Familial prion protein (PrP) mutants undergo conversion from soluble and protease-sensitive to insoluble and partially protease-resistant proteins. Cyclin-dependent kinase 5 (Cdk5) phosphorylation of wild type PrP (pPrP) at serine 43 induces a conversion of PrP into aggregates and fibrils. Here, we investigated whether familial PrP mutants are predisposed to Cdk5 phosphorylation and whether phosphorylation of familial PrP mutants increases conversion. PrP mutants representing three major familial PrP diseases and different PrP structural domains were studied. We developed a novel in vitro kinase reaction coupled with Thioflavin T binding to amyloid structure assay to monitor phosphorylation-dependent amyloid conversion. Although non-phosphorylated full-length wild type or PrP mutants did not convert into amyloid, Cdk5 phosphorylation rapidly converted these into Thioflavin T-positive structures following first order kinetics. Dephosphorylation partially reversed conversion. Phosphorylation-dependent conversion of PrP from α-helical structures into β-sheet structures was confirmed by circular dichroism. Relative to wild type pPrP, most PrP mutants showed increased rate constants of conversion. In contrast, non-phosphorylated truncated PrP Y145X (where X represents a stop codon) and Q160X mutants converted spontaneously into Thioflavin T-positive fibrils after a lag phase of over 20 h, indicating nucleation-dependent polymerization. Phosphorylation reduced the lag phase by over 50% and thus accelerated the formation of the nucleating event. Consistently, phosphorylated Y145X and phosphorylated Q160X exacerbated conversion in a homologous seeding reaction, whereas WT pPrP could not seed WT PrP. These results demonstrate an influence of both the N terminus and the C terminus of PrP on conversion. We conclude that post-translational modifications of the flexible N terminus of PrP can cause or exacerbate PrP mutant conversion.

Growing evidence indicates a general mechanism by which misfolded proteins can convert normally folded proteins into misfolded β-sheet-enriched, protease-resistant, and aggregated proteins (1, 2); prion protein (PrP) is the prototype of this type of mechanism. Prion diseases are either inherited through mutations of the PRNP gene, arise sporadically, or are acquired through transmissible prions (PrPSC). Although the acquired form of prion diseases is induced by PrPSC disease-propagating seed, the underlying molecular mechanism for the conversion of normal α-helical, soluble, and protease-sensitive cellular PrP (PrPc) into a β-sheet, insoluble, and protease-resistant PrPSC is unclear (1). Despite mutant PrPs being expressed early in development and often being prone to conversion in vitro, the disease is only manifested in the second to fifth decade of life, suggesting that conversion requires an age-dependent event (3). Possibly, PrP mutants undergo conversion at a low frequency, resulting in a cumulative increase of PrPSC over time. Alternatively, a cofactor is involved in the mutant PrP conversion as has been shown in vitro for recombinant wild type PrP conversion with RNA, proteoglycans, or lipids (4–9). However, one other possibility is that post-translational modifications of PrP promote its conversion. Protein phosphorylation has been linked to amyloid formation and aggregation in several neurodegenerative diseases. Tau phosphorylation has long been associated with the formation of Tau aggregates in neurofibrillary tangles of Alzheimer disease (10). Amyloid β peptide phosphorylation, observed in brains of transgenic mouse models of...
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Alzheimer disease and in human Alzheimer disease, promotes the formation of aggregates in vitro and induces age-dependent degeneration in the Drosophila eye (11). α-Synuclein and Huntington phosphorylation are associated with protein aggregation in Parkinson disease and Huntington disease, respectively (12, 13). PrP is phosphorylated by various kinases, including Fyn and casein kinase II (14). PrP is also phosphorylated at Ser43 (pPrPS43) by neuronal cyclin-dependent kinase 5 (CdK5), and pPrPS43 forms proteinase K-resistant aggregates and accumulates in scrapie-infected mouse brains (15). Cdk5 levels are Ni-nine(PrPWT-M) or valine(PrP WT-V) codon 129 was cloned into the XhoI and BamHI restriction sites of pET23b as described (16). QuikChange site-directed mutagenesis was used to generate the XhoI and BamHI restriction sites of pET23b as described (16). QuikChange site-directed mutagenesis was used to generate three independently prepared protein preparations and conducted in triplicate.

Western Blot Analyses—Proteins were separated by 15% SDS-PAGE, transferred to PVDF membranes, and immunostained with anti-PrP543 R48 antiserum (1:10,000) (15), anti-PrP109–112 3F4 antibody (1:3000) (22), anti-PrP36–56 (R155 antisera) (1:5000) (20), and anti-PrP79–92 SAF32 antibody (1:3000) (Bertin Pharma, Montigny, France).

In Vitro Kinase Assay and Densitometry—In vitro kinase (IVK) reactions were set up with 16.8 μM PrP, 1.84 pmol of CdK5/p25 (referred to as CdK5; SignalChem, Richmond, British Columbia, Canada), 3 mM ATP, 40 mM HEPES, pH 7.2, 50 μM EGTA, 50 μM EDTA, 4 mM MgCl2, 25 mM okadaic acid, and 4 μM NaF with freshly added protease inhibitors (38 μg/ml 4(2- aminoethyl)benzenesulfonyl fluoride, 0.1 μg/ml pepstatin, 0.1 μg/ml Nα-p-tosyl-L-lysine chloromethyl ketone, and 0.5 μg/ml leupeptin). We added 1 μCi of [32P]ATP (2 mCi/ml; 10 Ci/mmol) for radiolabeled IVK reactions. Reactions were incubated for 4 h at 37°C. Densitometry was done with a STORM phosphorimaging system (Amersham Biosciences), and quantification was done with ImageQuant (GE Healthcare). IVK with FynA (SignalChem) and casein kinase II (Calbiochem) were done as described for CdK5.

IKTA—IKTA was set up like the IVK with the addition of 40 μM Thioflavin T (Sigma) in black Costar microplates (BD Falcon) with flat clear bottoms. The reaction was started with the addition of CdK5, and fluorescence was measured at excitation and emission wavelengths of 440 and 482 nm, respectively. A bandpass of 9 nm was used as a filter. The reactions were measured every 15 min, with shaking for 10 s before each measurement, for 60 h on a Synergy H4 instrument (BioTek).

The rate constant of aggregation, F(t) = (F(0) - F∞)e(-kt) + F∞, where F(t) is fluorescence at time t, F∞ is the fluorescence level at the end of the IKTA reaction, F(0) is fluorescence at time 0, and k is the aggregation constant rate, was used for the hyperbolic curve fitting. The rate constant for Y145X and Q160X was measured with a sigmoidal data fit equation for nucleation-dependent polymerization: F(t) = (F(0) + F(∞) - F(0))/(1 + exp(t/tlag(k)) where tsub represents the time to achieve half of the maximal fluorescence and tlag is the time of the lag phase. The lag phase was calculated as tlag = tlag/(2/k) (23). Dephosphorylation was done by adding 1 unit of calf intestinal alkaline phosphatase (CIAP; New England Biolabs, Whitby, Ontario, Canada) or the equivalent volume in buffer to the 60-h IKTA reaction mixture, and the experiment continued for an additional 60 h.

Seeding Reactions—A 60-h IKTA reaction mixture lacking Thioflavin T was sonicated in a VibraCell sonicator (Sonics and Materials, Danbury, CT) for 10 s on/10 s off (three times) at 50% duty cycle and diluted 10% in another IKTA reaction mixture with Thioflavin T and PrP but lacking CdK5 and ATP. The reaction was analyzed as described above for IKTA. For Figs. 3, B and C, and 7, B and D, the seeds were added with 1 unit of roscovitine (BioMol, Burlington, Ontario, Canada).

Thioflavin T Fluorescence—The progression of PrPWT-M conversion to amyloid due to CdK5 phosphorylation was mon-

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis of Prion Protein cDNA—Human PrP cDNA encoding amino acids 23–231 with either a methionine (PrPWT-M) or valine (PrPWT-V) codon 129 was cloned into the XhoI and BamHI restriction sites of pET23b as described (20). QuikChange site-directed mutagenesis was used to generate the single point and truncated mutant PrPs. The N100X construct encodes amino acids 23–100 of PrPWT-M.

Purification of Recombinant Prion Protein—Escherichia coli BL21(DE3) pLySS (Stratagene) were freshly transformed with the PrP constructs. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside, and PrP was purified on a Ni2+–nitrilotriacetic acid fast flow Sepharose column (GE Healthcare) as described (21). Purified proteins were dialyzed against 10 mM Tris-HCl, 0.5% Triton X-100, pH 7.5 overnight at 4°C. Proteins were aliquoted, stored at −35°C, and only thawed once for any experiment. Each experiment was conducted on three independently prepared protein preparations and conducted in triplicate.

Thioflavin T Fluorescence—The progression of PrPWT-M conversion to amyloid due to CdK5 phosphorylation was mon-

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RESULTS

Familial PrP Mutants Are Equally and Efficiently Phosphorylated by Cdk5—To determine the impact of Cdk5-dependent phosphorylation at serine 43 on familial PrP diseases, familial single point mutations of PrP with their respective methionine or valine codon 129 were selected to represent each of the structural domains of PrP and different PrP diseases (Fig. 1A). The prokaryotically expressed purified recombinant C-terminally His-tagged mutant PrPs were over 98% pure as observed by Coomassie Blue-stained SDS-PAGE. As described previously (15), purified wild type PrP with a valine codon 129 (PrPWT-V) was phosphorylated by Cdk5, whereas mutant PrPWT-V/S43A was not (Fig. 1B), confirming phosphorylation of PrPWT at serine 43 (Ser43) by Cdk5. Before evaluating Cdk5-mediated phosphorylation of the familial PrP mutants, the time of maximal phosphorylation of PrP was determined to plateau at around 4 h (Fig. 1C). Under these conditions, no obvious differences were observed between the phosphorylation of PrPWT and PrPmutants by autoradiography (Fig. 1D) and semiquantitative densitometric analysis (Fig. 1E). Time course analyses of the phosphorylation levels indicated that the rate of phosphorylation was the same for all PrPWT and PrPmutants. Together, these results indicate that single point mutations of PrP associated with familial prion diseases do not alter the ability of Cdk5 to phosphorylate PrP at serine 43.

Cdk5-mediated Phosphorylation at Ser43 Induces a Thioflavin T-positive Conversion of PrP in IKTA—To evaluate the capacity of phosphorylated PrP to aggregate or convert into an amyloid-like structure, we developed automated IKTA to assess conversion of PrP in real time. To keep experimental conditions as close as possible to physiological conditions, the conversion of PrPWT-V was done at pH 7.0 and 37 °C. IKTA of Cdk5-phosphorylated PrPWT-V showed a rapid and time-dependent increase in Thioflavin T fluorescence that approached saturation after 40–50 h, indicating the conversion of PrP into Thioflavin T-binding amyloid (Fig. 2A). Thioflavin T did not interfere with Cdk5 phosphorylation of PrP (Fig. 2B). IKTA performed with PrPWT-V in the presence of 50 μM Cdk5 inhibitor rocscovitine, in the absence of ATP, in the presence of CIAP, with no Cdk5 (PrPWT-V), or with inactive Cdk5 (no p25 subunit), did not increase Thioflavin T fluorescence even after 60 h (Fig. 2, A and C). Furthermore, PrPWT-V did not increase Thioflavin T fluorescence in IKTA. Therefore, conditions that did not allow phosphorylation of PrPWT-V at serine 43 prevented the increased Thioflavin T fluorescence in the IKTA reaction. Thioflavin T fluorescence increased with the amount of PrP substrate in the IKTA reaction (Fig. 2D) and was accompanied with increasing levels of phosphorylated PrP (Fig. 2E). The rate constant of aggregation $F(t) = (F_c - F_0)e^{-kt} + F_c$ (23) correlated in a linear fashion with PrP concentrations (Fig. 2F). Similarly, Thioflavin T fluorescence increased with the levels of Cdk5 enzyme (Fig. 2G) and PrP phosphorylation levels (Fig. 2H). The rate constant of aggregation also correlated in a linear fashion with the amount of Cdk5 between 0.4 and 3.2 fmol (Fig. 2I).

To determine whether phosphorylation of alternate sites also causes amyloid conversion of PrP, IKTA was conducted with casein kinase II and Fyn kinase on PrPWT-V (14). Although each

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of these kinases was able to phosphorylate PrP, neither produced an increase in Thioflavin T fluorescence in contrast to Cdk5-phosphorylated PrP (Fig. 2). Lastly, an N terminus fragment of amino acids 23–100 did not form Thioflavin T-positive conformers with or without Cdk5 phosphorylation in the IKTA reaction (Fig. 2, K and L). The inability of the N100 to form Thioflavin T-positive conformers excludes His tag involvement in the phosphorylation-dependent conformational change. These results establish IKTA as a specific and quantitative measurement of Cdk5-dependent phosphorylated PrP structural change and indicate that the C terminus of PrP is essential to the Cdk5 phosphorylation-mediated conformational change.

The Cdk5-phosphorylated PrP<sub>WT-V</sub> Conversion Is Reversed upon Dephosphorylation, and Phosphorylated PrPs Do Not Exert a Conformational Change on Normal PrP in a Homologous Seeding Reaction—Previously, we have shown that the Cdk5-phosphorylated PrP was proteinase K-resistant and Congo Red-positive, and transmission electron microscopy revealed that Cdk5-phosphorylated PrP<sub>WT-V</sub> converted into aggregates composed of both anti-<sup>32</sup>P<sub>PrP</sub> immunopositive and immunonegative globules, which became compacted and generated rare fibrils with aging (15). The hyperbolic shape of the IKTA reaction with full-length wild type phosphorylated PrPs indicates a rapid conversion of these PrPs after Cdk5 phosphorylation (Fig. 2). To determine whether the conversion of phosphorylated PrP is reversible or irreversible, calf intestinal alkaline phosphatase was added to the reaction at the 60-h time point (Fig. 3A). The ThT fluorescence decreased to one-third within 15 min, indicating that two-thirds of the converted PrP can revert back to the non-amyloid form. However, one-third of the PrP remained ThT-positive.

The ability of converted PrP oligomers to exert a conformational change of normal cellular PrP in a homologous seeding reaction is paramount to prion transmissible disease. To determine whether the pPrP<sub>WT-V</sub> oligomers can seed non-phosphorylated recombinant PrP<sub>WT-V</sub>, samples of the 60-h time point IKTA reaction done without Thioflavin T were added as a 10% seed to a fresh IKTA reaction with Thioflavin T but lacking Cdk5 and ATP (Fig. 3B). Consistent with the first order rate kinetics of the IKTA reaction, which indicates that it is the phosphorylation of PrP that is responsible for the rapid increased Thioflavin T fluorescence (Fig. 2), the 60-h IKTA-generated pPrP<sub>WT-V</sub> was not able to act as a seed to promote non-phosphorylated PrP<sub>WT-V</sub> conversion. Similarly, pPrP<sub>WT-M</sub> was unable to seed non-phosphorylated PrP<sub>WT-M</sub> conversion (Fig. 3C). The amount of ThT fluorescence in the pPrP<sub>WT-V</sub> seeds decreased with time, indicating a time-dependent increase in aggregation of PrP as observed by others (26).

The Cdk5-phosphorylated PrP<sub>WT-M</sub> Reveals a Secondary Structure Rich in β-Sheets That Is Reversed upon Dephosphorylation—Because ThT staining can lead to artifacts, we verified the conversion of PrP from a mostly α-helical structure...
to a β-sheet by CD. Furthermore, because a recent study indicated that shaking alone could convert PrP into β-sheets (27), we opted to perform these experiments without shaking. Therefore, the IKTA experiment was carried out without shaking for 60 h. Only in the presence of Cdk5 did PrPWT-M show increased ThT fluorescence. In contrast, PrP without Cdk5 or PrP543A mutant incubated with Cdk5 showed no increase in ThT fluorescence (Fig. 4A). To determine whether the change in the secondary structure of PrPWT-M is due to the conversion into β-sheet structure, a CD technique was performed in the far-UV region. CD spectra for PrPWT-M in the absence of Cdk5 (Fig. 4B) or for PrP543A in presence of Cdk5 (Fig. 4C) showed a minimum at 208 nm and a shoulder at 222 nm, characteristic of a protein dominated by the α-helical secondary structure. Further analysis by CDPro (25) revealed a structure rich in α-helical region (~55%) with little β-sheet (~20%) content. In the presence of Cdk5, the PrPWT-M initially was dominated with α-helices (55%), similar to the structural arrangement of the non-phosphorylated form of PrPWT-M, but within 12 h, it converted to a structure rich in β-sheet with a single minimum close to the characteristic β-sheet minimum at 215 nm, culminating in a high of ~67% β-sheet with only 3% α-helical region after 60 h (Fig. 4D). To assess whether the conformational change due to phosphorylation is reversible, we measured ThT fluorescence of phosphorylated and non-phosphorylated forms of PrPWT-M in the absence and presence of CIAP from 60 to 120 h. The non-phosphorylated form of PrPWT-M when treated with CIAP did not change ThT fluorescence for up to 120 h of incubation, and the phosphorylated form of PrP (incubated with Cdk5 for 60 h) retained high ThT fluorescence. Only when CIAP was added to the phosphorylated form of PrPWT-M did we see a decrease in the ThT fluorescence, indicating a reversal back to the native state of PrPWT-M (Fig. 4E). These results were supported by CD spectra of non-phosphorylated PrPWT-M,
which retained the native structure rich in α-helical region in the presence and absence of CIAP (Fig. 4, F and G). The phosphorylated PrP<sup>WT-M</sup> retained a structure rich in β-sheet in the absence of CIAP for up to 120 h (Fig. 4H). However, when CIAP was added to the phosphorylated form of PrP<sup>WT-M</sup>, the CD spectrum showed a reversal of the β-sheet-rich structure to a structure rich in α-helix (~50%) and little β-sheet (~20%), highly similar to the native form of PrP<sup>WT-M</sup> dominated with α-helices (Fig. 4I). These results are entirely consistent with the ThT fluorescence levels obtained in the IKTA reaction and confirm that the ThT fluorescence observed in IKTA is not the result of shaking.

**Cdk5 Phosphorylation Increases Familial PrP Mutant Conversion**—A comparison of Cdk5-phosphorylated PrP<sup>WT-V</sup> with PrP<sup>WT-M</sup> in IKTA revealed that the pPrP<sup>WT-M</sup> induced higher Thioflavin T fluorescence levels than the pPrP<sup>WT-V</sup> (Fig. 5A). However, the rate constant at which conversion occurred did not differ between pPrP<sup>WT-M</sup> (35.6 ± 3.2 h<sup>-1</sup> × 10<sup>-3</sup>) and pPrP<sup>WT-V</sup> (38.6 ± 1.1 h<sup>-1</sup> × 10<sup>-3</sup>) (Table 1). Similarly, the pE200K<sup>Δ</sup> had an almost identical rate of conversion as the control PrP<sup>WT-M</sup>. In contrast, the rate constant of aggregation of Thioflavin T fluorescence for pD178N<sup>Δ</sup>, pT188A<sup>Δ</sup>, and pV210I<sup>Δ</sup> was higher than that of pPrP<sup>WT-M</sup>, but each mutant generated almost identical levels of maximal Thioflavin T fluorescence in the IKTA reaction (Fig. 5B and Table 1). Similarly, the rate of Thioflavin T fluorescence for pD178N<sup>Δ</sup> and pA117V<sup>Δ</sup> was also higher than that of pPrP<sup>WT-V</sup> (Fig. 5C and Table 1). We excluded an effect of protein concentration on the rate constant of aggregation because the PrP concentration in each reaction did not vary by more than 20% (3–17%).

to our previous findings with PrP<sup>WT</sup>, a 10-kDa fragment of the PrP<sup>WT-M</sup>, V210I<sup>Δ</sup>, and D178N<sup>Δ</sup> 60-h IKTA were resistant to proteinase K digestion, confirming conversion of the PrPs (Fig. 5D). Overall, these results show that Cdk5 phosphorylation of both PrP<sup>WT</sup> and PrP<sup>mutants</sup> increases the rate constant of Thio-

**FIGURE 3.** The Cdk5-phosphorylated PrP<sup>WT-V</sup> forms SDS-resistant oligomers, but these do not exert a conformational change on normal PrP in a homologous seeding reaction. A, Thioflavin T fluorescence of an average of three independent experiments for PrP<sup>WT-V</sup> phosphorylated with (red I<sup>2</sup> = 0.96) and without Cdk5 (black and yellow) for 60 h at which point calf intestinal alkaline phosphatase was added (blue, black, and yellow). The lower panel shows an immunoblot with anti-pPrP<sup>S43</sup> (pPrP) or the Coomassie Blue-stained control (PrP). B, Thioflavin T fluorescence measured for up to 60 h in a PrP<sup>WT-V</sup> IKTA reaction lacking Cdk5 and ATP but seeded with 10% pPrP<sup>WT-V</sup> (green and light blue), 10% PrP<sup>WT-V</sup> (red and black) (from an IKTA with no Thioflavin T) on PrP<sup>WT-V</sup> (light blue and black), or no additional PrP (green and red). C, Thioflavin T fluorescence measured for up to 60 h in a PrP<sup>WT-M</sup> IKTA reaction lacking Cdk5 and ATP but seeded with 10% pPrP<sup>WT-M</sup> or 10% PrP<sup>WT-M</sup> (from an IKTA with no Thioflavin T), a.u., arbitrary fluorescence units.

**FIGURE 4.** Circular Dichroism (CD) of Cdk5-phosphorylated and CIAP-dephosphorylated PrP<sup>WT-M</sup> A, Thioflavin T fluorescence generated in an IKTA reaction with PrP<sup>WT-M</sup> (+Cdk5) and PrP<sup>ΔS43</supA (+Cdk5) for 60 h. Each data point represents the S.E. of three separate measurements. B, CD spectra of PrP<sup>WT-M</sup> in the absence of Cdk5 every 12 h up to 60 h. C, CD spectra of PrP<sup>WT-M</sup> in the presence of Cdk5 every 12 h up to 60 h. D, CD spectra of PrP<sup>WT-M</sup> in the presence of Cdk5 every 12 h up to 60 h. E, CD spectra of PrP<sup>WT-M</sup> in the absence of CIAP at 60, 96, and 120 h, respectively. F, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of Cdk5 every 12 h up to 60 h. G, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of Cdk5 every 12 h up to 60 h. H, CD spectra of phosphorylated PrP<sup>WT-M</sup> in the absence of CIAP at 60, 96, and 120 h, respectively. I, CD spectra of phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP starting at 60 h and every 12 h up to 120 h, respectively. J, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. K, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. L, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. M, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. N, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. O, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. P, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. Q, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. R, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. S, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. T, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. U, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. V, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. W, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. X, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. Y, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. Z, CD spectra of non-phosphorylated PrP<sup>WT-M</sup> in the presence of CIAP at 60, 96, and 120 h, respectively. a.u., arbitrary fluorescence units. Error bars represent S.E.
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flavin T-positive PrP conversion into a Thioflavin T-positive and proteinase K (PK)-resistant conformer.

Cdk5 Phosphorylation Shortens the Lag Phase of PrP Fibril Formation in Nucleation-dependent Polymerization—In contrast to the full-length familial PrP mutants, Y145X produced a sigmoidal curve of Thioflavin T fluorescence in the absence of Cdk5 phosphorylation (Fig. 6A). The sigmoidal curve indicates that a nucleation event occurred with the time of incubation even in the absence of phosphorylation by Cdk5. Phosphorylation of Y145X (pY145X) shortened the lag phase by half (t<sub>lag</sub> 36.56 ± 11.23 h for Y145X versus 16.25 ± 1.15 h for pY145X) but maintained the same rate constant of conversion (k, 4900 ± 4249 h<sup>-1</sup> × 10<sup>−3</sup> for Y145X versus 4010 ± 805 h<sup>-1</sup> × 10<sup>−3</sup> for pY145X). Similarly, the time at which Thioflavin T fluorescence was 50% of the maximum was reduced by half with pY145X<sup>M</sup> (t<sub>50</sub> 37.03 ± 11.05 h for Y145X versus 16.76 ± 1.06 h for pY145X). However, the maximal amount of fluorescence was the same in the Y145X<sup>M</sup> and pY145X<sup>M</sup>. Transmission EM did not reveal a significant difference in the Y145X<sup>M</sup> fibrils compared with pY145X<sup>M</sup> fibrils (Fig. 6B).

The non-phosphorylated Q160X<sup>M</sup> mutant showed a slight increase of Thioflavin T fluorescence only after 55 h of incubation in IKTAs, whereas the pQ160X<sup>M</sup> had a lag phase of only 22 h and reached a maximal Thioflavin T fluorescence by 30 h of incubation (Fig. 6C). Maximum fluorescence obtained with the pQ160X<sup>M</sup> was over 10-fold higher than that of pH11005<sup>WT-M</sup> but could not be measured precisely because the maximum detection by the instrument was achieved before reaching the plateau. A plateau could be observed with reduced sensitivity of the detector (Fig. 6C, inset). The t<sub>50</sub> was 43.22 ± 9.9 h, k rate was 7600 ± 927 h<sup>-1</sup> × 10<sup>−3</sup>, and t<sub>lag</sub> was 42.59 ± 10.08 h for pQ160X. It was not possible to measure these in non-phosphorylated Q160X. In contrast to Y145X<sup>M</sup>, non-phosphorylated Q160X<sup>M</sup> appeared as amorphous aggregates in transmission EM. However, pQ160X<sup>M</sup> fibrils and white globular proteins were readily apparent after the phosphorylation (Fig. 6D).

CIAP treatment of pY145X<sup>M</sup> resulted in a very gradual reduction of the ThT-positive fluorescence, achieving one-third ThT fluorescence only after 60 h of incubation (Fig. 6E). In contrast, untreated pY145X<sup>M</sup> and Y145X<sup>M</sup> maintained their level of ThT-positive fluorescence up to 120 h of incubation. As observed with the pH11005<sup>WT</sup> and single point PrP mutants, the phosphorylation of Q160X<sup>M</sup> with FynA and casein kinase II did not increase Thioflavin T fluorescence in the IKTAs (Fig. 6F).

Unfortunately, CD cannot be applied to fibril structures so we relied on other methods to analyze the truncated PrP mutants. Immunostaining of the IKTAS products with anti-pPrP<sup>34,35</sup> showed the presence of pH11005<sup>PrP</sup> monomers, dimers, and tetramers for both pY145X<sup>M</sup> and pQ160X<sup>M</sup> (Fig. 6G). However, staining with R155 anti-PrP<sup>36−56</sup> and SAF32 anti-PrP<sup>39−49</sup> showed that these oligomers were also present in non-phosphorylated Y145X and Q160X. Interestingly, the anti-PrP<sup>36−56</sup> antisera did not detect the phosphorylated PrP<sup>r</sup>, indicating that the phosphorylation at Ser<sup>43</sup> is hiding this epitope in the truncated pPrPs. Oligomers seem to decrease in pY145X<sup>M</sup> stained with SAF32 antisera, but this was not consistently observed.

The Y145X<sup>M</sup> and Q160X<sup>M</sup> were submitted to 10 μg/ml PK treatment for 1 h at 37 °C (Fig. 6H). The immunostaining with SAF32 showed the complete digestion of oligomers in non-phosphorylated PrP, whereas monomers and some smaller fragments of both Y145X and pY145X were very resistant to PK. In contrast, no PK-resistant oligomers of pQ160X were observed, whereas the monomers exhibited slightly higher resistance to PK. Congo Red staining revealed high amounts of apple green birefringent fibrils under polarized light in Y145X<sup>M</sup> and pY145X<sup>M</sup> (Fig. 6I). In contrast, none were observed in Q160X<sup>M</sup>. However, large Congo Red-positive pQ160X<sup>M</sup> fibrils showed some green birefringence under polarized light.

| TABLE 1 |
| --- |
| Rate constants of aggregation of wild type and familial PrP mutants |
| PrP | Rate k |
| Wild type<sup>M</sup> | 35.6 ± 3.2 |
| D178N<sup>M</sup> | 52.0 ± 5.2* |
| T188A<sup>M</sup> | 50.0 ± 2.0* |
| E200K<sup>M</sup> | 34.5 ± 3.5 |
| V210<sup>M</sup> | 63.6 ± 4.7* |
| Wild type<sup>V</sup> | 38.6 ± 1.1 |
| D178N<sup>V</sup> | 62.0 ± 19 |
| A117V<sup>V</sup> | 50.0 ± 2.0* |

<sup>*p < 0.05.</sup>
Together, these results indicate that phosphorylation at Ser^43 amplifies the nucleation-dependent polymerization of Y145X and Q160X PrP into Thioflavin T-positive and PK-resistant fibrillar amyloid.

**Homologous Seeding of pY145XM and pQ160XM Amplifies Conversion of Non-phosphorylated PrP**—The sigmoidal curve in the Y145X and Q160X IKTA reactions indicates that an intermediate product must be generated before rapidly inducing the conversion of these proteins. Therefore, the Cdk5-phosphorylated truncated mutants would be expected to induce conversion of non-phosphorylated protein in a homologous seeding reaction. To determine whether the truncated pPrP mutants can act as a seed to convert non-phosphorylated PrP, a 60-h IKTA reaction mixture lacking Thioflavin T was sonicated and diluted to 10% in a new IKTA reaction lacking Cdk5 and ATP. Non-phosphorylated Y145X^M PrP seeds allowed conver-
The phosphorylation of Y145X and Q160X significantly shortened the lag phase for amyloid conversion and fibril formation in a homologous seeding reaction. However, the seeding reactions are quite different with the Y145X, requiring a lag phase of several hours to induce the conversion, whereas the Q160X seed induces an immediate conversion.

**DISCUSSION**

Our data indicate that Cdk5 phosphorylation of PrP at serine 43 promotes the conversion of PrP and that the type of conversion is dependent on the C terminus of PrP. Phosphorylated full-length wild type and mutant PrPs undergo a rapid Thioflavin T-positive conversion that follows a first-order reaction, which requires the C terminus of PrP. CD confirmed the conversion of pPrP from α-helical to β-sheet structures within 12 h of phosphorylation. In contrast, the phosphorylated PrP N100 fragment does not undergo conversion. The converted full-length PrPs oligomerize and become PK-resistant but do not seed a homologous seeding reaction. These results are consistent with a model in which the pPrPS43 is interacting with the C terminus of PrP to promote its conversion. Concordantly, the truncated Y145X and Q160X mutants, which lack the C terminus of PrP, convert spontaneously with a profile consistent with nucleation-dependent polymerization where a certain amount of time is required to form nucleating factors that initiate a rapid conversion of the proteins. Cdk5 phosphorylation clearly exacerbates this conversion by significantly reducing the lag phase required for the nucleating event. Furthermore, the seeding ability of the pY145X and pQ160X supports nucleation-dependent polymerization. Because the N100 PrP protein does not undergo conversion, it appears that amino acids 101–144 are essential to this type of conversion. The difference in conversion between full-length and truncated PrPs raises the possibility that endogenous proteolysis of PrP in a cellular setting could possibly generate truncated PrP species that are predisposed to the formation of fibrils as shown for Y145X and Q160X.

In these experiments, familial mutant PrPs are not more susceptible to Cdk5-mediated phosphorylation at the N-terminal serine 43 than WT PrP. However, the Cdk5-dependent phosphorylation has a major influence on the aggregation and conversion of PrP into an amyloid-like Thioflavin T-positive structure, and this response is exacerbated by most C-terminal familial mutations. Indeed, although the steady state level and the rate of Cdk5-mediated phosphorylation were not greater in full-length PrP mutants than in wild type PrP, phosphorylation increased the rate constant of aggregation in the D178N, T188A, V210I, and A117V mutant PrPs compared with full-length wildtype and mutant PrPs under go a rapid Thioflavin T-positive conversion, and the N100 fragment does not undergo conversion.
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N terminus of PrP is also clearly involved in familial and transmissible prion diseases. Additional octapeptide repeats are observed in familial human PrP diseases (33), and mice lacking the N-terminal amino acids 32–93 and 23–31 are less susceptible to transmissible prion diseases (34, 35). Furthermore, the N terminus of PrP has been associated with numerous cellular features such as lipid rafts (36), RNA binding (37), micropinocytosis (38), and neuroprotection (39, 40). Cdk5 phosphorylation provides another physiological molecular mechanism whereby the N terminus of PrP regulates the C-terminal conformation.

Whether the Cdk5-mediated change of PrP conformation leads to physiological or pathological functions is debatable. Functional amyloids (proteins that physiologically form amyloid structures to affect or enhance a normal cellular function) have been well described in the recent years, and several features of the Cdk5-phosphorylated full-length wild type and mutant PrPs are similar to these functional amyloid proteins. The conversion of full-length wild type and mutant Cdk5-phosphorylated PrPs occurs at physiological pH and temperature and in non-denaturant conditions, similar to that observed for the human Pmel17 Ma fragment, which readily converts into amyloid and forms the core of melanosomes (41). The IKTA reaction indicates first order kinetics for the Cdk5-mediated conversion of wild type and mutant full-length PrPs, similar to that obtained with other functional amyloid-forming proteins from fungi to mammals (41–47). Furthermore, the ThT-positive amyloid in PrP^{WT-V} or Y145X^{M} and the seedings properties of phosphorylated Q160X^{M} were partially reversible upon dephosphorylation, similar to the reversibility of secretory granule peptide hormone amyloids as they convert to a monomeric α-helical form when released (45). None of the phosphorylated full-length PrP mutants investigated showed fibril formation initially and were observed by electron microscopy to possess a globular structure of aggregated proteins (data not shown) as observed previously for wild type PrP (20). Furthermore, the phosphorylated WT PrP did not have the ability to seed PrP conversion in a homologous reaction over a period of 60 h. Lastly, the phosphorylation at Ser^{43} of PrP has been shown to affect or enhance a normal cellular function as observed previously for wild type PrP (20). Further- more, the phosphorylated WT PrP did not have the ability to seed PrP conversion in a homologous reaction over a period of 60 h. Lastly, the phosphorylation at Ser^{43} of PrP has been detected in normal adult mouse brain synaptic membranes (18). Interestingly, PrP is localized at the synapse, and synaptic membrane preparations support conversion of PrP in vitro (48, 49). Cdk5 is also localized in presynaptic and postsynaptic compartments and is involved in numerous pathways that are implicated in synaptic plasticity and function, including memory (50). This situation is reminiscent of the memory-associated conversion of the cytoplasmic polyadenylation element-binding protein in Aplysia (46, 51, 52). Together, these findings suggest that Cdk5-mediated phosphorylation of PrP could be involved in a physiological function.

However, this physiological mechanism could become pathogenic. The phosphorylated PrPs may convert irreversibly with time. We have shown by electron microscopy that aging of the phosphorylated wild type PrP resulted in a change from globular aggregates to a more compact structure (15). The increased rate constant of Cdk5-mediated PrP conversion in the familial PrP mutants may explain the age-dependent manifestation of the prion diseases associated with those mutations. Cdk5-phosphorylated PrP is also increased in mouse transmissible prion disease (20). Furthermore, selective PrP immunoprecipitation from scrapie-infected brains with the 6A12 antibody mapping to amino acids 41–47 (30) suggests an alteration of the N terminus of PrP in prion disease conditions. Recently, a thorough evaluation of PrP in cerebrospinal fluid revealed a significantly increased level of pPrP^{S43} in sporadic and familial Creutzfeldt-Jakob disease (19). Five isoforms of pPrP^{S43} were observed in two-dimensional gel analyses. In the case of the truncated Y145X and Q160X mutants, the formation of fibrils is likely to promote pathogenesis, and this effect would only be exacerbated by Cdk5 phosphorylation despite being somewhat reversed upon dephosphorylation.

A large amount of work has been done on the Y145X protein. This mutation, first identified in a Japanese patient (53), is deposited as cerebrovascular amyloid and is associated with neurofibrillary tangle formation (54). Its structure is composed of a highly flexible large N terminus spanning amino acids from 23 to around 111 and amino acids 140–144 and a core rigid region spanning amino acids 115–120 that forms a parallel β-sheet core of the amyloid via an intermolecular arrangement of the β-strands (55–57). Residues 138 and 139 are especially critical for the amyloid production because their deletion diminishes amyloid production (58, 59). However, deletion of residues 113–120 still allows conversion of Y145X into amyloid, indicating complex structural polymorphisms within this protein (60). These results indicate that the amino acids that are part of the flexible region of the Y145X influence its ability to convert into an amyloid structure. Our results add to these observations in that post-translational phosphorylation at the N terminus of Y145X exacerbates the conversion of this protein into amyloid.

The non-phosphorylated truncated Q160X mutant does not rapidly form fibrils, but Cdk5 phosphorylation induces fibril formation as observed by transmission electron microscopy and Congo Red staining, reduces the lag phase of Thioflavin T fluorescence by more than 50% in IKTA reactions, and significantly increases the level of Thioflavin T fluorescence. Our data seem at odds with an elegant and thorough study published by Watzlawik et al. (61) in which they showed that Q160X aggregates more rapidly than Y145X and readily forms fibrils. The pH used in the reactions may explain the difference in the results. The data presented here were obtained at physiological pH, whereas the conditions of Watzlawik et al. (61) were at pH 6.5. Indeed, acidic pH can induce conformational changes in human PrP that may promote aggregation (62). Furthermore, the purification method differs slightly and could also influence the conversion of the Q160X protein. Although there appeared to be large differences in the conversion curves between full-length PrP and the Y145X or Q160X mutants, the absence of shaking showed that even full-length PrP conversion can follow a sigmoidal curve.

Whether Cdk5 can exacerbate Y145X and Q160X PrP conversion in vivo remains to be determined but is highly plausible. In cellular models, Y145X and Q160X accumulate in the cytosol (63, 64) and could thus co-localize with cytosolic Cdk5 (65). Cdk5 is expressed in vascular tissues and has also been implicated in angiogenesis (66). Furthermore, the Q160X mutant has...
been reported in the nucleus (67) where Cdk5 can reside (68–71).

Lastly, we provide evidence for the specific involvement of Cdk5 in the conversion of PrP. Phosphorylation of full-length PrP or truncated Q160X with casein kinase II and FynA kinase does not induce conversion in PrP. The sites of Fyn and casein kinase II phosphorylation are not identified but are predicted to be in the C terminus of PrP. Therefore, it is interesting to note that only the neuronal Cdk5-dependent phosphorylation had a strong effect on the conversion of PrP.

The exact mechanism by which phosphorylation induces conversion of PrP is unclear and difficult to determine because the N terminus of PrP is inherently flexible and the Cdk5-mediated aggregates are composed of a mixture of phosphorylated and non-phosphorylated PrP (15). Nevertheless, others have observed that phosphorylation-induced conversion of several proteins is likely due to 1) the negative charge of the phosphoryl moiety, which can impart a repulsive charge, intramolecularly resulting in conversion of the protein (72, 73); 2) the exposure of hydrophobic regions of the phosphorylated protein, leading to aggregation (74); or 3) a phosphorylation-dependent stabilization of a β-sheet structure (11).

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