Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2878–2882, April 1992
Neurobiology

A protein kinase associated with paired helical filaments in Alzheimer disease

(Alz-50 reactive antigen/casein kinase I/hemin/microtubule-associated proteins τ)

INEZ J. VINCENT* AND PETER DAVIES

Departments of Pathology and Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461

Communicated by Dominick P. Purpura, December 26, 1991 (received for review September 19, 1991)

ABSTRACT **We have identified a protein kinase in immunoaffinity-purified preparations of paired helical filaments from brain tissue of individuals with Alzheimer disease. The kinase phosphorylates the filament proteins *in vitro* in a manner independent of second messenger regulation or of modulation by heparin and polyamines. Physiological concentrations of hemin, an oxidized heme porphyrin, inhibit the kinase and abolish Alz-50 immunoreactivity of the proteins. Since paired helical filaments are composed of hyperphosphorylated proteins, association of a protein kinase with the filaments provides a mechanism for abnormal processing of the proteins in disease.**

Aberrant phosphorylation has emerged as a biochemical hallmark of the Alzheimer disease (AD) brain (reviewed in ref. 1). Paired helical filaments (PHFs), the abnormal ultrastructural unit in neurofibrillary tangles, dystrophic neurites, and neuropil threads (2–4) are composed predominantly of hyperphosphorylated τ protein (5–7). On a more quantitative basis, PHFs have been reported to contain 9 or 10 phosphate groups more than are found in normal adult τ (8). Some of these sites have been identified by antibodies tau-1 (9), PHF-1 (10), and anti-T3P (7), while the remainder have yet to be located. Determination of the contribution of each of these phosphorylated sites to the genesis of PHFs poses a remarkable challenge and is of utmost importance in understanding the pathology of AD. Of equal importance is information regarding the enzymes responsible for these atypical posttranslational events. Although more general alterations in levels and distribution of certain kinases in AD have been reported (1), there is no evidence to implicate their action in phosphorylation of PHFs. A tubulin-dependent kinase from rat brain has recently been reported to phosphorylate human τ resulting in generation of a PHF epitope (11). While the corresponding human enzyme has yet to be identified, the role of this phosphorylation in PHF formation is still undetermined.

It is known that kinases often copurify with their physiologically active substrates as a result of being structurally linked to them. The best known examples include the cAMP-dependent protein kinase that copurifies with microtubules (12) and the cAMP-independent kinase that copurifies with neurofilament proteins (13). With this in mind, we have examined purified PHF preparations for enzyme activities that might be significant in their formation. Such studies have previously been impossible because of the harsh detergent and protease treatments used in isolation of PHFs. To obtain PHFs in a form amenable to functional characterization, we have resorted to affinity chromatography with the monoclonal antibody (mAb) Alz-50 used as the affinity ligand.

From among the spectrum of antibodies that have been used in studies of Alzheimer brain pathology, Alz-50 has emerged as a particularly useful probe. The diagnostic potential of the antibody (14–16) stems from its ability to detect a τ epitope that is abundant in the brains of individuals with AD but is not detected in similar regions of normal adult brain (17). Alz-50 immunoreactivity is also observed in the brains of patients with Down syndrome, Pick disease, and progressive supranuclear palsy (14, 20, 21).

The purification of PHFs in the present study exploits the ability of Alz-50 to discriminate between native forms of normal τ and PHF τ. Although PHFs obtained by this method have a protein composition similar to that of PHFs isolated by other procedures (7, 22, 23), they are distinguished by the presence of an endogenous protein kinase that transfers stoichiometric amounts of labeled phosphate from ATP to the PHF proteins. This kinase is characterized here both biochemically and with respect to its relationship with PHFs. The properties of this kinase do not resemble those of the known protein kinases implicated in τ phosphorylation. Additional experiments indicate that the protein kinase is very tightly associated with PHFs and may play a role in their formation.

EXPERIMENTAL PROCEDURES

Tissue Preparation. Cases of AD, Down syndrome, and Pick disease were selected by clinical and neuropathological criteria. Normal patients had no history of psychiatric or neurologic disease and no significant findings on neuropathologic examination. After a postmortem interval that averaged 11 hr, the brain tissues were frozen at −70°C until used. Twenty grams of cortical tissue was homogenized in 10 vol of Tris-buffered saline (TBS; 0.01 M Tris base/0.14 M NaCl, pH 7.4) using a Polytron operating at a setting of 5 for two 30-s bursts. The homogenate was centrifuged at 27,000 × *g* for 30 min at 4°C, and the supernatant was used for immunoaffinity purification of PHFs, as described below.

mAbs. The mAb used for affinity purification of PHFs was Ab42 (IgG), a class switch clone of Alz-50 (IgM) produced according to ref. 24. mAbs 38 and 126 were raised against 100,000 × *g* pellet preparations from AD brain that were washed with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (25). PHF-1 was raised against denatured preparations of nonaggregated populations of PHFs (10).

Immunoaffinity Isolation of PHFs. Approximately 20 mg of protein A-purified Ab42 was dialyzed into 0.1 M sodium phosphate buffer (pH 8.1) and mixed with 10 ml of washed Affi-Gel 10 (Bio-Rad). The mixture was incubated on a rotary shaker for 30 min at room temperature. At the end of this time, a volume of 0.1 M ethanolamine (pH 8.1) equal to that of the gel/antibody mixture was added and the incubation

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AD, Alzheimer disease; PHF, paired helical filament; mAb, monoclonal antibody; ADAP, AD-associated protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HRI, heme-regulated inhibitor.

*To whom reprint requests should be addressed.

was continued for 1 hr. The coupling efficiency was generally >90%. The immunoadsorbent was poured into a column and washed with TBS. The column was maintained at 4°C and all chromatography steps were conducted at this temperature. Before application of sample, the column was treated with at least 2 bed vol of eluting buffer (3 M potassium thiocyanate) followed by 5 bed vol of TBS. The 27,000 × *g* supernatant was loaded onto the immunoaffinity column at a flow rate of ≈25 ml/hr. Nonspecific binding was reduced by washing the immunoadsorbent with at least 30 bed vol of TBS. Subsequently, adsorbed protein was eluted with eluting buffer. Fractions were assayed for protein concentration with the Quantigold protein reagent (Bio-Rad). Peak fractions were dialyzed against TBS and aliquots were stored at −70°C until used. As determined by ELISA, ≈100% of the Alz-50 immunoreactivity of the 27,000 × *g* supernatant was recovered in the eluate. The typical protein yield was 60–80 μg per ml of eluate, representing an ≈10,000-fold enrichment of the antigen relative to the homogenate.

Samples of AD-associated proteins (ADAPs) were provided by H. Ghanbari and B. Miller (Abbott Laboratories). In this preparation, a 100,000 × *g* pellet from AD brain was further enriched for Alz-50 reactivity by washing with CHAPS and by extraction with 3.5 M guanidine hydrochloride. The resulting ADAP preparation contains the three major Alz-50-positive bands and is similar to the immunoaffinity PHF preparation with respect to all other cross-reactive mAbs (25).

Electron Microscopic Studies. Two microliters of a 1:10 dilution of the PHF preparation was loaded onto a Formvar carbon-coated, glow-discharged grid. The grids were stained with 5% uranyl acetate for 20 min. Structural parameters were obtained from 60 determinations encompassing immunoaffinity-purified PHF preparations from nine different cases.

Standard Phosphorylation Assay. Twenty-microliter aliquots of the PHF preparation containing ≈1.5 μg of protein per μl of TBS was incubated with 1 μCi (10 nM) of [γ-^{32}P]ATP (1 Ci = 37 GBq) (Amersham) and 2 mM $MgSO_4$ for 10 min at room temperature. The incubation was terminated by addition of 5 μl of electrophoresis sample buffer containing 1 M Tris base (pH 6.7), 5% SDS, 5% 2-mercaptoethanol, 50% (vol/vol) glycerol, 0.5% bromophenol blue. Samples were boiled for 5 min before electrophoresis on SDS/10% polyacrylamide gel, with a 4% stacking gel. The resolved proteins were transferred from gel to nitrocellulose membrane. Alz-50 staining of blotted proteins was performed as described (14). Alternatively, gels were fixed and stained with silver reagent or Coomassie blue. Either gels or nitrocellulose blots, as indicated, were exposed to Kodak X-Omat AR film with a Lightning Plus screen (DuPont) at −70°C, generally overnight.

In kinetic experiments involving variations in ATP concentration, the amount of [γ-^{32}P]ATP in the incubation mixture was kept fixed (4 μCi), while the concentration of unlabeled ATP was varied.

When casein or histone was used as substrate for phosphorylation, the incubation conditions were as follows: 5 μg of casein (Sigma) or 25 μg of histone (type II-AS) (Sigma), 3 μl (0.15 μg of protein) of immunoaffinity PHF preparation, 2 mM $MgSO_4$, and 1 μCi of [γ-^{32}P]ATP in a final vol of 20 μl. The incubation was conducted at room temperature for 10 min. Samples were resolved by electrophoresis on SDS/12% polyacrylamide gel and were further processed according to standard procedures.

Quantitation of Incorporated ^{32}P into PHFs. Gels were dried and subjected to autoradiography. PHF proteins (57–68 kDa only) were cut out from the gels using the autoradiogram as a trace. The gel bands were digested with 200 μl of 30% hydrogen peroxide for 4 hr at 50°C, and radioactivity was estimated by liquid scintillation counting.

Immunoprecipitation with mAbs 38 and 126. The respective purified mAbs (IgG1s) were coupled to goat anti-mouse IgG-coated inert microspheres (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and were used to precipitate PHF protein as described (26).

RESULTS

Electron Microscopic Studies. Electron microscopic examination of immunoaffinity-purified preparations revealed an ultrastructure consisting predominantly of twisted ribbon-like filaments, with an average maximum width of 19.2 ± 4 nm and an average minimum width of 7.2 ± 2 nm occurring at a periodicity of 80 ± 12 nm (Fig. 1). Untwisted filaments of 10.3 ± 2 nm diameter are occasionally observed.

Endogenous Phosphorylation of PHFs. Incubation of an immunoaffinity-purified PHF preparation with [γ-^{32}P]ATP and $MgSO_4$ for 10 min at room temperature results in phosphorylation of all Alz-50-reactive components, as well as other proteins visualized by silver staining or reactivity with the PHF-specific mAb PHF-1 (Fig. 2*A*). Such high and low molecular weight species have been previously described in purified PHF preparations and correspond to aggregates or endogenously derived degradation products (22, 27). The PHF-1 immunoreactive and phosphorylated material in the stacking gel probably represents insoluble PHFs, another feature commonly observed with PHF preparations (22, 23). That the various phosphorylated bands of the immunoaffinity preparation are derived from PHFs was verified by analyzing the phosphorylated preparation under nondenaturing conditions (Fig. 2*B*). The silver-stained gel shows that no protein enters the gel. This is consistent with the immunoblots and corresponding autoradiograms, which reveal that all the immunoreactive and phosphorylated proteins remain excluded from the gel (Fig. 2*B*). Boiling the PHF preparation before incubation with ATP prevents incorporation of labeled

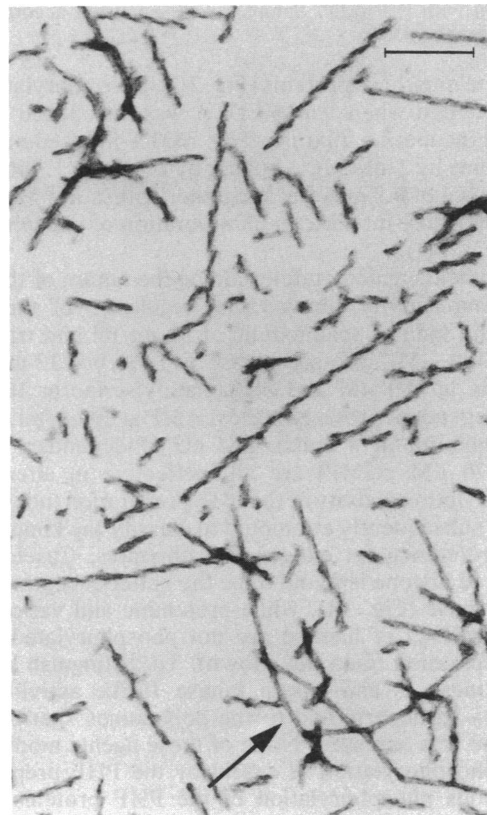

FIG. 1. Electron microscopic studies. The immunoaffinity purified PHF sample was loaded onto grids and negatively stained with 5% uranyl acetate for 20 min. Arrow indicates untwisted filament. (Bar = 0.25 μm.)

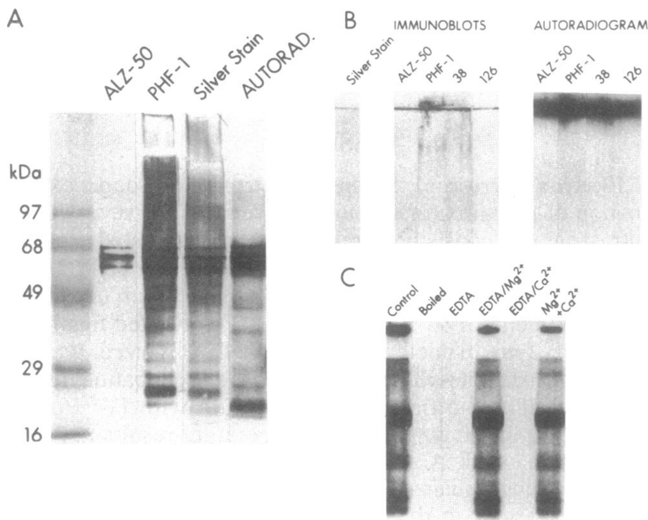

FIG. 2. Endogenous phosphorylation of PHFs. (*A*) Phosphorylation of PHF proteins. Immunoaffinity preparations of PHFs were incubated with [γ-^{32}P]ATP under standard conditions. Samples were analyzed by SDS/PAGE, immunoblotting, and autoradiography. The figure represents a composite of blotted samples stained with Alz-50 and PHF-1, a silver-stained sample, and an autoradiogram. (*B*) Analysis of phosphorylated PHF proteins under nondenaturing conditions. The PHF preparation was phosphorylated as in *A* but subjected to PAGE under nondenaturing conditions—i.e., without SDS and 2-mercaptoethanol. One gel strip was silver stained and the remainder was transferred to nitrocellulose, immunostained with the indicated mAbs, and subjected to autoradiography. (*C*) Effects of divalent cations. Immunoaffinity preparations of PHFs were incubated with [^{32}P]ATP under standard conditions (control) or in the presence of 1 mM EDTA, 2 mM Mg^{2+}, or 2 mM Ca^{2+} as indicated. The second lane represents a sample that was boiled for 5 min before incubation with [^{32}P]ATP. Samples were analyzed according to a standard protocol.

phosphate into these proteins (Fig. 2*C*). Phosphorylation was also prevented when 1 mM EDTA was introduced into the standard incubation mixture. This EDTA-induced inhibition is overcome by 2 mM Mg^{2+} but not by 2 mM Ca^{2+}. Moreover, the presence of 0.2 mM Ca^{2+} together with 2 mM Mg^{2+} does not produce any increase in incorporation of phosphate into PHFs (Fig. 2*C*).

Experiments aimed at determining the nature of the phosphorylating activity showed that regulators of the Ca^{2+}/calmodulin kinases (calmodulin at 50 μg/ml and trifluperazine at 100 μM), protein kinase C (phorbol 12-myristate 13-acetate at 100 μM and phosphatidylserine at 100 μM), cAMP-dependent protein kinase (cAMP at 20 μg/ml, and the Walsh inhibitor at 4 units), and cGMP-dependent protein kinase (20 μM cGMP) are all ineffective in altering the phosphorylation activity of the PHF preparation (not shown).

It was subsequently attempted to classify the kinase based on its preference for exogenous substrates. Casein rather than mixed histone is found to be the better acceptor protein for the kinase (Fig. 3*A*), while protamine and various individual fractions of histone are not phosphorylated by the PHF preparation (data not shown). To distinguish between casein kinase I- and casein kinase II-like activities, the sensitivity to heparin and to the polyamines spermine and protamine was assessed. None of these agents modifies the rate of phosphorylation of casein by the PHF preparation. Endogenous phosphorylation of the PHF proteins is also unaffected by heparin, spermine, or protamine (Fig. 3*B*).

PHF-Associated Kinase Activity Is Sensitive to Hemin (Ferriprotoporphyrin IX Chloride). One compound found to exert an inhibitory effect on the kinase of the PHF preparation is the blood porphyrin hemin. At 10 μM, hemin totally abolishes phosphorylation of the PHF proteins (Fig. 3*B*) as well as phosphorylation of casein by the PHF preparation (Fig. 3*A*). In addition, incubation of PHF with 10 μM hemin results in inhibition of Alz-50 immunoreactivity with PHF on electrophoretic blots (Fig. 3*B*).

The ADAP preparation was also observed to contain a protein kinase activity that exhibits properties similar to the kinase of the immunoaffinity PHF preparation. Thus, the ADAP-associated kinase preferentially phosphorylates casein, and this phosphorylation is effectively reduced by 10 μM hemin. Incubation with 10 μM hemin also inhibits endogenous phosphorylation of the ADAP proteins, as well as subsequent Alz-50 immunoreactivity with the proteins on electrophoretic blots (Fig. 3*C*).

Analysis of specific phosphorylation sites in the PHF proteins reveals that serine is the predominant acceptor of labeled phosphate, although some incorporation of label into threonine residues is also observed (Fig. 4*A*).

Kinetic Properties of the Kinase. Kinetic experiments were conducted to obtain estimates of the affinity of the kinase for ATP and the stoichiometry of phosphorylation. In the first case, aliquots of the PHF preparation were incubated with concentrations of ATP ranging from 50 μM to 2 mM, and the incorporation of label into the major triplet proteins was quantitated

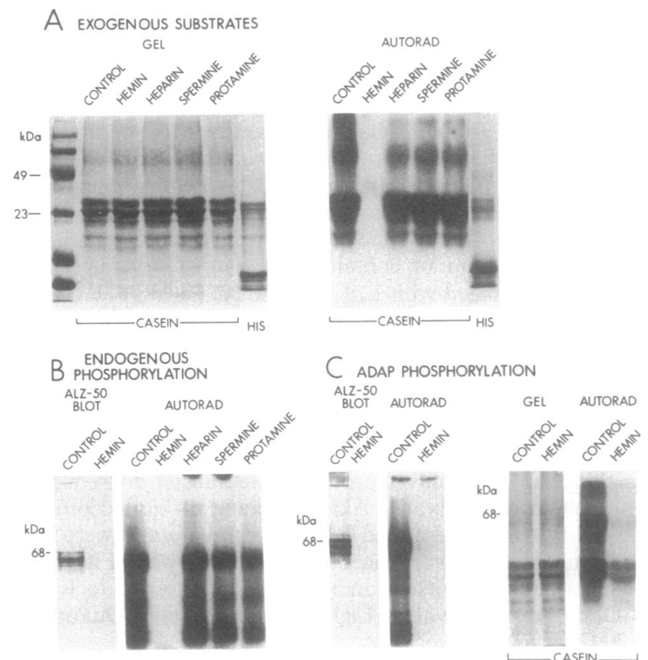

FIG. 3. Characterization of the kinase of the PHF preparation with respect to exogenous substrates and inhibition by hemin. (*A*) Exogenous substrates. Casein or histone (His) was incubated with [γ-^{32}P]ATP and the immunoaffinity PHF preparation under standard conditions (control) or with TBS containing 10 μM hemin, heparin (0.5 μg/ml), 2 mM spermine, or 0.5 mM protamine. The samples were electrophoresed on SDS/12% polyacrylamide gel; the gels were stained with Coomassie blue and subjected to autoradiography. (*B*) Endogenous phosphorylation. PHF preparations were incubated with [γ-^{32}P]ATP under standard conditions (control) or in the presence of 10 μM hemin, heparin (0.5 μg/ml), 2 mM spermine, or 0.5 mM protamine. SDS/PAGE, immunoblotting with Alz-50, and autoradiography were conducted according to standard procedures. (*C*) ADAP phosphorylation. Ten microliters ($\approx$1 μg of protein) of the ADAP sample was incubated with [^{32}P]ATP in the absence (control) or presence of 10 μM hemin. The samples were processed according to a standard protocol. Alz-50 staining of the blotted ADAP samples is shown along with the autoradiogram. In other experiments, 10 μg of casein was incubated with 3 μl (0.3 μg of protein) of the ADAP preparation and [^{32}P]ATP in the absence or presence of 10 μM hemin. The samples were resolved on SDS/12% polyacrylamide gels. The Coomassie-stained gel and autoradiogram are shown.

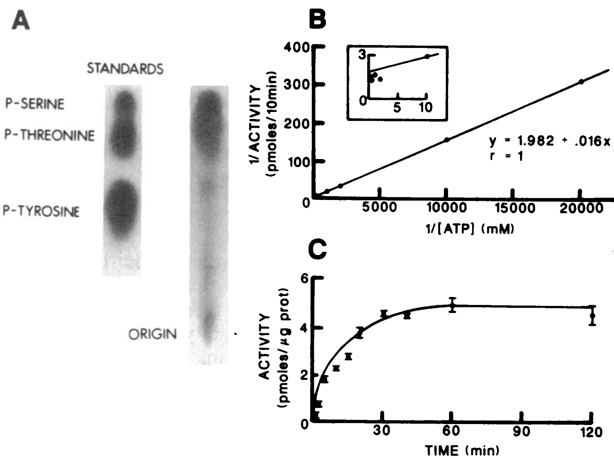

FIG. 4. Identification of phosphorylated amino acids and kinetic characterization. (*A*) ^{32}P-labeled PHF preparations were digested with chymotrypsin, hydrolyzed with 6 M HCl, and analyzed by TLC. The standards were visualized with ninhydrin and the labeled amino acids were visualized by autoradiography. (*B*) Twenty microliters of the PHF preparation was incubated with 4 μCi of [γ-^{32}P]ATP and various concentrations of unlabeled ATP. The concentration of Mg^{2+} in each case was maintained at 5-fold that of the respective ATP concentration. The samples were resolved by SDS/PAGE and the radioactivity incorporated into the bands corresponding to the PHF triplet proteins (i.e., 57–68 kDa) was quantitated as described. An example of a Lineweaver–Burk plot of the data from a total of six such determinations is shown. (*Inset*) Enlargement of the lower end of the plot indicating the intercept on the ordinate. (*C*) PHF preparations were incubated with 4 μCi of [^{32}P]ATP and 200 μM unlabeled ATP for the indicated times. ^{32}P incorporation into the triplet PHF proteins was quantitated as described.

as described. The K_m for ATP was estimated from a Lineweaver–Burk plot of the data to be 6 μM (Fig. 4*B*). The dependence of the degree of phosphorylation on the duration of exposure to [γ-^{32}P]ATP was examined at 200 μM unlabeled ATP. A plot of the extent of phosphorylation vs. time of incubation is shown in Fig. 4*C*. Phosphorylation approaches a maximum at 40 min and then it plateaus. Based on an average molecular mass of 64 kDa, the stoichiometry of phosphorylation was determined to be 0.64 mol per mol of PHF.

Kinase Is Tightly Associated with PHF. As a first step toward evaluating the relationship between the kinase and PHFs, the occurrence of the kinase was examined in immunoaffinity preparations from cases of Pick disease and Down syndrome, where Alz-50 immunoreactivity is detected, and in normal brain tissue, which lacks Alz-50 reactivity. As expected, no Alz-50-positive bands are observed in the preparation from normal brain and no endogenous phosphorylation is detected (Fig. 5*A*). Moreover, the normal brain preparation does not phosphorylate casein (data not shown). Immunoaffinity preparations from Pick disease, Down syndrome, and AD, respectively, contain increasing amounts of Alz-50 antigen. Endogenous phosphorylation of these preparations is observed and demonstrates an increase in parallel with the increase in abundance of PHF protein (Fig. 5*A*).

To further examine the association of the kinase with the PHF proteins, the immunoaffinity preparation was subjected to immunoprecipitation using two additional mAbs (mAb 38 and 126) reacting at independent epitopes on PHFs. The immunoprecipitates and the supernatants were then incubated with [^{32}P]ATP and the phosphorylated products were analyzed by a standard procedure. With both mAbs, the kinase activity as measured by phosphorylation of the PHF proteins was found to coprecipitate with the immunoreactive PHF proteins (Fig. 5*B*). In contrast, the nonspecific IgG1 did not precipitate PHFs or the phosphorylating activity (Fig. 5*B*).

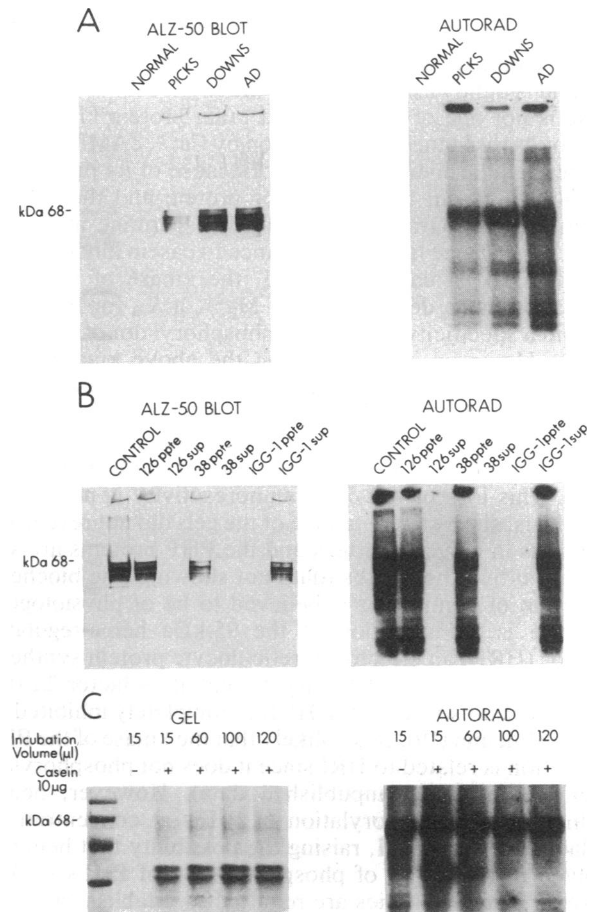

FIG. 5. Association of the protein kinase with PHFs. (*A*) Distribution of kinase relative to the occurrence of Alz-50 immunoreactivity. Brain tissue from normal, Pick disease, Down syndrome, and AD was chromatographed on the Ab42 affinity column, and the retained proteins were eluted as described. Fifty microliters of the normal preparation (protein was not detected in this sample), 50 μl of the Pick disease sample (1 μg of protein), 50 μl of the Down syndrome sample (2 μg of protein), and 20 μl of the AD sample (2 μg of protein) were incubated with [γ-^{32}P]ATP under standard conditions. Samples were analyzed by a standard procedure. (*B*) Immunoprecipitation with mAbs 38 and 126. The immunoaffinity PHF preparation was subjected to immunoprecipitation with mAb 38, mAb 126, or an irrelevant IgG1. The immunoprecipitates (126ppte, 38ppte, and IgG-1ppte) and supernatants (126sup, 38sup, and IgG-1sup) were incubated with [γ-^{32}P]ATP under standard conditions. The samples were then subjected to SDS/PAGE, immunoblotting with Alz-50, and autoradiography. (*C*) Effects of dilution on phosphorylation of PHFs. Five micrograms of casein was incubated with 8 μl (0.4 μg of protein) of the PHF preparation in increasing amounts of TBS so that the final reaction volume varied from 15 to 125 μl. The concentrations of [γ-^{32}P]ATP and Mg^{2+} were maintained at their standard values at each dilution. The samples were resolved on a SDS/12% polyacrylamide gel; the gel was stained with Coomassie blue and subjected to autoradiography by a routine procedure.

Another approach used to establish a physical association between PHFs and the kinase was to study the effects of dilution on phosphorylation of PHFs. This was accomplished by incubating a fixed amount of the PHF preparation with [^{32}P]ATP and increasing volumes of TBS in an attempt to reduce the interaction between kinase and substrate. Casein was included in the reaction as a positive control. It was found that phosphorylation of casein, being dependent on stoichiometric interaction with the kinase protein, was markedly reduced when the volume of the incubation mixture was increased 4-fold; it was not detected at further dilutions (Fig. 5*C*). In contrast, phosphorylation of the PHF proteins was sustained at all dilutions (Fig. 5*C*).

DISCUSSION

We have discovered a protein kinase that phosphorylates PHF and is tightly associated with the twisted filaments. This kinase is not one of the abundant brain protein kinases by virtue of its resistance to modulation by Ca^{2+}, cAMP, cGMP, or other cellular messengers (28). Because of its preference for utilizing casein as an acceptor protein and the lack of inhibition by heparin or activation by spermine and protamine, the enzyme has a resemblance to casein kinase I (29). Like the 37-kDa casein kinase I, the kinase of the PHF preparation has a dependence on Mg^{2+}, a K_m for ATP of 6 μM, and a specificity for ATP as phosphoryl donor (data not shown). However, it differs from the above kinase in its apparent ability to modify threonine residues in addition to serine. An intriguing aspect of the PHF kinase is its sensitivity to hemin. Accompanying this inhibition of phosphorylation is the loss of immunoreactivity of the proteins with Alz-50. This loss of Alz-50 immunoreactivity is not due to proteolysis, since silver staining of the gels did not reveal any alterations in banding pattern and the PHF proteins are still recognized by other mAbs (data not shown). One biochemical action of hemin that is believed to be of physiological relevance is its inhibition of the 95-kDa heme-regulated inhibitor (HRI), an effector of reticulocyte protein synthesis (18). Phosphorylation of eukaryotic initiation factor 2α (the only known substrate for the HRI) is completely inhibited by 10–20 μM hemin (19). It is unlikely that the kinase of the PHF preparation is related to HRI since it does not phosphorylate initiation factor 2α (unpublished data). However, hemin eliminates the phosphorylation of PHFs at concentrations similar to those of HRI, raising the possibility that hemin is involved in regulation of phosphorylation of PHFs in AD. However, further studies are required to establish this relationship and elucidate its significance.

Additional experiments in the present paper provide cogent evidence for a functional relationship between the hemin-sensitive protein kinase and PHFs: the activity varies in direct proportion to the abundance of PHFs in various neurological diseases and is not found in preparations from normal brain that are deficient in PHFs; Alz-50 antigens prepared according to an entirely independent protocol (i.e., ADAP) also have a similar hemin-sensitive kinase activity; the kinase remains associated with PHFs even after a second round of immunoselection with mAbs 38 and 126; phosphorylation of PHFs is unaffected by dilution, whereas the phosphorylation of added casein is affected by dilution; the activity is also observed when PHFs are isolated by affinity chromatography through a column of PHF-1 (unpublished data). Furthermore, the properties of protein kinase preclude the likelihood of contamination from classified kinases, especially those that phosphorylate τ (i.e., protein kinase C, Ca^{2+}/calmodulin kinase, and casein kinase II). In view of the resistance of phosphorylation of the ADAP preparation to treatment with CHAPS and 3.5 M guanidine hydrochloride, two detergents capable of eliminating protein–protein interactions, it is suggested that the kinase is a component of PHFs. This contention is strengthened by the observed coincidence of the kinase with PHFs after ion-exchange chromatography on Mono Q or Mono S columns and phosphocellulose chromatography (data not shown).

In light of the multiplicity of phosphorylation sites in PHFs, a general imbalance in phosphorylation/dephosphorylation homeostasis in neurodegenerative disease might be alleged. Thus, PHF may be the ultimate result of sequential addition of phosphates into normal τ. An alternative possibility is that only one phosphorylation site is sufficient to generate PHFs, while the remainder are introduced post hoc. In this regard, an unusual protein kinase associated with PHFs may be a reasonable culprit for posttranslational modification of these proteins. We have subsequently observed that prior dephosphorylation greatly enhances phosphorylation of PHFs by the endogenous kinase (unpublished data), indicating that this site is phosphorylated in PHFs *in vivo*. However, it has yet to be determined whether the action of the hemin-sensitive PHF kinase is imperative for precipitation of the tangled mass of protein that destines neurons for death.

We thank Drs. Hossein Ghanbari and Barney Miller for providing mAbs and the ADAP preparation, Dr. D. J. Dickson for neuropathological evaluation of brain samples, Dr. Y. Kress for assistance with electron microscopy, and Dr. Samuel Gandy for assistance with phosphoamino acid analysis. We also thank Dr. F.-C. Chiu, Dr. S.-H. Yen, and Tobias Kollmann for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants MH38623 and AG06803.

1. Saitoh, T., Masliah, E., Jin, L.-W., Cole, G. M., Wieloch, T. & Shapiro, I. P. (1991) *Lab. Invest.* **64**, 596–616.
2. Kidd, M. (1963) *Nature (London)* **197**, 192–194.
3. Terry, R. D., Gonatas, N. F. & Weiss, M. (1964) *J. Neuropathol. Exp. Neurol.* **44**, 269–297.
4. Braak, H., Braak, E., Grundke-Iqbal, I. & Iqbal, K. (1986) *Neurosci. Lett.* **65**, 351–355.
5. Grundke-Iqbal, I., Iqbal, K., Tung, Y.-C., Quinlan, M., Wisniewski, H. M. & Binder, L. I. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4913–4917.
6. Wood, J. G., Mirra, S., Pollock, N. J. & Binder, L. I. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4040–4043.
7. Lee, V. M.-Y., Balin, B. J., Otvos, L. & Trojanowski, J. Q. (1991) *Science* **251**, 675–678.
8. Ksiezak-Reding, H., Lieu, W.-K., Si, V. C. & Yen, S.-H. (1991) *J. Neuropathol. Exp. Neurol.* **50**, 361 (abstr. 217).
9. Kosik, K. S., Orecchio, L. D., Binder, L., Trojanowski, J. Q., Lee, V. M.-Y. & Lee, G. (1988) *Neuron* **1**, 817–825.
10. Greenberg, S. G., Davies, P., Schein, J. D. & Binder, L. I. (1992) *J. Biol. Chem.* **267**, 564–569.
11. Ishiguro, K., Omori, A., Sato, K., Tomizawa, I. & Uchida, T. (1991) *Neurosci. Lett.* **128**, 195–198.
12. Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L. & Greengard, P. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 177–181.
13. Pant, H. C., Shecket, H., Gainer, H. & Lasek, R. J. (1978) *J. Cell Biol.* **78**, R23–R25.
14. Wolozin, B. & Davies, P. (1987) *Ann. Neurol.* **22**, 522–526.
15. Hyman, B. T., Van Hoesen, G. T., Wolozin, B., Davies, P., Kromer, L. J. & Damasio, A. R. (1988) *Ann. Neurol.* **23**, 371–379.
16. Ghanbari, H. A., Miller, B. E., Haigler, H. J., Arato, M., Bissette, G., Davies, P., Nemeroff, C. B., Perry, E. K., Ravid, R., Swaab, D. F., Whetsell, W. O. & Zemlan, F. P. (1990) *J. Am. Med. Assoc.* **263**, 2907–2910.
17. Wolozin, B., Pruchnicki, A., Dickson, D. W. & Davies, P. (1986) *Science* **232**, 648–650.
18. Gross, M. (1980) *Mol. Cell. Biochem.* **31**, 25–32.
19. Trachsel, H., Ranu, R. S. & London, I. M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3654–3658.
20. Tabaton, M., Whitehouse, P. J., Perry, G., Davies, P., Autilio-Gambetti, L. & Gambetti, P. (1988) *Ann. Neurol.* **24**, 407–413.
21. Love, S., Burrola, P., Terry, R. D. & Wiley, C. A. (1989) *Neuropathol. Appl. Neurobiol.* **15**, 223–231.
22. Greenberg, S. G. & Davies, P. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5827–5831.
23. Iqbal, K., Thompson, C. H., Mertz, P. A. & Wisniewski, H. M. (1984) *Acta Neuropathol.* **62**, 167–177.
24. Spira, G., Bargellesi, A., Teillaud, J.-L. & Scharf, M. D. (1984) *J. Immunol. Methods* **74**, 307–313.
25. Ghanbari, H. A., Riesing, S., Miller, B. E. & Tribby, I. (1989) *Soc. Neurosci. Abstr.* **15**, 1042.
26. Vincent, I. & Davies, P. (1990) *Brain Res.* **531**, 127–135.
27. Ksiezak-Reding, H., Binder, L. I. & Yen, S.-H. (1990) *J. Neurosci. Res.* **25**, 420–430.
28. Nestler, E. J. & Greengard, P., eds. (1984) *Protein Phosphorylation in the Nervous System* (Wiley, New York).
29. Hathaway, G. M. & Traugh, J. A. (1982) *Curr. Topics Cell Biol.* **21**, 101–127.