

Identification and Characterization of Two Huge Protein Components of the Brush Border Cytoskeleton: Evidence for a Cellular Isoform of Titin

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Abstract. Two extremely high molecular weight proteins were found to be components of the intestinal epithelial cell brush border cytoskeleton. The largest brush border protein, designated T-protein, migrated on SDS gels as a doublet of polypeptides with molecular weights similar to muscle titin T I and T II. The other large brush border protein, designated N-protein, was found to have a polypeptide molecular weight similar to muscle nebulin. In Western analysis, a polyclonal antibody raised against brush border T-protein reacted specifically with T-protein in isolated brush borders and cross-reacted with titin in pectoralis and cardiac muscle samples. T-protein was distinguished from the muscle titins by an anti-cardiac titin mAb. A polyclonal antibody raised against N-protein was specific for N-protein in brush borders and cross-reacted with nothing in pectoralis muscle. Immunolo-

calization in cryosections of intestinal epithelia and SDS-PAGE analysis of fractionated brush borders revealed that both T-protein and N-protein are concentrated distinctly in the brush border terminal web region subjacent to the microvilli, but absent from the microvilli. EM of rotary-replicated T-protein samples revealed many of the molecules to be long (912 ± 40 nm) and fibrous with a globular head on one end. In some of the molecules, the head domain appeared to be extended in a fibrous conformation yielding T-protein up to 1,700-nm long. The brush border N-protein was found as long polymers with a repeating structural unit of ~ 450 nm. Our findings indicate that brush border T-protein is a cellular isoform of titin and suggest that both T-protein and N-protein play structural roles in the brush border terminal web.

THE brush border array of microvilli at the apical end of intestinal epithelial cells is supported by one of the most extensively organized actin-based cytoskeletons found in nonmuscle cells (for review of brush border structure, see references 1, 12, 18). This cytoskeleton is a useful model system with which to identify structural components, because it can be isolated intact and in amounts sufficient for biochemical analysis. Moreover, the multiple domain structure of the brush border cytoskeleton aids in determining the functional and structural contributions of each component.

In the brush border, each of the numerous microvilli is supported by a core bundle of parallel actin filaments that is rooted in the underlying terminal web. Numerous filaments of myosin II and fodrin cross-link the microvillar bundle rootlets. This interrootlet domain is encompassed by a circumferential ring of anti-parallel actin filaments. Several major brush border proteins have been characterized and their localization in these brush border domains has yielded insight into their possible functions. Many of the proteins in this integrated system are related to components of various cytoskeletal organizations in other cells (12).

We report here the discovery of two huge proteins in the

brush border terminal web. These cytoskeletal proteins tentatively have been designated T-protein and N-protein to signify that their unusually high molecular weights are markedly similar to those of muscle titin and nebulin.

In skeletal muscle, titin and nebulin contribute to assembly and maintenance of the sarcomere by interacting with the thick and thin filaments (15, 26). Titin (also known as connectin, see reference 15) is a huge protein ($2-3 \times 10^6$ D molecular weight) that spans the distance between each thick filament and the Z-line in each half sarcomere (17). As an elastic component, titin is postulated to maintain the thick filaments in register and resist overstretching of the sarcomere (6, 7, 16, 23, 26). Nebulin ($6-8 \times 10^5$ D molecular weight), which is coextensive with each thin filament in skeletal muscle, is postulated to dictate the uniform thin filament length by providing a template for actin polymerization (8, 13, 28).

Titin-like molecules have been reported to exist in various nonmuscle systems (3, 4, 16), but the structure and function of these proteins remain uncharacterized. Nebulin has been found only in skeletal muscle and is absent even from cardiac muscle (28).

Our characterization of the immunoreactivity, molecular morphology, and cellular localization of brush border T-protein indicates that it is a cellular isoform of titin that might mediate the function of myosin II in the terminal web. Similar characterization of brush border N-protein has revealed properties that appear to differ from those of muscle nebulin, but are consistent with N-protein playing an important structural role in the brush border terminal web. The presence of T-protein and N-protein in the brush border cytoskeleton raises the possibility that they are structural components of actin-based cytoskeletons in other cells.

Materials and Methods

Isolation of Brush Borders, Microvilli, and Myofibrils

Brush borders were isolated from chicken intestinal epithelial cells by the method of Keller and Mooseker (11), with the following modifications: the intestinal segments were slit lengthwise and washed extensively in saline with 1 mM β -mercaptoethanol which appears to reduce mucin contamination, and the protease inhibitors leupeptin (0.05 mM) and pepstatin (0.1 mM) were used in addition to PMSF (0.2 mM) and Aprotinin (0.1 mM) in all of the solutions except the saline washes. To minimize proteolysis, the isolated brush borders were used immediately after purification. Microvilli were sheared from isolated brush borders and purified by differential centrifugation according to Mooseker and Howe (20).

Fresh chicken pectoralis and cardiac muscle was diced, snap frozen in liquid nitrogen, and pulverized under liquid nitrogen with a mortar and pestle. The resulting muscle powder was stored at -80°C . SDS-PAGE samples were made by resuspending the muscle powder in sample buffer and heating to 80°C for 2 min (25).

Extraction of Isolated Brush Borders

Brush borders were extracted, as described previously (9), by incubation for 10 min on ice as 10% suspensions in solutions containing ATP. The brush borders were extracted first in 0.15 M KCl + ATP (BSBB containing a total of 0.15 M KCl and 4 mM ATP) and pelleted by centrifugation at 12000 g for 10 min. The extraction was repeated, and the supernatants were combined as the 0.15 M KCl + ATP extract. The pelleted cytoskeletons were further extracted with 0.6 M KCl + ATP, with centrifugation and recovery of the supernatant and pellet as before.

Hydroxyapatite Column Purification of Brush Border T-protein and N-protein

To partially purify brush border T-protein, 0.15 M KCl + ATP extracts were loaded directly onto a hydroxyapatite column (HAP, 2.5-cm diam \times 16.5 cm) that was equilibrated with 0.6 M KCl-BSBB. The column was eluted with a 0–400 mM phosphate gradient made in the equilibration buffer. T-protein elution was most readily detected by silver-staining SDS-gels of the column fractions. The T-protein fractions were pooled and dialyzed into the column equilibration buffer. The T-protein then was concentrated by chromatography through a second HAP column (1.5 \times 7.5 cm). This concentrating column was eluted in the reverse direction with a step of the 400 mM phosphate elution solution.

N-protein was partially purified from 0.6 M KCl + ATP extracts of brush borders with a similar protocol. The peak of N-protein elution usually overlapped the elution of TW 260/240 (chicken brush border fodrin). N-protein peak fractions were dialyzed into BSBB before preparation for EM.

Gel Electrophoresis and Electroelution

To lessen smearing at the top of the gels, isolated brush borders, microvilli, and extracted brush borders were washed briefly with 0.1% NP-40 in BSBB, followed by BSBB with no NP-40, before dissolving in SDS-sample buffer. Muscle samples were made by dissolving the frozen powder directly in SDS-sample buffer. All samples were warmed immediately to 80°C for 2 min. Undissolved material was pelleted before loading the supernatant onto

the gel. Boiling or reheating the samples caused detectable breakdown of the T-protein and was avoided.

Electrophoresis was performed on high porosity SDS-polyacrylamide gels. For these gels, a stock solution of 30% acrylamide–0.4% bis-acrylamide was diluted using the Laemmli buffer system (14), to make straight 5% gels or 2–20% gradient running gels topped with 3% stacking gels. After electrophoresis, the gels were stained either with Coomassie blue (2) or silver (21). For immunoblot analysis, the gels were electroblotted onto nitrocellulose (27).

T-protein and N-protein were electroeluted from gels by first silver-staining diagnostic strips cut from the ends of 5% curtain gels and then excising the regions corresponding to the T-protein and N-protein bands from the unstained portions of the gels; the gel slices were equilibrated in electroelution buffer (0.015 M Tris-Cl, diluted from 1.5 M Tris-Cl stock solution, pH 8.8), diced, placed in an Isco electroelution chamber (Isco, Inc., Lincoln, NE), and electroeluted for 14 h at 1 W, according to the manufacturer's instructions. The current was reversed for 30 s before recovering the fluid in the collection chamber. The N-protein samples were dialyzed against 50 mM ammonium acetate before rotary replication with platinum for EM.

Electron Microscopy

The procedure used for platinum replication was a modification of the layering technique described by Nave et al. (22). Briefly, a drop of the sample solution containing 50% glycerol was applied to freshly cleaved mica and allowed to sit for 30 s. Filter paper was drawn across the mica to remove excess liquid. The mica was adhered with double-sided adhesive tape to a circular piece of cardboard at a radius of 5 cm. The cardboard was placed on top of the rotor of a microcentrifuge (model 235C; Fisher Scientific Co., Pittsburgh, PA) and spun very briefly. The sample then was shadowed with platinum at an angle of 5° and carbon coated at 90° (Edwards coating system E306A equipped with a Cressington Power Supply EB 602 PC). Replicas were floated from the mica onto the surface of distilled water and collected with 400-mesh copper grids. Grids were viewed using a transmission electron microscope (1200 EX; JEOL USA, Peabody, MA) at an accelerating voltage of 80 kV.

Morphometry

The images of T-protein molecules on EM negatives were traced manually and enlarged xerographically. Length contours were measured with a Bioquant II Image Analysis System (R & M Biometrics, Nashville, TN).

Antibodies and Immunoblotting

Polyclonal antibodies to brush border T-protein and N-protein were raised in rabbits. For the initial injections, the T-protein band in gels of the 0.15 M KCl + ATP extract of brush borders and the N-protein band in 0.6 M KCl + ATP extracts of brush border were excised from a silver-stained SDS-polyacrylamide gel, frozen in liquid nitrogen, pulverized, and emulsified in Freund's complete adjuvant. The T-protein and N-protein used for subsequent boosts were electroeluted from SDS-gel bands and emulsified in Freund's incomplete adjuvant.

Both antibodies were affinity purified using antigen electroblotted from SDS gels onto nitrocellulose. Crude antisera were diluted 1:5 in a block solution containing BSA and incubated with the nitrocellulose blots. The blots were washed extensively and specifically bound antibodies were eluted using 100 mM glycine, pH 2.5. The affinity-purified antibody solutions were neutralized with Tris base before storage.

The reactivity of anti-T-protein (1:2,500 dilution of the blot-purified antibody as eluted from the nitrocellulose) and anti-N-protein (1:500 dilution of the blot-purified antibody as eluted from the nitrocellulose) antibodies was demonstrated by Western analysis. We also tested an anti-cardiac titin mAb (1:2,000 dilution of antibody 9 D10, reference 29) that was purchased from the Developmental Studies Hybridoma Bank (Johns Hopkins University School of Medicine, Baltimore, MD). Primary antibody binding was detected with either anti-rabbit IgG (1:8,000 dilution in Buffer N, reference 26) or goat anti-mouse (1:2,000 dilution) secondary antibodies that were conjugated to alkaline phosphatase.

Immunolocalization of T-protein and N-protein in Cryosections of Intestinal Epithelium

Fixation and processing of isolated brush borders and cryosections of adult chicken small intestine for Differential Interference Contrast microscopy

1. *Abbreviation used in this paper:* HAP, hydroxyapatite.

and immunofluorescence microscopy were performed as described previously (10). The affinity-purified anti-brush border T-protein (1:100 dilution of the antibody as eluted) and N-protein (1:50 dilution of the antibody as eluted) antibodies were diluted in block solution (10% nonfat dry milk) before incubation with the tissue. Purified rabbit IgG (Sigma Chemical Co., St. Louis, MO), diluted to 7 $\mu\text{g}/\text{ml}$ in block solution was used as a nonimmune control.

Results

Identification and Biochemical Localization of Brush Border T-protein and N-protein

Electrophoresis of isolated brush borders in high porosity SDS-polyacrylamide gels revealed the presence of extremely high molecular weight polypeptides (Fig. 1, lane B). The largest of the brush border polypeptides, designated T-protein, migrated as a doublet with apparent molecular weights similar to those of the titin doublet (T) in SDS-extracts of snap frozen chicken pectoralis muscle (Fig. 1, lane A). The other major high molecular weight polypeptide in the brush border sample, designated N-protein, migrated with an apparent molecular weight similar to, but slightly greater than, that of pectoralis muscle nebulin (N).

To investigate the localization of T-protein and N-protein in the brush border cytoskeleton, isolated brush borders were fractionated into microvilli and terminal webs. SDS-PAGE analysis of microvilli that were sheared from isolated brush borders and purified free of the terminal webs by differential centrifugation revealed that the microvilli were devoid of both T-protein and N-protein (Fig. 1, lane C). The terminal webs, which retained significant numbers of un-

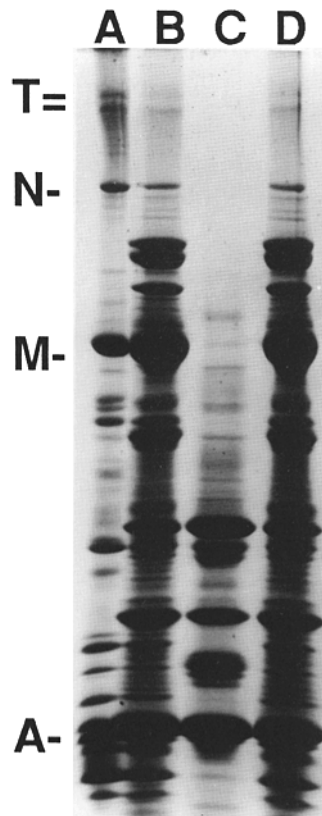


Figure 1. Identification of intestinal epithelial cell brush border T-protein and N-protein and comparison to muscle titin and nebulin by SDS-PAGE. SDS-PAGE analysis (2–20% gradient gel) of isolated brush borders (lane B), sheared microvilli (lane C), and terminal webs (lane D), all demembrated with NP-40 detergent treatment to lessen smearing at the top of the gel, reveals the presence of high molecular weight proteins in the brush border sample and the terminal web sample that migrate at rates similar to those of the titin doublet (T) and nebulin (N) standards in an SDS extract of chicken pectoralis muscle (lane A). M, myosin; A, actin.

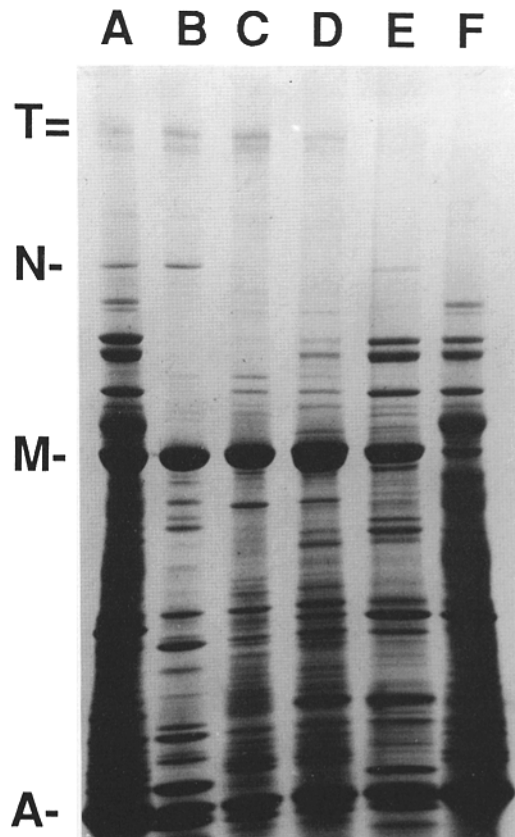


Figure 2. Extraction of T-protein and N-protein from isolated brush borders. SDS-PAGE analysis of proteins extracted from isolated brush borders (A) by incubation with 0.15 M KCl+ATP solution (D) and subsequently with 0.6 M KCl+ATP (E). Also shown are the residual brush border pellet following extraction (F) and pectoralis (B) and cardiac (C) muscle samples. The isolated brush borders and extracted brush borders were resuspended and loaded onto the gel in volumes equivalent to those of the extracts. The migration positions of the (T) titin and T-protein doublets; (N) nebulin and N-protein; and (M) myosin are designated.

sheared microvilli, contained all of the original T-protein and N-protein (Fig. 1, lane D).

Extraction of T-protein and N-protein from Isolated Brush Borders

To further characterize the interactions of T-protein and N-protein with the brush border cytoskeleton, solutions containing ATP (4 mM) at moderate (0.15 M KCl) and high (0.6 M KCl) ionic strength were tested for their ability to extract these proteins from isolated brush borders. The 0.15 M KCl + ATP solution extracted a subset of the T-protein with a subset of the myosin but little of the N-protein (Fig. 2, lane D). Densitometry of the Coomassie blue-stained gel revealed that $\sim 70\%$ of both the T-protein and myosin were extracted under these conditions. Consistent with this, the ratio of T-protein/myosin staining in the 0.15 M KCl + ATP extract was similar to that in the isolated brush border (Fig. 2, lane A). In both samples, there was a 1:10 optical density ratio of T-protein/myosin. Assuming a molecular weight of 3×10^6 D for T-protein and 4×10^5 D for the two myosin heavy chains in each myosin molecule, this ratio converts to a T-protein/myosin molar ratio of 1:75. Using similar crite-

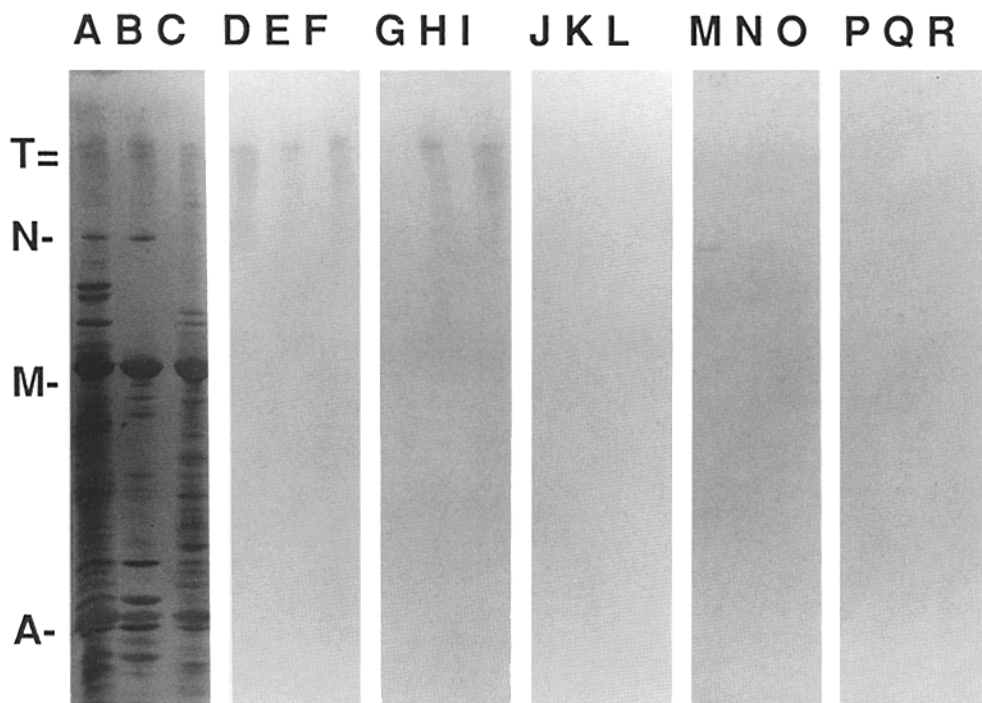


Figure 3. Immunoblot analysis using rabbit polyclonal antibodies raised against brush border T-protein and N-protein. Samples of isolated brush borders (A, D, G, J, M, and P) pectoralis muscle (B, E, H, K, N, and Q), and cardiac muscle (C, F, I, L, O, and R) were subjected to SDS-PAGE and transferred to nitrocellulose. The blots were stained with India ink to visualize the proteins (A-C) or probed with primary antibodies as follows: (D-F) blot-purified anti-T-protein; (G-I) mouse anti-bovine cardiac titin mAb; (M-O) and blot-purified anti-N-protein antibody. For controls, lanes J-L were probed with only the alkaline phosphatase-conjugated goat anti-mouse polyvalent antibody used as the secondary probe in G-I, and lanes P-R were probed with only the alkaline

phosphatase-conjugated goat anti-rabbit IgG used as a secondary for the other blots. The positions of (T) titin and T-protein; (N) nebulin and N-protein; (M) myosin; and (A) actin are indicated.

ria, we estimated a titin/myosin molar ratio of 1:44 in the pectoralis sample in this gel.

Subsequent incubation of the extracted brush borders in 0.6 M KCl + ATP solubilized most of the remaining myosin and T-protein and a substantial portion of the N-protein (Fig. 2, lane E). This extraction left little, if any, of these proteins associated with the residual brush border pellet (Fig. 2, lane F). The 200-kD band remaining in the residual brush border appears to be microvillar protein that migrates with the myosin (for example see Fig. 1, lane C). The N-protein, but not the T-protein or myosin, also was extracted with 0.6 M KCl in the absence of ATP (data not shown).

Production and Characterization of Brush Border T-protein and N-protein Antibodies

Polyclonal antibodies were raised in rabbits against SDS-PAGE-purified chicken brush border T-protein and N-protein to investigate their relationship to muscle proteins and immunolocalize the brush border proteins in intestinal epithelial cells. Specific anti-T-protein and anti-N-protein antibodies were isolated from immune sera by blot purification.

Western analysis of isolated brush borders revealed that the anti-T-protein antibody reacted exclusively with the brush border T-protein doublet and a smear of what appear to be T-protein degradation products running below the doublet (Fig. 3, lane D). This smear varied between brush border preparations. The anti-T-protein antibody also cross-reacted specifically with titin in pectoralis and cardiac muscle samples (Fig. 3, lanes E and F). In some blots, the antibody reacted with additional bands in both the brush border and muscle samples. These included a group of discrete bands occasionally present in the smear between the T-protein and N-protein (see for example, Fig. 5, lane A) and a band that occasionally underlies the myosin band in both

brush border and muscle samples (data not shown). The variable presence of these lower molecular weight reactive bands appears to be due to variable proteolytic degradation of the T-protein and titin into relatively stable products.

An anti-cardiac titin antibody displayed a distinct difference in its reactivity to T-protein and the muscle titins. This antibody reacted with both cardiac and pectoralis muscle titins (Fig. 3, lanes H and I) but not with brush border T-protein (Fig. 3, lane G). The reactivities of the anti-T-protein polyclonal antibody and anti-cardiac titin mAb indicate that T-protein and the muscle titins share some but not all epitopes.

Western blot analysis with the blot-purified anti-N-protein antibody demonstrated specific reactivity with the N-protein in brush borders (Fig. 3, lane M), but no cross-reactivity with nebulin or any other protein in the muscle samples (Fig. 3, lanes N and O).

Immunolocalization of T-protein and N-protein in Intestinal Epithelium

The polyclonal antibodies were used to immunolocalize T-protein and N-protein in cryosections of intestinal epithelium. T-protein immunofluorescence was localized distinctly in the brush border terminal web and absent from the microvilli (Fig. 4 A). This pattern of immunolocalization was consistent with the brush border fractionation data demonstrating that isolated microvilli lack T-protein. The cell body also displayed anti-T-protein immunofluorescence above background levels (Fig. 4 E), but the cell body localization was much less overall and much more diffusely spread than that in the brush border terminal web.

Immunolocalization of N-protein in isolated brush borders confirmed that N-protein also was concentrated in the terminal web region of the brush border in intestinal epithelial cells and absent from the microvilli (Fig. 4 C). When viewed

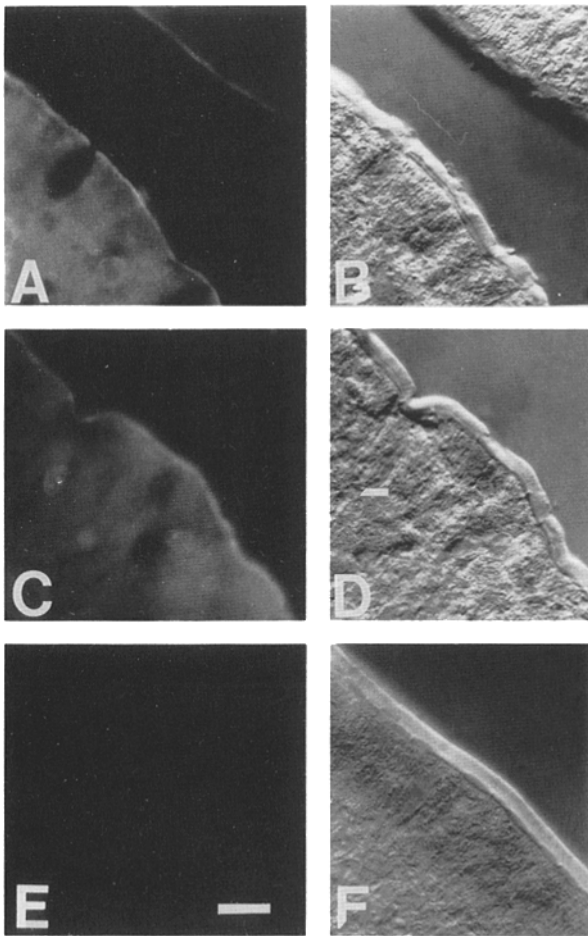


Figure 4. Immunolocalization of T-protein and N-protein in cryosections of adult duodenal intestinal epithelium. Cryosections of intestinal epithelium were incubated with anti-T-protein antibody (A), anti-N-protein antibody (C), or 7 $\mu\text{g/ml}$ nonimmune rabbit IgG as a control (E), followed by a fluorescein-conjugated goat anti-rabbit immunoglobulin secondary antibody. B, D, and F are Differential Interference Contrast micrographs of A, C, and E, respectively. Bar, 10 μm .

through the microscope, the N-protein immunofluorescence consistently appeared as a broader band than the T-protein immunofluorescence, suggesting that the N-protein is distributed through the entire depth whereas the T-protein may be confined to the basal region of the terminal web.

Partial Purification and Molecular Morphology of Brush Border T-protein

For morphological analysis, T-protein was extracted from isolated brush borders with 0.15 M KCl + ATP and partially purified by hydroxylapatite chromatography. Fractions that were enriched in T-protein but still contaminated with myosin and small amounts of other proteins (Fig. 5, lane A) were used for the morphological analysis. The cluster of bands between the T-protein and myosin in these fractions varied from preparation to preparation and appear to be T-protein breakdown products, because they reacted positively with the anti-T-protein antibody (data not shown).

EM of platinum rotary-replicated replicas of the T-protein fractions revealed numerous thin, fibrous molecules that

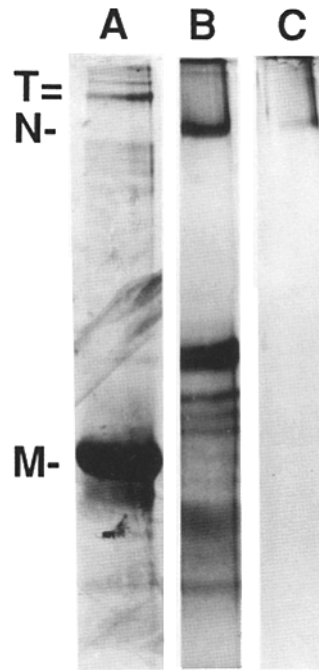


Figure 5. Partial purification of T-protein and N-protein. SDS-PAGE analysis of T-protein (A) and N-protein (B) that were partially purified by hydroxylapatite chromatography and N-protein that was purified by electroelution from an SDS gel (C). T, T-protein; N, N-protein; and M, myosin.

were easily distinguished from the double-headed brush border myosin molecules (not shown) and thus deemed to be brush border T-protein. Three representative T-protein morphologies are shown (Fig. 6). Regardless of the overall length, one end of each molecule typically was either beaded or slightly splayed. This apparent splaying may indicate that T-protein is a side-by-side association of more than one fibrous component, but more likely it reflects an unwinding of small globular domains of a single polypeptide chain. Adjacent to the splayed end in each molecule was a fibrous domain of $\sim 500\text{--}600$ nm in length; many of the molecules were kinked in this region. Approximately 30% of the measured molecules were capped by a globular head (as in Fig. 6 A) and measured 912 ± 40 nm in length (Fig. 7). Shorter molecules (600–850 nm, Fig. 7) with the characteristic beaded tails lacked globular heads (morphology not shown).

Approximately half of the molecules measured were significantly longer than the molecules with globular heads (Fig. 7). Although it is possible that these longer molecules are associations of multiple molecules, it appears that the increase in length results from an unwinding of the globular head of T-protein into an extended conformation composed of two or three additional fibrous domains (Fig. 6, B and C). The first of these domains is a 300-nm span in which several strands of protein are evident. Although this also could indicate that the protein is composed of multiple polypeptides, it appears more likely to be the splaying of a domain in which a single polypeptide had been folded back on itself several times. Adjacent to this section is another 300-nm-long strand, that in some molecules has a small globular head at the end (Fig. 6 B). In other molecules, an additional very thin fiber extended ~ 75 nm from that head. The longest of these conformations measured up to 1,700-nm long (Fig. 6 C).

Partial Purification and Molecular Morphology of Brush Border N-protein

N-protein that was enriched by two different approaches was

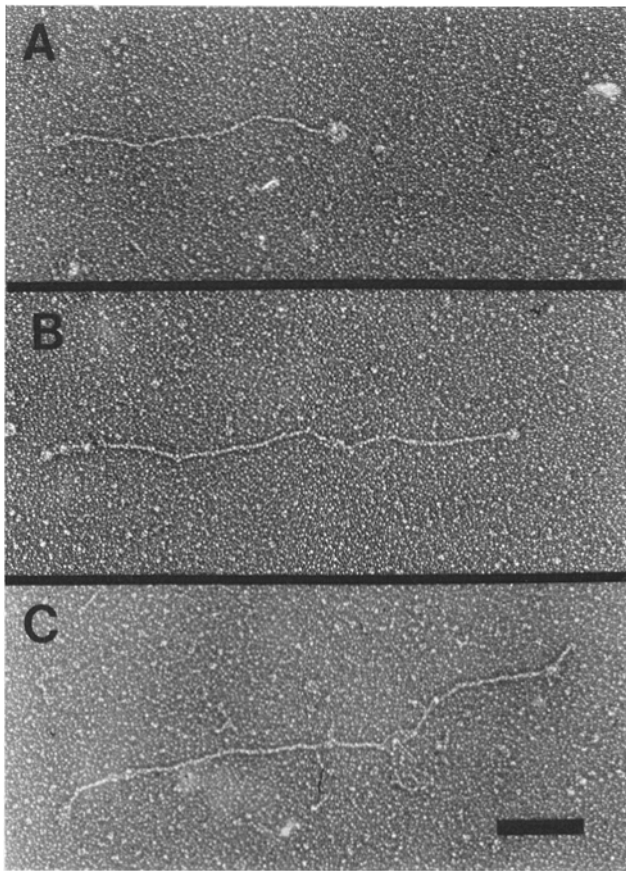


Figure 6. Molecular morphology of brush border T-protein. Brush border titin was partially purified by hydroxylapatite column chromatography and rotary-replicated with platinum. *A–C* are three representative morphologies. Bar, 200 nm.

similarly investigated by EM of rotary-shadowed replicas. To initiate this investigation, we visualized what we anticipated would be fully denatured N-protein; this N-protein was purified by electroelution from SDS-gel bands and dialyzed into a low ionic strength solution. Re-electrophoresis of this protein revealed it to be pure (Fig. 5, lane *C*). We were surprised to find that the SDS-PAGE-purified N-protein in the dialyzed samples was assembled into long filaments (Fig. 8, *A–C*). Periodic structures along the length of the filaments suggested that these filaments were end-to-end assemblies of structural units ~ 400 – 450 -nm long (Fig. 8 *C*).

N-protein also was partially purified by hydroxylapatite column chromatography of 0.6 M KCl + ATP-extracts of isolated brush borders. The peak fraction of N-protein elution (Fig. 5, lane *B*) was contaminated by TW 260/240 (chicken brush border terminal web fodrin), which was identified in the replicas by its characteristic tetrameric morphology (not shown). Dialysis of N-protein-enriched fractions into low ionic strength buffer yielded long polymers of native N-protein similar to those present in the SDS-purified samples. These polymers displayed apparent overlap structures at 400 – 450 -nm intervals along their length (Fig. 8 *D*).

Discussion

We have discovered two huge, fibrous proteins, designated

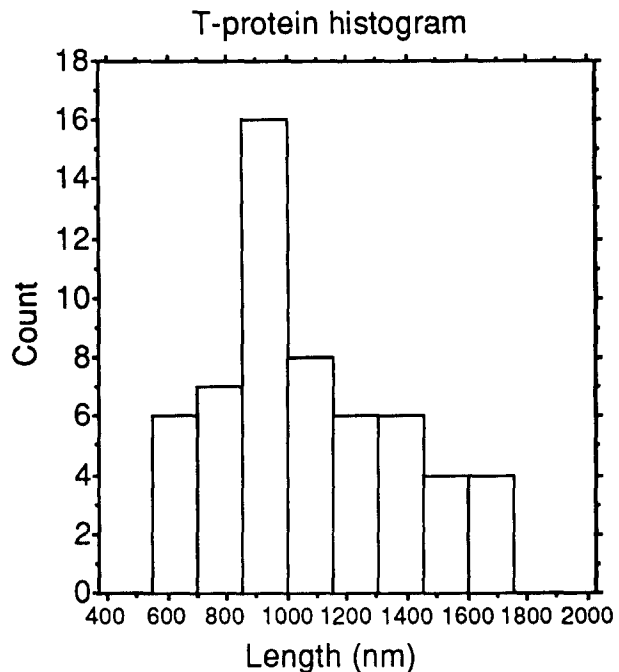


Figure 7. Histogram of T-protein lengths. EM images of 57 rotary-replicated T-proteins were enlarged and measured.

T-protein and N-protein, as components of the intestinal epithelial cell brush border cytoskeleton. SDS-PAGE analysis of these brush border proteins indicates that the polypeptide molecular weights of T-protein and muscle titin (2 – 3×10^6 D) (17) and N-protein and muscle nebulin (8×10^5 D) (28) are similar. Fractionation of isolated brush borders and immunolocalization in intestinal epithelial cells has revealed that both T-protein and N-protein are concentrated distinctly in the terminal web region of the brush border and absent from the microvilli. Our discovery and localization of these proteins in this well-characterized model system yields greater insight into the complexity of the brush border cytoskeleton and should facilitate elucidating the roles of T-protein and N-protein in this and possibly other actin-based cytoskeletons.

Brush Border T-protein

Although molecular weight often is a poor criterion for judging relatedness, the similarity of T-protein and titin in this characteristic is significant, because these proteins share the distinction of being the largest yet found. Moreover, brush border T-protein also resembles muscle titin in several other key properties, including immunocross-reactivity and molecular morphology. Cross-reactivity of our anti-T-protein polyclonal antibody with titin from both skeletal and cardiac muscle demonstrates the relatedness of these proteins, but lack of T-protein reactivity with an anti-titin mAb indicates that T-protein and titin share only certain epitopes. Together, its molecular weight and immunoreactivity suggest that T-protein is a cellular isoform of titin.

This possibility is supported further by the similarity between T-protein and muscle titin in molecular morphology. The predominant form of T-protein that we found in rotary-replicated samples was a 900 -nm (912 ± 40 nm) long fibrous molecule with a globular end. This length and morphology

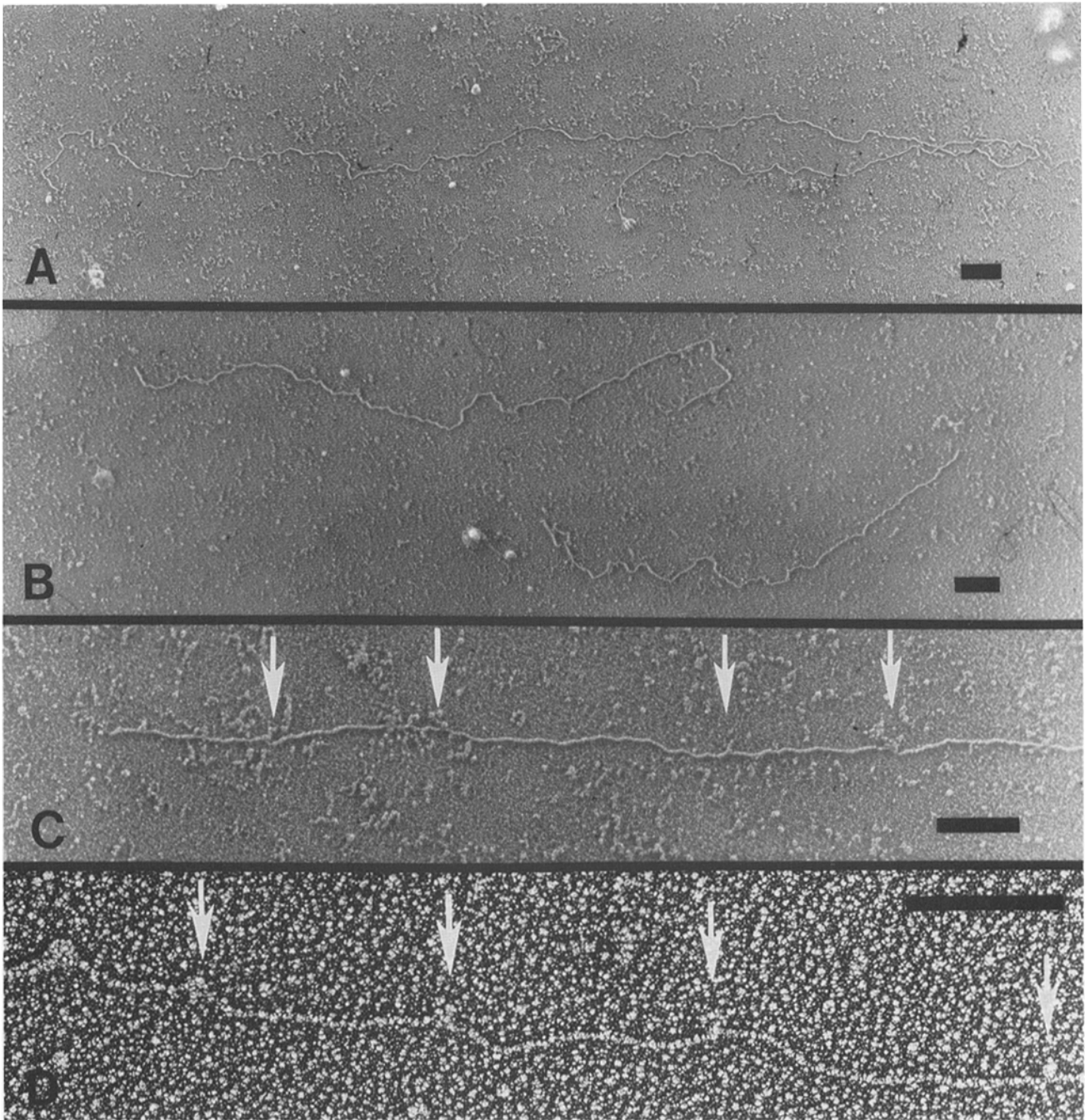


Figure 8. Molecular morphology of native and electroeluted N-protein. Rotary replicas of the protein electroeluted from N-protein bands that were excised from SDS gels and dialyzed into 50 mM ammonium acetate, *A-C*, and N-protein partially purified by hydroxylapatite chromatography, *D*. Arrows indicate apparent overlap regions at ~ 450 -nm intervals along the long polymers. Bars, 200 nm.

are similar to that of muscle titin (22). Also, the apparent extensibility of the globular head region that we have observed in some of the T-protein molecules may be related to the elastic property proposed for a region of the muscle titin molecule (6, 7, 16, 23, 24, 26).

In muscle, titin mediates interaction of myosin with the sarcomere by maintaining the position of the thick filament in the center of each contractile structure. In brush borders, T-protein is localized in the terminal web where it could mediate the association of myosin with both the interrootlet and circumferential ring domains of the cytoskeleton. Consistent with this is our observation that subsets of T-protein

are coextracted at a constant molar ratio (1 T-protein:75 myosins) with previously defined subsets of myosin (9). One of the myosin subsets is extractable from isolated brush borders with ATP at physiological ionic strength. Because this myosin is unnecessary for brush border contraction, it is thought to comprise the filaments that crosslink the microvillar rootlets in the interrootlet domain of the terminal web (9). The other myosin subset, extracted by high ionic strength-ATP incubation of isolated brush borders, is necessary and sufficient for brush border contraction and thought to comprise the circumferential ring myosin (9).

If T-protein is associated with myosin filaments in the

brush border terminal web, the size and organization of the coassembly would be expected to be significantly different from that of titin and thick filaments in muscle sarcomeres. In some skeletal muscle, each bipolar filament is composed of 294 myosin molecules (31), and may have six to twelve associated titin molecules (30). Our estimate of the titin/myosin ratio in pectoralis muscle SDS extracts (1:44) is most consistent with 12 titins per thick filament. This is significantly different from the T-protein/myosin ratio (1:75) that we find in brush border samples, although this ratio may be an underestimate because of difficulty in maintaining T-protein intact during brush border purification. A ratio of 75 myosins:1 T-protein is difficult to reconcile with evidence that brush border myosin filaments in situ may be as small as two to four molecules (6, 19). Nevertheless, our preliminary investigation of T-protein brush border myosin coassembly in vitro indicates that the number of myosins comprising the coassembled bipolar filaments exceeds 20 and could be as many as 75 (K. Eilertsen and T. Keller, unpublished observations).

Brush Border N-protein

In contrast to the similarity of T-protein with titin, brush border N-protein differs significantly from muscle nebulin in several properties other than molecular weight. In muscle, mAb mapping studies have demonstrated that individual nebulin molecules are coextensive with each thin filament. The observation that variations in nebulin molecular weight consistent with thin filament length in different muscles suggests that the length of the nebulin molecule specifies the length of actin filament (8, 13, 28).

Although the morphology of brush border N-protein has yet to be fully characterized, periodicities along the length of polymers suggest that a 450-nm-long subunit has the ability to form various length filaments, at least in vitro. Moreover, localization of N-protein in the brush border terminal web, where it has access to only the rootlet portions of the microvillar actin filaments, precludes a role for N-protein in regulating the overall length of the microvillar bundle of actin filaments by a mechanism similar to that postulated for nebulin control of skeletal muscle thin filament length (8, 13, 28).

Clearly, further investigation of the molecular characteristics of the brush border T-protein and N-protein and their interactions with other brush border components will yield greater insight into the functions of these two huge proteins in the brush border cytoskeleton. In addition, investigation of their synthesis and localization during embryonic brush border assembly and differentiation of progenitor crypt cells into mature enterocytes in the adult intestine should demonstrate whether T-protein and N-protein play key roles in organizing the brush border cytoskeleton or stabilizing an organization established primarily by other factors.

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