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Immunochemical Demonstration of Tropomyosin in the Neurofibrillary Pathology of Alzheimer's Disease

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The focus of research on the neurofibrillary pathology (NFP) of Alzheimer disease has been not only to determine the component forming the paired helical filaments but also to determine whether they result from abnormal processes affecting a single protein. Therefore, although these studies have lead to controversy concerning the respective contribution of components of microtubules and neurofilaments, there has been essentially no consideration of whether other cytoskeletal systems might also be involved and of what are the common features for the incorporated components. Particularly relevant to this issue is our finding that several antisera raised to either skeletal or smooth muscle tropomyosin, a microfilament component, intensely recognize NFP. These antibodies continue to recognize NFP after affinity purification to tropomyosin or paired helical filament fractions. We show that the antibodies do not recognize NFP due to cross-reactivity with the previously identified NFP components related to neurofilaments and microtubules, τ, and MAP2, or neurofilament proteins because the antisera did not recognize these proteins on immunoblots or were not adsorbable by the proteins. Ultrastructural analysis of the immunoreaction showed that tropomyosin-related epitopes were clustered rather than uniformly distributed along paired helical and straight filaments. Although the distribution suggests that tropomyosin is an NFP-associated protein, its retention by paired helical and straight filaments after detergent extraction indicates that it is an integral component strongly and specifically associated with the filaments characteristic of NFP. These findings indicate that NFP involves the three primary neuronal cytoskeletal filament systems, microtubules, neurofilaments, as well as microfilaments, and therefore that NFP probably results from the reorganization of these normal filaments that interact to comprise the cytomatrix and may continue this interaction under the pathologic condition of Alzheimer's disease to generate novel, abnormal polymers. (Am J Pathol 1990 137: 291–300)

Neurofibrillary pathology (NFP), the characteristic intraneuronal filamentous lesion of Alzheimer's disease (AD), has been studied with the methods available since its description by Alzheimer in 1906.[1] Even with an intense effort, the cellular origin of NFP has remained a topic of considerable controversy.[1,2] The structural similarity of the abnormal filaments constituting NFP, paired helical and straight filaments, have suggested a relationship to normal neuronal filaments, ie, the cytoskeleton (the neurofibrils of Alzheimer's era). Application of several immunochemical approaches to NFP have indicated the sharing of epitopes with proteins that comprise either neurofilaments[3–7] or microtubules[5,7–13] along with considerable controversy regarding the respective contribution of each.[14–16] The contrasting views are either that NFP results from an abnormality of the microtubule-associated protein τ,[9,10,17] with possible involvement of other proteins,[18] or reorganization and possible collapse of the microtubule–neurofilament network of interacting filaments in affected neurons.[19–21] The implication of the apparent controversy is whether NFP occurs as a result and thus

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can be understood through the study of a single component.

While studying the Hirano body,[22,23] a microfilament-derived intraneuronal inclusion found in normal aging as well as in AD, we found that NFP is recognized by antibodies to the microfilament protein tropomyosin. Our findings suggest that the cytoskeletal reorganization of AD is not limited to τ or the microtubule–neurofilament network and thus may be best studied as a pervasive reorganization of interacting proteins in the cytomatrix.

Materials and Methods

Tissue

The hippocampal gyrus was taken from eight confirmed cases of AD (patient age range, 63 to 84 years)[24] and two age-matched controls, fixed in either Bouin's fixative or 10% formalin with phosphate buffer, pH 7.0, for 24 to 72 hours before dehydration and embedding in paraffin. Sections were cut at 6 μm.

Antibodies

The following antibodies were used: rabbit antisera raised to 1) chicken gizzard tropomyosin (gifts of Drs. R. Goldman and F. Matsumura),[22] and 2) rabbit skeletal muscle tropomyosin.

Production of Antiserum

One New Zealand rabbit (Hazelton Research Products Inc., Denver, PA) was immunized subcutaneously with 100 μg of rabbit skeletal muscle tropomyosin (Sigma Chemical Co., St. Louis, MO) at 14-day intervals. The initial immunogen (in 1 ml) was prepared in Freund's complete adjuvant (Sigma), and all subsequent boosts were prepared in Freund's incomplete adjuvant (1 ml).

Tropomyosin-sepharose Chromatography

Chicken gizzard tropomyosin (5 mg; Sigma) was used as the affinity ligand by coupling to cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ) by using the procedure recommended by the supplier. The tropomyosin-sepharose was placed in a column and rinsed with 10% bovine serum albumin in TRIS-buffered saline (TBS) (50 mmol/l [millimolar] TRIS, pH 7.6, and 150 mmol/l NaCl). After rinsing with TBS, the antiserum (100 μl, undiluted) was applied to the column at room temperature and slowly run through. Antibodies not retained (run-off) were collected, and the column was rinsed with 50 ml of TBS. Specifically retained antibodies were eluted with 0.1 mol/l (molar) glycine pH 2.5 (eluant). These antibodies were adjusted to pH 8 with 1 mol/l TRIS and dialyzed against TBS before they were characterized.

Immunostaining

Sections were immunostained with the antibodies by using the peroxidase–antiperoxidase procedure[25] with 3,3′ diaminobenzidine (DAB) as cosubstrate. Photographs (4 × 5 negative) were taken with a Zeiss Axiophot (Carl Zeiss, Inc., Thornwood, NY) equipped with planapochromatic objectives.

Immunoblotting

Immunoblots were prepared from proteins electrophoresed in 10% SDS-polyacrylamide gels (SDS-PAGE). Proteins used were: 1) bovine heat-stable microtubule-associated proteins,[26] ie, MAP2 and τ; 2) one-time–cycled bovine microtubule protein;[27] 3) a rat spinal cord cytoskeletal preparation;[28] 4) postmortem human cauda equina neurofilament proteins;[29] 5) bovine erythrocyte ubiquitin (Sigma); 6) rabbit skeletal muscle tropomyosin (Sigma); 7) chicken gizzard tropomyosin (Sigma); 8) chicken skeletal muscle tropomyosin (Sigma); and 9) postmortem human hippocampal grey matter of an AD and control case. The electrotransfer to Immobilon (Millipore Corp., Bedford, MA) was performed according to Towbin.[30] The protein transfers were blocked in 10% nonfat dry milk (Carnation, Los Angeles, CA) for 1 hour before application of the primary antibody. After incubation with the primary antibody for 16 hours at 4°C, the blots were rinsed and incubated with goat anti-rabbit IgG labeled with peroxidase 1:1000 (BioRad Laboratories, Richmond, CA). After 1-hour incubation at room temperature, the blots were washed and developed in 50 mmol/l TRIS, pH 7.6 containing 0.05% H_2O_2 and 0.75 mg/ml DAB. Molecular weights of the blotted proteins were determined by transferred prestained markers (Bethesda Research Laboratories) or by marker proteins obtained from Sigma (see below).

Immunoadsorption

Affinity-purified or total antisera were incubated with the potential adsorbent or ovalbumin for 16 hours at 4°C. After centrifugation, the supernatant was used to immuno-

stain tissue sections containing NFP as well as immunoblots containing rabbit skeletal muscle tropomyosin.

Immunogold Staining

Vibratome sections were treated for 10 minutes at room temperature with 0.01% Triton X-100 in 0.15 mol/l NaCl, 50 mmol/l TRIS, pH 7.6. After rinsing, they were incubated in primary antibody for 2 days at 4°C, followed by rinsing and subsequent incubation for 2 days at 4°C with the appropriate colloidal gold-antibody complex. Colloidal gold was prepared by citrate reduction (20 nm) and complexed to affinity-purified anti-rabbit IgG (Cappel Research Products, Durham, NC).[5] Sections were subsequently fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.2) for 2 hours, followed by postfixation in 2% OsO_4 and flat-embedded in Spurr's medium (Electron Microscopy Sciences, Fort Washington, PA). After electron contrasting in uranyl and lead salts, the sections were viewed at 60 kV in a JEOL 100CX electron microscope (JEOL USA, Inc., Peabody, MA).[5]

Immunodecoration of SDS-treated NFT Fractions

Grey matter from the hippocampus of an AD patient that was stored unfixed at −70°C was homogenized and treated with SDS as previously described.[5] The insoluble material was applied to carbon-coated nickel grids and incubated for 1 hour with the primary antibodies or controls outlined above and followed by a 1-hour incubation with secondary antibodies conjugated to colloidal gold. All the incubations were at room temperature and were followed by three rinses with 1% bovine serum albumin (BSA) in 0.15 mol/l NaCl, 50 mmol/l TRIS HCl, pH 7.6. Immunostained preparations were negatively stained with 2% phosphotungstic acid. Grids were viewed at 80 kV in a JEOL 100CX electron microscope.[5]

Immunoaffinity Purification by Using a Paired Helical Filament Fraction

An enriched PHF fraction (approximately 100 μg of protein) prepared from an AD case, using the protocol of Iqbal and coworkers,[31] was used as an affinity ligand by applying it to a nitrocellulose filter (0.22 μm, 2.5 cm, Millipore) set on a sintered glass support of a filtration apparatus (Millipore). The PHF-containing membrane was incubated with 10% BSA in TBS (50 mmol/l TRIS, pH 7.6, and 150 mmol/l NaCl). After rinsing with TBS, the antiserum (100 μl, undiluted) was incubated with the filter for 18 hours at room temperature. Antibodies not retained (run-off) were collected, and the filter was rinsed with 50 ml of TBS. Specifically retained antibodies were eluted with 0.1 mol/l glycine pH 2.5 (eluant). These antibodies were adjusted to pH 8 with 1 mol/l TRIS and dialyzed against TBS before they were characterized.

Immunochemical Characterization of Tropomyosin Peptide Maps

Fragments of β and γ chicken gizzard tropomyosin[32] were produced by specific peptide cleavage with chemical reagents or trypsin. Tropomyosin was subjected to either trypsin (0.1 μg/ml for 5 minutes at 40°C according to Ueno[33]); cyanogen bromide (at a $1:10^4$, 10^5 or 10^6 molar ratio), which cleaves C-terminal to methionine residues,[34] 2-nitro-5-thiocyanatobenzoic acid (TNB-CN) (Kodak, Rochester, NY) (at a 1:7.5 or 100 molar ratio), which cleaves N-terminal[35] to cysteine residues; N-bromosuccinimide (NBS) (Sigma), which (under the conditions used for tropomyosin of a 1:50 molar ratio) cleaves C-terminal to tyrosine residues.[36] In addition, β and γ tropomyosin were resolved on a 14% SDS-PAGE and each band was excised and electroeluted (Biorad Electroeluter, BioRad Laboratories) and individually subjected to cleavage. Peptides were resolved on 14% SDS-PAGE[37] and either Coomassie blue or silver stained,[38] or transferred to Immobilon (Millipore). Molecular weight standards (Sigma) consisted of β-galactosidase (116 kd); BSA (66 kd); ovalbumin (45 kd); carbonic anhydrase (29 kd); trypsinogen (24 kd); trypsin inhibitor (20.1 kd); β-lactoglobulin (18.4 kd); α-lactalbumin (14.2 kd); and ubiquitin (8.5 kd). The protein transfers were immunostained by using the crude antisera prepared to chicken gizzard tropomyosin or the antisera affinity purified to either tropomyosin or paired helical filaments.

Results

While studying the components of Hirano bodies, which are derived from microfilaments,[22,23,39] it was noted that an antiserum raised to chicken gizzard (smooth muscle) tropomyosin, in addition to recognizing Hirano bodies[22] and the smooth muscle contained within large vessels, also recognized neurofibrillary tangles as well as the dystrophic neurites characteristic of NFP in AD. Although the immunoreaction was apparently specific, there was considerable concern that the observed reaction may have resulted from nonspecific immune reaction with adherent

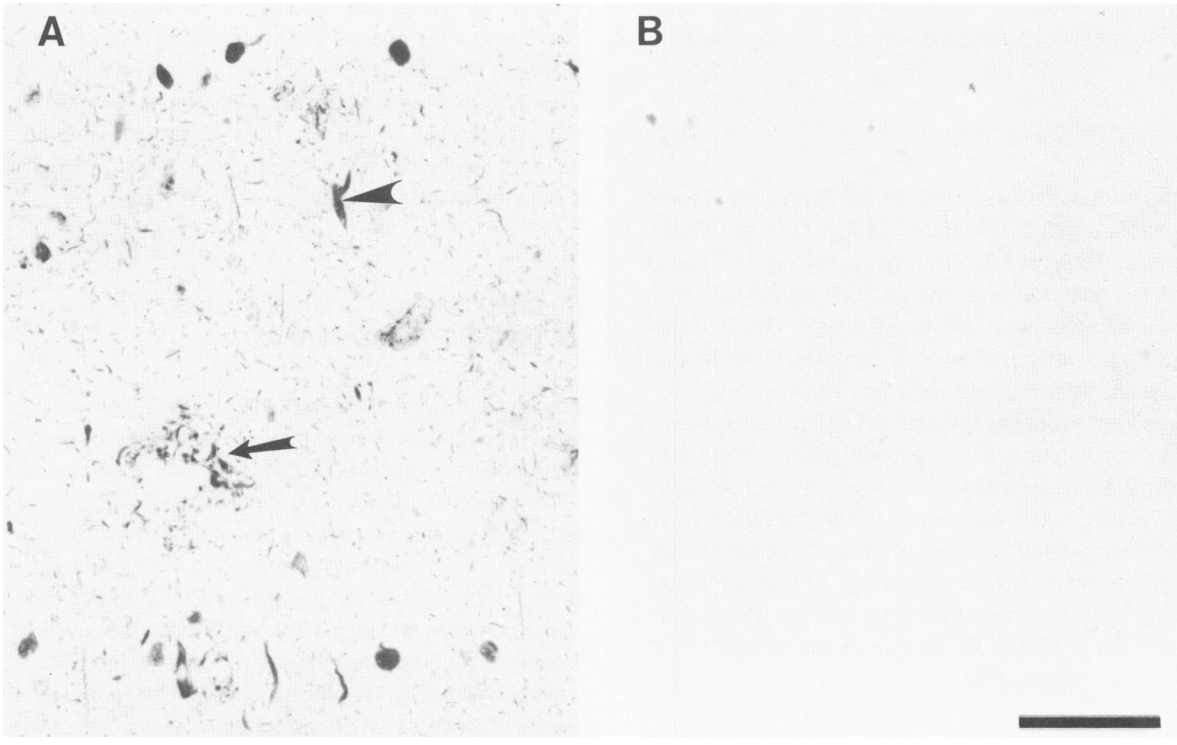

Figure 1. *The affinity-purified antiserum to tropomyosin recognizes neurofibrillary tangles (arrowhead), neuropil threads (thread-like immunostained structures), as well as dystrophic neurites surrounding senile plaques (arrow),* A: *Adsorption of the antiserum with tropomyosin specifically blocks recognition of these structures,* B: *Peroxidase–antiperoxidase. Scale bar = 100 μm.*

elements or cross-reactivity of the tropomyosin antibody, with one of the previously established NFP components.

To rule out cross-reactivity of the antiserum with epitopes not related to tropomyosin, we first affinity purified it with chicken gizzard tropomyosin-sepharose chromatography. Neurofibrillary pathology was still recognized by the affinity-purified antibodies (Figure 1A). Furthermore adsorption of the total or affinity-purified antiserum with tropomyosin blocked its recognition of both NFP (Figure 1B) and vascular smooth muscle in tissue sections as well as tropomyosin on immunoblots (not shown). To determine whether our results were unique to the antigens (smooth muscle tropomyosin), we produced antisera to the structurally similar tropomyosin found in skeletal muscle. We found that this antisera also recognized NFP (not shown). The results of these experiments indicate that both smooth and skeletal muscle forms of tropomyosin share epitopes with NFP.

We addressed the issue of whether the tropomyosin-related antigen(s) were integral components of the abnormal filaments comprising NFP by immunoelectron microscopy. First, NFP was immunodecorated with the antiserum to smooth muscle tropomyosin followed by colloidal gold labeled secondary antibody. Although the antibodies definitely recognized both straight and paired helical filaments, the gold labeling was punctuated along the filaments (Figure 2A) and thus differed from that noted with antibodies raised to paired helical filaments, τ, or neurofilaments that more uniformly label the abnormal filaments.[5,19,21,40,41] The peripheral localization raised the hypothesis that the tropomyosin epitopes found in NFP may be associated elements, and thus dissociable from the filaments by denaturants. This possibility was tested by immunodecorating an SDS-extracted paired helical filament fraction with the tropomyosin antiserum. We found that the tropomyosin antiserum (Figure 2B) yielded variable labeling of paired helical filaments (shown), as well as straight filaments (not shown), with many strongly labeled filaments (as shown), while others showed near background levels (not shown). Therefore the tropomyosin-related epitopes found in NFP, although integral components[5] closely associated with paired helical filaments, may not be represented in each filament.

Several experiments were used to address the issue of the cross-reactivity of the tropomyosin antibody with NFP due to the sharing of epitopes between previously identified proteins of NFP and tropomyosin. First, the affinity-purified antibody was applied to immunoblots containing heat-stable microtubule-associated proteins (τ and MAP2), neurofilament proteins, ubiquitin, or tropomy-

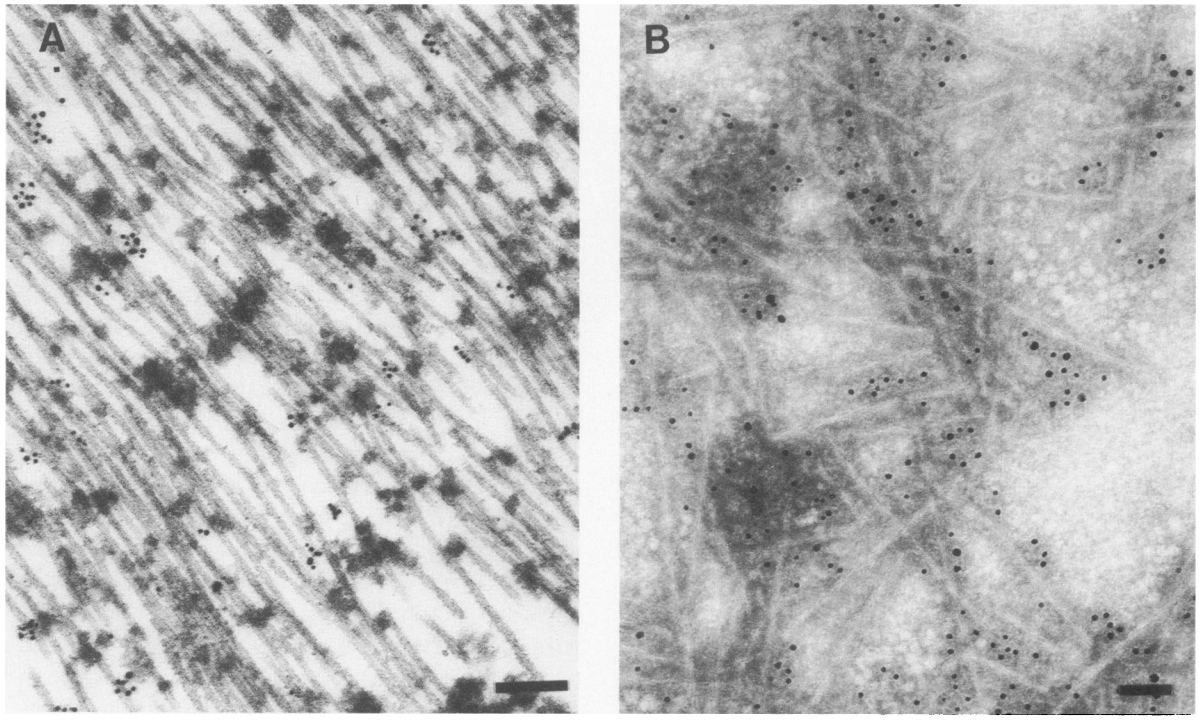

Figure 2. *The antiserum to chicken gizzard tropomyosin specifically labels the abnormal filaments found in the neurofibrillary tangle.* **A:** *The gold particles found in clusters along neurofibrillary tangle filaments were noted to be associated with material at a distance from the filament proper. Interestingly the tropomyosin epitopes remain associated with the abnormal filaments, even after isolation and ionic detergent extraction,* **B. A:** In situ *pre-embedding decoration of tissue section, indirect immunogold.* **B:** *Negative stain, indirect immunogold. Scale bar = 100 nm.*

osin. With the exception of tropomyosin, there was no reactivity of the antiserum with any of the proteins on the blots (Figure 3). When applied to immunoblots containing gray matter proteins prepared from an AD or control case, the antibodies recognized bands corresponding in molecular weight to tropomyosin (not shown), while on immunoblots of an enriched PHF fraction it recognized a band not entering the stacking gel on SDS-PAGE (not shown). The specificity of the antibody only for tropomyosin, or nonsolubilizable PHF protein excluded from SDS-PAGE gels, was further attested to by the inability of heat-stable microtubule-associated proteins, τ, neurofilament pro-

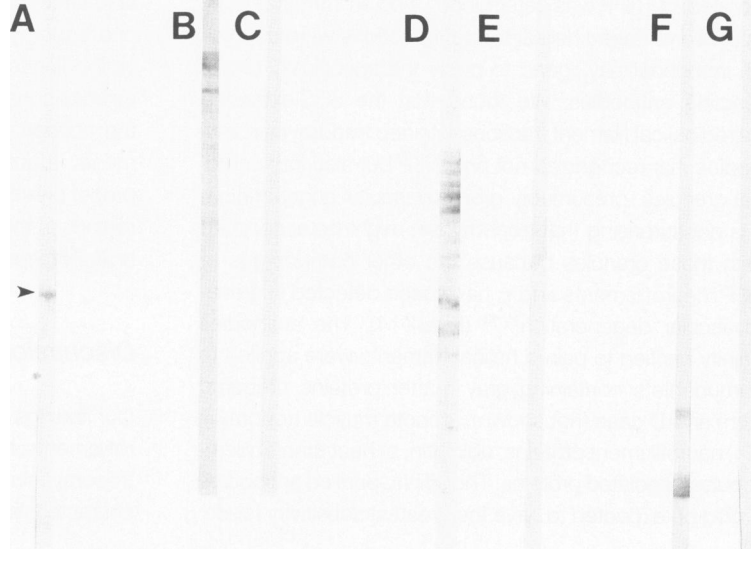

Figure 3. *The affinity-purified antiserum to tropomyosin recognizes a band corresponding to tropomyosin (arrowhead) in a protein transfer of chicken gizzard tropomyosin,* **A:** *The tropomyosin antibody shows no reaction with protein transfers containing neurofilament protein* **C,** *heat-stable microtubule associated proteins,* **E** *or ubiquitin G. In blots* **B, D,** *and* **F,** *the transfers were stained by antibodies directed to neurofilaments, τ and ubiquitin, respectively. Indirect peroxidase.*

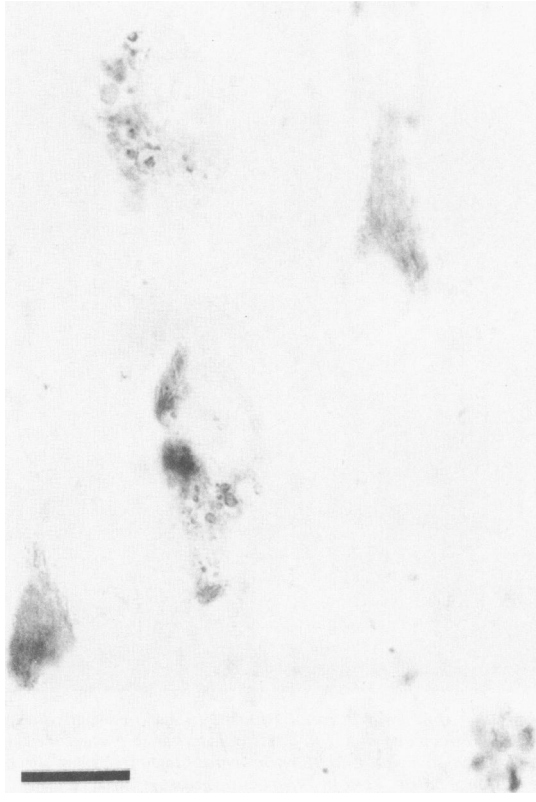

Figure 4. *The tropomyosin antisera affinity purified to paired helical filaments recognizes not only neurofibrillary tangles but also intraneuronal granules, presumably granulovacuolar degeneration, which are prominently shown in this micrograph. Peroxidase–antiperoxidase. Scale bar = 20 μm.*

teins, or ubiquitin to reduce immunostaining of NFP by the affinity-purified tropomyosin antiserum in adsorption experiments (not shown).

The nature of the specific tropomyosin epitopes incorporated into NFP was determined using an immunoaffinity approach.[7] Paired helical filament fractions were used as an immunoaffinity ligand to purify the specifically cross-reacting antibodies. We found that the SDS-extracted paired helical filament fractions retained tropomyosin antibodies that recognized not only NFP but also intraneuronal granules, presumably granulovascular degeneration. It is not surprising that tropomyosin might be associated with these granules because the other components of NFP, neurofilaments and τ, have been detected in granulovascular degeneration[42,43] (Figure 4). The antibodies affinity purified to paired helical filaments were applied to immunoblots containing gray matter proteins prepared from an AD case (not shown), smooth muscle tropomyosin, neurofilament proteins, ubiquitin, or heat-stable microtubule-associated proteins. The affinity-purified antibodies would be expected to have the greatest sensitivity for detecting the cross-reacting proteins, yet they recognized only tropomyosin on immunoblots (Figure 5). Interestingly dephosphorylation of the blots and sections with alkaline phosphatase[44,45] enhanced the recognition of tropomyosin in blots and NFP in tissue sections by the antiserum to tropomyosin as well as the antisera affinity purified to either tropomyosin or PHF (data not shown). Because it is the phosphatase labile epitopes found in NFP that are shared between τ, neurofilaments heavy subunit, and MAP 2,[14–16] the phosphatase stability (or possibly enhancement) of the tropomyosin epitopes provides additional evidence for the uniqueness of the tropomyosin epitope and the shared epitopes found in these other proteins.

These experiments establish a new component to NFP that shares at least one epitope with tropomyosin. To determine the location of the tropomyosin-related epitopes found in NFP on tropomyosin, peptide mapping was performed. When the antisera to tropomyosin were applied to protein transfers containing the various peptides generated from cleavage of tropomyosin, we found that the original antisera to chicken gizzard tropomyosin recognized the same fragments before and after affinity purification to paired helical filaments (as shown for TNB-CN and cyanogen bromide in Figure 6). This finding suggested that paired helical filaments share with tropomyosin all the epitopes recognized by the original antisera. Unfortunately it was not possible to define the exact tropomyosin domains recognized by the antisera because the antisera did not recognize fragments smaller than 15 kd, even though fragments representing all domains smaller than this are generated by the treatments performed (Figure 6).[33–36] We suggest that fragments smaller than 15 kd do not define any of the epitopes recognized by the tropomyosin antisera because the epitopes are localized over large regions of the molecule that require considerable secondary structure. The participation of more than one tropomyosin domain in the epitopes recognized by antibodies to tropomyosin might be expected because it exhibits considerable coiling. These findings indicate that the epitope tropomyosin shares with paired helical filaments requires considerably secondary structure for proper presentation and, in all probability, involves several regions of the tropomyosin peptide chain and therefore is unlikely to result from the sharing of a single epitope.

Discussion

Our findings demonstrate that a protein other than τ, neurofilaments, MAP2, or ubiquitin and antigenically related to tropomyosin is incorporated into the abnormal filaments characteristic of the NFP of AD. It is likely that this protein

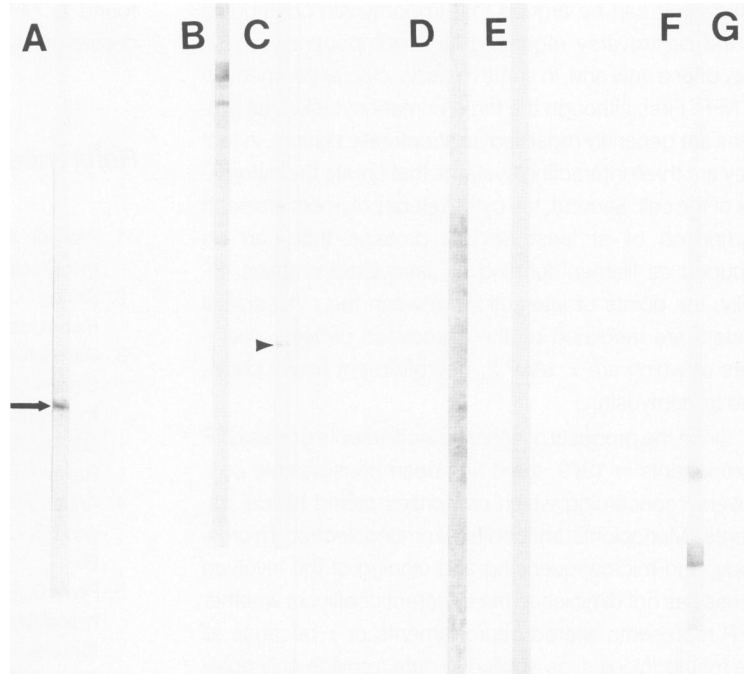

Figure 5. *The tropomyosin antiserum purified to an enriched paired helical filament fraction recognizes tropomyosin in a preparation of chicken skeletal muscle tropomyosin (arrow),* **A**, *yet did not recognize bands on protein transfers of neurofilament proteins,* **C**, *heat-stable microtubule associated proteins,* **E** *or ubiquitin G. The weak band stained (arrowhead) on the neurofilament protein transfer,* **C**, *corresponds in molecular weight to tropomyosin. In blots* **B, D,** *and* **F**, *the transfers were stained by antibodies directed to neurofilaments, tau and ubiquitin, respectively. Indirect peroxidase.*

is tropomyosin because it apparently shares homology with epitopes defined by large regions of β and γ chicken gizzard tropomyosin rather than reflecting the incorporation of an individual epitope.[46] The relationship of tropomyosin to NFP structure is indicated by immunoelectron microscopy. Instead of uniformly labeling paired helical filaments, as observed for antibodies to neurofilaments,[5,47] τ,[21] or ubiquitin,[48] the tropomyosin antibody intensely labeled discrete regions of the filaments. Furthermore it appeared that the label was often separated from the filament structure. These observations are consistent with tropomyosin being an NFP-associated protein that occurs at intervals along the abnormal filaments. Although structural observations indicate that tropomyosin is an NFP-associated protein, the interaction is strong because it is maintained after denaturant extraction, making tropomyosin an integral PHF component.[5]

Tropomyosin is a microfilament-associated protein found in muscle and nonmuscle cells that plays a role in cell motility and shape maintenance (reviewed in Payne and Rudnick[49]). Finding tropomyosin in NFP therefore involves a third cytoskeletal system, microfilaments, in NFP.

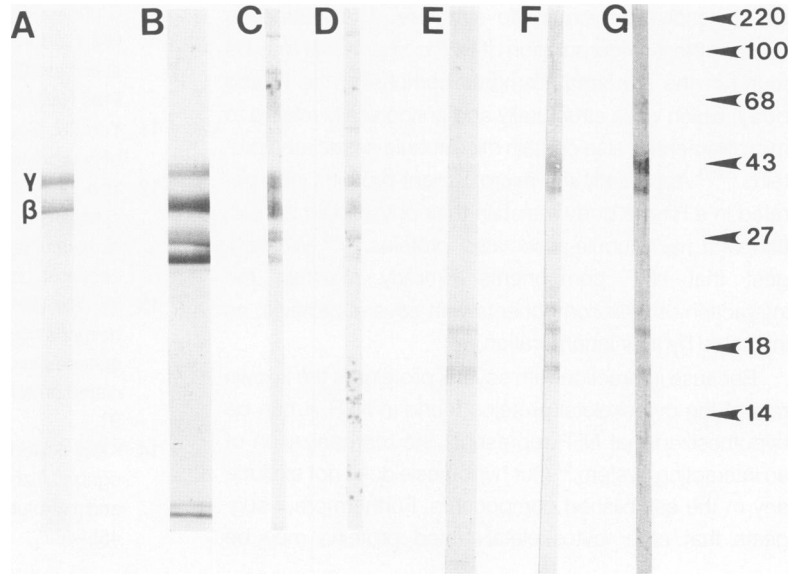

Figure 6. *Cleavage of β and γ (32) chicken gizzard tropomyosin* **(A)** *with TNB-CN* **(B)** *yielded unfragmented β and γ tropomyosin as well as two large fragments that were recognized by the antisera raised to chicken gizzard tropomyosin before* **(C)** *or after* **(D)** *immunoaffinity purification to a paired helical filament fraction. Similar results were obtained with cyanogen bromide cleavage* **(E)** *of the same tropomyosin preparation in which the antisera before* **(F)** *and after* **(G)** *immune affinity purification to paired helical filaments recognized the same fragments. Of interest is that the antibodies did not recognize any fragments generated by these treatments that were smaller than 15 kd (see text). Peptides resolved on 14% SDS-PAGE. Molecular weight markers indicated at right* **A, B,** *and* **E** *Coomassie blue.* **C, D, F,** *and* **G** *indirect peroxidase.*

Although it can be argued that tropomyosin contributes to the controversy regarding NFP components, it also may offer a new and, in some regards, clearer perspective of NFP. First, although the three primary cytoskeletal systems are generally regarded as structurally distinct, in fact they are three interacting systems that create the cytomatrix of the cell. Second, the cytoskeletal polymers are each comprised of at least several proteins that can be grouped as filament-forming or associated proteins. Finally, the points of interaction between the cytoskeletal system are mediated by the associated proteins, members of which are τ, MAP 2, neurofilament heavy chain, and tropomyosin.

Since the proposal of either neurofilaments or τ as NFP components in 1979, there has been considerable controversy concerning which comprises paired helical filaments. Monoclonal antibodies, immunoelectron microscopy, and microsequencing and cloning of the involved genes has not diminished the apparent conflict of whether NFP represents altered neurofilaments or τ because all the methodologies, as applied to date, provide only qualitative assessments of NFP and not a rigorous reconstruction of the relationship of each component to the observed structure. The findings here, along with those previously reported identifying neurofilaments,[4-7] τ,[9,10,50] MAP 2,[17] and ubiquitin,[51,52] implicate the view of NFP as a generalized alteration and transformation of cytoskeletal elements. Therefore knowledge of a single element of NFP provides only a part of the information necessary to understand the nature and mode of formation of NFP. An analogy is the microtubule, for while the primary structural element is the tubulin dimer, it is composed also of a multitude of specifically associated proteins. Although identification of these components defined microtubules, understanding the relationship of the components was critical to resolving microtubule structure. The interaction responsible for incorporation of NFP components may be found in the abnormal filaments comprising the Hirano body, which while structurally and antigenically related to microfilaments[22] also contain microtubule-associated proteins.[23,53] Apparently the microfilament proteins incorporated in a Hirano body maintain their physiologic interaction with microtubule-associated proteins.[54-56] We suggest that NFP components similarly maintain the interaction of their components with several proteins, as indicated by their incorporation.

Because interaction with several proteins is the known role of the cytoskeletal proteins found in NFP, it can be hypothesized that NFP represents the reorganization of an interacting system.[57] Our hypothesis does not exclude any of the established components. Furthermore it suggests that other cytoskeletal-related proteins may be found in NFP and emphasizes understanding the processes involved in inclusion formation.

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