin has a mobility of 95 kD on Laemmli gels.

Chicken breast muscle actin was purified according to Spudich and Watt (1971). Actin was dialyzed against 2 mM Tris, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl<sub>2</sub>, 0.5 mM mercaptoethanol, clarified by centrifugation at 100,000 g, its concentration determined, and then polymerized by bringing salt to 50 mM KCl, ATP to 1 mM, and MgCl<sub>2</sub> to 2 mM. Actin stocks were diluted to 5-10  $\mu$ M immediately before use.

### Competition Assays

Villin and TM stocks were dialyzed against the binding assay buffer (150 mM KCl, 2.7 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 20 mM imidazole, pH 7.0) overnight and were clarified by centrifugation in an airfuge (Beckman Instruments, Inc., Fullerton, CA) at 150,000 g to remove any aggregated protein before the determination of protein concentration. All binding assays were performed at 23°C in a total volume of 100  $\mu$ l. In competition assays using pure proteins, the actin concentration was constant at 3  $\mu$ M and in the case of microvillar cores the actin concentration was 10  $\mu$ M. Assays were performed as follows. Binding assay buffer was added to a tube, villin or TM was added and mixed by pipetting, and actin added and mixed by pipetting to initiate the reaction or preincubation. Concentrations of proteins were used such that when diluted to 100  $\mu$ l they were at the desired level. Varying concentrations of villin or TM were added to F-actin for a 1-h incubation period to derive normal saturation

curves. In competition assays, actin filaments were preincubated for 30 min with saturating concentrations of one protein, as determined by the saturation studies, and then increasing amounts of the competing protein were added for a 1-h incubation period. After incubation, tubes were spun at 27 psi (150,000 g) for 35 min in an airfuge (Beckman Instruments, Inc.) for actin-binding assays and tubes were spun in a microfuge (12,000 g; Beckman Instruments, Inc.) for 15 min for actin-bundling assays. After sedimentation, a 50- $\mu$ l aliquot of each supernatant was removed and the remaining supernatant carefully wicked away. A volume of 16.7  $\mu$ l of 4 $\times$  SDS sample buffer was added to the 50- $\mu$ l supernatant. The pellet was mixed with 100  $\mu$ l of binding buffer and 33.4  $\mu$ l of 4 $\times$  SDS sample buffer. The samples were denatured in a boiling water bath for 2 min before gel electrophoresis. Equal volumes of supernatants and pellets were electrophoresed in 12% Laemmli SDS microslab gels (Matsudaira and Burgess, 1978).

Before the initiation of the study, quantitation of protein by scanning densitometry (Zeineh scanning densitometer, tungsten lamp) was tested by running known amounts of protein per lane on a microslab gel that was subsequently stained with Coomassie Brilliant Blue R-250 as previously described (Matsudaira and Burgess, 1978). The linear range of dye binding was determined experimentally; all gels were loaded within these limits, and each experiment was monitored for the linearity and slope of the recorder response. For instance, the linear range for TM on microslab gels using Coomassie stain was determined to be 5–500 ng per lane. Each experimental set was loaded on the same gel, and the percentage of total protein bound to actin (or microvillar cores) or that protein displaced into the supernatant was quantitated by scanning the protein band both vertically and horizontally. The calculated results from separate scans were averaged for each sample. The percentage of total protein in the pellet was then used to calculate the concentration bound to F-actin. The points in the binding, bundling, and competition studies were derived from at least three different experiments using at least three different protein or microvillus preparations.

### Protein Determination

The concentration of intestinal TM was measured using the Bradford assay (Bio-Rad Laboratories, Richmond, CA) with purified gizzard TM ( $E_{280}^{1\%} = 2.2$ , [Woods, 1969]) as a standard for intestinal TM. Skeletal muscle actin and villin concentration were determined spectrophotometrically using  $E_{278}^{1\%} = 11.1$  and  $E_{280}^{1\%} = 13$ , respectively (Houk and Ue, 1974; Glenney et al., 1981b). Microvillus core protein concentration was determined according to Lowry using BSA as a standard. The actin concentration in cores was estimated by densitometric scanning of Coomassie-stained gels of cores. Approximately 40% of the core cytoskeletal protein was actin. Actin concentration was therefore used as 40% of core protein concentration. This measure is only an estimate, since it assumes that all proteins bind the dye Coomassie equivalently.

### Electron Microscopy

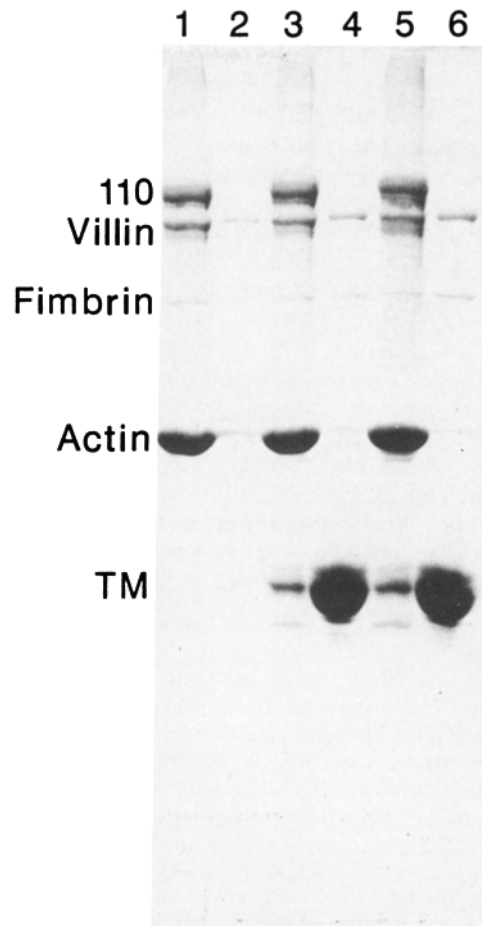
Samples were negatively stained on Formvar- and carbon-coated 300 mesh grids using 1% uranyl acetate and viewed in a JEOL 100CXII electron microscope operated at 60 kV.

## Results

### TM-Microvillus Core Competition

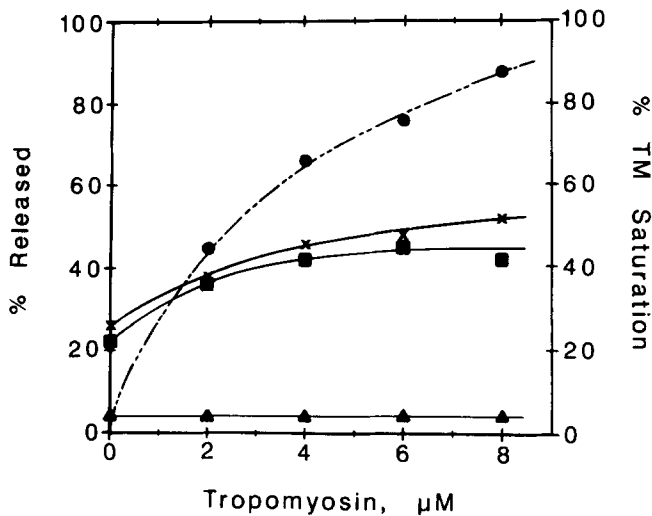
Since TM is absent from the microvillus core but present on the rootlet portion of the microvillus actin filament bundle, it was thought that one or more of the core actin-binding proteins might prevent TM's binding to the microvillus domain. Therefore, microvillar actin cores were isolated and a series of binding and bundling assays were performed with added exogenous TM. Previous studies have shown that villin-actin bundles, but not single actin filaments, are sedimented at low speed (12,000 g), whereas both single filaments and bundles are sedimented at high speed (>100,000 g) (Glenney et al., 1981b). By using both sedimentation assays the effects of TM on binding and on bundling of actin could be analyzed.

High speed centrifugation actin-binding assays with isolated core bundles revealed that TM bound effectively to the



**Figure 1.** SDS slab gel electrophoresis of isolated microvillar cores showing the displacement of villin and fimbrin by TM in high speed centrifugation assays. Demembrated microvilli (10  $\mu$ M actin) were incubated with increasing concentrations of intestinal epithelial cell TM in a buffer containing 150 mM KCl, 2.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM DTT, and 20 mM imidazole, pH 7.0, for 1 h at 23°C before high speed centrifugation. Supernatant and pellet samples were solubilized and electrophoresed in SDS gels as described in Materials and Methods. Lane 1, high speed pellet of isolated untreated cores showing protein composition. Note that no TM is present in the isolated core. Lane 2, high speed supernatant of isolated untreated cores reveals that very little actin or any of the actin-binding proteins are released in the control. Lane 3, high speed pellet of isolated cores incubated for 1 h with 6  $\mu$ M TM showing that TM effectively binds to the cores. Lane 4, high speed supernatant corresponding to the pellet shown in lane 3 demonstrating that villin and fimbrin are displaced from the microvillar core bundle by TM while 110 kD is not. Lane 5, high speed pellet of cores incubated for 4 h with TM (6  $\mu$ M), again showing binding of TM to the cores. Lane 6, high speed supernatant of the pellet shown in lane 5 showing dissociation of villin and fimbrin from the core by TM but no effect on the binding of 110 kD.

core actin filaments and, in addition, competed off both fimbrin and villin (Fig. 1). Increasing amounts of TM were incubated with isolated cores for 1–4 h, under conditions where 6–8  $\mu$ M total TM is required to fully saturate the actin ( $\sim 10$   $\mu$ M) in the bundle, and the amount of each protein released into the supernatant was determined. In this experiment, higher concentrations of core actin than those in experiments with pure proteins described later were necessary

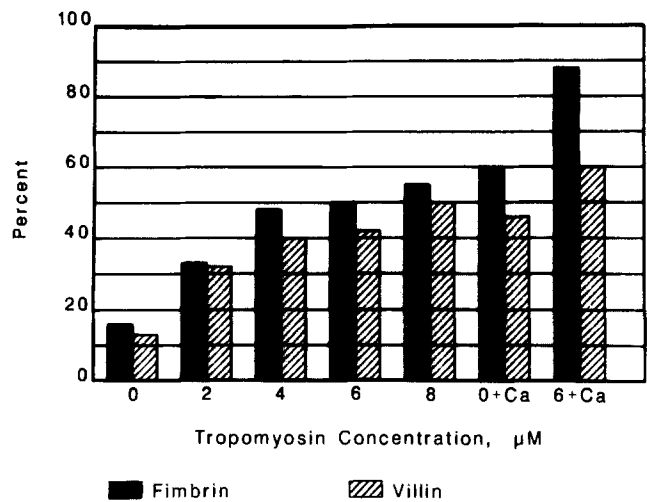


**Figure 2.** Quantitation of effects of TM on binding to isolated microvillar cores in high speed centrifugation assays. The percent of the microvillar core actin that is saturated with TM (solid circle) as determined by densitometry of the supernatant and pellet samples (described in Materials and Methods) is shown on the right ordinate axis. The percent of villin (solid square), fimbrin, (X), or actin (solid triangle) released to the supernatant in control and experimental samples is plotted on the left ordinate. Tropomyosin binds to microvillar core actin filaments, and approaches full saturation with a concomitant increase in the villin and fimbrin released into the supernatant. However, <5% of the actin is present in the supernatant in control or TM-treated microvillar cores.

because the concentration of various actin-binding proteins in the core is low (i.e., villin, fimbrin, and 110-kD are all found at a 1:10 molar ratio with actin) and is not visible on gels if actin is used at 3 μM. Consequently, higher concentrations of TM are required to saturate the actin present. More TM is required for full saturation of actin with isolated cores than that required to saturate pure actin, indicating inhibition of TM binding to core actin. To achieve full saturation of pure actin with TM (bound ratio of 1:6 TM/actin) a total molar ratio of 1:4 (TM/actin) is required (see below), whereas a total molar ratio of 1:1 (TM/actin) is necessary for saturation to core actin.

Up to 30% over control levels of both villin and fimbrin were released into the supernatant by increasing the level of TM from 2 to 8 μM for a 1-h incubation period (Fig. 2). Increasing the length of incubation time up to 4 h did not significantly increase the amount of villin or fimbrin released from the core (data not shown). Some villin and fimbrin in controls was released from the cores, most likely due to the fact that there was no free villin and fimbrin in the assay. Interestingly, no 110-kD cross-filament protein was found to be displaced into the supernatant by TM.

Low speed centrifugation assays were performed to determine the effects of TM on core bundling. Increasing amounts of TM caused a proportional increase in release into the low speed supernatant of villin and fimbrin from the core of up to 40–50% of each binding protein (Fig. 3); again, however, no release of the 110-kD cross-filament protein was detected. Treating isolated cores with >1 mM free Ca<sup>++</sup>, to cause severing of actin filaments by villin, released significantly more villin and fimbrin into the supernatant than did TM (Fig. 3), a result that is not surprising since this is the method

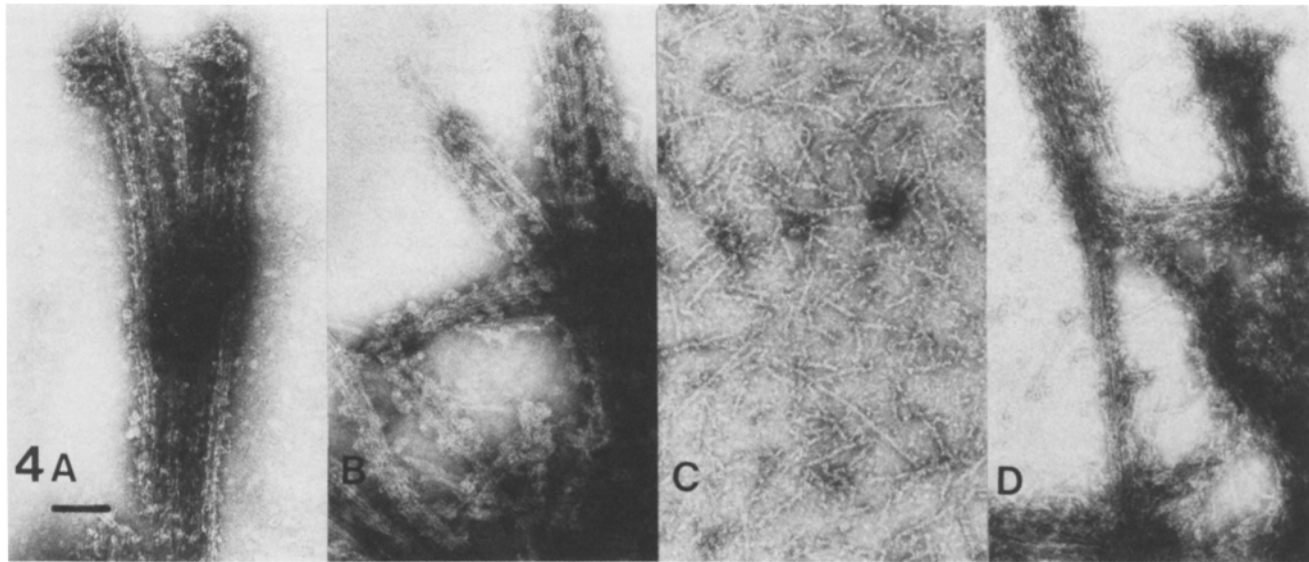


**Figure 3.** Quantitation of villin and fimbrin release from microvillar cores by addition of TM and/or calcium. The percent of villin and fimbrin released into the supernatant by each treatment was quantitated by densitometry as described in Materials and Methods. As shown in the high speed assay, increasing amounts of villin and fimbrin are released into the supernatant as TM binds to the actin of the microvillar cores. Microvillar cores were incubated for 1 h in the absence or presence of TM, and then were treated with 1 mM free Ca<sup>++</sup> for an additional 20 min before low speed sedimentation. Addition of Ca<sup>++</sup> causes the release of 60% of the fimbrin and 46% of the villin in the absence of TM. In the presence of 6 μM TM and Ca, 88% of the fimbrin and 60% of the villin are released into the low speed supernatant.

employed to disrupt cores for isolation of these bundling proteins (Bretscher and Weber, 1980). An additive effect on villin and fimbrin release from the cores was noted when TM preincubated cores were then treated with >1 mM free Ca<sup>++</sup> (Fig. 3).

The effects of added TM on the structure of isolated microvillus cores were also examined by negative stain EM (Fig. 4). Upon isolation, the core bundle remained intact with 10–15 core filaments in a tight bundle from which extended the periodically spaced cross-filaments (Fig. 4 A). The cores tended to aggregate together, as has been noted previously. When saturating amounts of TM were added to cores, most bundles appeared to retain the periodic cross-filaments. However, some core bundles appeared to be more loosely packed than control cores after the addition of TM (Fig. 4 B). As in control microvilli, the microvillar cores tended to aggregate in the presence of TM.

It has been reported that brush border TM protects pure actin filaments from severing by villin (Mooseker et al., 1982). To confirm that TM is indeed binding to microvillus core actin filaments, and not just adventitiously associating, we tested whether the core filaments were protected by TM from Ca<sup>++</sup>-induced actin-severing mediated by villin. We confirmed that Ca<sup>++</sup> causes the complete disruption of isolated control microvillar cores as has been reported before several times (Glenney et al., 1980; Mooseker et al., 1980; Burgess and Prum, 1982). In the presence of millimolar levels of free Ca<sup>++</sup>, isolated cores were severed, leaving behind large numbers of uniform short actin filaments averaging 0.15 μm in length (n = 19); no intact cores were observed (Fig. 4 C). However, when microvillar cores were first



**Figure 4.** Transmission electron micrographs of negatively stained isolated cores treated with TM,  $>1$  mM free  $\text{Ca}^{++}$ , or cores preincubated with TM followed with  $\text{Ca}^{++}$ . (A) Isolated intact cores showing well bundled cores with periodically arranged cross-filaments. (B) Isolated cores treated with saturating amounts of TM showing that while cores remain bundled, they are somewhat looser than those shown in A and reveal that TM has no effect on cross-filaments. (C) Isolated cores treated with high free  $\text{Ca}^{++}$  showing complete disruption of core bundles into short filaments. (D) Protection of isolated cores from  $\text{Ca}^{++}$ -treatment by preincubation with TM. As in TM-treated cores in the absence of  $\text{Ca}^{++}$ , the bundles are more loosely arranged than in controls; but with  $\text{Ca}^{++}$  the cross-filaments are not as distinct, although many are still seen. However, many cores are disrupted as evidenced by the presence of numerous short filaments in the field. Bar,  $0.1 \mu\text{m}$ .

preincubated with saturating amounts of TM and then treated with millimolar amounts of free  $\text{Ca}^{++}$ , many appeared to be protected from villin-induced severing (Fig. 4 D). These cores retained many cross-filaments, some appearing periodically spaced while others appeared as irregular protrusions from the bundles. Interestingly, while many cores remained intact and well-bundled, other core bundles were more loosely packed than in control bundles or in TM-treated bundles. Moreover, other TM-treated cores were fully disrupted by  $\text{Ca}^{++}$ , since the grids were covered with large numbers of actin filaments that averaged  $0.29 \mu\text{m}$  in length ( $n = 22$ ).

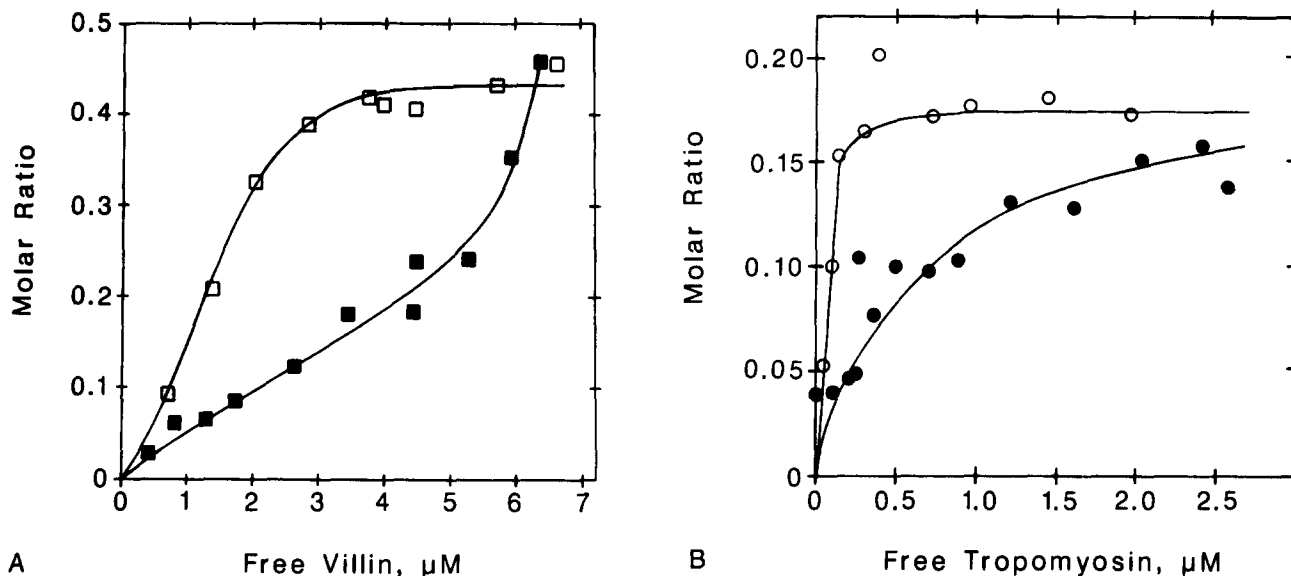
#### **TM-Villin Competition for Actin Binding**

Purified intestinal epithelial cell villin and TM were used in a series of *in vitro* actin-binding studies to further investigate their competitive binding to F-actin. Villin is normally found in the microvillar core at a bound molar ratio of 1:10 with actin (Matsudaira and Burgess, 1979) and has also been localized to the rootlet, along with TM, although its molar ratio to actin is unknown in the rootlet. Villin bound to and saturated pure F-actin at a molar ratio of 1:2.5 (Fig. 5 A), a finding similar to that reported in the original paper characterizing villin (Bretscher and Weber, 1980). Under these conditions of approximate physiological ionic strength and magnesium level and with  $3 \mu\text{M}$  F-actin, villin saturated actin at  $\sim 4 \mu\text{M}$  free villin. The calculated apparent  $K_a$  under these conditions, using the free villin concentration at half-saturation, was  $7 \times 10^5 \text{ M}^{-1}$ .

We then tested the effect of TM on villin's actin-binding properties. Intestinal epithelial cell TM has an apparent  $K_a$  for actin of  $8 \times 10^6 \text{ M}^{-1}$  (Broschat and Burgess, 1986) under the same conditions used to determine the apparent  $K_a$  of villin. Furthermore, like other nonmuscle TMs, it satu-

rated at a molar ratio of 1:6 with actin (Fig. 5 B). At approximate physiological ionic strength and magnesium level with  $3 \mu\text{M}$  actin, TM saturated F-actin at  $\sim 1 \mu\text{M}$  free TM. Therefore, for competition studies, actin was first saturated with  $1.25 \mu\text{M}$  TM and the binding of villin to the TM-actin was assayed. Tropomyosin significantly depressed villin's binding to actin such that the free villin required for one-half saturation was shifted from  $1.4 \mu\text{M}$ , for binding to pure F-actin, to  $6 \mu\text{M}$  for binding to actin presaturated with TM (Fig. 5 A).

The converse experiment, in which the effect of villin on the binding of TM to F-actin was analyzed, was also performed. Presaturating actin filaments with  $4 \mu\text{M}$  villin, to make bundles, had a moderate inhibitory effect on the binding of TM to F-actin (Fig. 5 B). The free TM concentration required for one-half saturation of F-actin shifted from  $\sim 0.15 \mu\text{M}$  for binding to pure actin to  $\sim 0.35 \mu\text{M}$  for binding to villin-saturated F-actin. This result is comparable to the previous result with isolated microvillar cores. Since the actin concentration was  $10 \mu\text{M}$  in the microvillar core experiment and  $3 \mu\text{M}$  in the purified villin and actin-binding study, the absolute TM concentration required to saturate F-actin was lower in the latter experiments. However, comparison of the total TM/actin ratio in assays that exhibited maximal TM binding indicates that the results of the two experiments are similar. When a total molar ratio of 1:2 TM/microvillar core actin was incubated, TM bound at a level of 60% saturation. At the same molar ratio with villin-saturated actin, TM bound at 56% saturation. Although the bound villin/actin ratio was higher in the latter experiment than that in the microvillar core experiment, the similar level of inhibition of TM binding suggests that other core proteins may inhibit TM binding to the microvillar core.



**Figure 5.** Binding of villin and TM to F-actin presaturated with the competing protein. (A) The binding of villin to 3  $\mu\text{M}$  F-actin (open square) was measured at increasing villin concentrations by high speed sedimentation assays. The molar ratio of villin/F-actin pelleted is plotted versus the free villin concentration. Villin saturates F-actin at a villin/actin ratio of 0.42. Binding of villin to F-actin preincubated with 1.25  $\mu\text{M}$  TM was measured at increasing villin concentrations (solid square). Binding is inhibited when compared with the control curve, but saturable binding is recovered at a higher free villin concentration. (B) Binding of TM to F-actin and F-actin preincubated for 30 min with a saturating concentration of villin is shown. Increasing concentrations of TM were incubated for 1 h with 3  $\mu\text{M}$  F-actin (open circle) and the bound TM was quantitated by the high speed sedimentation assay. The molar ratio of bound TM to F-actin is plotted versus the free TM concentration. Tropomyosin saturates F-actin at a bound molar ratio of 1:6. Binding of TM to F-actin that was preincubated with 4  $\mu\text{M}$  villin for 30 min is shown (solid circle). Binding of TM is inhibited when compared with the control curve.

Analysis of the binding and competition data according to Scatchard reveals more details about the actin-binding properties of these proteins (Fig. 6 A). Tropomyosin has long been known to bind to actin cooperatively, as is shown for intestinal epithelial TM (Fig. 6; Broschat and Burgess, 1986). The corresponding plot of TM binding to villin-saturated actin indicates that the presence of villin on actin completely abolishes the typical cooperativity of TM binding to actin. When the competition data are fitted with linear least squares, the  $x$  intercept is at 0.172 molar ratio of TM/actin, virtually identical to that of 0.169 determined experimentally (Broschat and Burgess, 1986). The Scatchard plots of villin binding to either actin or TM-saturated actin are more complex. Since these plots are the result of binding by both of villin's actin-binding sites, they must be interpreted with caution (Fig. 6 B). The plot of villin binding to pure actin is strongly suggestive of cooperative binding. The cooperativity could be due to one molecule of villin enhancing binding by other molecules due to alignment of actin filaments in a bundle. The plot is dramatically different for villin binding to TM-saturated actin. The apparent cooperativity of villin binding to pure actin is at least partially lost when villin binds to TM-saturated actin.

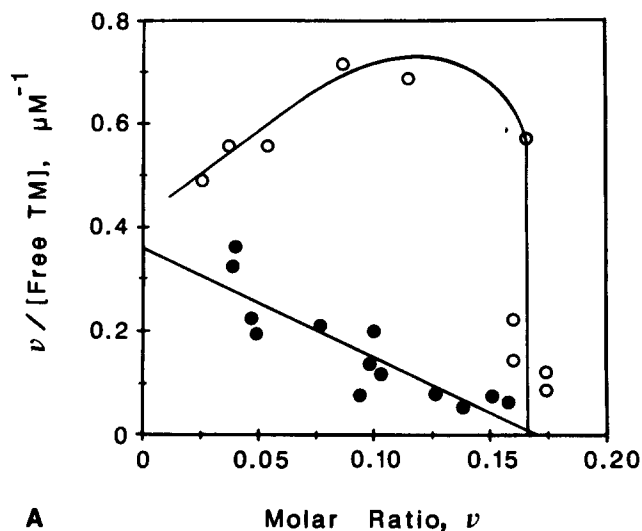
Another way of demonstrating the competition between villin and TM for binding to the actin filament is to determine the amount of the presaturated actin-binding protein that is displaced as the competing protein binds saturably to the actin filament (Fig. 7). Saturating levels of TM were found to displace  $\sim 50\%$  of the bound villin from actin filament bundles, whereas saturating amounts of villin could maximally displace only  $\sim 25\%$  of the bound TM from actin filaments.

#### TM-Villin Competition for Actin Bundling

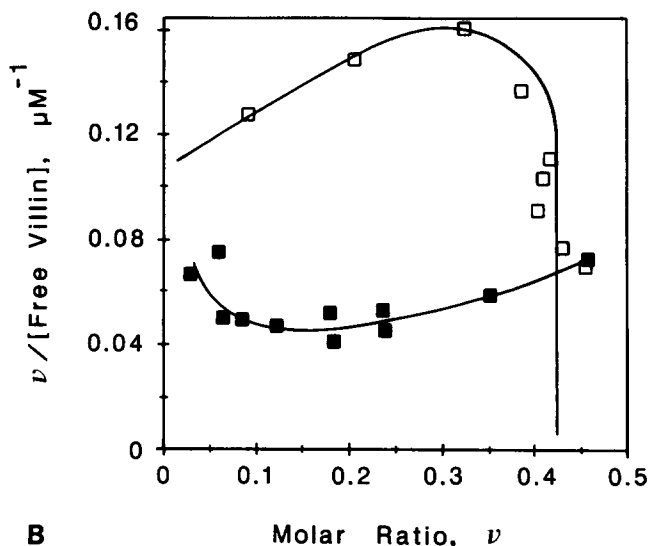
The binding data presented above suggest that TM has a significant effect on the binding of villin to actin. Since villin has two actin-binding sites, both required for actin filament-bundling (Glenney et al., 1981b), a series of low speed sedimentation assays were performed to quantitate the effects of TM on the bundling of actin filaments by villin. As a control, increasing amounts of villin were mixed with pure actin and the percentage of actin pelleting at low speed was determined (Fig. 8). Subsaturing amounts of villin (1  $\mu\text{M}$ ) caused  $\sim 30\%$  of the F-actin to be sedimented, whereas maximal bundling was achieved by 2.5  $\mu\text{M}$  villin, which is a subsaturating level, as shown in Fig. 5 A. The apparent  $K_d$  for bundling was calculated from the free villin concentration at one-half maximal bundling to be  $1 \times 10^6 \text{ M}^{-1}$ , a slightly higher value than that for binding.

Actin filaments were also preincubated with saturating amounts of TM and then increasing levels of villin were added for a 1-h period. After low speed centrifugation, the amount of actin sedimenting as a measure of bundling ability was determined (Fig. 8). Control actin saturated with TM did not sediment at low speed. Increasing the villin concentration from 1 to 9  $\mu\text{M}$  ( $\sim 0.7\text{--}7.3 \mu\text{M}$  free villin) resulted in detectable bundling only at the highest villin concentrations. While the size of the actin pellets was small, the pellets did contain TM, and determinations of the ratio of TM bound to actin in the pellets indicated that the actin was still near saturation with TM (data not shown).

The converse low speed centrifugation bundling assay was also performed, in which villin-saturated actin bundles were incubated with increasing amounts of TM. Measuring the



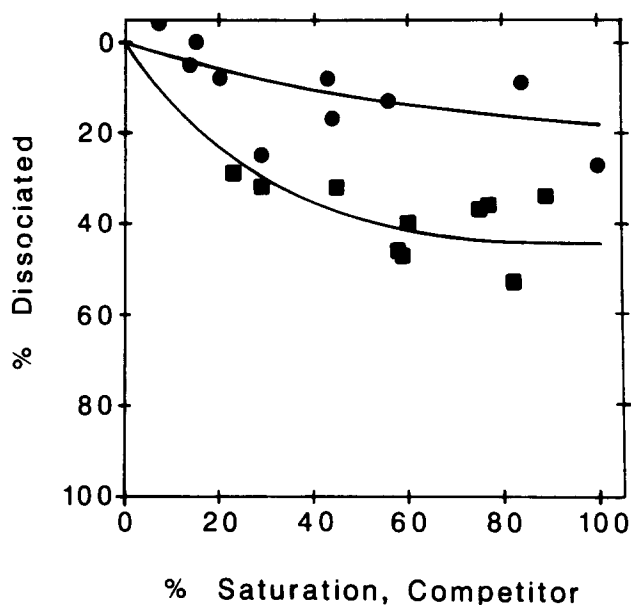
A



B

Figure 6. Scatchard plots of TM and villin high speed actin binding curves. (A) Scatchard plots of TM binding to F-actin and F-actin presaturated with villin. The bound TM/actin molar ratio,  $v$ , is plotted vs.  $v/\text{free TM}$ ,  $\mu\text{M}^{-1}$ . Tropomyosin binding to F-actin (open circle) follows a highly convex curve, which indicates that it binds cooperatively to F-actin. Tropomyosin binding to F-actin presaturated with villin (solid circle) falls upon a line that intersects the x axis at 0.172, the same as found with the control curve. The cooperative binding of TM to F-actin is abolished by the presence of villin. (B) Scatchard plots of villin binding to F-actin and F-actin presaturated with TM. The bound villin/actin molar ratio,  $v$ , is plotted vs.  $v/\text{free villin}$ ,  $\mu\text{M}^{-1}$ . Villin binding to F-actin (open square) follows a convex curve, suggesting cooperative binding. Villin binding to F-actin presaturated with TM (solid square) follows a concave curve, suggesting that TM interferes with the cooperative nature of villin binding to F-actin.

amount of actin pelleting showed that even a subsaturating amount of TM,  $1.0 \mu\text{M}$  ( $\sim 0.7 \mu\text{M}$  free TM), inhibited bundling by  $\sim 50\%$  (Fig. 9). Higher TM concentrations, including levels at and above saturating concentrations ( $2 \mu\text{M}$  equals  $\sim 1.6 \mu\text{M}$  free TM) were even more effective in inhibiting bundling. The calculated molar ratio of TM and villin to actin in the low speed pellet at the highest concentra-



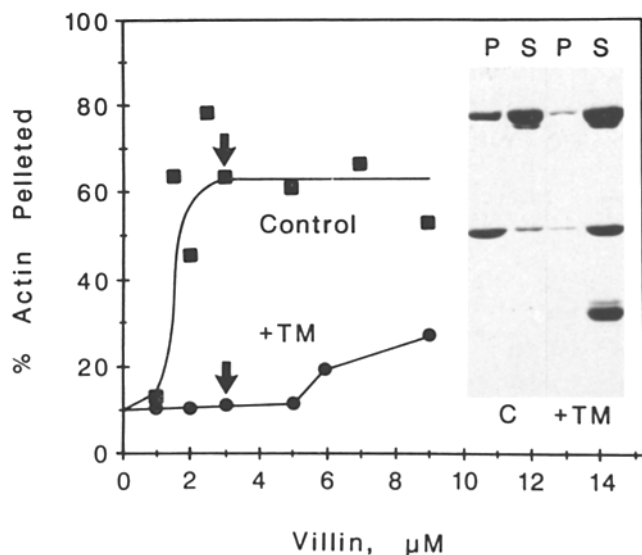
tion of TM added ( $5 \mu\text{M}$  equals  $\sim 4.3 \mu\text{M}$  free) showed both binding proteins to be near full saturation of the actin.

Figure 7. Dissociation of villin or TM from F-actin during binding of the competitor. The percent of villin dissociated (solid square) from the F-actin in high speed sedimentation assays of TM binding to F-actin presaturated with villin was quantitated and is plotted versus the percent of TM saturation. Initially, the F-actin is saturated with villin ( $4 \mu\text{M}$  villin with  $3 \mu\text{M}$  actin), but as TM binds to the actin, up to 50% of the bound villin is displaced from the actin filament. In the converse experiment where villin is added to F-actin ( $3 \mu\text{M}$ ) presaturated with TM ( $1.25 \mu\text{M}$ ), some TM (solid circle) is displaced from the F-actin as villin approaches saturation, but TM is still bound at 80% of saturation, even when villin saturates the F-actin. In both cases a percentage of the preincubated protein remains bound to the F-actin even when it is saturated with the competitor protein.

tion of TM added ( $5 \mu\text{M}$  equals  $\sim 4.3 \mu\text{M}$  free) showed both binding proteins to be near full saturation of the actin.

### Structure of Bundles

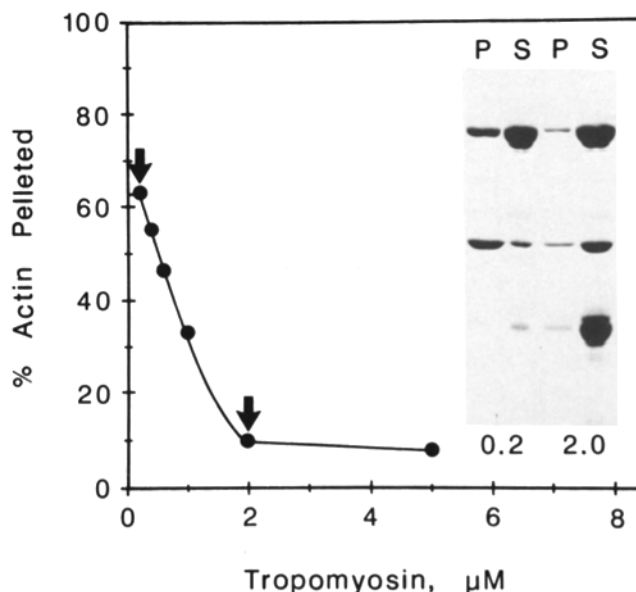
The structure of actin bundles was analyzed by both light and electron microscopy in an effort to determine if there were any morphological correlates to the binding and bundling data. Negative stain electron microscopy showed villin-actin bundles as thick, coarsing, loose, intermeshed bundles that were generally not straight (Fig. 10). Most of the bundles formed with saturating amounts of villin consisted of at least 10 filaments, with some of the bundles merged into larger arrays. Aliquots of both the whole sample before centrifugation and the 12,000-g supernatant from the low speed competition bundling assays were also analyzed by electron microscopy (Fig. 10). The trends described were noted in each of the samples, regardless of the molar ratio of the competitor added. The whole sample of TM-saturated actin to which villin was added had many single actin filaments, along with a small population of thin fascicles consisting of fewer than seven filaments each. These thin bundles were straighter than those formed with villin and actin. The low speed supernatant of this sample was almost entirely composed of single actin filaments with an occasional thin fascicle interspersed. The almost exclusive presence of single actin filaments in these samples is consistent with the bundling data shown in



**Figure 8.** Villin bundling of F-actin or F-actin presaturated with TM. Increasing amounts of villin were added to F-actin (*solid square*) or TM-saturated actin (*solid circle*) as described in Materials and Methods and bundling was monitored by measuring the percent of actin sedimented at low speed. Increasing the amount of villin causes an increase in the amount of actin pelleted to a maximum of 62% pelleted. However, when the actin is presaturated with TM (1.25  $\mu\text{M}$ ), bundling is detected only when the villin concentration approaches that known to saturate actin (5  $\mu\text{M}$  total villin equals 4.4  $\mu\text{M}$  free villin; 9  $\mu\text{M}$  total equals 7.8  $\mu\text{M}$  free). The inset shows an SDS gel of the pellet and supernatant fractions of the two points indicated on the curves and reveals the dramatic inhibitory effect of TM upon the bundling of actin by villin.

Fig. 8, where only minimal bundling occurred with high concentrations of added villin. On the other hand, the whole sample of villin-presaturated actin with added TM was composed of single filaments along with actin bundles ranging in size from thin fascicles to bundles. The presence of some fascicles and small bundles is consistent with the bundling data shown in Fig. 9, where bundling is shown to be inhibited but not abolished by added TM. Again, all bundles were much straighter than villin-pure actin bundles. The low speed supernatant from villin-presaturated actin with added TM contained single actin filaments along with many fine, straight, thin fascicles but few bundles. These results confirm that low speed centrifugation is a rough measure of bundling, since large bundles, but not thin fascicles, were sedimented. The finding that TM-containing bundles are straighter than villin-actin bundles supports previous biochemical work indicating that TM stiffens the actin filament.

The bundling data indicate that all bundles formed in competition assays contained TM and villin. As another means of confirming that TM was present on the bundled F-actin, and was not just aggregating, we performed immunofluorescence microscopy using antibodies against intestinal epithelial TM (data not shown). The antibodies were purified and characterized as described in Broschat and Burgess (1986). Phase-contrast microscopy of villin-presaturated actin bundles to which saturating levels of TM were added revealed large aggregates of fine thin filaments. All fine filaments observed by phase microscopy were uniformly fluorescent with no detectable periodicity or banding. Phase microscopy of



**Figure 9.** Effects of TM upon villin-actin bundles. Villin-actin bundles were formed by incubating villin (4  $\mu\text{M}$ , a saturating concentration) and F-actin (3  $\mu\text{M}$ ) for 30 min before TM addition. Increasing amounts of TM were added to the villin-actin bundles and incubated for 1 h before low speed sedimentation. Bundling, as measured by the percent of actin pelleted, appears to be disrupted by even subsaturating amounts of TM, and appears fully inhibited by saturating concentrations of TM (1.0  $\mu\text{M}$  total TM equals 0.7  $\mu\text{M}$  free TM; 2  $\mu\text{M}$  total TM equals 1.6  $\mu\text{M}$  free TM). The inset shows an SDS gel of the pellet and supernatant fractions from two points on the curve indicated by arrows. This gel shows the dramatic difference in the amount of actin pelleted as a result of adding TM to villin-actin bundles.

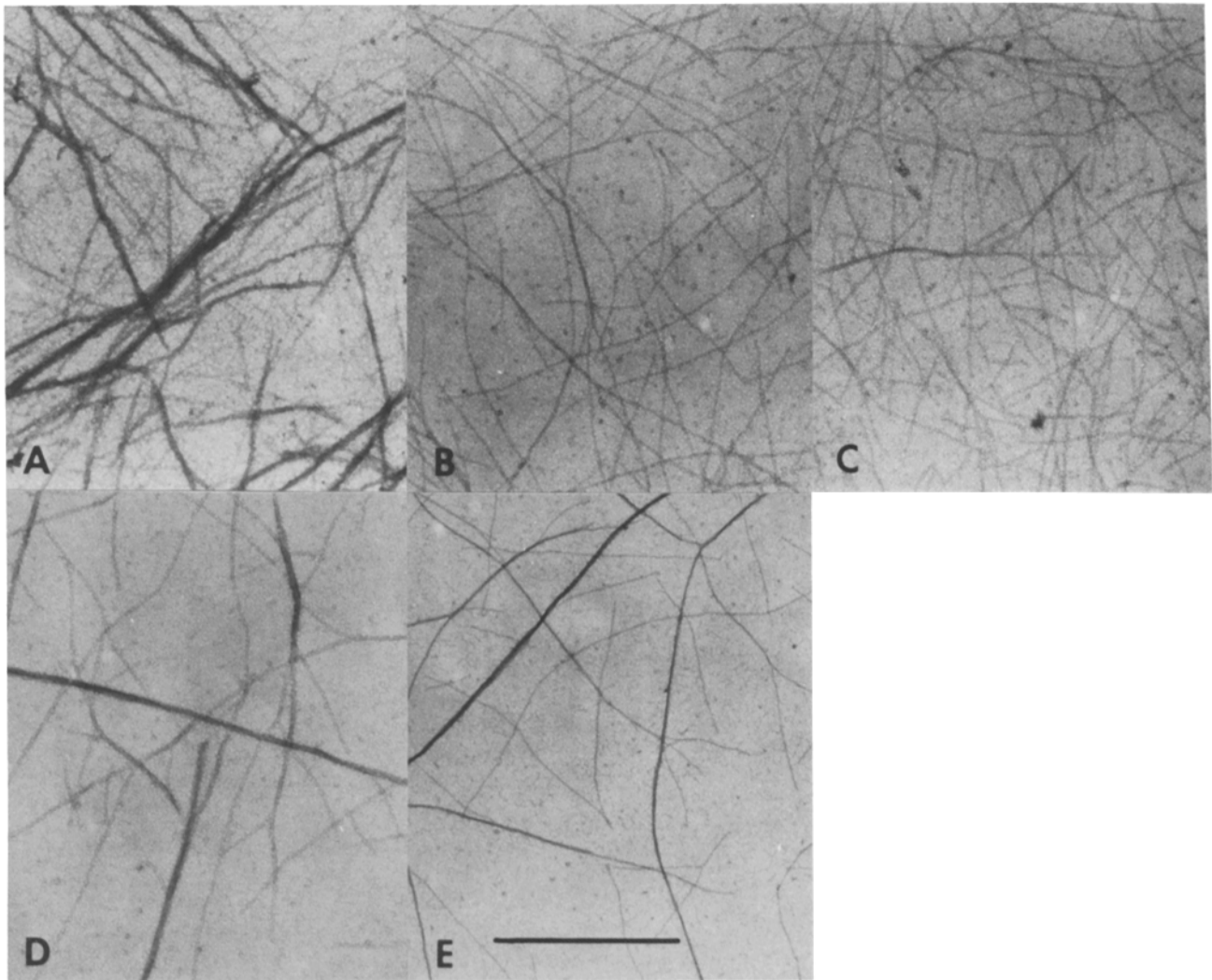
TM-presaturated F-actin to which saturating amounts of villin were added showed not only fine filaments but larger clumps of very fine cottony masses of material. Upon observation by immunofluorescence microscopy, all of these TM-presaturated bundles and fine masses were also uniformly fluorescent. Therefore, the immunofluorescence data support the bundling data in that all villin-actin bundles formed contained TM.

### Discussion

There are possibly two ways, not mutually exclusive, to account for inhibition of actin binding by another bound actin-binding protein. First, one protein could, upon binding to actin, change the filament conformation to lower the apparent affinity of the second actin-binding protein. Second, two actin-binding proteins could share the same binding site or have very close sites, such that the protein with the highest binding affinity would be favored to bind. It is well known that TM strengthens or stiffens the actin filament (Fujime and Ishiwata, 1971; Wegner, 1982) such that it may alter the filament structure, and thus when bound may inhibit binding by another protein.

Our data support the hypothesis that TM and villin share at least one common binding domain on F-actin. We find that intestinal TM's association constant for actin is 10-fold higher than that of villin, while both proteins bind to actin in a cooperative manner. Further, TM effectively diminishes





**Figure 10.** Transmission electron micrographs of samples from the low speed bundling assays. An aliquot was removed before low speed centrifugation at 12,000 g or from the 12,000 g supernatant and was negatively stained. Shown are samples from assays at the highest level of competitor in the bundling experiments shown in Figs. 8 and 9. (A) Control villin-actin bundles with 7  $\mu\text{M}$  villin and 3  $\mu\text{M}$  actin before centrifugation. Large bundles of actin that tend to branch and aggregate are formed. Most of the bundles are not linear but appear as if they are flexible. Occasional single filaments are present between the bundles that thickly cover the grid. (B) Sample before centrifugation of 9  $\mu\text{M}$  villin incubated 1 h with TM-presaturated actin showing a large field of single actin filaments with an occasional small fascicle composed of two or three filaments. (C) Sample of the supernatant after low speed centrifugation of 9  $\mu\text{M}$  villin incubated with TM-presaturated actin, revealing that the supernatant fraction contains a uniform population of single actin filaments. A rare fascicle of two filaments is observed. (D) The sample before centrifugation of 5  $\mu\text{M}$  TM incubated with villin-presaturated actin contains single filaments, small fascicles, and medium-sized bundles. All fascicles and bundles are much straighter than control villin bundles. (E) The supernatant fraction after centrifugation of the sample shown in D contains large numbers of single filaments and fine fascicles. Only rarely is a medium-sized bundle observed. Bar, 1.0  $\mu\text{m}$ .

villin's cooperative binding to actin, and also its F-actin-bundling ability, indicating that the proteins could be competing for the same binding sites. More support of the idea that TM and villin have similar F-actin-binding sites lies in the results wherein either protein is competed off actin filaments by the other. The sites on the actin molecule where TM binds have been localized through biochemical labeling studies to be between amino acids 50–80 and 325–375 (Johnson and Blazyk, 1978; Curmi et al., 1982). These same regions have been shown to be the binding domains for several other actin-binding proteins, including myosin subfragment 1 (Sutoh, 1982), profilin (Malm, 1984), and depactin (Sutoh and Mabuchi, 1984; also see Hambly et al., 1986 for review).

The presence of common binding domains on actin for many actin-binding proteins may explain why many actin-binding proteins compete in binding assays. Only more completely detailed structural and domain mapping work, and/or analysis of crystal structure will determine the precise domains on the actin molecule for villin. However, the kind of competition study used in this work provides an alternative way of analyzing and confirming binding sites for actin-binding proteins. This kind of competition analysis has an advantage over the use of fragments of binding proteins generated by proteolysis in that it makes use of the intact protein whose tertiary structure has not been modified, avoiding a potential problem in studies using fragmented proteins.

To date, studies on villin's two actin-binding sites have been directed at analyzing the actin-binding domains on the villin molecule and show that the two sites are located at different ends of the protein (Glenney et al., 1981b; Matsudaira et al., 1985a, b), both of which are required for actin filament-bundling (Glenney et al., 1981b). The present work is the first to indicate that villin's two binding sites may bind to different domains on actin. Surprisingly, while villin has been shown to possess a very high affinity actin barbed-end site (Walsh et al., 1984) the association constant of intact villin or of the two major villin proteolytic fragments for F-actin have not been reported, although villin fragments possessing the two actin-binding domains have been isolated and studied (Glenney et al., 1981b; Matsudaira et al., 1985a, b; Hesterberg and Weber, 1986). We now show that villin binds actin in a cooperative manner, with apparent  $K_a$ s for binding of  $7 \times 10^5 \text{ M}^{-1}$  and for bundling of  $1 \times 10^6 \text{ M}^{-1}$ , although how this cooperativity is manifested is not known at this time. The apparent  $K_a$ s we calculate for intact villin are obviously the products of the two actin-binding sites on villin, and yet intestinal TM competes with both of these sites, as shown by the release of villin from villin-presaturated bundles when TM is added into the supernatant in both binding and bundling competition assays. The binding and bundling data are complemented by negative stain electron microscopy that shows that many bundles are unbundled or significantly reduced by adding TM. Further, the presence of TM on actin significantly alters the cooperative binding of villin to actin. Comparison of the binding curves with the bundling curves in the case where increasing amounts of villin are added to TM-saturated actin indicates that one of villin's actin-binding sites is much more sensitive to TM than the other. Using the same amount of villin needed to saturate TM-preincubated F-actin (Fig. 5 A) results in less than half the actin bundling, as shown in Fig. 8. The presence of large numbers of single actin filaments observed in the supernatants of competition assays is consistent with this result. A simple explanation for such a result is that villin's two actin-binding sites bind to different regions on actin, one of which binds to the same domain as does TM, but which has a lower apparent  $K_a$  than that of TM, and a second that binds to a site different from the TM site. In such a case one could still have fully saturated but unbundled actin filaments. That saturation of F-actin with either villin or TM does not cause 100% displacement of the other bound protein, which would be predicted if both villin sites overlapped with the TM sites, further supports this hypothesis. Alternatively, both of villin's actin-binding sites could bind to identical domains on actin as does TM, but with one of the sites having a higher affinity and one having a lower affinity than that of TM. These hypotheses are being directly tested by actin-binding competition studies with TM, using two proteolytic fragments of villin, 51T and 44T, which each possess one binding site. A priori since the amino terminal villin fragment, 44T, retains the  $\text{Ca}^{++}$ -sensitive actin-severing ability and TM inhibits such severing, it is likely that TM and the amino-terminal actin-binding domain on villin bind to the same site on actin.

Little is known about the actin-binding sites of either fimbrin or the 110-kD cross-filament protein. Structural data suggest that fimbrin is the primary actin filament bundler of

the core, since it forms more ordered actin bundles than does villin (Matsudaira et al., 1983). While villin and fimbrin bind actin synergistically in vitro (Glenney et al., 1981a), they exist at well below saturation levels in the microvillus core (Matsudaira and Burgess, 1979) and therefore they should not affect each other's binding in the core. Like villin, fimbrin must contain two actin-binding sites to be able to bundle filaments and, also, like villin, TM is effective in displacing both sites as shown by the experiments performed in this study with cores. On the other hand, TM does not displace the 110-kD cross-filament protein from the core, indicating that it must bind to a different region on the actin filament than does villin or fimbrin. Like the actin-binding site for myosin, the 110-kD protein's site is ATP sensitive but not sensitive to inhibition by TM.

In terms of the intestinal brush border cytoskeleton where TM is found only in the rootlet portion of the microvillar bundle, our results would argue for a lower villin concentration in the rootlet than in the core, where villin is found in a 1:10 molar ratio with actin. To achieve such a molar ratio in vitro, we found that twice as much free villin must be added when the filaments are presaturated with TM as that necessary to achieve a 1:10 ratio on pure actin. Since all the cytoskeletal proteins of the brush border are turning over (Stidwill et al., 1984; Cowell and Danielson, 1984), our results do not explain why TM is excluded from the microvillar core. One possible explanation for the restriction of TM to the rootlet is that another rootlet protein enhances the binding of TM to the rootlet and that levels of TM in the cell are at subsaturating levels for actin. One candidate for a rootlet protein that might enhance TM's actin binding is caldesmon, which has been localized to the rootlet region (Bretscher and Lynch, 1985) and which enhances the binding of TM to actin (Sobue et al., 1982; Bretscher, 1984). In terms of other terminal web proteins, we have also analyzed the effects of TM on binding of TW260/240 to actin. TW260/240 has been shown to be unique among the spectrin family in that it does not bind well to actin, inside out red blood cell membranes, or to band 4.1 under physiological conditions (Howe, 1985), and yet in situ it is associated with the rootlets (Hirokawa et al., 1983; Glenney and Glenney, 1983). We found that TM did not enhance the very weak binding of TW260/240 (courtesy of Dr. Mark Mooseker, Yale University, New Haven, CT) to actin. Unfortunately, we still do not understand how TW260/240 is associated with the terminal web cytoskeleton and can only speculate that some other protein mediates its binding.

Recently, there has been speculation that various nonmuscle TM isoforms may play a role in modulating the organization of microfilaments in cells, both by stabilizing filaments and by regulating the distribution of other actin-binding proteins along filaments (Matsumura and Yamashiro-Matsumura, 1986). Our results tend to support this hypothesis. These authors found that TM competes with a fibroblast 55-kD actin filament-bundling protein. Similarly, Fattoum et al. (1983) found that muscle TM blocks the actin binding of macrophage gelsolin, a  $\text{Ca}^{++}$ -activated actin-severing protein related to villin (Matsudaira et al., 1985a). Interestingly, we also found that TM tends to make actin bundle into very straight or linear bundles rather than into coarsening bundles. Since intestinal epithelial TM was found to modulate

the binding of microvillar actin-binding proteins, and both blocks the severing abilities and appears to protect the rootlets from villin severing (Burgess and Prum, 1982), this TM may have a critical role in the regulation of cytoskeletal structure in the intestinal epithelial cell.

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