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Hyperphosphorylation of Human TAU by Brain Kinase PK40^{erk} beyond Phosphorylation by cAMP-dependent PKA: Relation to Alzheimer's Disease

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Abnormal hyperphosphorylation of the cytoskeletal protein TAU is seen in the characteristic paired helical filaments [neurofibrillary tangles] of Alzheimer's disease [AD]. A recently described protein kinase, PK40^{erk}, (1) a member of the ERK family of kinases, can produce in vitro many of the properties of Alzheimer-like hyperphosphorylated TAU. cAMP-dependent protein kinase A [PKA] phosphorylates TAU to a lesser extent; however, the product is not like the hyerphosphorylated TAU of AD in several important respects. We now report that in vitro PK40^{erk} , a candidate for the enzyme responsible for TAU hyperphosphorylation in AD, will further phosphorylate TAU that was previously saturated by protein kinase A, provided that the concentrations of free uncomplexed ATP are low. Interestingly, the actions of different kinases on TAU are not independent, but may depend on the order in which they work on TAU; i.e.,prior phosphorylation by PKA partially inhibits the action of PK40^{erk}.

Frequent intracellular neurofibrillary tangles in postmortem brains are characteristic of Alzheimer's disease. Their frequency is said to correlate with the degree of dementia observed in the Alzheimer patient (2,3). Clearly, the protein nature and the origin of these tangles are of paramount importance, if we are to understand the etiology of this disease. The cytoskeletal protein TAU is a major constituent of tangles (4,5,6,7,8,9). Since then attention has focused on defining the difference between TAU as found in Alzheimer tangles [PHF-TAU]

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Abbreviations: PHF = paired helical filaments; PHF-TAU = TAU protein as isolated from PHFs; S/T-P = serine/threonine-proline motif; hTau40 = recombinant human TAU, largest isoform; AD = Alzheimer's Disease.

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and "normal" TAU. Whereas normal adult brain TAU is phosphorylated at only a few of the 17 known serine/threonine-proline sites (10), the abnormal PHF-TAU is phosphorylated at a larger number of sites. This could be due to increased kinase activities or decreased phosphatase activities, or both (8,9,11,12). Normally phosphorylated TAU interacts with the tubulin of microtubules (13), presumably stabilizing the axonal cytoskeleton. However, hyperphosphorylated TAU appears to have a lower binding affinity for tubulin, at least in vitro (10). Consequently, axonal transport and synaptic function are expected to be weakened or destroyed; self-assembly to tangles might be promoted. It is not clear how many of these in vitro properties of phosphorylated TAU are also operative in vivo. Since the abnormal hyperphosphorylation of TAU is likely to be one of the major causes of neuronal dysfunction and degeneration in Alzheimer's disease, if not the major cause, we need to understand the biochemistry of this process.

It is not known which protein kinases are responsible in vivo for the normal phosphorylation of TAU. Several serine/threonine-proline kinases [S/T-P kinases] phosphorylate recombinant and isolated TAU in vitro. For example, mitogen-activated protein (MAP) kinase introduces up to 12-14 phosphate residues (14,15), and glycogen synthase kinase-3 (16) and proline-directed protein kinase (17) up to 4 residues in each case. A porcine brain S/T-P kinase (14) can phosphorylate to add 14-16 PO_4 residues per molecule. Roder et al. reported (1) that the brain protein kinase $PK40^{erk}$, being a member of the ERK family of S/T-P kinases, incorporates approximately 14 phosphate residues per molecule of hTau40. In addition cAMP-dependent kinase PKA also phosphorylates TAU in vitro, and perhaps also in vivo (18). However, PKA is not a S/T-P specific kinase and phosphorylates at other sites (19).

$PK40^{erk}$ (1,20) was originally characterized by its ability to phosphorylate Lys-Ser-Pro (KSP) sites in neurofilament proteins and also certain sites on TAU protein. This kinase is strongly inhibited by mM concentrations of free ATP, not complexed with Mg^{++}. We now report further experiments which document in vitro the additional phosphorylation of PKA-phosphorylated TAU by $PK40^{erk}$, but only when levels of free ATP are low.

Materials and Methods

Phosphorylation reaction: The TAU phosphorylation reaction was performed in 25mM HEPES pH7, 2mM $MgCl_2$, 1mM ATP (Sigma A-2383), 1mM DTT, 5μM okadaic acid (GIBCO BRL), 3μg hTau40 in a total volume of 30μl at 37°C for 3 hrs.or 5 hrs. $PK40^{erk}$ was used at 50-80 units (picomoles/min.) per 30μl reaction, cyclic-AMP protein kinase catalytic subunit (PKA_{cat}) from bovine heart (Sigma) at 10 units and cyclic-AMP protein kinase regulatory subunit (PKA_{reg}) (Sigma) at 12 units per 30μl reaction. Reactions were stopped with SDS sample buffer, boiled for 5 min., separated on 10% SDS-PAGE, stained with Coomassie Blue or transferred for immunoblotting to a nitrocellulose membrane (0.22mM Schleicher & Schuell) in 20mM Tris-HCl, 150mM glycine, 0.03% SDS, 20% methanol.

Western blots: Nitrocellulose blots were blocked by 1 hr. incubation with 3% BSA in 10mM PBS pH7.2. Antibodies were diluted in 10mM PBS pH7.2, 0.5% TritonX-100 and 2% NGS

(normal goat serum) for primary antibodies, 5% NGS for secondary antibodies. Blots were incubated at least 2 hrs. with SMI monoclonal antibodies (Sternberger Monoclonals, Inc.): SMI-31, SMI-33 and SMI-34 [1:500]. Both SMI-31 and SMI-34 respond to phosphorylated epitopes (31). Under the circumstances used by us, SMI-31 responds to phosphorylation at $KS396_{PO_4}P$ (1); $S404_{PO_4}P$ may be necessary, but not sufficient [numbers refer to hTau40, the largest human TAU isoform]. SMI-34 recognizes two phosphorylated epitopes on either side of the sequence repeats in hTau40; one is KS396P, the other is not mapped. It has been suggested that SMI-34 reacts with a conformational epitope (31). SMI-33, on the other hand, reacts only with the non-phosphorylated epitope KS235P.

After incubation, blots were washed in 10mM PBS pH7.2, 0.5% TritonX-100. Incubation with secondary Abs was overnight: goat anti-mouse IgG, IgM and IgA peroxidase conjugate (Boehringer Mannheim cat.#60517) 1:200 and avidin horseradish peroxidase conjugate (Biorad) 1:1,500 added for detecting the biotinylated SDS-PAGE standards (Biorad). Blots were developed with 0.06% 4-chloro-1-napthol (Sigma) and 0.06% of H_2O_2 in 50mM Tris-HCl pH7.5, 40% ETOH for 20min. All incubations and washes were at room temperature.

Protein purification: $PK40^{erk}$ was prepared as described (1,20). An expression clone of human tau isoform, hTau40 was a gift from Dr. M. Goedert. The human cDNA clone for hTau40 protein was in pRK172 vector and expressed in the BL21 (DE3) E. coli system (21). hTau40 was prepared as follows: BL21 cells containing the hTau40 cDNA were grown in LB medium at 37°C to an optimal OD_{600} of 0.6-1.0, then induced with 0.4mM IPTG and harvested after 2 hrs. The cell pellet from a 500ml culture was lysed in 15ml of 50mM PIPES pH6.8 containing 1mM DTT, 1mM EDTA, 0.2mM PMSF and 0.5mg/ml lysozyme. Cells resuspended in buffer were sonicated 3 x 1 min. at full speed. The supernatant was chromatographed on a phosphocellulose column (3 x 1mm) equilibrated with 50mM PIPES, pH6.8 containing 1mM DTT, 1mM EDTA, 0.2mM PMSF. Fractions containing TAU protein were pooled, dialyzed against 50mM MES pH6.25, 1mM DTT, 1mM EDTA, 0.2mM PMSF, and then concentrated to 1ml in a Centricon 30 tube. After chromatography on a MonoS column in a Pharmacia FPLC system using the same buffer as for dialysis and eluting with a 0-500mM NaCl gradient, 1ml fractions were analysed on 10% SDS-PAGE. Peak hTau40 fractions were assayed for protein concentration using the Pierce dye binding assay kit.

Results

Phosphorylation of hTau40 by $PK40^{erk}$ in the presence of excess [free] ATP. Concentrations of $PK40^{erk}$ and PKA_{cat} which give the maximum slowing of electrophoretic mobility of hTau40, are used in the standard reaction at 1mM ATP/2mM $MgCl_2$ for 3 or 5 hours as described in Methods. For example, phosphorylation of human recombinant hTau40 with $PK40^{erk}$ in 1mM ATP shows a large electrophoretic mobility shift after 3 hrs. at 37°C (Fig.1A). The addition of excess [free] ATP to a final concentration of 6mM at zero time inhibits the mobility shift, as expected (1). Adding additional [free] ATP to a total of 6mM (at 2mM $MgCl_2$) at 20, 40 and 60 min. during a 3 hr. phosphorylation reaction decreases phosphorylation of

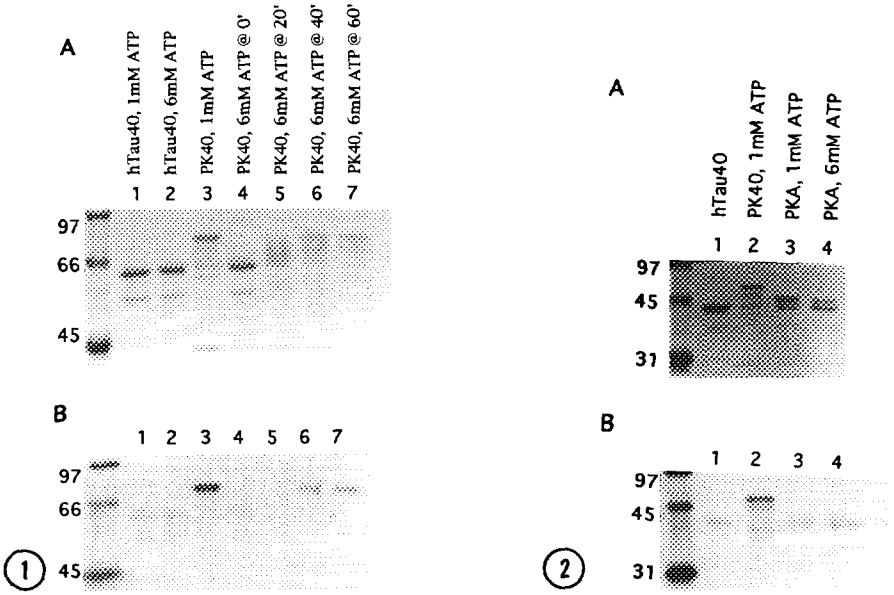


FIG.1. Effect of excess [free] ATP on hyperphosphorylation of hTau40 by $PK40^{erk}$. 10% SDS-PAGE of 3μg hTau40 phosphorylated in 2mM $MgCl_2$, as described in Methods, with 80 units $PK40^{erk}$ for 3 hrs at 37°C. Lane 1: hTau40 incubated alone in 1mM ATP; lane 2: hTau40 incubated alone in 6mM ATP; lane 3: hTau40+$PK40^{erk}$ in 1mM ATP; lanes 4, 5, 6, 7: hTau40+$PK40^{erk}$ in 1mM ATP with the addition of 5mM ATP at time 0, 20, 40, 60 min , respectively.

(A) Coomassie blue stain; (B) Western blot with mAb SMI-31.

FIG.2.Phosphorylation of hTau40 by PKA_{cat} 10% SDS-PAGE of 3μg hTau40 phosphorylated as described in Methods at 37°C for 3 hrs. Lane 1: hTau40, no kinase; lane 2: $PK40^{erk}$, in 1mM ATP; lane 3: PKA_{cat} in 1mM ATP; lane 4: PKA_{cat} in 6mM ATP.

(A) Coomassie blue stain; (B) Western blot with SMI-34.

hTau40 and shows bands with intermediate levels of phosphorylation (Fig.1A, lanes 5, 6, 7). The mAb SMI-31 is specific for the phosphorylated KSP epitopes in neurofilament proteins and also for phosphorylated KS396P in hTau40 (1). A Western blot with the mAb SMI-31 gives a strong positive reaction only with the fully phosphorylated hTau40 when reaction was with $PK40^{erk}$ in 1mM ATP (Fig.1B, lane 3). This antibody gives only a weak reaction with hTau40 phosphorylated by $PK40^{erk}$ for 40 or 60 min in 1mM ATP, followed by further incubation in 6 mM ATP for a total of 3 hours. Addition of ATP to 6mM at zero time and at 20 minutes (Fig.1B, lanes 4,5) completely abolishes the immunoreactivity of hTau40 towards SMI-31. The intermediate bands (intermediate phosphorylation products) seen with Coomassie blue staining show no reactivity to SMI-31. These observations indicate that the phosphorylation of KS396P in hTau40 may be a late step in the hyperphosphorylation of TAU.

Phosphorylation of hTau40 by PKA_{cat} (catalytic subunit of cyclic-AMP dependent protein kinase A).

We find that hTau40 is phosphorylated by PKA_{cat} under standard conditions (as described in Methods) and shows reduced electrophoretic mobility on SDS-PAGE compared with the unphosphorylated protein(Figs. 2,3). The electrophoretic retardation is, however, much smaller than with $PK40^{erk}$ (Fig.2A), indicating a lower degree of phosphorylation. Tme course experiments indicate that phosphorylation by PKA_{cat} goes to completion during these 3 hr. reactions. At saturation levels of $PK40^{erk}$ and PKA_{cat}, large and complete mobility shifts of hTau40 were seen at 60 min. for PK40erk and smaller, but complete mobility shifts were seen with PKA_{cat} at 40 min. (data not shown).

The decreased mobility shift of recombinant TAU after phosphorylation by PKA has also been reported by Scott et al.(22) and for bovine TAU by Litersky and Johnson (23). This mobility shift is inhibited, but only partially, by the addition at zero time of ATP to 6mM (Fig.2A, lane 4). Immunoblotting with SMI-34, an SP-phosphorylation dependent mAb, shows positive reactivity with hTau40 phosphorylated by $PK40^{erk}$ at low ATP concentration and no reactivity with TAU phosphorylated by PKA_{cat} at low or high ATP concentrations (Fig.2B).

Phosphorylation of hTau40 by PKA_{cat}, followed by hyperphosphorylation with $PK40^{erk}$. Phosphorylation of hTau40 with PKA_{cat} for 2 hrs. at 37°C shows the expected mobility shift on SDS-PAGE (Fig.3A, lane 3), which is less than with $PK40^{erk}$ (Fig.3A, lane 6). Adding 20% excess PKA_{reg} to effectively stop PKA_{cat}, then adding $PK40^{erk}$ and continuing incubation for another 3 hours decreases electrophoretic mobility of hTau40 still further (Fig.3A, lane 4), but not quite to the expected extent. The additional phosphorylation which is clearly responsible for the decrease in electrophoretic mobility is inhibited by the addition of ATP to a total of 6mM (Fig.3A, lane 5), as expected. Immunoblotting with SMI-33, a mAb specific for the non-phosphorylated KS235P epitope, shows strong reactivity towards hTau40 even after phosphorylation with PKA_{cat} alone, and only very weak reactivity after phosphorylation byPKA, followed by $PK40^{erk}$ at 6mM ATP (Fig.3B lanes 1,2,3,and 5). The control reaction of $PK40^{erk}$ alone at 1mM ATP shows no reaction with SMI-33 (Fig.3B lane 6) and a strong positive reaction with SMI-34 (Fig.3C lane 6). On the other hand, prior phosphorylation with PKA clearly produces a phosphorylated hTau40 protein with altered substrate properties: $PK40^{erk}$ at low ATP (1mM) can still reduce the electrophoretic mobility of hTau40, but the SMI-34 epitope is hardly produced any more. Evidently, prior phosphorylation with PKA_{cat} interferes with further phosphorylation by $PK40^{erk}$, so that some S/T-P sites (among them KS396P) are only weakly phosphorylated and the maximum reduction in mobility seen with $PK40^{erk}$ alone is not produced. KS235P, however, seems to be accessible to $PK40^{erk}$. It remains to be seen in future work where the $PK40^{erk}$ phosphorylation sites are when native hTau40 is the substrate or when PKA-phosphorylated hTau40 is used.

Conclusions

SMI-31 and SMI-34 are phosphorylation-dependent monoclonal antibodies for serine/proline residues at positions KS396P (1) and other related sites in hTau40 (24,25). These

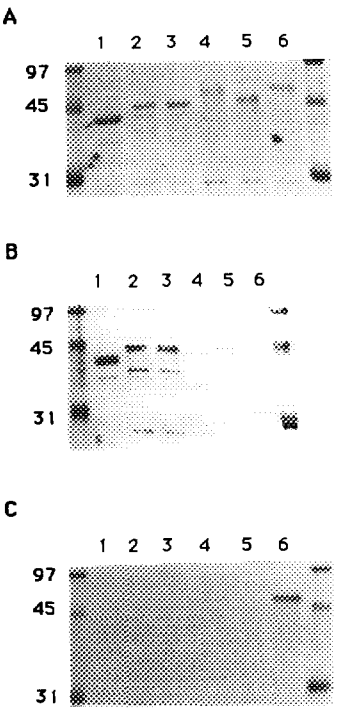
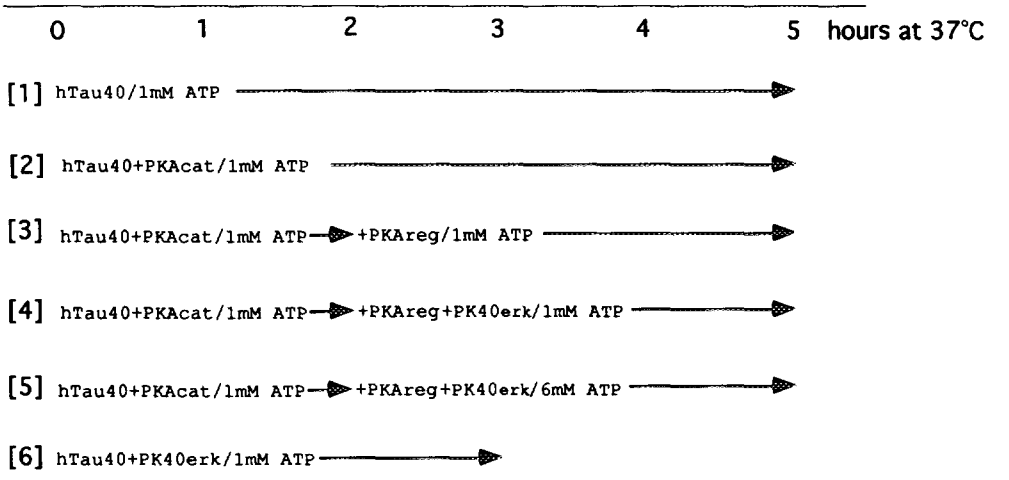


FIG.3. Phosphorylation of hTau40 by PKA_{cat}, followed by PKA_{reg}, followed by $PK40^{erk}$. hTau40 (3mg) was subjected to phosphorylation schedules as shown in the diagram; reactions [1] - [6] were analyzed by 10% SDS-PAGE in lanes 1-6. For PKA_{cat} - 10 units of enzyme were used, for PKA_{reg} - 12 units, for $PK40^{erk}$ - 50 units (20).



(A) Coomassie blue stain; (B) Western blot with mAb SMI-33; (C) Western blot with mAb SMI-34.

sites are apparently phosphorylated by $PK40^{erk}$, in keeping with our previous findings (1). The formation of these SMI phosphoepitopes by $PK40^{erk}$ is inhibited by excess [free] ATP not complexed by Mg^{++}.

Several studies have shown that TAU protein is a substrate for PKA (26,27,28,29,30). Scott et al.(22) have identified five phosphorylation sites on recombinant human TAU by PKA. However, none of these serine residue sites are followed by proline residues and are therefore not phosphoepitopes specific to the SMI series of monoclonal antibodies. Neither should they be sites for $PK40^{erk}$ and other MAP-kinases, which are all SP or TP specific.

$PK40^{erk}$ phosphorylates one or more phosphoepitopes of TAU specific to SMI-34, as well as to SMI-31. Human TAU has two KSP sites at KS235P and KS396P. It would seem that $PK40^{erk}$ phosphorylates the KSP site at KS396P,since mAbs SMI-34 and SMI-31 stains this phosphoepitope strongly when hTau40 was phosphorylated with $PK40^{erk}$ alone in 1mM ATP. The KS235P site is only identified by the monoclonal antibody SMI-33, specific for non-phosphorylated epitopes.(25,31) Since saturation phosphorylation by $PK40^{erk}$ (maximum mobility shift) gives no reaction with SMI-33, it seems that $PK40^{erk}$ phosphorylates this KS235P site as well.

Addition of $PK40^{erk}$ to recombinant hTau40 pre-phosphorylated by PKA_{cat} leads to the phosphorylation of additional sites. There is a further reduction in electrophoretic mobility, but not to the maximum extent as seen with $PK40^{erk}$ alone. The formation of SMI-31 and SMI-34 phosphoepitopes, a late event in the TAU phosphorylation by ERKs (1,31) and an abnormal property of AD-TAU, is strongly impaired after pretreatment with PKA_{cat}. Clearly the actions of different kinases are not independent; the action of one, e.g. PKA, can alter the substrate properties of TAU towards another kinase, $PK40^{erk}$, in this case.

$PK40^{erk}$ is known to be strongly inhibited by free ATP (20) in initial rate assays as well as in its ability to reconstitute the neurofilament phosphoepitopes of SMI-31 and SMI-34. The present study shows that this inhibition indeed prevents alterations of TAU associated with ERK hyperphosphorylation, such as gel mobility reduction and the AD-like phosphorylation of KS396P. The observation of a similar but less pronounced effect of free ATP on TAU phosphorylation by PKA_{cat} correlates with a relatively weak inhibition of initial rate PKA activity by free ATP (H. Roder, unpublished). The inhibitory effect of ATP on TAU phosphorylation, shown in our experiments, may be related to the regulation of $PK40^{erk}$ by ATP concentration in the aged or degenerating brain. This novel type of regulation, particularly effective for $PK40^{erk}$, may also be operative for other kinases.

Such a mechanism would invoke a decline in ATP production in degenerative conditions, such as reported for AD (32). Constitutive $PK40^{erk}$ activity could be released from a "normal" inhibition by excess free ATP in younger neurons. This late-onset process may play a role in the etiology of AD and may explain age as a risk factor.

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