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Tau Phosphorylated at Tyrosine 394 is Found in Alzheimer's Disease Tangles and can be a Product of the Abl-Related Kinase, Arg

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Abstract

Tau is a microtubule-associated protein and a main component of neurofibrillary tangles, one of the pathologic hallmarks of Alzheimer's disease. The paired helical filaments (PHF) that comprise neurofibrillary tangles contain an abnormally hyperphosphorylated form of tau. Historically, most of the tau phosphorylation sites that have been characterized are serine and threonine residues. Recent reports state that tau can be phosphorylated at tyrosine residues by kinases including Fyn, Syk, and c-abl (Abl). Proteomic analyses show that tau phosphorylated at tyrosine 394 (Y394) exists within PHF samples taken from Alzheimer's disease brains. This study also confirms phosphorylation of Y394 as an Alzheimer's disease-specific event by immunohistochemistry. To date, only Abl is known to phosphorylate this particular site on tau. We report, for the first time, that Arg, the other member of the Abl family of tyrosine kinases, also phosphorylates tau at Y394 in a manner independent of Abl activity. Given the reported role of Arg in oxidative stress response and neural development, the ability to phosphorylate tau at Y394 implicates Arg as a potential player in the pathogenesis of Alzheimer's disease and other tauopathies.

Keywords

Abl2; Alzheimer's disease; Arg; phosphorylation; tangle; tau; tyrosine

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease leading to memory loss and profound deficits in multiple spheres of cognitive function. The major pathologic hallmarks of AD include senile plaques and neurofibrillary tangles (NFTs). NFTs are aggregates of paired helical filaments (PHF) composed of a hyperphosphorylated form of the microtubule associated protein tau [1–3]. Tau is normally involved in microtubule assembly and cytoskeletal dynamics [4]. However, abnormal phosphorylation of certain residues of the tau protein leads to a loss of microtubule binding and subsequent loss of microtubule stability [5].

Historically, most investigations of tau phosphorylation focused on serine/threonine kinases and corresponding phosphorylation sites [6]. A potential role for tyrosine kinases in the pathogenesis of AD has only recently emerged. Tau contains 5 tyrosine residues (Y18, Y29, Y197, Y310, and Y394), numbered according to the longest CNS isoform of tau, containing 441 amino acids. Proteomic studies have noted tau phosphorylation at tyrosine residues in pathological brain extracts from AD patients. Specifically, PHF samples from AD brains were found to contain tau phosphorylated at tyrosine residue 394 (Y394) [7–9].

Additionally, exposure of cultured neurons to various forms of multimeric amyloid- β (A β) peptide led to increased tyrosine kinase activity and subsequent tyrosine phosphorylation of tau [10–12]. In a recent study, both oligomeric A β peptide and soluble A β derived from AD brains induced hyperphosphorylation of tau in cultured neurons [11]. It was observed that this aberrant tau phosphorylation and subsequent cell death could be prevented by treatment with tyrosine kinase inhibitors, specifically Src kinase inhibitors PP1, PP2, and the Abl family kinase inhibitor, imatinib (Gleevec) [10–12].

The identification of tyrosine kinases responsible for phosphorylation of tau is a work-in-progress. The first tyrosine kinase established as a tau kinase was Fyn, a member of the Src family of tyrosine kinases [13]. Fyn phosphorylates tau primarily at tyrosine 18, a phosphorylation site whose relevance to AD and tau pathology remains to be demonstrated. More recently, Syk, another member of the Src family of tyrosine kinases, was also found to phosphorylate tau at tyrosine 18 [14]. However, there is no evidence to suggest that Fyn or Syk phosphorylate tau at Y394, the only reported AD-relevant tyrosine phosphorylation site. In 2005, Derkinderen and colleagues identified c-Abl (Abl) as a tyrosine kinase capable of phosphorylating tau at Y394 [9]. The ability of Abl to phosphorylate tau at Y394, as well as Y197 and Y310 was confirmed by our laboratory (unpublished data). Prior to this report, no other kinases responsible for Y394 phosphorylation had been identified.

Arg (Abl-related gene, also known as Abl2) is a nonreceptor tyrosine kinase, first discovered in a search for mammalian Abl homologues [15]. Arg is expressed as a ~140 kDa protein and shares extensive homology with Abl in the Src-homology domains, particularly the SH1, or kinase domain [15,16]. However, while Abl is expressed fairly ubiquitously throughout the body, Arg is most abundant in the brain. Arg has been demonstrated as an important player in a number of processes that suggest it may have a role in the pathogenesis of neurodegeneration and AD. The role of Arg in neural development and synaptic plasticity has been well established [17–21]. Arg is also involved in pathways of oxidative stress response and subsequent apoptosis [22–27].

Through the development of phospho-specific monoclonal antibodies, we confirmed the findings of earlier proteomic studies suggesting that tyrosinephosphorylated species of tau are abnormal and localize to AD pathology. A series of biochemical experiments allowed us to demonstrate that Arg is capable of phosphorylating tau, primarily at the Y394 site. Arg-mediated tau phosphorylation occurred in the absence of Abl activity, indicating direct phosphorylation by Arg rather than a signaling event between kinases. Our findings confirm Y394 as an AD-specific tau phosphorylation site for which Abl and Arg are the only known kinases, providing further support for the involvement of Abl kinases in the underlying mechanisms of AD and tau pathology.

MATERIALS AND METHODS

Antibodies

The monoclonal anti-phosphotyrosine antibody (4G10) was obtained from Millipore, formerly Upstate Cell Signaling. Monoclonal phospho-tau antibodies specific to dual phosphorylation of Y394 and S396 (YP4) were generated in mice immunized with a

synthetic KLH-conjugated tau phosphopeptide: DHGAEIVpYKpSPVVSGDT. Monoclonal anti-phospho-tau (pS202) antibody CP13 was used for immunohistochemical staining of human hippocampal sections. Monoclonal anti-Arg antibody (AR11) was generated in mice by immunization with a recombinant GST fusion protein containing the C-terminus of Arg (residues 766–1182). Monoclonal anti-tau (total) antibodies (CP27 and DA9) were generated against human tau preparations [28]. CP27 was used for detecting total tau in ELISA experiments, while DA9 was used for all total tau immunoblotting. Anti-tubulin (Sigma-Aldrich) antibodies were used in immunoblotting as loading controls.

Immunohistochemistry

Blocks of formalin fixed human hippocampus were obtained from the Albert Einstein/Montefiore Brain Bank, under protocols approved by the IRB. Cases were classified by standard neuropathologic criteria. Cases classified as AD had numerous NFTs and neuritic plaques in cortical regions; cases classified as controls were essentially free of either lesion. Immunohistochemistry was performed on free-floating 50 μ m sections from formalin fixed brains. Sections were incubated for 30 min in 3% H₂O₂/0.25% Triton X-100/TBS at room temperature. Non-specific binding was blocked by 1 h incubation in 5% non-fat dried milk/TBS at room temperature. Sections were incubated in YP4 antibody diluted 1:500 in 5% milk/TBS overnight at 4°C, followed by rinsing 4 $\times$ 5 min in 0.05% Triton-X/TBS. Anti-LPS IgM antiserum (Southern Biotech) was diluted 1:300 for control immunohistochemistry. Next, sections were incubated in biotinylated goat anti-mouse IgM secondary antiserum diluted 1:1000 in 20% Superblock/TBS (Pierce Biotechnology) for 2 h at room temperature. Following another 4 $\times$ 5-min rinse in TBS, sections were incubated for 1 h in HRP-labeled streptavidin (Southern Biotech) diluted 1:1000 in 20% Superblock/TBS. Following a 3 $\times$ 5-min rinse in TBS, immunoreactivity was visualized by reacting sections for 8 min in a solution containing 0.3 mg/mL diaminobenzidine, 100 mM Tris (pH 7.4), and 0.018% H₂O₂. Following diaminobenzidine reaction, sections were rinsed in TBS and mounted on microscope slides for analysis.

Indirect immunofluorescence

Procedures for immunofluorescence are the same as described for immunohistochemistry until the incubation of tissue sections with primary antisera. In addition to YP4, sections were incubated in anti-pTau (S202) (CP13) diluted 1:500 in 5% milk/TBS overnight at 4°C. Following this rinsing, sections were incubated in Alexafluor568-conjugated anti-mouse IgG1 secondary antisera (Molecular Probes) diluted 1:1000 in 20% Superblock/TBS for 2 h at room temperature. Following 4 $\times$ 5-min rinse in TBS, sections were incubated with biotinylated anti-mouse IgM secondary antisera (Southern Biotech) diluted 1:1000 in 20% Superblock/TBS for 2 h at room temperature. Following another rinse, sections were incubated in Alexafluor488-conjugated Streptavidin (Molecular Probes) diluted 1:1000 in 20% Superblock/TBS for 1 h at room temperature. Sections were finally treated with 0.3% Sudan black (Sigma) in 70% ethanol for 10 min at room temperature and mounted with 80% Tris-buffered glycerol. Sections were imaged using a Leica AOBS confocal microscope. Images were taken with a 63.3x oil immersion objective with sequential laser excitation of fluorophores. Merged images were created using ImageJ64 software.

In vitro kinase assay

Direct phosphorylation of tau by Arg was examined by *in vitro* kinase assay. 5 ng of active recombinant human Arg (Millipore) was incubated with 5 μ g of re-combinant2N4R tau (rPeptides, www.rpeptide.com) in kinase buffer (20 mM HEPES, 1 mM MnCl₂, 1 mM MgCl₂, 1 mM DTT, 100 μ M sodium orthovanadate, 1 mM Na₂ATP) with a final volume of 50 μ L for 30 min at 30°C. Abltide-GST (Millipore) was used included in some reactions as a control Arg substrate. Kinase reactions were terminated by addition of 5X Laemmli buffer

and boiling of samples. Phosphorylation of tau was assessed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using anti-phosphotyrosine (4G10) and anti-total tau (DA9).

Immunoprecipitation

Immunoprecipitation of phosphotyrosine was used to assess the efficiency of kinase reactions. Kinase reactions were performed as previously described, with a control reaction in which no ATP was present in the kinase buffer. Reaction products were diluted to a total volume of 400 μ L in kinase buffer and incubated overnight with 50 μ L of washed 4G10-conjugated agarose beads (Millipore) at 4 °C. Following incubation, agarose beads were centrifuged at 5000 rpm for 1 min. Supernatant was collected and boiled in Laemmli sample buffer. After 3 washes with TBS, beads were boiled in a volume of 1X sample buffer equivalent to that of dilution present in supernatants. Both supernatant and immunoprecipitated samples were analyzed by SDS-PAGE and immunoblotting for total tau and phosphotyrosine.

ELISA analysis of tau phosphorylation kinetics

Michaelis-Menton kinetics of *in vitro* tau phosphorylation were investigated by combining kinase reactions of varying tau concentration and a sandwich enzyme-linked immunosorbent assay (ELISA) technique. Kinase reactions were performed as described, except tau was added in concentrations ranging from 0 to 2 μ M. Reactions were terminated by diluting reaction products 1:4000 in Superblock/TBS. Tau capture was performed by coating 96-well immuno-plates (Nunc) with purified anti-tau antibody DA9 [2 μ g/mL] in coating buffer (20 mM K_2HPO_4 , 20 mM KH_2PO_4 , 0.8% NaCl, 1 mM EDTA, 0.05% NaN_3 , pH 7.2). Following coating, plates were rinsed with TBS containing 0.05% Tween-20 and incubated with undiluted Starting Block (Pierce) for 1 h at room temperature. Following blocking step, diluted (1:8000 in 20% Superblock/TBS) kinase reaction products were added to plates for overnight incubation at 4 °C. Following incubation, kinase reaction products were discarded, and plates rinsed 5 times with TBS-Tween. Primary antisera were added to the plates for 1 h at room temperature on a shaker (purified CP27 for total tau detection, and 4G10 [1:20,000] for phospho-tau detection). Plates were again rinsed 5 times with TBS-Tween, followed by 1 h incubation with HRP-conjugated isotype-specific secondary antisera (1:1000, Southern Biotech) adsorbed against mouse IgG₁. Plates were again washed with TBS-Tween, at which time 100% Ultra TMB (Pierce) was added to plates for 15 min. TMB reaction was terminated with 4N sulfuric acid. Optical density at 450 nm was measured with an Infinite M200 microtiter plate spectrophotometer (Tecan). Phospho-tau was quantified using signal from 4G10 detection. Kinetic measures were calculated using Graph-pad Prism 4.0 (<http://www.graphpad.com>).

Microtubule binding assays

Microtubules were prepared from purified tubulin as specified by the manufacturer (Cytoskeleton, Inc.). Purified tubulin (5 mg/mL) was incubated 20 min at 35 °C in buffer containing 80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EGTA, 5% glycerol, and 1 mM GTP. Microtubules were diluted 1:100 in PIPES buffer containing 2 μ M taxol before proceeding with the binding assay. Phospho-tau was prepared for microtubule binding assays by 1 h *in vitro* phosphorylation of 1 mM tau with 25 ng of recombinant Arg in the previously described kinase buffer for a final volume of 50 μ L. Unphosphorylated tau was prepared in the same manner, only in the absence of ATP. 20 μ L of preformed microtubules were incubated with 10 μ L of kinase reaction product in a final volume of 50 μ L for 30 min at room temperature. Following incubation, reaction mixtures were centrifuged at 100,000 g for 40 min through 150 μ L of PIPES buffer containing 50% glycerol. Supernatants and pellets were collected for SDS-PAGE and immunoblotted with DA9 [1:500], 4G10 [1:2000], and anti-tubulin [1:10,000] for tau, phosphotyrosine, and tubulin, respectively.

Tubulin polymerization assay

Phospho-tau and unphosphorylated tau were prepared in the same manner described in microtubule binding experiments. Tubulin was prepared on ice in the same buffer as described in microtubule binding experiments. Kinase reaction products were added to tubulin to achieve a final tubulin concentration of 3 mg/mL. Kinase buffer with or without ATP, as well as recombinant Arg were included in control polymerization reactions. 100 μ L of each reaction mixture was added to a 96-well plate. Reaction mixtures were placed into a spectrophotometer at 37 °C. Tubulin polymerization was followed by measuring optical density at 340 nm every minute for 90 min.

Tau cDNA constructs

For mammalian expression of *tau*, cDNA for the longest isoform (2N4R) was transiently transfected. Additional tau cDNAs were constructed in which each of the five tyrosine residues were mutated to phenylalanine, as described [9]. The mutants cDNAs are referred to as TauY18F, TauY29F, TauY197F, TauY310F, and TauY394F.

Arg (Abl2) cDNA constructs

Co-transfection experiments with *arg* were performed using cDNA for isoform B, supplied in the pEBB vector, driven by the *EF1 α* promoter (gift from Dr. Bruce Mayer). A constitutively active *arg* construct was created by mutating two proline residues in the SH3-SH2 linker region to alanines (ArgP269A/P276A herein referred to as ArgPP). Construction of cDNA for expression of gleevec-resistant Arg was achieved by mutation of residue 361 from threonine to isoleucine (ArgT361I).

Cell culture and transfection

Transfection experiments were performed using a glioblastoma cell line (U138) expressing a 695 amino acid isoform of the amyloid- β precursor protein (A β PP695), herein referred to as U138/695. U138/695 was selected for ease of transfection of cDNAs and subsequent protein expression level. U138/695 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% non-essential amino acids. Stable expression of A β PP695 was maintained by G418 selection (50 μ g/mL, Invitrogen). Transfection of cDNAs was carried out at 90% confluency using 1 μ g of DNA per 30 mm well and 6 μ L of Lipofectamine-2000 (Invitrogen) in DMEM without supplements for 6 h. Cells were rinsed with ice cold TBS containing 1 mM sodium orthovanadate 48 h after transfection. Cell lysates were harvested in TBS-based homogenizing buffer (pH 7.4) containing 10 μ M NaF, 1 μ M sodium orthovanadate, 2 μ M EGTA, and Complete Mini protease inhibitor cocktail (Roche Diagnostics). Cells were scraped from the well, placed on ice for 10 min, homogenized with a Powergen 125 polytron (Fisher Scientific), centrifuged at 13,000 rpm for 10 min. Supernatants were collected and frozen at -80 °C or boiled for 5 min in Laemmli sample buffer for SDS-PAGE and subsequent immunoblotting. Immunoprecipitation was performed using 4G10-conjugated agarose beads as previously described, except 50 μ g of protein from total lysates was diluted in homogenizing buffer to a final volume of 400 μ L.

Heat-stable lysate preparation

In order to concentrate tau from total cell lysates, many of the normal protein constituents were removed by denaturation. In preparing heat-stable fractions, 5% β -mercaptoethanol and 200 mM NaCl were included in lysate solution. Lysates were then heated at 100 °C for 10 min, cooled at 4 °C for 30 min. Insoluble proteins were pelleted by centrifuging at 13,000 rpm in a tabletop microcentrifuge at 4 °C for 15 min. Supernatants were collected and Laemmli sample buffer added for SDS-PAGE and western blotting. Membranes were

probed with 4G10 [1:2000] and DA9 [1:500] for detection of phosphotyrosine and total tau, respectively.

Quantification of cellular tau phosphorylation

The sandwich ELISA described in our kinetic analysis was used to quantitatively assess tyrosine phosphorylation of tau following cotransfection of U138/695 cells with tau and Arg cDNAs. All aspects of procedure were performed in the same manner as previously described, except that total cell lysates were used in place of kinase reaction products. Tau phosphorylation was quantified by normalizing the phosphotyrosine signal to total tau signal. Statistical analysis was performed using Graphpad Prism 4.0 software.

Statistical analysis

All statistical analyses were performed using Graph-pad Prism 4.0 software. A p-value of 0.05 was used as a threshold for identifying significant differences between groups in all t-tests and ANOVA post-hoc testing. An unpaired t-test was used for comparison of YP4 labeling between normal and AD brain sections. All *in vitro* experiments were repeated a minimum of 3 separate times. Representative figures were selected. All quantitative analyses included at least 3 replicates per data point, as specified in figure legends. Michaelis-Menton kinetic constants were calculated for kinase reactions by fitting data to a curve with the equation: $Y = V_{max}(X)/K_m + X$. One-way ANOVA was performed for all subsequent *in vitro* studies presented with Bonferroni post-hoc tests for comparison between all pairings of sample groups.

RESULTS

With the knowledge that tau phosphorylation at Y394 had been observed in AD brain samples using mass spectrometry, an effort was made to develop tools to confirm the presence of tau phosphorylation at Y394 in AD [7–9]. To this end, hybridomas were generated from mice immunized with tau phosphopeptides. A monoclonal antibody (YP4) was generated that specifically recognizes a tau species that is duallyphosphorylated at Y394 and S396. Specificity of the antibody was confirmed by an ELISA analysis of YP4 binding to phospho-tau peptides (Fig. 1A). YP4 bound only to the dually-phosphorylated peptide. No binding was detected for individual phosphotyrosine sites (pY18, pY29, pY197, pY310, or pY394). More importantly, YP4 did not recognize the singly phosphorylated peptide containing phospho-S396, which is prevalent in AD, and is the pathologic antigen recognized by the PHF1 antibody [29].

The newly generated antibody provided useful information about the phosphorylation state of tau in the AD brain. Immunohistochemical staining of hippocampal sections using YP4 revealed that, while the duallyphosphorylated tau species is fairly abundant in AD, it is nearly undetectable in non-AD samples (Fig. 1B). While a few thread-like areas of staining in the entorhinal cortex were depicted by Fig. 1B, this illustrates the greatest density of staining observed in any control sample. In order to rule out a non-specific affinity of IgM immunoglobulins for tau aggregates, control (anti-LPS) mouse IgM staining of AD brains was performed with no labeling detected. YP4-labeled neurons were counted in AD ($n = 5$) and non-AD ($n = 3$) hippocampal sections (Fig. 1C). As expected, YP4 labeling of both CA1 and entorhinal regions was significantly greater in AD.

In addition, two patterns of YP4 staining were observed in AD hippocampi. Within many CA1 and entorhinal neurons, YP4 labeling appeared as obvious tangles, but some CA1 pyramidal neurons contained YP4-positive cytoplasmic granules (Fig. 1B+D). Even within a single hippocampal section, both patterns of staining could be found simultaneously, often

in adjacent neurons. Interestingly, granular staining appeared to correlate with milder pathology. YP4 labeling in the CA1 region of non-AD brains was present only as faint granular labeling, with nearly complete absence of tangles. However, granular YP4 staining was uncommon in severely diseased brains, despite being abundant in many AD cases, suggesting tyrosine phosphorylation as an early event in the disease process, preceding tangle formation. Specimens in which neuronal loss was most severe exhibited almost exclusively tangle labeling, with occasional labeling of neuritic plaques (Fig. 1B). Immunohistochemical staining with anti-phospho-tau (S202) antibody (CP13) revealed that YP4 labels some, but not all tangles (Fig. 1C). Also, granular YP4 staining did not localize as well to areas of CP13 labeling as YP4-positive tau present in tangles, again suggestive of different timing for Y394 and S202 phosphorylation. From the immunohistochemical data, it can be concluded that the duallyphosphorylated species of tau exists within neurons of affected AD brains, are specific to diseased neurons, and likely present early in tau pathology.

Having confirmed the presence of Y394-phosphorylated tau in tangles, and given reports that Abl was capable of tau phosphorylation, Arg seemed a likely candidate to phosphorylate tau at Y394. This hypothesis was initially tested by performing *in vitro* kinase reactions. Recombinant 2N4R tau and/or a control substrate, abltide-GST, were incubated with Arg in the presence of ATP for 30 minutes. Western blotting with anti-phosphotyrosine antibody (4G10) revealed substantial tyrosine phosphorylation at 64 kDa and 25 kDa, corresponding to tau and abltide-GST, respectively (Fig. 2A). Immunoprecipitation with 4G10 was performed following *in vitro* phosphorylation in an effort to ascertain what proportion of total tau was phosphorylated in the reaction. Immunoblots of the immunoprecipitated material suggested as much as 20–30% of the recombinant tau was phosphorylated in a one-hour reaction (Fig. 2B). It was difficult to get an accurate estimate, because 4G10 immunoprecipitation was not 100% efficient. Based on the presence of phospho-tau in unbound fractions, the proportion of phospho-tau was likely an underestimate. However, the data suggest that the kinase reaction was reasonably efficient, and led to the phosphorylation of a substantial proportion of the total tau protein.

Further analysis was required to better understand the kinetics of the reaction. An ELISA approach was used to evaluate the kinetics of Arg-mediated tau phosphorylation. A sandwich ELISA utilizing an anti-total tau antibody (DA9) for capturing tau and anti-phosphotyrosine (4G10) antisera for detection of phospho-tau was used to quantify phosphorylation in kinase reactions with varying concentrations of tau. The observed K_m value for tau as an Arg substrate was about 450 nM. Since previous reports estimated that the intraneuronal tau concentration is in the range of 5–10 μ M [30], this K_m value suggested that tau is likely to be a physiologically relevant Arg substrate (Fig. 2C).

Given the efficiency of Arg-mediated tau phosphorylation *in vitro*, the next objective was determining whether tyrosine phosphorylation altered the physiological interaction between tau and microtubules. First, experiments were conducted to determine whether Arg-mediated tyrosine phosphorylation altered the ability of tau to bind microtubules synthesized *in vitro*. Following incubation with microtubules and ultracentrifugation, most of the tau protein was found in the pellet, indicating that it was bound to microtubules, regardless of phosphorylation (Fig. 3A). A subsequent experiment examined whether Arg-mediated tyrosine phosphorylation altered the ability of tau to facilitate *in vitro* tubulin polymerization (Fig. 3B). Both phospho-tau and unphosphorylated tau demonstrated equal capacity to potentiate the process of tubulin polymerization. In addition, the rate of tubulin assembly also appeared to be nearly identical regardless of tau phosphorylation (Fig. 3C). Two-way ANOVA failed to demonstrate a significant effect of phosphorylation on tubulin assembly

rate. These data suggested that tyrosine phosphorylation alone may not significantly affect the role of tau in microtubule dynamics.

The next set of experiments sought to determine whether co-expression of Arg and tau in a cellular context resulted in substantial phosphorylation. To this end, glioblastoma cells (U138/695) expressing A β PP were transfected with a 2N4R human tau construct in addition to a constitutively active Arg construct (ArgPP). Heat-stable samples were prepared from total cell lysates in order to limit the number of phosphotyrosine bands seen in immunoblotting, thus increasing resolution (Fig. 4A). A phosphotyrosine band was detected at the approximate molecular weight of tau (64kD) in lysates from cells expressing tau with constitutively active Arg, while no such band was detected from lysates lacking active Arg. The identity of this phosphoprotein was confirmed by immunoprecipitation of cotransfected lysates with anti-phosphotyrosine antibody (4G10) and subsequent immunoblotting (Fig. 4B). Tau was detected in the immunoprecipitated material from lysates transfected with ArgPP. No tau signal was evident in 4G10 immunoprecipitated protein from lysates lacking Arg, despite higher levels of tau in the unbound fraction, suggesting that endogenous kinase activity was insufficient for tau phosphorylation at tyrosine sites.

Since tau phosphorylation by Abl was previously reported [9], a set of experiments was performed to determine whether Abl activity was necessary for Arg-mediated tau phosphorylation. Imatinib mesylate (Gleevec) has been reported as a relatively potent inhibitor of both Abl and Arg [31,32]. A gleevec-resistant Arg construct (ArgT361I) was made based on a homologous mutation in *BCR-abl*, known to confer gleevec resistance [33,34]. Both wild-type Arg (ArgWT) and ArgT361I were transfected alongside 2N4R Tau constructs in glioblastoma cells. Suppression of wild-type Arg and endogenous Abl activity was achieved by gleevec treatment at a dose of 5 μ M, which is ~10–20 times the reported IC₅₀ for cellular treatment [35]. Minimal reduction in phospho-tau was observed in treated cells expressing gleevec-resistant Arg (Fig. 4C). Quantitative analysis of phospho-tau from gleevec-treated samples was performed using a sandwich ELISA approach in which tau from total lysates was captured and phospho-tau was detected with 4G10 (Fig. 4D). Treatment with 5 μ M gleevec led to a significant reduction in tau phosphorylation by wild-type Arg (approximately 70%), but had almost no effect on tau phosphorylation resulting from expression of drug-resistant Arg. From this data, it can be concluded that Arg phosphorylates tau directly, without need for endogenous Abl activity.

Having concluded that Arg is capable of directly phosphorylating tau in a physiological context, the next objective was to determine which tyrosine sites Arg phosphorylates. For the purposes of phosphorylation site identification, Arg was co-transfected with tau constructs in which individual tyrosine residues had been mutated to phenylalanine (Y18F, Y29F, Y197F, Y310F, and Y394F). Immunoblots of the heat-stable preparations revealed a substantial decrease in phosphotyrosine signal when residues Y197, Y310, and Y394 were altered (Fig. 5A). In particular, mutation of the Y394 site nearly eliminated the phosphotyrosine signal, implicating this residue as the primary site of Arg-mediated phosphorylation. Changes in tau phosphorylation were quantified by performing the previously described phospho-tau ELISA on total lysates. As expected, significant reductions in phospho-tau were observed in the absence of Y197, Y310, and Y394 (Fig. 5B). In addition, the most prominent reduction in phospho-tau was observed when Y394 was mutated. In fact, tau phosphorylation in the absence of the Y394 site was similar to levels observed in the absence of active Arg kinase. Thus, it was concluded that Y394 is the primary site of Arg-mediated tau phosphorylation.

The observations of this investigation suggested that Arg should be the target of further research on tau pathology and AD pathogenesis. Tau was demonstrated as a physiologically

relevant Arg substrate. The primary tau phosphorylation site upon which Arg acted was tyrosine 394. The presence of Y394-phosphorylated tau was observed almost exclusively in affected neurons of AD brains. Taken together, these observations suggest a potential role for Arg in the pathogenesis of AD.

DISCUSSION

Our investigation identifies Arg as a tyrosine kinase capable of phosphorylating tau. More importantly, Arg primarily phosphorylates tau at Y394, a site reported here and in previous studies as present in AD pathology [7,8]. Our findings also provide additional support for tyrosine phosphorylation of Y394 as a marker of tau pathology. Previous to this report, Abl was the only tyrosine kinases known to phosphorylate this particular site [9].

While we can conclude from our investigation that Arg phosphorylates tau, and that tyrosine phosphorylation is present in tangle pathology, further investigation is required to ascertain the physiological consequences of Arg-mediated tau phosphorylation. Through a series of *in vitro* studies we were able to demonstrate that tyrosine phosphorylation has little effect on microtubule binding of tau, as well as the ability of tau to facilitate microtubule assembly. However, these observations cannot predict the additive effect of tyrosine phosphorylation on tau physiology in the presence of other physiological or pathological tau modifications. The possibility remains that tyrosine phosphorylation increases the propensity of tau to form aggregates and NFTs.

In a recent study, an activated form of Abl (phospho-AblY412) was found localized to granular structures within hippocampal pyramidal neurons of AD brains [36]. Based on morphological characteristics, the authors of the study believe the granular pattern to represent the granulovacuolar degeneration bodies (GVDs) often seen in AD. The granular staining pattern of YP4 in CA1 pyramidal neurons bears a strong resemblance to the pattern seen with phospho-AblY412, suggesting that tyrosine-phosphorylated tau may be sequestered within GVDs in early stages of AD. In our experience, antibodies against phospho-AblY412 do not discriminate between Abl and Arg, because of the extensive homology between these related kinases, particularly in the catalytic domain (unpublished observation). Thus the combination of these findings lends support to the role for Abl family kinases in AD pathogenesis. However, further research is necessary to pinpoint a particular family member as the culprit.

Given the findings of this study, and the observations of many previous investigations we suggest that Abl and/or Arg may play a role in the pathogenesis of AD. Unfortunately, most of the previous research linking Abl family kinases to AD-related processes has focused only on Abl. One connection between Abl kinases and AD pathogenesis is the neuronal response to A β . Multiple studies have observed an activation of tyrosine kinases resulting from neuronal A β treatment, as mentioned previously [10–12,37]. In only two reports did investigators specifically examine the role of Abl kinases in A β toxicity [10,37]. In these studies, inhibition of Abl kinase activity by gleevec or RNA interference was effective in preventing tyrosine phosphorylation and cell death. Src kinases were implicated in other investigations of A β -induced tyrosine phosphorylation and neuronal toxicity [11,12]. However, both studies involved treatment with Src inhibitors PP1 or PP2 at concentrations greater than 30 times the IC₅₀ for Abl, suggesting that the effects of treatment may have been due to inhibition of a number of tyrosine kinases, including Arg. While none of the investigations mentioned specifically address the role of Arg, compounds that inhibit Abl, particularly gleevec, also inhibit Arg at similar concentration [32,38].

A sparse collection of literature also hints at a role for Abl family kinases in regulating the physiology of A β PP. Abl interacts with A β PP and Fe65, an A β PP-interacting protein that associates with the C-terminal domain of A β PP [39,40]. Investigators first reported that Abl interacts with Fe65, phosphorylates A β PP at tyrosine residue 682 (A β PP695), and is capable of forming a stable complex with A β PP following phosphorylation [40]. A later study found that Abl phosphorylates Fe65 at tyrosine residue 547 and is capable of enhancing nuclear signaling and transcription associated with Fe65-A β PP interaction [39]. In an investigation of A β PP metabolism, researchers observed inhibition of γ -secretase mediated A β PP cleavage in a cell-free system, as well as in N2a cells in the presence of gleevec [41]. Interestingly, while gleevec was capable of inhibiting cleavage of A β PP, it did not affect Notch cleavage. The inhibitory effect could not be attributed to Abl activity, because A β PP cleavage was inhibited by gleevec in *abl*^{-/-} fibroblasts. However, as stated by the authors of that study, this finding does not preclude a role for Arg, which is also effectively inhibited by gleevec treatment [41].

For reasons ranging from oxidation of protein, DNA, and lipids to altered levels of antioxidant enzymes in aging and demented brains, researchers have long postulated a role for oxidative stress in the etiology of AD [42]. The role of Arg in the oxidative stress response serves as another potential connection between Arg and AD pathogenesis. Abl was the first of the Abl family kinases to be implicated in mediating an apoptotic response to oxidative stress [43]. Arg was later implicated when it was found to phosphorylate Siva-1 in response to hydrogen peroxide treatment, which subsequently increased levels of apoptosis [27]. Since then, it has also been discovered that Arg is involved in regulating other proteins involved in oxidative stress response, including glutathione peroxidase 1 and catalase [22,23,25]. In addition, Abl and Arg interact with one another, with Abl phosphorylating Arg in response to oxidative stress [24]. In a later study, the same group found that oxidative stress appears to regulate Arg activity in response to different doses of reactive oxygen species [26]. Abl kinases may represent a cellular switch involved in deciding whether to increase the physiologic response to oxidative stress or initiate apoptosis.

It is obvious from the findings of this study and the observations of previous research that Arg has the potential to connect a number of seemingly unrelated hypotheses about the mechanisms underlying the neurodegeneration responsible for AD. Arg is involved in oxidative stress, tau phosphorylation, and may be involved in the metabolism of A β PP and neuronal response to A β . Further investigation is required to determine whether Arg is capable of causing pathologic changes in AD-relevant brain regions.

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Fig. 1.

Dual phosphorylation of tau at Y394 and S396 is present in AD pathology. A) Monoclonal anti-phospho-tau (Y394/S396) antibody specificity was tested by ELISA assessing binding to synthetic phosphopeptides. B) Immunohistochemical staining of hippocampal sections from age-matched AD and non-AD brains using an antibody specific to tau species phosphorylated at both Y394 and S396 (YP4) (scale bars = 100 μm). YP4 labeling was very specific to AD neurons, and found mostly in tangles. However, some labeling of cytoplasmic granules (arrowhead) and neuritic plaques (arrow) was observed. Control staining was performed on AD sections using anti-LPS mouse IgM. C) Histogram of YP4-positive neurons in the CA1 region and entorhinal cortex of AD ($n = 5$) and non-AD ($n = 3$) brains (All values expressed as means $\pm$ SEM; *** indicates $p < 0.001$, unpaired t-test). D) Confocal micrographs of YP4 (green) and anti-phosphoserine 202 tau antibody (CP13) (red) in the CA1 region of AD hippocampus, demonstrating two patterns of labeling: tangles and granular cytoplasmic staining.

Fig. 2.

Arg phosphorylates tau *in vitro*. Kinase reactions were performed using purified recombinant 2N4R tau, purified recombinant Arg and ATP. Abltide-GST was included in some reactions as a positive control for Arg kinase activity. A) Immunoblotting of kinase reaction product with anti-phosphotyrosine antibody (4G10) revealed substantial phospho-tau, 64 kDa band. B) Immunoblots of tau from kinase reactions with ATP (p-Tau) and without ATP (Tau) following immunoprecipitation with 4G10 revealed phosphorylation of a substantial proportion of the total tau. C) The Michaelis-Menton kinetics of the kinase reaction were measured using a sandwich ELISA approach to quantify phospho-tau from reactions with varying tau concentrations. A K_m for tau of ~450 nM was calculated by non-linear regression using Graphpad Prism 4.0 (All values expressed as mean $\pm$ SEM; $n = 3$ for each data point).

Fig. 3.

Arg-mediated tyrosine phosphorylation has little or no effect on tau physiology *in vitro*. Recombinant tau was incubated with recombinant Arg in a one-hour *in vitro* kinase reaction with ATP (Tau) and without ATP (p-Tau) before examining effects of tyrosine-phosphorylated tau on microtubule binding and tubulin polymerization *in vitro*. A) Kinase reaction products were assessed for microtubule-binding by ultracentrifugation. Immunoblots of supernatant (S) and pellet fractions (P) revealed similar proportions of tau in the pellet fraction, regardless of tyrosine phosphorylation. B) *In vitro* microtubule assembly using recombinant tubulin and GTP was analyzed by measuring optical density at 340 nm over time with and without ATP, Arg, and kinase reaction products. The effect of tau was assessed using products of kinase reactions with (phospho-Tau) and without (Tau) ATP present (bars represent mean $\pm$ SEM; $n = 4$ per group). C) Histogram demonstrating rate of tubulin polymerization between 20 and 40 minutes under the previously described conditions (bars represent mean $\pm$ SEM; $n = 4$ per group). Statistical analysis included one-way ANOVA with post-hoc test for comparison between groups with and without ATP. No significant phosphorylation effect was detected.

Fig. 4.

Arg phosphorylates Tau when co-expressed in cells. Glioblastoma cells were transiently transfected with Tau (2N4R) and constitutively active Arg (ArgPP). **A)** Heat-stable samples were prepared from total lysates for immunoblotting with anti-phosphotyrosine (4G10) and anti-tau antibodies. Total lysates were blotted with anti-Arg (AR11) and anti-β-tubulin as controls for kinase expression and loading, respectively. **B)** Phosphotyrosine immunoprecipitation was performed on total lysates from Tau transfected glioblastoma cells with and without co-transfection of a constitutively active Arg construct (ArgPP). Anti-tau immunoblots developed using enhanced chemiluminescence (ECL) and 4-chloronaphthol (4-CN) demonstrated tau in the 4G10-bound fraction only when active Arg was present. **C)** Cells were co-transfected with Tau (2N4R) and either wild-type (Arg-WT) or gleevec-resistant (ArgT361I) constructs. Following transfection, cells were incubated in normal DMEM (U) or DMEM containing 5 μM gleevec (T). Heat-stable samples were prepared from total cell lysates for immunoblotting with 4G10 to assess phospho-tau levels. **D)** Phospho-tau was quantified from total cell lysates from the above stated conditions by a sandwich ELISA (values expressed as mean ± SEM, ***indicates significant difference; $p < 0.001$, $n = 6$ per group). Statistical analysis consisted of one-way ANOVA with post-hoc test comparing each pair of groups.

Fig. 5.

Arg phosphorylates tau at three residues: Y197, Y310, and Y394. Cells were co-transfected with wild-type Arg and Tau constructs in which individual tyrosine residues were mutated to phenylalanine (Y-to-F). A) Immunoblots of heat-stable preparations were used to examine the levels of phospho-tau (4G10) present in each sample relative to total tau. Total lysates were blotted with anti-Arg (AR11) and anti- β -tubulin for kinase expression and loading controls, respectively. B) Quantification of phospho-tau by sandwich ELISA using total lysates indicated that reductions in phosphorylation in the absence of Y197, Y310, and Y394 were all significant (Values expressed as mean $\pm$ SEM, ***indicates significant difference; $p < 0.001$, $n = 4$ per group). Statistical analysis consisted of one-way ANOVA with post-hoc tests comparing levels of phospho-tau with wild-type transfection to each mutated construct.