(BPDK), which was purified from bovine brain extract as a neurofilament kinase. These enzymes, TPK II and nclk, are considered to be identical.

Phosphorylation of tau with TPKI and TPKII

The phosphorylation sites in tau protein by TPKI and TPKII were first examined chemically. Protease digests of tau maximally phosphorylated by the kinase in the presence of [γ-^{32}P]ATP, were separated by HPLC using a C4 column. In the case of TPKI, tau which had first been fully phosphorylated with TPKII and cold ATP was used. The sequences of phosphopeptides were determined using an improved protocol in a gas-phase sequencer (*24*), and the phosphorylation sites of tau by TPKII were identified as Ser202, Thr205, Ser235, and Ser404 (*25*), while those by TPKI were Ser199, Thr231, Ser396, and Ser413 (numbering of amino acids is based on the longest human tau) (*26*) (Table I and Fig. 1). We omitted ambiguous sites which exhibited very low levels of phosphorylation or which were localized in the extreme C-terminal region of long peptide fragments.

Phosphorylation sites by TPKII have a common sequence of SerPro or ThrPro, indicating that TPKII is a proline-directed kinase, though not all Ser/ThrPro sequences in tau protein, were phosphorylated. Although TPKI/GSK3β is also regarded as a member of the class of proline-directed kinases, one of the 4 TPKI sites, Ser413, is non-proline-directed site.

As a next step, we aimed to prepare antibodies specific for these phosphorylation sites of TPKI and TPKII by using chemically synthesized phosphopeptides as antigens, and to confirm that TPKI and TPKII act at the phosphorylation sites determined chemically (*12, 27*). Antigen-dodecapeptides containing phosphoserine or phosphothreonine in the center were synthesized by the use of a phenyl group (*28*) or a cyclohexyl group (in the case of aromatic or sulfur-containing amino acids) (*29*) as a protecting group for the phosphate group. In addition, antibodies raised against peptides phosphorylated at Ser262 or Ser422 were prepared in the method described above. Each phosphopeptide was named based on the species and the number of the phosphorylated amino acid, *e.g.*, PS199 or PT205. The obtained antibodies against the phosphorylated 4 TPKI sites, 4 TPKII sites, Ser262 and Ser422 were specific for the corresponding phosphopeptide, and did not cross-react with other phosphopeptides. Human recombinant tau (htau40) phosphorylated by TPKII, reacted with all antibodies specific for the 4 TPKII sites, anti-PS202, anti-

TABLE I. **Phosphorylation sites of tau *in vivo* (A) and those generated by various kinases reported so far as candidates to act in PHF formation (B).**

A	TP 181	S 198	SP 199	SP 202	TP[d] 205	S 208	S 210	TP 212	S 214	TP 217	TP 231	SP 235	S 262	S 324	S 356	SP 396	S 400	T 403	SP 404	S 409	S 412	S 413	S 416	SP 422
PHF-tau[a]		◎	◎	◎		○	○	○	○	○	◎	◎	○			◎	○	○	◎	◎	○	◎		◎
Fetal tau[a]	○	○	◎	◎						○	◎	◎				◎	○		◎	○		○		
Adult tau[b]	○		○	◎							○								◎					
B																								
TPK I/GSK3 β			*								*					*						*		
GSK3α											*	*							*					
TPK II/cdk5+p23				*	*							*							*					
cdc2				*	*						*	*												
MAPK	*		*	*			*					*				*			*					*
MARK[c]													*		*									
PKA								*					*	*	*					*			*	
PKC														*										
CMPK II																							*	

[a]Cited from Ref. *33*. [b]Cited from Ref. *34*. [c]Cited from Ref. *38*. [d]Ser205 was reported as a PHF-site by others (*35, 36*).

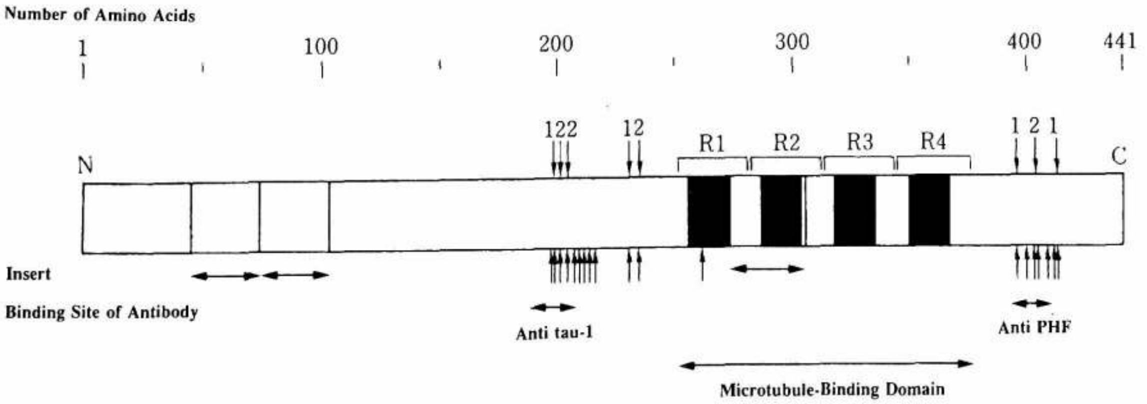

Fig. 1. **Phosphorylation sites on tau by TPKI and II.** The primary structure of the longest human tau is presented here. ↓: Phosphorylation sites by TPKI and TPKII are indicated by 1 and 2, respectively. ↑: Phosphorylation sites in PHF-tau (*33*). Rn: 31 or 32 amino acid repeat region. ■: 18 amino acid microtubule-binding element.

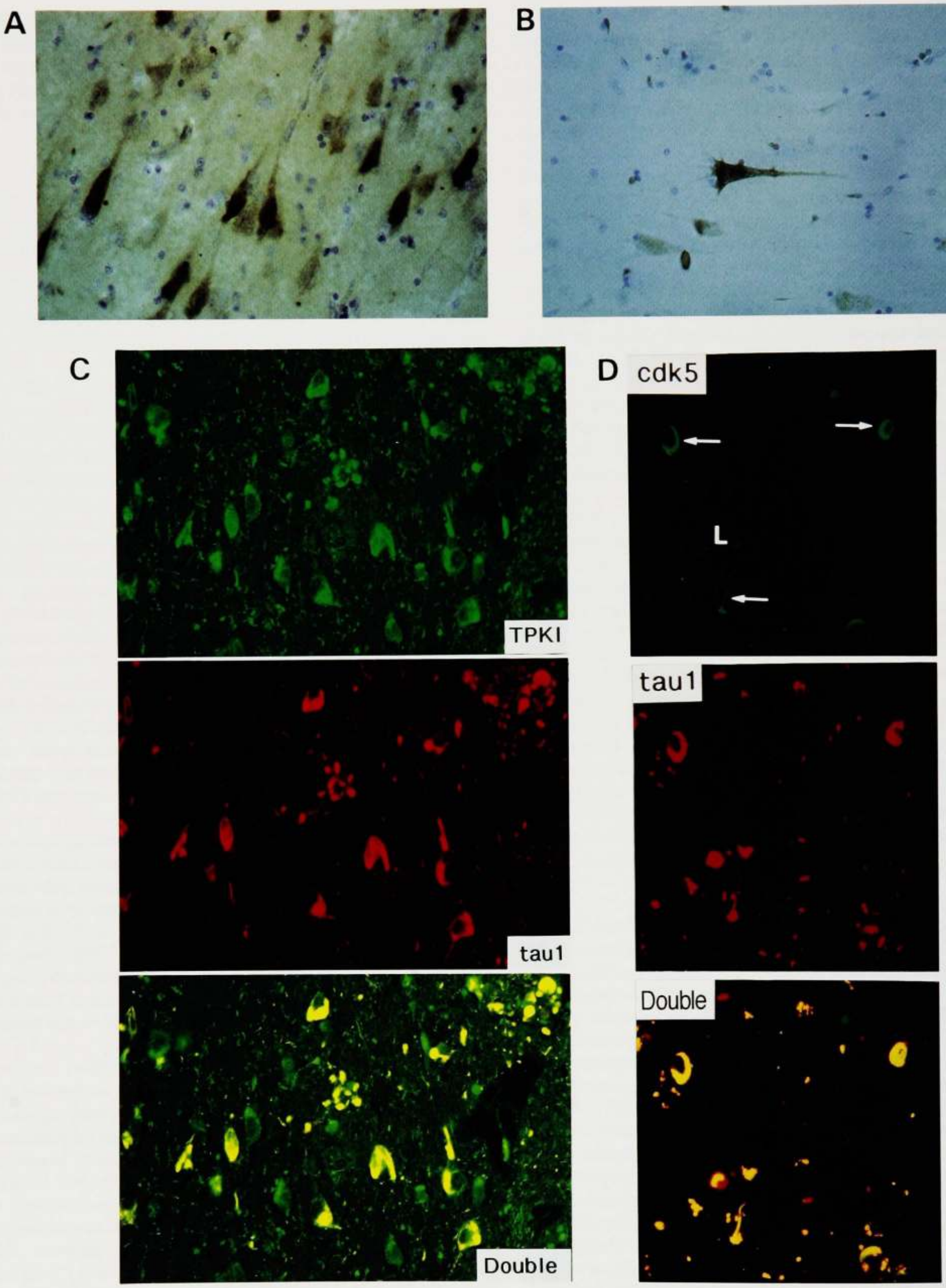

Fig. 2. **Immunocytochemistry of anti-TPKs in the human hippocampal CA1 subfield.** (A) AD brain stained with anti-TPKI antibody. (B) Control brain with slight AD-like changes stained with anti-TPKI antibody. In pyramidal cells, the cell body, apical dendrite, basal neurite and proximal axon are stained. (C) Staining of Down's syndrome brain with anti-TPKI antibody (top), tau-1 (middle), and double exposure (bottom). (D) Staining of Down's syndrome brain with anti- CDK5 antibody (top), tau-1 and double exposure (bottom). Tau-1 stained dephosphorylated NFT. Overlapping of blue and red gave yellow.