Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6070–6074, October 1982
Neurobiology

Brain transglutaminase: *In vitro* crosslinking of human neurofilament proteins into insoluble polymers

[ε-(γ-glutamyl)lysine/isopeptide crosslinks/brain filaments/aging brain/Alzheimer disease]

DENNIS J. SELKOE, CARMELA ABRAHAM, AND YASUO IHARA

Ralph Lowell Laboratories, Mailman Research Center, McLean Hospital, and Department of Neurology and Neuropathology, Harvard Medical School, Belmont, Massachusetts 02178

Communicated by Richard L. Sidman, May 26, 1982

ABSTRACT The accumulation in aged human neurons of insoluble, high molecular weight filamentous polymers apparently linked by nondisulfide covalent bonds led us to examine human brain for the presence of transglutaminase (EC 2.3.2.13) and endogenous protein substrates for this crosslinking enzyme. We demonstrate the presence in brain of a transamidating enzyme that can covalently crosslink brain proteins into insoluble polymers *in vitro* by forming γ-glutamyl-ε-lysine intermolecular bridges. Brain transglutaminase is Ca^{2+} dependent, has an electrophoretic mobility similar to that of erythrocyte transglutaminase, and is active in human postmortem brain from aged normal individuals and patients with Alzheimer disease (senile dementia). Brain neurofilament fractions incubated in the presence of transglutaminase, Ca^{2+}, and the fluorescent amine dansylcadaverine form a fluorescent, nondisulfide-bonded insoluble polymer; this process is associated with a decrease in the amount of soluble neurofilament polypeptides in the preparation. Electron microscopy of the polymeric material reveals an extensive network of connecting filaments, which can be immunostained with various neurofilament antisera. Cystamine, an inhibitor of transglutaminase, prevents the neurofilament crosslinking. Glial filaments and myelin basic protein can also serve as substrates of brain transglutaminase *in vitro*. Although Alzheimer disease-type paired helical filaments are not formed under the specific *in vitro* conditions employed, the data suggest one possible mechanism for the covalent crosslinking of filaments into insoluble polymers during human neuronal aging.

Transglutaminases (TGases; R-glutaminyl-peptide:amine γ-glutamyltransferases, EC 2.3.2.13) are Ca^{2+}-dependent enzymes that catalyze the intermolecular crosslinking of certain proteins by γ-glutamyl-ε-lysine side chain bridges (1, 2). The crosslinks thus formed are covalent bonds that link protein molecules into rigid high molecular weight polymers that are insoluble in $NaDodSO_4$, reducing agents, and urea. Crosslinks of this type have been found in numerous mammalian tissues. Examples of extracellular proteins that are known to be crosslinked by ε-(γ-glutamyl)lysine isopeptide bonds are fibrin during the final stages of blood clotting (3–5) and keratin in hair and wool (6, 7). Among intracellular proteins, ε-(γ-glutamyl)lysine-crosslinked polymers are formed in erythrocyte membranes (8, 9), during senile cataract formation in human lens (10), and in the cornified envelope of terminally differentiated keratinocytes in human epidermis (11). The latter examples have suggested a role for TGases in the rigidification of some structural proteins during cell aging (11). It has also been proposed that the extent of protein crosslinking in the plasma membrane may correlate with the proliferative state of the cell (12). The concept of an increase in isopeptide bonds as a cell moves from a proliferative to a nonproliferative state is supported by the finding of an increase in proliferation of cultured human lung fibroblasts after inhibition of TGase (13). In this context, neurons, which are in general postmitotic throughout postnatal life, have not been examined for the presence of covalently crosslinked proteins and TGase activity.

In human neurons, abnormal pairs of helically wound intermediate filaments (PHF) accumulate progressively during brain aging and, to a much greater extent, in presenile and senile dementia of the Alzheimer type (Alzheimer disease) (14–18). The PHF occur in large cytoplasmic bundles, referred to as neurofibrillary tangles, within the perikarya of neurons in hippocampus, amygdala, cerebral cortex, and certain deep gray nuclear structures. PHF are also present in the abnormal cortical neurites, principally axonal terminals, that form the neuritic, or senile, plaque. Although these lesions represent the principal structural alteration of human neurons during aging and develop in great abundance in Alzheimer disease, little is known about their molecular nature or pathogenesis. We recently partially purified PHF from postmortem human cortex and showed that they are very high molecular weight complexes with unusual solubility properties, including insolubility in $NaDodSO_4$, urea, reducing agent, guanidine·HCl, 0.2 M HCl, or 0.2 M NaOH (19). These findings suggest that PHF contain nondisulfide covalent bonds that bind filaments into a rigid intracellular polymer. Although the type(s) of chemical bond(s) holding these polymers together is unknown, we raised the possibility that ε-(γ-glutamyl)lysine crosslinks formed by the action of TGase could serve this function (19). To investigate this hypothesis further, we have carried out studies to detect and characterize TGase in human brain and examine its ability to crosslink brain proteins.

MATERIALS AND METHODS

Brains from five neurologically normal patients (age range 39–83 years) and eight patients with Alzheimer disease (38–86 years) were obtained at autopsy. One cerebral hemisphere from each brain was immediately frozen at −70°C. Histopathological examination of the other hemisphere revealed abundant neurofibrillary tangles and neuritic plaques in the neocortex of Alzheimer disease specimens; virtually none were seen in control neocortex. Postmortem intervals ranged from 3 to 24 hr in Alzheimer disease patients and from 3 to 24 hr in controls.

To detect TGase activity, 0.5–2.0 g (wet weight) of human gray matter was dissected from frozen coronal brain sections. Samples (0.5 g) of freshly frozen rabbit forebrain were also used. The tissue was homogenized in 2 vol of 50 mM Tris·HCl, pH

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Abbreviations: TGase, transglutaminase; PHF, paired helical filaments; NF, neurofilament; GFA, glial fibrillary acidic protein; kDal, kilodalton(s).

7.5/1 mM EDTA. For initial detection of TGase, low-speed (1,000 × *g*) supernates of this homogenate were assayed. Thereafter, homogenates were spun at 200,000 × *g*, and the high-speed supernate was used as the source of enzyme in all subsequent studies. TGase was detected by the gel assay of Lorand *et al.* (20), in which the fluorescent amine donor, dansylcadaverine, is covalently bound to an exogenous protein substrate, *N,N'*-dimethylcasein, the glutaminyl side chains of which serve as the amine acceptors. Aliquots (20–50 μl) of brain supernates (6–7 mg of protein per ml) as well as the supernates of washed human erythrocyte lysates were electrophoresed in 1% agarose gels containing 0.5% dimethylcasein at 3 mA/cm for 4 hr at 4°C. Thereafter, the gel was overlayed with 5 ml of 2 mM dansylcadaverine/10 mM $CaCl_2$/2 mM dithiothreitol/50 mM Tris·HCl, pH 7.5, for 2 hr at 37°C. After gel fixation and washing, TGase activity was visualized under UV light as a fluorescent spot where dansylcadaverine had been bound by the enzyme in the gel to the dimethylcasein substrate. The technique is both sensitive and specific for transamidases of the endo-γ-glutamine:ε-lysine transferase type (20, 21).

To identify protein substrates of brain TGase, we examined the products of the TGase reaction by $NaDodSO_4$/polyacrylamide gel electrophoresis. Brain supernates (50 μl) containing TGase activity were incubated with 0.2% dimethylcasein, 2 mM dansylcadaverine, and 10 mM $CaCl_2$ for 2 hr at 37°C. Alternatively, preparations of various human brain structural proteins (see below) were substituted for dimethylcasein as substrate in this reaction. After incubation, the reaction mixture was made 2% (wt/vol) in $NaDodSO_4$, 5% (vol/vol) in 2-mercaptoethanol, 10% (vol/vol) in glycerol, and 32 mM in Tris·HCl, pH 6.8. This sample was heated to 100°C for 2 min and electrophoresed (15 mA, 90 min) on a 0.1% $NaDodSO_4$/13% acrylamide gel (22) in a minislab gel apparatus. Fluorescent bands in the unstained gels were viewed and photographed under UV light. The gel was then stained with 0.1% Coomassie blue and photographed conventionally.

Brain filament fractions were prepared from frozen human subcortical white matter (23); their composition, shown in Fig. 2*B*, is similar to that of previously described partially purified filament fractions from mammalian central nervous system (23–25). In addition to the neurofilament (NF) triplet polypeptides [approximate molecular masses: 68, 160, and 200 kilodaltons (kDal)], the preparation contains α- and β-tubulin (55 and 53 kDal), glial filament protein (glial fibrillary acidic protein, GFA) (50 kDal), actin (45 kDal), and myelin basic protein (20 kDal). Such preparations were dissolved in 50 mM Tris·HCl, pH 7.5 (18–20 mg of protein per ml) and 25-μl aliquots were used as substrate in the TGase reaction in place of dimethylcasein. In all cases, leupeptin (Sigma), 40 μg/ml, was added to the incubation mixture to inhibit the proteolysis of NF by Ca^{2+}-activated neutral proteinases (26). Incubations of NF fractions and Ca^{2+} with and without leupeptin (37°C, 2 hr, pH 7.5) showed that leupeptin prevented any appreciable proteolysis of the NF triplet proteins. Leupeptin had no effect on the TGase assay.

For electron microscopic examination of TGase-treated NF fractions, the reaction mixture was centrifuged (110,000 × *g*, 60 min) and the pellet was resuspended in 2% $NaDodSO_4$/5% mercaptoethanol and heated to 100°C for 5 min. After respinning, the $NaDodSO_4$/mercaptoethanol-insoluble residue was fixed in 2.5% (wt/vol) glutaraldehyde/10 mM sodium phosphate, pH 7.5, overnight at 4°C and then postfixed, dehydrated, embedded, and sectioned according to conventional methods.

Immunostaining of the TGase-treated NF preparations was carried out on the $NaDodSO_4$/mercaptoethanol-insoluble residue prepared as for electron microscopy. The insoluble pellet was chopped into small pieces, smeared on albumin-coated glass slides, and incubated (38°C, 1 hr) with various rabbit antisera (or their IgG fractions) to brain intermediate filament proteins (see *Results*). Conventional staining with fluorescein-conjugated goat anti-rabbit IgG was then employed (27).

RESULTS

For initial detection of TGase in human brain, low-speed (1,000 × *g*) supernates of cortical homogenates were electrophoresed on dimethylcasein-containing agarose gels and allowed to react with dansylcadaverine (20). A fluorescent spot indicating the presence of TGase activity was readily apparent. Enrichment of the enzyme was then achieved by high-speed centrifugation of the homogenates; the enzyme activity remained in the 200,000 × *g* supernate, which was used as the source of enzyme in all further studies. Electrophoresis of supernates from various regions of several brains revealed a characteristic fluorescent spot for human brain TGase migrating close to the human erythrocyte enzyme (Fig. 1). TGase from freshly frozen rabbit brain migrated slightly ahead of the human enzyme (Fig. 1). Thus, brain TGase has electrophoretic mobility similar to the mobilities of other mammalian TGases (9, 20, 21, 28). No fluorescent spot appeared in the absence of exogenous Ca^{2+} and in the presence of 5 mM EDTA, indicating the calcium dependence of brain TGase. Magnesium could not be substituted for Ca^{2+} in this reaction. Appreciable TGase activity was present in frontal and temporal neocortex, hippocampus, cerebellum, and white matter of human brain frozen as long as 24 hr postmortem, although the intensity of dansylcadaverine incorporation appeared to decline after 6 hr. For comparison, we incubated rabbit forebrain at room temperature for 0, 3, 6, or 24 hr postmortem before freezing; a moderate decrease in TGase activity occurred (data not shown). No consistent difference in TGase activity staining between samples of equal protein concentration from six normal and eight Alzheimer disease brains matched for age and postmortem interval was apparent with the assay employed. Confirmation of this initial finding will require further study.

To examine the substrate specificities of brain and erythrocyte TGase, we incubated supernates from each source with

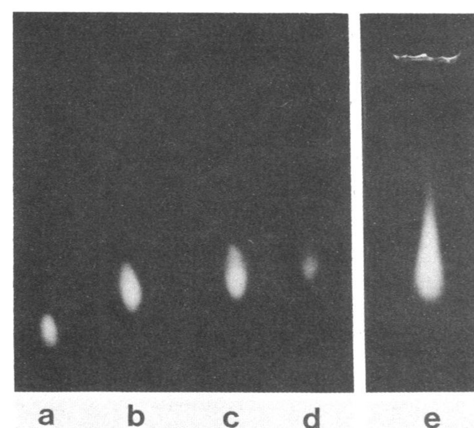

FIG. 1. TGase activity staining of human and rabbit brain high-speed supernates in dimethylcasein/agarose electropherograms. Lane a, rabbit forebrain; lane b, normal human frontal cortex; lane c, normal human inferior temporal cortex; lane d, human inferior temporal cortex from an Alzheimer disease patient; lane e, for comparison, human erythrocyte lysate. Differences in the intensities of the spots do not reflect consistent differences in quantitative TGase activity among the various brain samples.

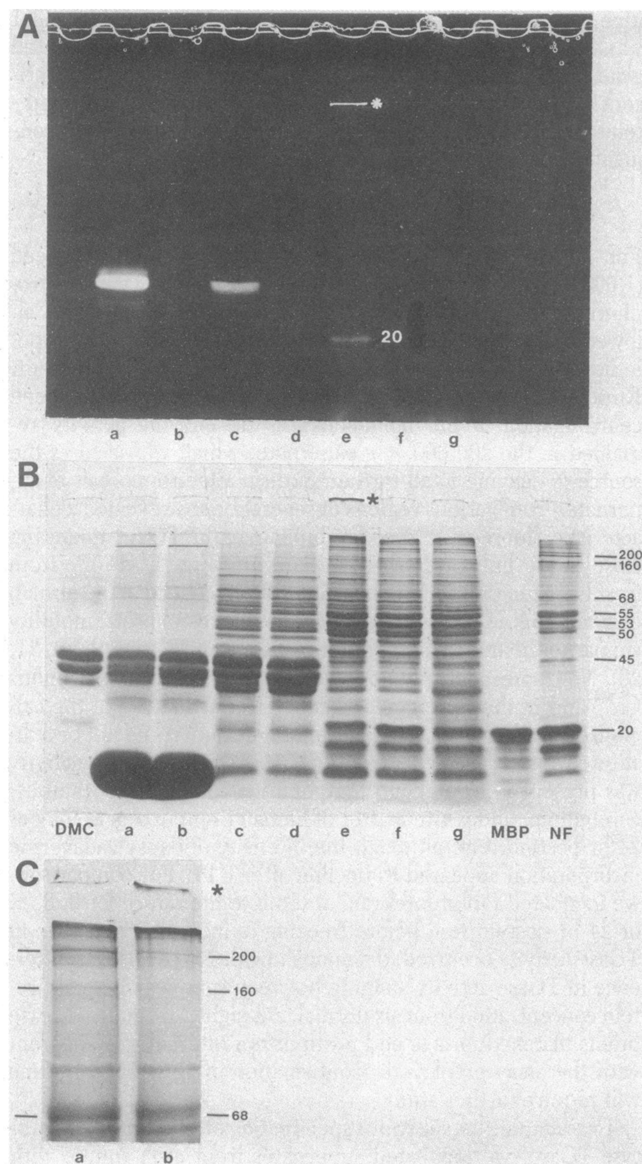

FIG. 2. (*A*) Fluorescence (UV) photograph of NaDodSO$_4$/polyacrylamide gel electropherogram of various human TGase reaction mixtures (see text) all containing dansylcadaverine. Lane a, erythrocyte TGase, dimethylcasein (as substrate), Ca^{2+}; lane b, erythrocyte TGase, dimethylcasein, no Ca^{2+}; lane c, brain TGase, dimethylcasein, Ca^{2+}; lane d, brain TGase, dimethylcasein, no Ca^{2+}; lane e, brain TGase, human brain NF fraction (as substrate), Ca^{2+}; lane f, brain TGase, NF fraction, no Ca^{2+}; lane g, brain TGase preincubated with 10 mM cystamine, NF fraction, Ca^{2+}. The * indicates fluorescent crosslinked polymer that fails to enter gel; 20 indicates 20-kDal myelin basic protein band in NF fraction. (*B*) Coomassie blue staining of gel shown in *A*; lanes are marked as in *A*. DMC, dimethylcasein; MBP, purified human myelin basic protein. NF, human brain neurofilament fraction: NF triplet proteins (68, 160, and 200 kDal), tubulins (55 and 53 kDal), glial filament protein (50 kDal), actin (45 kDal), MBP (20 kDal). *, Crosslinked polymer. (*C*) Upper half of a Coomassie blue-stained gel of TGase-NF reaction mixtures incubated without (lane a) and with (lane b) Ca^{2+}. Note formation of crosslinked high molecular weight polymer (*) and associated decrease in the 200-, 160-, and 68-kDal NF proteins in the Ca^{2+} sample.

dimethylcasein, dansylcadaverine, and Ca^{2+} (37°C, 2 hr) and electrophoresed the reaction mixtures on NaDodSO$_4$/acrylamide slab gels (Fig. 2). Brain TGase incorporated dansylcadaverine into the smaller of the two major dimethylcasein proteins (approximately 31 kDal); the larger dimethylcasein protein (33 kDal) showed no incorporation of the fluorescent amine (Fig. 2*A*, lane c). Erythrocyte TGase showed preferential transamidation of the 31-kDal chain, although some dansylcadaverine incorporation into the 33-kDal protein also occurred (Fig. 2*A*, lane a). In the absence of Ca^{2+}, no fluorescent bands were seen (Fig. 2*A*, lanes b and d).

In order to detect potential endogenous substrate(s) of brain TGase, we prepared NF-enriched preparations from normal human cerebral white matter. NaDodSO$_4$/polyacrylamide gel electrophoresis of a NF fraction that had been incubated with dimethylcasein, dansylcadaverine, leupeptin (to inhibit Ca^{2+}-activated NF proteolysis), and Ca^{2+} in the absence of brain supernate showed no fluorescent bands, indicating the lack of intrinsic TGase activity in the NF fraction. A 25-μl aliquot of NF fraction was then incubated (37°C, 2 hr) with 2 mM dansylcadaverine, 10 mM Ca^{2+}, leupeptin at 40 μg/ml, and 50 μl of TGase-containing brain supernate. Electrophoresis of this reaction mixture after heating in NaDodSO$_4$/mercaptoethanol revealed a fluorescent band of very high molecular weight material that failed to enter the 4% acrylamide stacking gel and remained in the sample well at the top of the gel (Fig. 2*A*, lane e). No fluorescence was detected at the positions of the NF triplet proteins, GFA, or the other fibrous proteins in the NF fractions. However, a band in the NF preparation migrating at approximately 20 kDal did incorporate dansylcadaverine (Fig. 2*A*, lane e); this band comigrated with the major component of purified human myelin basic protein and has previously been identified as myelin basic protein by several criteria (24). When Ca^{2+} was excluded from the incubation mixture or when the brain supernate was heated to 60°C before incubation with the NF fraction, no fluorescent band was seen at the top of the gel (Fig. 2*A*, lane f). These results indicate that the NaDodSO$_4$/mercaptoethanol-insoluble material in the NF fraction did not fluoresce as a result of nonspecific adsorption of dansylcadaverine but had been covalently coupled with dansylcadaverine by the action of a Ca^{2+}-activated enzyme—i.e., TGase.

After fixation and Coomassie blue staining of the gel (Fig. 2*B*), the excluded high molecular weight band that had fluoresced under UV light was found to be intensely stained (Fig. 2*B*, lane e). Comparison of the polypeptide patterns of NF/TGase reaction mixtures incubated with and without Ca^{2+} (in the presence of leupeptin) showed a decrease of the 200- and 160-kDal NF proteins after incubation with Ca^{2+} (Fig. 2*C*); this change was highly reproducible. A quantitative change of the 68-kDal NF protein was less clearly seen, although some preparations showed a definite decrease of this band (Fig. 2*C*). No such decreases of NF proteins occurred in fractions incubated with Ca^{2+} and leupeptin in the absence of TGase. All other bands in the preparation appeared unchanged, with the exception of the 20-kDal band (myelin basic protein), which was also decreased in the Ca^{2+}-present sample compared to the Ca^{2+}-absent control. These experiments were repeated with NF fractions and TGase-containing supernates prepared from freshly frozen rabbit brain, and identical results were obtained.

In order to determine the structure of the high molecular weight polymer that resulted from the action of brain TGase on human NF fractions, we carried out electron microscopy examination of the NaDodSO$_4$/mercaptoethanol-insoluble pellets of such preparations. The most abundant constituent of these pellets was an extensive network of randomly oriented filamentous structures of various lengths and diameters, which formed an irregular latticework throughout the pellet (Fig. 3). Filaments of different diameters were contiguous with each other at frequent points of intersection and thus formed a virtually continuous filamentous network. The diameter of the thinnest filaments within this network was approximately 4–5

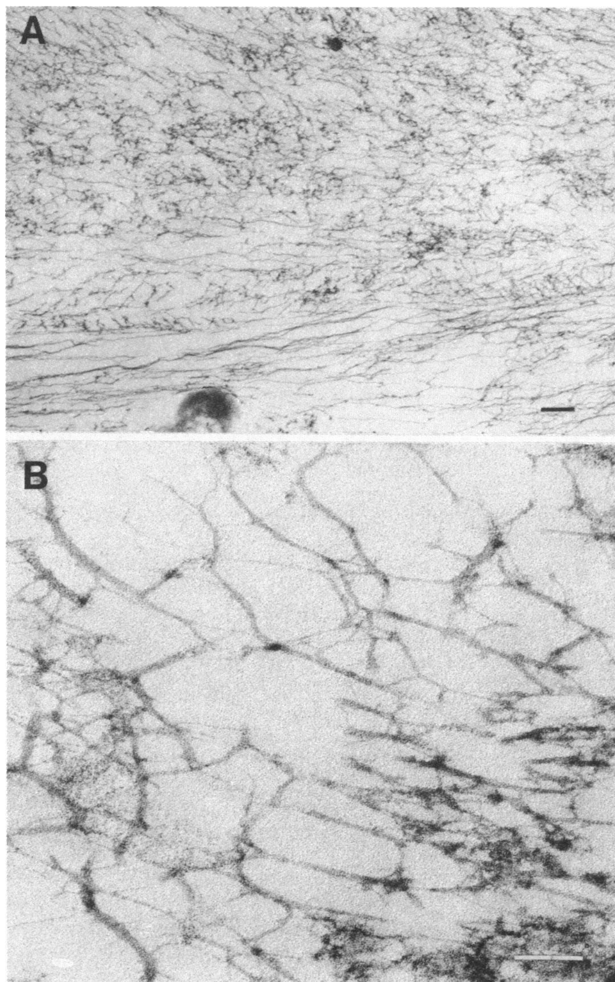

FIG. 3. Electron micrographs of $NaDodSO_4$/mercaptoethanol-insoluble filament network formed by allowing Ca^{2+}-activated brain TGase to react with normal human NF fraction *in vitro* (see text). (*A*) Low power; (*B*) higher power. Bars = 100 nm.

nm, but most filamentous structures ranged from 8 nm up to 20–25 nm. It appeared that several thinner filaments could converge to form the thicker elements. The $NaDodSO_4$/mercaptoethanol-insoluble pellets of NF fractions that had been incubated with TGase in the absence of Ca^{2+} were much smaller than the Ca^{2+}-present pellets, and extensive electron microscopy analysis revealed almost no filamentous network. Rarely, small fragments of a much less well-developed filamentous network consisting of shorter and thinner filaments were seen. Preparations incubated either with or without Ca^{2+} contained other $NaDodSO_4$/mercaptoethanol-insoluble, amorphous, electron-dense material, which was not readily identifiable and was similar in the two pellets. NF preparations incubated with Ca^{2+} in the absence of TGase and heated in $NaDodSO_4$/mercaptoethanol showed no filamentous network.

To confirm the participation of NF proteins in forming this crosslinked filamentous polymer, the $NaDodSO_4$/mercaptoethanol-insoluble material was allowed to react with various rabbit antisera to brain intermediate filament proteins and was examined by fluorescence microscopy. Preimmune rabbit IgG and fluorescein-conjugated goat anti-rabbit IgG employed as controls produced no fluorescent staining of the filamentous network. An antiserum (IgG fraction) to denatured chicken brain NF proteins (29) (the generous gift of D. Dahl) produced intense staining of the crosslinked network; at higher magnification, decoration of individual fibrous elements could be seen (Fig. 4). This antiserum has previously been shown to react with mammalian NF polypeptides by immunoaffinity chromatography (30) and to immunostain normal mammalian NF as well as Alzheimer neurofibrillary tangles in tissue sections (29, 31, 32). An antiserum (IgG fraction) to the gel-purified 200-kDal protein of rat spinal cord NF, which has also been shown to immunolabel normal NF and Alzheimer tangles (33), stained the filamentous network, as did an antiserum to the gel-purified 160-kDal protein of a human brain NF preparation. Preabsorption of these various NF antibodies with normal human NF fraction (23) markedly decreased the immunostaining in each case. An antiserum to GFA (34) also produced bright immunostaining of the filamentous complex.

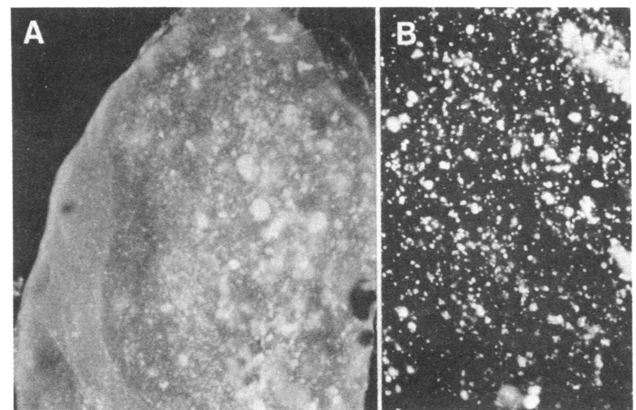

FIG. 4. Fluorescent immunostaining of insoluble filamentous network. (*A*) Staining with IgG fraction of a rabbit antiserum to chicken NF proteins (30). (*B*) Staining with IgG fraction of preimmune rabbit serum. The bright white granules in each specimen represent autofluorescent lipofuscin granules. (×370.)

Finally, we examined the inhibition of the brain TGase reaction by cystamine, an active site inhibitor of TGase in erythrocytes and lung fibroblasts (13, 35). When human brain TGase was incubated with 10 mM cystamine (13) in the presence of Ca^{2+} (30 min, 37°C, pH 7.5), the incorporation of dansylcadaverine into the dimethylcasein test substrate was abolished. Similarly, when a NF fraction was employed as substrate, preincubation of TGase with cystamine completely inhibited the formation of a fluorescent high molecular weight protein polymer at the top of the gel and prevented the incorporation of dansylcadaverine into myelin basic protein (Fig. 2*A*, lane g).

DISCUSSION

We became interested in detecting and characterizing brain TGase as a result of our recent studies of brain tissue from patients with Alzheimer disease (19). We found that the PHF that accumulate progressively in selected human neurons during aging and particularly in Alzheimer disease are high molecular weight polymers apparently containing nondisulfide covalent bonds which bind certain fibrous proteins into rigid insoluble complexes (19). The similarity of these properties to those of certain mammalian intracellular proteins known to undergo crosslinking by ε-(γ-glutamyl)lysine isopeptide bonds (1, 2, 8–13, 28, 35) raised the possibility that such bonds could be involved in PHF formation. Although the subunit protein(s) composing PHF have not been identified, several recent immunocytochemical studies suggest that PHF may contain antigens shared with neurofilaments (31–33, 36) and perhaps with other intermediate filaments (37). We therefore asked whether normal neuronal intermediate filaments could serve as a po-

tential natural substrate of brain TGase.

The results of this study demonstrate the presence in brain of a calcium-dependent transamidating enzyme that apparently can crosslink brain proteins into high molecular weight insoluble polymers by forming γ-glutamyl-ε-lysine intermolecular bridges. This TGase is active in various regions of postmortem human and rabbit brain. Its mobility, as judged by agarose gel electrophoresis, and its substrate affinity for dimethylcasein are similar to those of human erythrocyte TGase. TGase activity remains in the 200,000 $\times$ g supernatant of brain homogenates, providing the initial step for purification of the brain enzyme. TGase in rabbit forebrain has substrate affinities similar to those of the human brain enzyme.

The following findings support the conclusion that human NF can be crosslinked by the action of brain TGase. (*i*) Partially purified brain filament fractions containing abundant NF (in addition to other filament proteins) form, upon incubation with TGase-containing supernates, a $NaDodSO_4$/mercaptoethanol-insoluble, high molecular weight protein polymer that fails to enter gels. (*ii*) In the absence of added Ca^{2+} or after heat inactivation of enzymes, no such polymer forms. (*iii*) Dansylcadaverine is incorporated into this polymer in the presence of TGase and Ca^{2+}, indicating that some available glutaminyl residues in the proteins being crosslinked bind dansylcadaverine. (*iv*) Formation of the high molecular weight polymer is accompanied by a decrease in the amount of 200-, 160-, and 68-kDal NF proteins in the preparations. (*v*) Electron microscopy of the $NaDodSO_4$/mercaptoethanol-insoluble material demonstrates an extensive network of connecting filaments of various diameters that is not formed in the absence of Ca^{2+} or TGase. (*vi*) The filaments of this network can be decorated by various anti-NF antisera. (*vii*) Cystamine, an inhibitor of TGase, prevents the formation of the insoluble filament complex.

The immunostaining of this polymerized network with GFA antiserum suggests that glial filaments present in our preparation can also be crosslinked by TGase. Under these *in vitro* conditions, glial filaments (and perhaps myelin basic protein) probably form heterogeneous polymers with NF. This heterogeneity provides one possible explanation for why Alzheimer disease-type PHF do not appear to be formed in this particular *in vitro* system. The purification from frozen human brain of intact neuronal intermediate filaments entirely free of contamination with other fibrous proteins (tubulin, actin, GFA) has not yet been described. Studies of TGase-catalyzed crosslinking of reconstituted human NF prepared from chromatographically purified NF triplet polypeptides should allow us to define the ultrastructure of homogeneous polymers of isopeptide-linked human NF.

It is important to emphasize that there is currently no evidence for the presence of ε-(γ-glutamyl)lysine isopeptide bonds in human PHF or in any other brain cytoskeletal protein. The *in vitro* crosslinking of human neurofilaments by TGase reported here provides a model for certain electrophoretic, immunochemical, and solubility properties of PHF. It should be informative to attempt to detect ε-(γ-glutamyl)lysine bonds in purified PHF preparations. Although such crosslinks are one candidate for the bonds holding PHF together, a different type of intermolecular covalent bond, perhaps not previously described, may well serve this function.

We thank W. Hamilton for preparation of electron microscopy sections and W. Busse for photographic assistance. Drs. M. Shelanski, K. Kosik, and A. Pope provided helpful discussion. This work was supported in part by National Institutes of Health Grants AG 02126 and NS 16367 (Huntington's Disease Research Center Without Walls). D.J.S. is the recipient of a Teacher-Investigator Award from the National Institute of Neurological and Communicative Disorders and Stroke.

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