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Mechanism of Inhibition of PP2A Activity and Abnormal Hyperphosphorylation of Tau by I_2^{PP2A}/SET

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Abstract

Protein phosphatase-2A (PP2A) activity, which is compromised in Alzheimer disease brain, is regulated by two endogenous inhibitors, one of them being I_2^{PP2A}, a 277 amino acid long protein also known as SET. Here we report that both the amino terminal fragment (I_{2NTF}; aa 1–175) and the carboxy terminal fragment (I_{2CTF}; aa 176–277) of I_2^{PP2A} inhibit PP2A by binding to its catalytic subunit PP2Ac and cause hyperphosphorylation of tau. The C-terminal acidic region in I_{2CTF} and Val 92 in I_{2NTF} are essential for their association with PP2Ac and inhibition of the phosphatase activity.

1. Introduction

Abnormal hyperphosphorylation and aggregation of microtubule associated protein tau into paired helical filaments/neurofibrillary tangles is a hallmark of Alzheimer disease (AD) and related neurodegenerative disorders, called tauopathies [1–3]. The hyperphosphorylated tau sequesters normal tau, MAP1 and MAP2, which results in the breakdown of the microtubule network and, probably in a progressive retrograde degeneration of the affected neurons [4].

Protein phosphatase 2A (PP2A) is a heterotrimeric enzyme, consisting of one catalytic C subunit, one scaffolding A subunit, and one of several structurally distinct, regulatory B subunits [5]. The phosphorylation of tau that suppresses its microtubule binding and assembly activities in adult mammalian brain is primarily regulated by PP2A [6]. PP2A regulates tau phosphorylation, both directly as tau phosphatase and indirectly by regulating the activities of several tau kinases which include CaM Kinase II, PKA, MAP kinase kinase (MEK 1/2), extracellular regulated kinase (ERK 1/2), GSK-3β, and P70S6 kinase [7]. PP2A activity in AD brain is decreased [8].

The activity of PP2A is regulated by an inhibitor protein, I_2^{PP2A} [9–11]; This protein, also known as SETα, TAF-1β, and PHAPII protein [12–14] was discovered as a translocated

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Structured summary of protein interactions
I2PP2A physically interacts with PP2A-C by anti tag coimmunoprecipitation (View Interaction 1, 2)
I2PP2A physically interacts with PP2A-C by pull down (View Interaction 1, 2)
PP2A-A physically interacts with PP2A-B and PP2A-C by pull down (View interaction)

gene fused to the CAN gene in a patient with acute undifferentiated leukemia [14]. I_2^{PP2A} is a 277 amino acid long polypeptide with an apparent molecular weight of 39 kDa in SDS-PAGE. This protein is widely expressed in various tissues and localizes primarily in the nucleus [12,15,16] where it has been reported to block DNase activity and acetylation of histones [17]. Previously, we showed that I_2^{PP2A} is selectively cleaved at N175 into I_{2NTF} (N-terminal fragment) and I_{2CTF} (C-terminal fragment) and translocated from the neuronal nucleus to the cytoplasm, co-localized with neurofibrillary tangles in the affected areas of AD brain [18]. However, the exact molecular mechanism of the inhibition of PP2A activity and generation of abnormal hyperphosphorylation of tau by I_2^{PP2A} in the cell was not understood. The present study shows that I_2^{PP2A} inhibits PP2A activity dose-dependently and cleavage of this inhibitor into I_{2NTF} and I_{2CTF} not only results in its translocation from the cell nucleus to the cytoplasm but also potentiates its inhibitory activity, for each of these two fragments can interact with the PP2A catalytic subunit PP2Ac. I_{2CTF} interacts with PP2Ac through its highly acidic C-terminal domain. Interaction of I_{2NTF} with PP2Ac and its ability to inhibit the phosphatase activity is completely inhibited when valine 92 in the inhibitor is mutated to alanine.

2. Materials and Methods

For generation of I_2^{PP2A} and its deletion mutant plasmids, GST pull-down assays, coimmunoprecipitation, PP2A activity assays, and immunocytochemical staining, see Supplementary Data.

3. Results

3.1 I_2^{PP2A} interaction with PP2Ac subunit

I_2^{PP2A} was previously shown to inhibit PP2A activity in vitro [9,11]. To date, it is not clear whether I_2^{PP2A} can decrease PP2A activity in cells and whether this reduction involves inhibition of the PP2A expression level. To address to this question, we carried out transient transfection of NIH3T3 cells with different amounts of pCMV2B-I_2^{PP2A}. After 48 hours transfection the cell lysates were collected and one half of the sample was employed for Western blots (Fig. 1A, B), whereas the other half was subjected to immunoprecipitation with anti-PP2Ac, followed by PP2A activity assay (Fig. 1C). The FLAG-tagged I_2^{PP2A} expression in transfected cells increased with the amount of DNA used for transfection (Fig. 1A). The expression of PP2Ac, however, was not altered (Fig. 1B), suggesting that the decrease in PP2A activity in transfected cells (Fig. 1C) was due to PP2A inhibition and not to a reduction of the PP2A expression level.

I_2^{PP2A} was previously shown to inhibit PP2A1 (the ABC complex), PP2A2 (the AC dimer), and PP2Ac (the C subunit) in a non-competitive manner [9,19]. However, no direct evidence was reported to confirm this association. To study the interaction between PP2A and I_2^{PP2A}, in vitro pull-down assays were carried out using bacterially expressed GST alone (negative control), GST-PP2A-A (positive control), or GST-I_2^{PP2A}. Rat brain extract as the source of PP2A holoenzyme was added to purified GST-PP2A-A, GST-I_2^{PP2A}, or GST alone, on glutathione Sepharose 4B beads and the interactions were detected by Western blots developed with antibodies to PP2A subunits A, B, or C. We found that I_2^{PP2A} could pull down PP2Ac but not PP2A-A or PP2A-B subunits (Fig. 1D). These findings suggest that I_2^{PP2A} interacts with the PP2A catalytic subunit rather than the PP2A regulatory subunits PP2A-A or PP2A-B.

I_{2FL}, I_{2NTF} and I_{2CTF} localize differently in HEK293FT cells

Previously we reported that in AD brain I_2^{PP2A} is selectively cleaved at N175 and is translocated from the neuronal nucleus to the cytoplasm [18]. To test whether the N-terminal fragment (I_{2NTF}, aa1–175) or C-terminal fragment (I_{2CTF}, aa176–277) or both can translocate from the cell nucleus to the cytoplasm, we generated three constructs corresponding to I_2^{PP2A} full length (I_{2FL}), I_{2NTF}, and I_{2CTF}, each tagged with myc, and inserted them in pcDNA3.1 vector (Fig. 2A) for expression in eukaryotic cells. To confirm the integrity of the constructs, the plasmids were multiplied in dH5α E. coli, purified and digested by BamHI and Xhol restriction enzymes. The resulting DNA fragments were separated and extracted from 1% agarose gel (Fig. 2B). The accuracy of the DNA fragments was attested with subsequent sequencing. Finally, the plasmids were transfected either in HEK293FT cells or NIH3T3 cells and by Western blot analysis we confirmed the expression of myc-tagged I_{2FL}, I_{2NTF}, and I_{2CTF}, at 39 kDA, 22 kDa, and 20 kDa apparent molecular weight, respectively (Fig. 2C).

To assess the intracellular localization, we co-transfected HEK293FT cells with pcDNA3.1-I_{2FL} and its fragments with an exclusively cytoplasmic GFP fused tau protein. Then, 72 hours post-transfection, the HEK293FT cells were fixed with 4% paraformaldehyde solution and analyzed by immunocytochemistry. The antibody to myc recognized the exogenously expressed I_2^{PP2A}. While as expected I_{2FL} was found within the nucleus, I_{2NTF} and I_{2CTF} were found diffused both in the nucleus and the cytoplasm (Fig 2D).

3.2 PP2Ac co-immunoprecipitates with I_{2FL}, I_{2NTF}, and I_{2CTF}

Employing GST-pull down assays we showed above (Fig. 1D) that in vitro I_{2FL} could interact with PP2Ac but not with PP2A-A or PP2A-B. To confirm this interaction in cells, HEK293FT cells were transfected with myc-tagged I_{2FL}, I_{2NTF}, or I_{2CTF} for 72 hours, followed by immunoprecipitation with anti-myc from these cell lysates and analysis by Western blots. PP2Ac co-immunoprecipitated with I_{2FL} as well as I_{2NTF} and I_{2CTF} (Figs. 3A, B). These findings suggested that cleavage of I_2^{PP2A} into I_{2NTF} and I_{2CTF} could result in an increased inhibition of PP2A activity.

3.3 Inhibition of PP2A activity and generation of hyperphosphorylated tau by I_{2FL}, I_{2NTF}, and I_{2CTF}

Next we proceeded to assess the effect of interactions of I_{2FL}, I_{2NTF} and I_{2CTF} with PP2A on the phosphatase activity and on phosphorylation of tau. A previous study had reported that I_2^{PP2A} inhibits PP2A through its amino terminal region [12]. We, therefore, first examined whether I_{2NTF} could inhibit PP2A activity. We transfected HEK293FT cells with pcDNA3.1-I_{2FL}, -I_{2NTF}, or vector alone for 72 hours, and then assayed okadaic acid sensitive PP2A activity towards pSer199 tau [20] in the cell lysates. We found that PP2A activity was reduced to ~70% and ~50% in I_{2FL} and I_{2NTF} transfected cells, respectively (Fig. 4A). In order to study the effect of this PP2A inhibition on abnormal hyperphosphorylation of tau, we expressed in tau_{441}-stably transfected PC12 cells [21] I_{2FL} or I_{2NTF} for 72 hours. We then examined the phosphorylation of tau at multiple known pathological sites by Western blots of the cell lysates developed with various phospho-specific antibodies. We found both in I_{2FL} and in I_{2NTF} transfected cells abnormal hyperphosphorylation of tau at M4 (Thr231/Ser235), pS404 (Ser404), PHF-1 (Ser396/404), and 12E8 (Ser262/356) sites. The tau hyperphosphorylation at M4, PHF-1, and 12E8 sites, which are believed to be major sites in Alzheimer-type neurofibrillary degeneration, was quite robust in cells transfected with I_{2NTF} (Fig. 4B,C).

Since both in the GST-pull down assays as well as the co-immunoprecipitation assays we had found that not only I_{2NTF} but also I_{2CTF} interacted with PP2Ac (see above), we studied

the effect of I_{2CTF} in comparison to I_{2FL} on PP2A activity and hyperphosphorylation of tau in cells. In lysates of NIH3T3 cells which were transfected with I_{2FL}, I_{2CTF}, or vector alone, we found a ~31% and ~53% reduction in PP2A activity, respectively (Fig. 5A). Tau_{441} stably transfected PC12 cells transfected with pcDNA3.1-I_{2FL} or -I_{2CTF} showed a marked increase in abnormal hyperphosphorylation of tau at M4 (Thr231/Ser235), pS404 (Ser404), pS262 (Ser262), and 12E8 (Ser262/346) sites (Fig. 5B,C).

3.4 Domains of I_2^{PP2A} involved in its binding to PP2A catalytic subunit

Finally, based on the structural features of I_2^{PP2A} (TAF-Iβ) characterized by a subtype specific N-terminal region, a coiled-coil region involved in dimerization of TAF-I, a putative nuclear localization signal, and a long stretch of acidic amino acids at C-terminal ends [12,22], we attempted to define the PP2Ac binding domain in I_2^{PP2A}. For this purpose, based on the structure, a series of the deletion mutants of I_2^{PP2A}, I_{2NTF}, I_{2CTF} and F4 corresponding to the C-terminal acidic region were generated (Fig. 6A). During these studies Val 92 in I_{2NTF} was accidently mutated to Ala. While we corrected this mutation by mutagenesis, we also decided to study the effect of this point mutation on interaction of I_{2NTF} with PP2A. *In vitro* pull-down assays were carried out with bacterially expressed GST fusion I_2^{PP2A} full length protein or its mutants. Rat brain extract was added to purified GST fusion proteins or GST alone on glutathione Sepharose 4B beads and PP2A catalytic subunit that was pulled down by these beads was detected by Western blots. The results showed that I_2^{PP2A} and its mutants containing C-terminal acidic region (I_2^{PP2A}F4 and I_{2CTF}) had the ability of binding with PP2Ac, whereas, the mutation of valine 92 to alanine in the leucine rich region of I_{2NTF} lost its PP2Ac binding ability (Fig. 6B and C). Thus, the minimal region of I_{2CTF} required for the binding of PP2Ac is localized at the C-terminal acidic region (amino acid 225–277), and Val 92 in I_{2NTF} is not only necessary for the binding but also for its inhibitory activity (Fig 6D).

4. Discussion

I_2^{PP2A}/SET is a multifunctional protein. It is believed to have a nuclear localization signal between amino acid residues 168–181 and is imported in the cell nucleus mainly by importin alpha-3 [23]. In its primary location in the cell nucleus it protects cells both by inhibiting DNAse [24] and acetylation of histones [17]. Translocation of I_2^{PP2A} from the nucleus to the cytoplasm has been reported to be cytotoxic and exacerbates DNA damage [23]. Generation of intracytoplasmic juxtamembrane domains of APP and APLP2 by caspase has also been shown to lead to the translocation of I_2^{PP2A} from the nucleus to the cytoplasm and cell death [25]. In vitro studies had shown that I_2^{PP2A} was also a potent inhibitor of PP2A [19] and that this inhibitory activity was localized in its amino terminal region [26]. Previously, we discovered that in AD brain I_{2FL} is selectively cleaved at N175 into I_{2NTF} and I_{2CTF}, and translocated from the neuronal nucleus to the cytoplasm where it colocalizes with abnormally hyperphosphorylated tau/neurofibrillary tangles [18]. A subsequent study showed that the cleavage of I_2^{PP2A} at N175 can be catalyzed by a lysosomal/endosomal enzyme, asparaginyl endopeptidase (also known as legumain) which occurs during cerebral acidosis caused by ischemic stroke or seizures [27]. However, neither the exact molecular mechanism by which I_2^{PP2A} inhibits PP2A activity nor how its cleavage into I_{2NTF} and I_{2CTF} contributes to abnormal hyperphosphorylation of tau were understood. The present study for the first time shows that I_2^{PP2A} decreases PP2A activity in the cell by directly interacting with and inhibiting, and not by affecting the expression of PP2Ac. Furthermore, while I_{2FL} localizes in the cell nucleus, both I_{2NTF} and I_{2CTF} are diffused into the cytoplasm where they interact with PP2A through PP2Ac and inhibit the phosphatase activity. I_{2NTF} and I_{2CTF} appear to be quite robust in inhibiting PP2A activity and causing abnormal hyperphosphorylation of tau. I_{2CTF} interacts with PP2Ac through its carboxy

terminal acidic region. In I_{2NTF} valine 92 is required for its interaction with PP2Ac and inhibition of the phosphatase activity. The inhibition of PP2A activity by I_{2FL}, I_{NTF}, and I_{2CTF} was observed in all three cell lines, i.e. NIH3T3, HEK293FT, and PC12 cells, studied.

Because of the small sizes of I_{2NTF} and I_{2CTF} as compared with I_{2FL}, these proteins transferred to the PVDF membrane at quite variable rates and, therefore, it was not possible to normalize the inhibition of PP2A activity or hyperphosphorylation of tau from the intensity of the protein bands seen in Western blots. However, hyperphosphorylation of tau at various sites observed in I_{2NTF} or I_{2CTF} as compared with I_{2FL}-transfected cells indicated that the two fragments of I_2^{PP2A} probably inhibited PP2A activity towards phosphotau in a substrate-specific manner. Relative to I_{2FL}, the expression of I_{2NTF} caused a high level of hyperphosphorylation of tau at Thr231/Ser235 (M4 site), Ser262/356 (12E8 site), and Ser396/404 (PHF-1 site) but not at Ser404, and I_{2CTF} at Thr231/Ser235 (M4 site). Previously, in an in vivo study in rat, we showed that I_{2CTF} caused hyperphosphorylation of tau at Ser396 [28].

In AD brain the activity of PP2A is compromised [8] and tau is abnormally hyperphosphorylated [1,3]. The AD abnormally hyperphosphorylated tau probably disrupts the microtubule network and leads to neurodegeneration by sequestration of normal tau as well as microtubule associated proteins MAP1 and MAP2 [4]. In the present study, the PP2A inhibition by I_2^{PP2A}, especially I_{2NTF} and I_{2CTF}, resulted in abnormal hyperphosphorylation of tau at Thr231/Ser235, Ser262/356, and Ser396/404 in cultured cells. These phosphorylation sites are known to inhibit binding of tau to microtubules [7] and cause neurodegeneration, and cognitive impairment [29]. Since the inhibition of PP2A by the carboxy terminal region of I_2^{PP2A} was not previously known, based on the present study, we expressed I_{2CTF} in the brain and found inhibition of PP2A activity, neurodegeneration associated with abnormal hyperphosphorylation of tau, and intraneuronal accumulation of Aβ and impairment in spatial learning and memory in rats [28]. These in vivo findings on the PP2A inhibitory activity of I_{2CTF} are in agreement with the present study.

PP2A, which accounts for ~70% of the total phosphoseryl/phosphothreonyl protein phosphatase activity in human brain, is the major tau phosphatase [30]. PP2A regulates phosphorylation of tau both directly and by regulating the activities of several tau protein kinases which include PKA, CaMKII, MEK1/2, ERK1/2, and p70S6 kinase [7]. I_2^{PP2A} binds the neuronal cdk5 activator p35 and enhances the ckd5/p35 activity [31]. Knockdown and overexpression of I_2^{PP2A} in the brain have been found to decrease and increase GSK-3β activity, respectively [28,32]. Thus, a dysregulation of I_2^{PP2A} can have a profound effect on affected neurons, both through activation of several protein kinases and abnormal hyperphosphorylation of tau.

Besides I_2^{PP2A}, PP2A activity is also regulated by methylation and phosphotyrosinylation of PP2Ac and by I_1^{PP2A}, also known as PHAPI (putative histocompatibility leukocyte antigen class II associated protein-1), mapmodulin, pp32, and LANP [9]. Both mRNA and protein expressions of I_1^{PP2A} are elevated and the inhibitor is co-localized with neurofibrillary tangles in AD brain [18]. Demethylation of PP2Ac, which decreases its activity, was reported in AD brain [33,34]. Phosphorylation of PP2Ac at tyrosine 307, which inhibits its activity, was found to be elevated in AD brain [35]. Microarray analyses showed an upregulation of the expression of SET genes in AD hippocampus [36]. While the mRNA and protein expressions of I_2^{PP2A} are elevated in AD brain, reported by us previously [18], the dual action of I_{2NTF} and I_{2CTF} in inhibiting PP2A activity shown in the present study shows how the cleavage and translocation of this inhibitor could work as the last straw which broke the camel's back in producing neurofibrillary degeneration in AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AD	Alzheimer disease
PP2A	protein phosphatase 2A
MEK1/2	MAP kinase kinase
ERK 1/2	extracellular regulated kinase
PHAP1	putative histocompatibility leukocyte antigen class II associated protein-1

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Highlights

- Molecular mechanism of protein phosphatase-2A activity by I_2^{PP2A}/SET
- Role of cleavage of I_2^{PP2A} into I_{2NTF} and I_{2CTF} in inhibition of PP2A activity

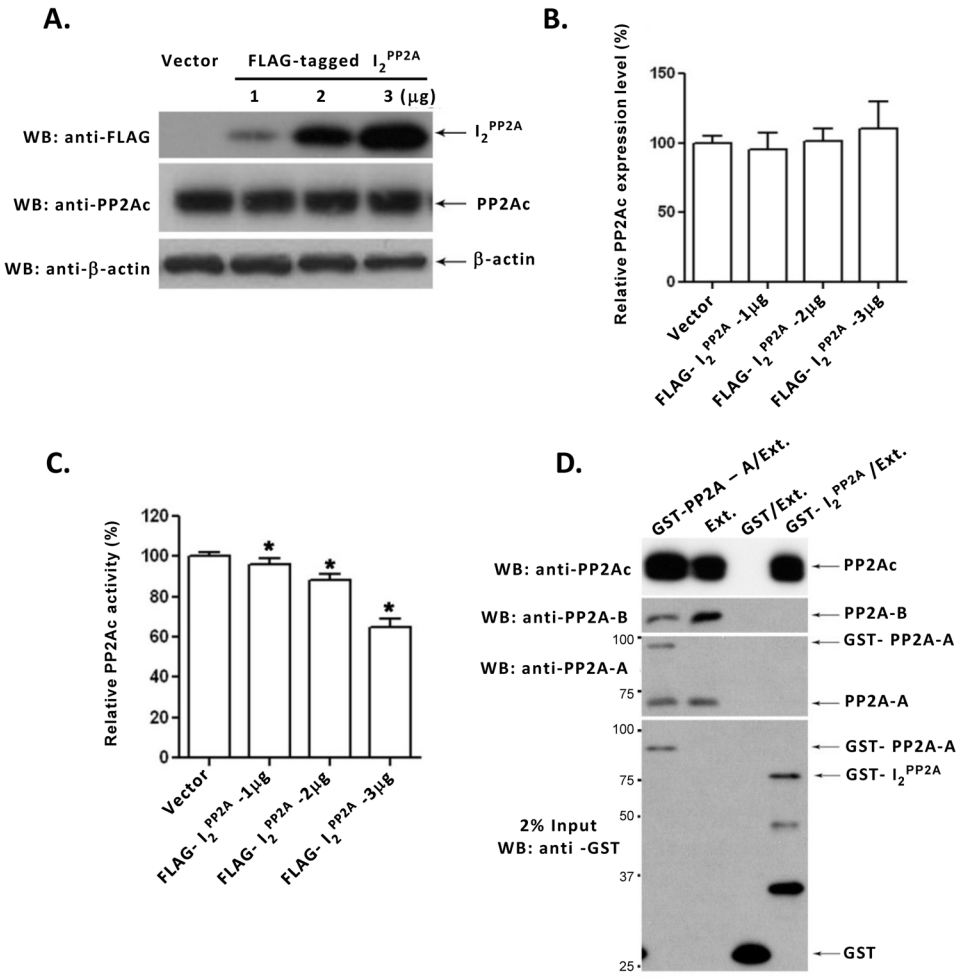

Fig. 1. Inhibition of PP2A activity by I_2^{PP2A} and its interaction with PP2Ac

(A) NIH3T3 cells were transfected with different amounts of pCMV2B-I_2^{PP2A} to express FLAG-tagged I_2^{PP2A} or, as a control, with vector alone. After 48 hours transfection, cells were lysed and expression of PP2Ac, I_2^{PP2A}, and, as a loading control, β-actin were determined by Western blots. **(B)** The relative PP2Ac expression level was normalized by the expression of β-actin. PP2Ac expression level did not show any significant difference among different amounts of expression of I_2^{PP2A} in NIH3T3 cells. One way ANOVA, p = 0.1369; t-test, vector vs. 1 μg, p = 0.2189; vector vs. 2 μg, p = 0.5031; vector vs. 3 μg cDNA, p = 0.1992. **(C)** PP2A was immunoprecipitated from cell lysates with anti-PP2Ac and the phosphatase activity assayed colorimetrically using pNPP as a substrate. I_2^{PP2A} inhibited the PP2A activity in a pCMV2B-I_2^{PP2A} dose-dependent fashion. *p<0.05 compared with control. **(D)** GST-pull down assay. Rat brain extract (Ext.), used as a source of PP2A holoenzyme, was incubated with Sepharose 4B beads bearing GST, GST-I_2^{PP2A}, or GST-PP2A-A. After washing, bound PP2Ac, PP2A-B, and PP2A-A were detected by Western blots. The GST, GST-I_2^{PP2A} and GST-PP2A-A used in pull down assay are shown in the lowest panel. GST-I_2^{PP2A} pulled down PP2Ac (PP2A catalytic subunit) but not PR55α (PP2A Bα regulatory subunit) or PR65α (PP2A A regulatory subunit). From left to right, Lane 2 (Ext.) is input, and Lane 3 (GST/Ext.) is a negative control. Positions of protein size markers are indicated on the left of the panel. Error bars in panels B and C indicate means ± SE; n=3.

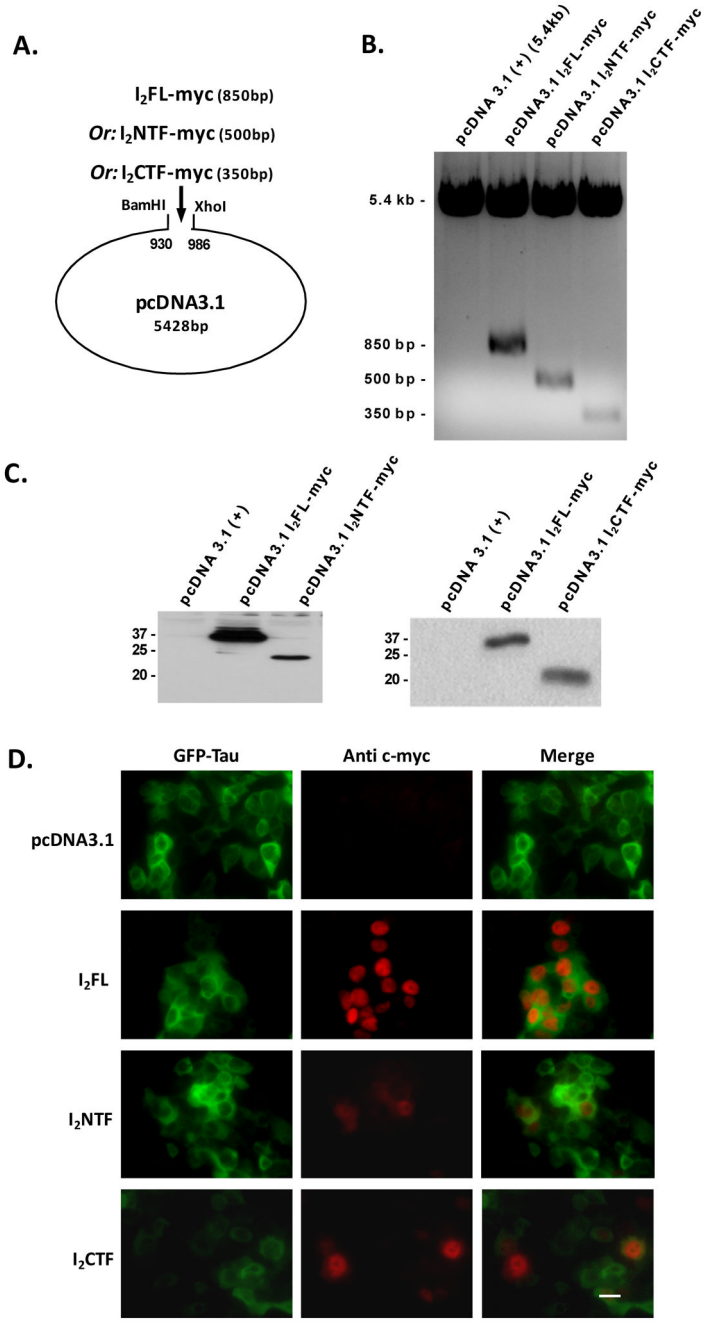

Fig. 2. Intracellular localization of I_{2FL}, I_{2NTF}, and I_{2CTF} with reference to tau

(A) $I_{2FL\text{-}myc}$, $I_{2NTF\text{-}myc}$, and $I_{2CTF\text{-}myc}$ cDNA were generated using pEGFP-N3/I_2^{PP2A} (wt) as a template. The PCR inserts as well as pcDNA3.1 were digested with BamHl and Xhol. The products were subsequently ligated into pcDNA3.1 vector and transformed into E. Coli DH4-α. **(B)** After amplification, the purified plasmids were digested with BamHl and Xhol, separated by electrophoresis, and sequenced. **(C)** Left panel: Western blots specific for the C-terminal myc tag of each protein confirmed the expression of I_{2FL} and I_{2NTF}. Right panel: Western blots specific for the C-terminal myc tag of each protein confirmed the expression of I_{2FL} and I_{2CTF}. **(D)** Immunocytochemical staining of HEK293FT cells transfected with GFP-Tau and pcDNA3.1 or pcDNA3.1-I_{2FL}, -I_{2NTF}, or -I_{2CTF}. I_{2FL} tagged with c-myc was

localized exclusively in the nucleus of HEK293FT cells. I_{2NTF} tagged with c-myc was diffused all over the HEK293FT cells. I_{2CTF} tagged with c-myc, although concentrated in the nucleus, was also found distributed within the cytoplasm. Magnification bar = 10 μm.

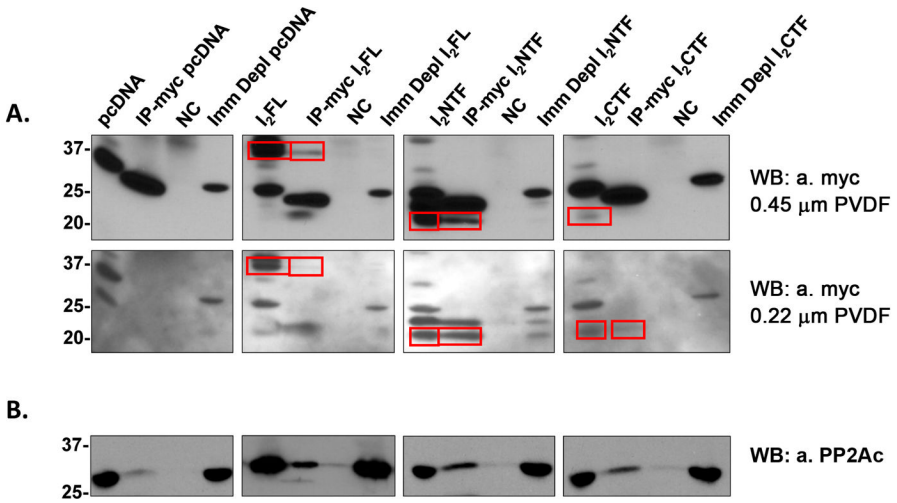

Fig. 3. Co-immunoprecipitation of PP2Ac with I_{2FL}, I_{2NTF}, or I_{2CTF}

HEK293FT cells transfected to overexpress c-myc-tagged I_{2FL}, I_{2NTF} or I_{2CTF} were lysed and c-myc tag antibody was used for immunoprecipitation. The pulled down proteins were separated on SDS-polyacrylamide gels, then transferred onto two stacked PVDF membranes of decreasing pore size and analyzed by Western blots. **(A)** Upper row: Western blots on the 0.45 µm PVDF membrane developed with c-myc tag antibody. Lower row: Western blots on the 0.22 µm PVDF membrane developed with c-myc tag antibody. I_{2FL}, I_{2NTF}, and I_{2CTF} were all immunoprecipitated with the c-myc antibody (see framed bands). **(B)** Western blots developed with anti-PP2Ac. NC=negative control, i.e. non-treated with the vector. Positions of protein molecular markers are shown on the left of the blots.

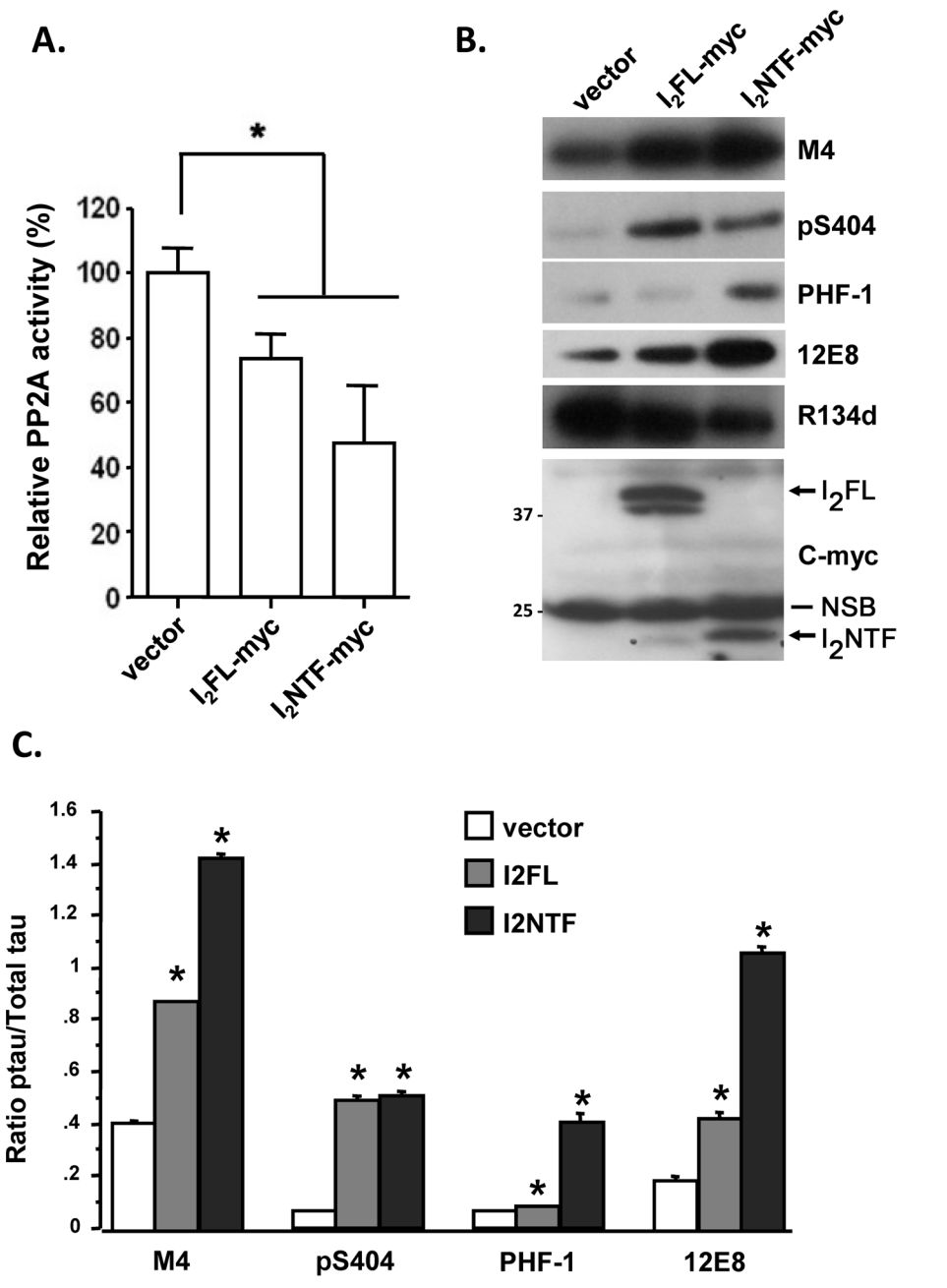

Fig. 4. Inhibition of PP2A activity by I_{2FL} and I_{2NTF}

(A) Relative PP2A activity in transiently transfected HEK293FT cells with N-terminal myc tagged I_{2FL} and I_{2NTF}. I_{2FL} and I_{2NTF} reduced PP2A activity to ~70% and ~50%, respectively, in HEK293FT cells. *p<0.05 compared with the vector transfection control. **(B)** Tau_{441} stably transfected PC12 cells were transiently transfected to express myc tagged I_{2FL} or I_{2NTF} and tau phosphorylation at M4 (pT231/pS235), pS404, PHF-1 (pS396/404) and 12E8 (pS262/356), total tau (R134d), as well as transfected I_2^{PP2A} were detected by Western blots from same amounts of lysates. The myc-tagged proteins were detectedwith anti-myc monoclonal 9E10 (1:100 in 5% defatted milk and 0.1% Tween-20) from Millipore/Chemicon. **(C)** The level of phosphorylated tau was quantified by densitometry and

normalized to the amount of total tau. *p<0.05 compared with the vector transfection control. Positions of protein size markers are indicated on the left of the blots. Positions of I_{2FL} and I_{2NTF} bands are labeled with arrows. NSB = non-specific band.

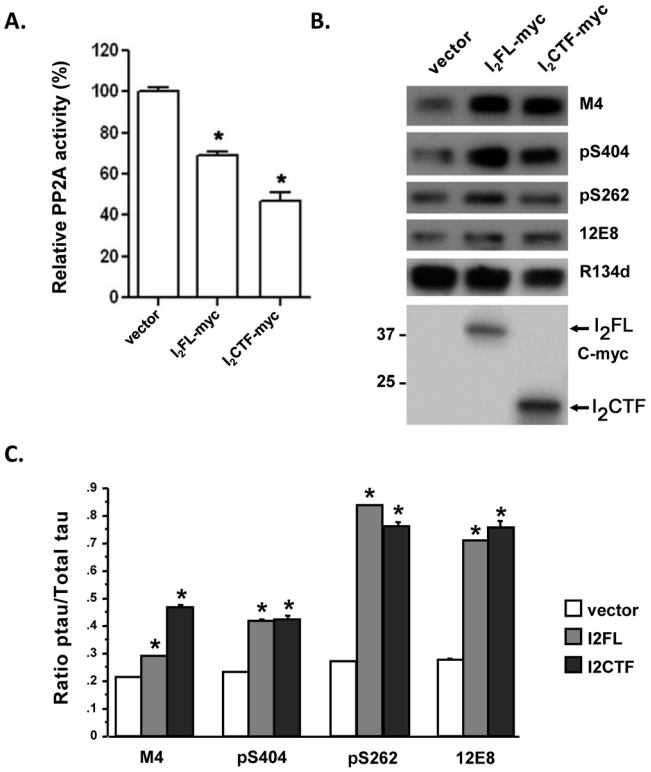

Fig. 5. Inhibition of PP2Ac activity by I_{2FL} and I_{2CTF}

(A) Relative PP2A activity in transiently transfected cells with C-terminal myc tagged constructs was detected as described in Fig. 1C. I_{2FL} reduced PP2A activity to ~69% whereas I_{2CTF} inhibited PP2A activity to ~47%. *$p<0.05$ compared with the vector transfection control. **(B)** Tau phosphorylation at M4 (pT231/pS235), pS404, pS262, 12E8 (pS262/356), total tau (R1334D) and I_2^{PP2A} were detected by Western blots from same amounts of lysates from I_{2FL} and I_{2CTF} transfected cells. The myc-tagged proteins were detected with anti-myc monoclonal 9B11 (1:1,000 in 5% defatted milk and 0.1% Tween-20). **(C)** The level of phosphorylated tau was quantified by densitometry and normalized using the amount of total tau. *$p<0.05$ compared with the vector transfection control. The rest of the details are the same as in Figure 4.

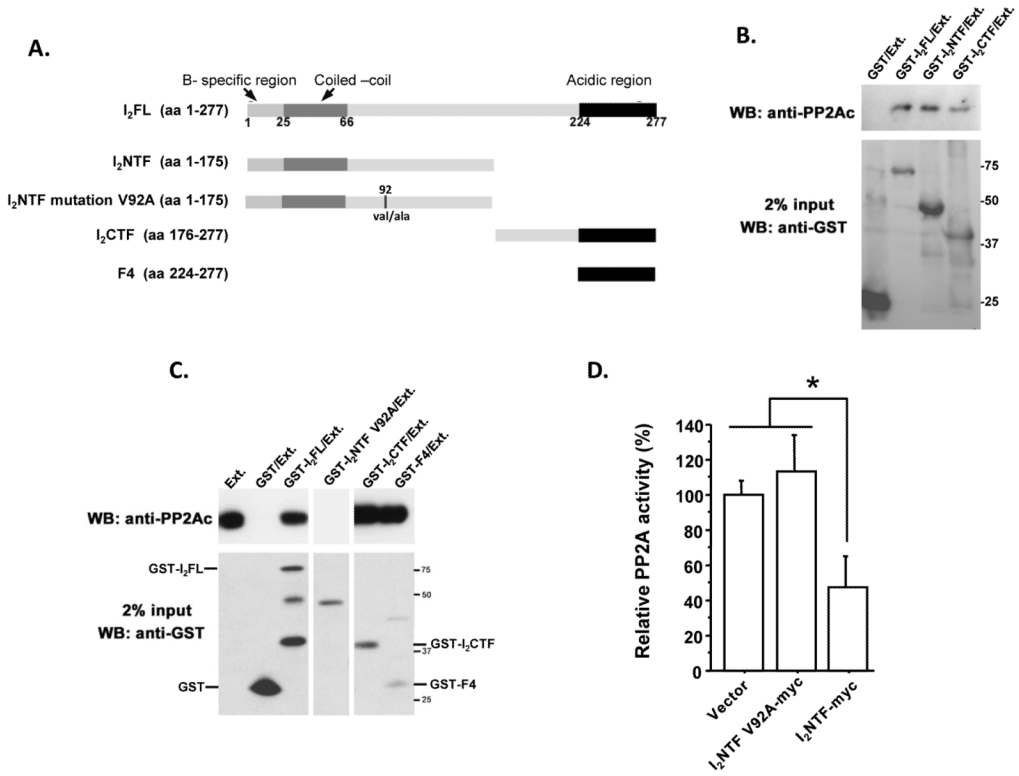

Fig. 6. Identification of minimal and critical regions of I_2^{PP2A} required for interaction with PP2Ac

(A) Schematic diagram of the constructs of functional domains of I_2^{PP2A} employed for the interaction studies. **(B, C)** GST-pull down assays with rat brain extract as a source of PP2A holoenzyme. The pull down assays were carried out using 0.5 μg of GST-fusion protein per mg brain extract, except in cases of GST-I_{2CTF} (0.1 μg was employed), and GST alone (double the amount, i.e. 1 μg was employed). I_{2CTF}, F4, and I_{2NTF} but not $I_{2NTFV92A}$ interacted with PP2Ac. **(D)** Relative PP2A activity in transiently transfected NIH3T3 cells with C-terminal myc tagged constructs $I_{2NTF\text{-}myc}$ can reduce PP2A activity and mutation of V92 to A in I_{2NTF} results in a complete loss of this inhibitory activity. *$p<0.05$.