Ser-262 in human recombinant tau protein is a markedly more favorable site for phosphorylation by CaMKII than PKA or PhK

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Abstract Several kinases have been shown to phosphorylate tau protein at Ser-262, an important site involved in the regulation of the binding of tau to microtubules. In this study we compared the phosphorylation of tau at Ser-262 by CaMKII, PhK and PKA in vitro as determined by radioimmunoblots developed by the monoclonal antibody 12E8 which recognizes P-Ser-262 and P-Ser-356; and Ab-262, a polyclonal antibody which is specific to unphosphorylated Ser-262 in tau. We found that the phosphorylation at Ser-262 was several times more effective by CaMKII than PKA or PhK. Employing rat brain extract as a source of all brain kinases and KN-62, a specific inhibitor of CaMKII, we found that CaMKII accounts for ~45% of phosphorylation at Ser-262. Furthermore, in rat brain slices kept metabolically active in oxygenated artificial CSF, phosphorylation of tau at Ser-262 was (i) increased up to 120% in the presence of bradykinin, a CaMKII activator, and (ii) inhibited by ~35% in the presence of KN-62. Thus, CaMKII is a major tau Ser-262 kinase in mammalian brain.

Key words: Calcium calmodulin kinase II; Protein kinase A; Phosphorylase kinase; Ser-262; Tau phosphorylation; Alzheimer's disease

1. Introduction

In Alzheimer disease (AD) the most characteristic neuropathological lesion is the accumulation of paired helical filaments (PHFs). These filaments are formed of tau, a neuronal-specific microtubule-associated protein [1]. Tau plays an important role in neuronal morphogenesis, the maintenance of axonal shape, and axonal transport through its ability to bind and regulate microtubule structure and dynamics [2,3]. There are six isoforms of human tau which are generated from a single gene by alternative mRNA splicing [4]. These isoforms differ from each other by the presence of three or four tandem repeats of 31–32 amino acids and no, one or two inserts of 29 amino acids each near the N-terminal. The tubulin binding repeats are rich in positively charged residues and are thought to interact with the negatively charged carboxy-terminal region of tubulin exposed on the microtubule structure, resulting in a tau-microtubule binding [3,5].

Phosphorylation of tau greatly reduces its affinity for microtubules [6–9]. Fetal tau, which is more phosphorylated than normal tau, can promote microtubule assembly but PHF-tau which also is abnormally hyperphosphorylated, cannot [10–13]. Dephosphorylation of PHF-tau restores its ability to promote the in vitro microtubule assembly and tubulin binding, suggesting that phosphorylation plays a key role in the regulation of binding of tau to microtubules. Fetal tau and PHF-tau are phosphorylated at 11 common sites. Of the additional 10 sites found only in PHF-tau, one is Ser-262. The phosphorylation of tau at this site is believed to be primarily responsible for decreasing the affinity of tau for microtubules [8,9]. Ser-262 is the only site located within the microtubule binding repeats of tau. Inhibition of assembly and or binding to microtubules is observed after tau is phosphorylated at Ser-262 by calcium calmodulin kinase II (CaMKII) [6,14], protein kinase A (PKA) [15,16], $MARK^{110}$ kinase [9,17], 100 kDa protein kinase [18,19], glycogen synthase kinase 3 [20], casein kinase 1 [20] and protein kinase C [20]. Recently it has been shown that phosphorylase kinase (PhK) can phosphorylate tau in a Ca^{2+}-dependent manner on five sites including Ser-262 [21]. However, to date, no studies have been reported in which the substrate affinity for this site by these three kinases have been compared quantitatively. In this study we have examined (i) the abilities of three kinases, PKA, CaMKII and PhK, to phosphorylate Ser-262; and (ii) the contribution of CaMKII in the phosphorylation of tau at Ser-262 in brain. We found (i) that of the three kinases investigated, CaMKII is most effective in phosphorylating Ser-262; and (ii) that at least one third of all phosphorylation of tau at Ser-262 in brain is carried out by CaMKII.

2. Materials and methods

2.1. Reagents

The human tau clone 39 (kindly supplied by Dr. Michal Goedert, MRC, Molecular Biology Labs, Cambridge, UK) which encodes for tau isoform 3L, tau_{410} [4], was subcloned in *Escherichia coli* and purified from cell extracts as described previously [22]. CaMKII was purified from rat brain by the method of Ohmstede et al. [23]. PhK from rabbit muscle was purified according to Cohen [24], PKA was purchased from Sigma (St. Louis, MO). The monoclonal antibody 12E8 which recognizes tau phosphorylated at Ser-262 and or Ser356 [25] was a generous gift from Dr. Dale Schenk of Athena Neurosciences (San Francisco, CA) and was used at a 1–2 μg/ml dilution; the rabbit antibody AbSer-262 was generated and affinity purified [26] and used 1:100 diluted in dot-blot assays; the monoclonal antibody Tau-1 that recognizes tau unphosphorylated at Ser-195/198/199/202 [10,27,28] was a gift from Dr. Lester Binder of Northwestern University, Chicago, IL and was used at 1:10 000 dilution for assaying total tau in radioimmunodot-blots pretreated with alkaline phosphatase (226 U/ml; Sigma, St. Louis, MO) for 6 h at 37°C. KN-62 which specifically inhibits CaMKII [29] at K_i 0.9 μM and bradykinin which specifically activates CaMKII [30] at 1 μM concentration were purchased from Sigma (St. Louis, MO).

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2.2. Phosphorylation of tau$_{410}$ by isolated protein kinases

Tau$_{410}$ was phosphorylated at 30°C for 20 min by different kinases (at concentrations indicated in each experiment) in reaction mixtures, unless otherwise indicated, containing 0.12 mg/ml tau, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.25 mM ATP and 40 mM HEPES (pH 6.8 or 7.5) as described previously [22,31]. In addition, 40 μg/ml calmodulin and 0.4 mM $CaCl_2$ were included in reaction mixtures catalyzed by CaMKII and 0.4 mM $CaCl_2$ for PhK. Reactions were initiated by addition of [γ-^{32}P]ATP (500–1000 cpm/pmol) and after 20 min of incubation, aliquots of the reaction mixtures were removed, spotted on phosphocellulose filter paper and processed as described previously [32].

2.3. Phosphorylation of tau$_{410}$ by brain extract

Rat brain extract was prepared by homogenizing the tissue in 1 volume of 100 mM Tris pH 8.6, 2 mM EGTA, 2 mM EDTA, 100 mM NaF, 1 mM PMSF, 2 mM benzamidine, 1 mM Na_3VO_4, 2 mM DTT, 0.06% Brij-35, and a cocktail of protease inhibitors (leupeptin, aprotinin, pepstatin A at 5 μg/ml each one) and centrifuged at 100 000×*g* for 30 min at 4°C. The supernatant was used for the phosphorylation assay in a ratio 1:30 of the reaction mixture as described above (Section 2.2) and contained 10 μM okadaic acid. In the phosphorylation experiments in which the CaMKII activity was to be inhibited, the brain extract was first incubated with 10 μM of KN-62 (to inhibit CaMKII) for 10 min at room temperature and then employed for the phosphorylation reaction as described above (Section 2.2) for 10 min.

2.4. Phosphorylation in rat brain slices

Rats, 7–15 day old, were killed and the brains were rapidly dissected and cooled in artificial CSF (aCSF) containing 126 mM NaCl, 3.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose and 25 mM $NaHCO_3$ at pH 7.4, and washed two times with the same buffer. The total brain was sliced into prisms (300×300 μm) using a McIlwain tissue chopper. Slices were dispersed in 20 ml of gassed (95% O_2/5% CO_2) artificial CSF at 37°C, and incubated in the presence of 1 μM bradykinin, 10 μM KN-62 or as control 1 μl/ml DMSO. After different periods of time slices were removed and washed two times and homogenized in 10 volumes of buffer containing 50 mM HEPES pH 7.5, 25 mM β-glycerophosphate, 10 mM BME, 10 mM NaF, 2 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 mM Na_3VO_4, and a cocktail of protease inhibitors (see above, Section 2.2). The homogenate was centrifuged 30 min at 100 000×*g*. The supernatant was heat treated for 10 min at 95°C and centrifuged again 30 min at 100 000×*g*. The pellet was discarded and the supernatant was dried in a Speed-Vac (Savant) and resuspended in an adequate volume and applied to nitrocellulose membrane for quantification by radioimmunoblot, as described below.

2.5. Quantitation of total tau and phosphorylation at Ser-262 by radioimmunodot-blot assay

Levels of site-specific phosphorylation on tau were determined by radioimmunodot-blot assay p [33] with two different antibodies, 12E8 and AbSer-262 [25,26]; in the same samples the total tau levels were determined by first dephosphorylating tau on the blots with alkaline phosphatase and then developing the blots with Tau-1 antibody (see Section 2.1). In the incubation system [γ-^{32}P]ATP was omitted and after different times of incubation at 30°C aliquots were removed and heated for 5 min at 95°C and centrifuged. Supernatants were diluted and applied to a nitrocellulose membrane, dried 30 min at 30°C and blocked 1 h in 5% BSA Tris saline buffer. Then the membrane was incubated overnight with the specific primary antibody and after that 2 h with the suitable secondary antibody labeled with ^{125}I. The polyclonal antibody AbSer-262 is more reactive with unphosphorylated tau than tau phosphorylated at Ser-262 and this antibody is a useful reagent for studying tau phosphorylation at the Ser-262 residue [26]. We used this antibody to determine the specific phosphorylation at this residue at 1:100 dilution. A computerized Fuji Bas-1500 video imaging system was used to scan and quantify the radioimmunoblots. At similar times other aliquots were removed from the incubation system, mixed with 1 volume of sample buffer and run in a 10% SDS polyacrylamide gel, transferred to Immobilon-P membranes and blots developed with 12E8 antibody. The ratio of the immunoreactivity of 12E8:Tau-1 (dephosphorylated blots) represented the phosphorylation of tau at Ser-262/356 per unit tau.

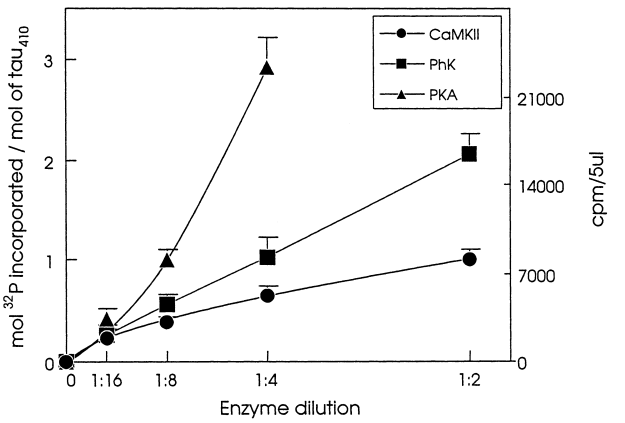

Fig. 1. Incorporation of γ-^{32}P in tau$_{410}$ by different concentrations of CaMKII, PhK and PKA preparations. Tau$_{410}$ was separately phosphorylated either by CaMKII (●), PhK (■) or PKA (▲) at the indicated dilutions. After 20 min at 37°C aliquots of the reaction mixtures were removed and γ-^{32}P incorporation was quantitated as described in Section 2.

3. Results

3.1. Phosphorylation of tau by different amounts of kinase

To compare the phosphorylation of tau at Ser-262 by various protein kinases, equal amounts of enzyme activity of each kinase must be used, because different enzymatic preparations have different specific activities. Hence, to determine the amount of each kinase that can incorporate 1 mol of γ-^{32}P per mol of tau in 20 min we phosphorylated the same amount of tau with increasing amounts of each kinase (Fig. 1). The CaMKII preparation had a low specific activity and we used a dilution of 1:2 to have an incorporation of about 1 mol γ-^{32}P/mol of tau. PhK showed a linear incorporation up to at least 2 mol of phosphate when it was used at 1:2 dilution. PKA preparation had a very high specific activity and a dilution 1:8 was able to incorporate 1 mol of γ-^{32}P/mol of tau$_{410}$ in 20 min. An assay without tau was used as a control to account for the autophosphorylation of the kinases. We defined 1 unit of each kinase as the amount of kinase able to incorporate 1 mol of γ-^{32}P/mol of tau$_{410}$ in 20 min at 30°C. We found that 1 unit of kinase activity corresponded to 10 μg/ml of PKA and 500 μg/ml of PhK and CaMKII.

3.2. Total phosphorylation of tau by CaMKII, PKA and PhK

We determined the total ^{32}P incorporation/phosphorylation of tau$_{410}$ by incubation in a reaction mixture containing [γ-^{32}P]ATP and 1 unit of CaMKII, PhK or PKA. At different times 5 μl aliquots from the incubation mixture were removed and spotted on a phosphocellulose membrane as described above. The total incorporation of ^{32}P to tau was quantitated and the result is presented in Fig. 2. The phosphorylation was similar for the three kinases until 20 min of incubation at 30°C, and another 80 min after that time PhK and PKA incorporated ∼20% more phosphate in tau than CaMKII and all the three enzyme reactivities were at a plateau. These results show that by 20 min all of the three kinases can incorporate the same amount of ^{32}P per mol of tau$_{410}$, but overall CaMKII is the least favorable of the three enzymes for phosphorylation of tau$_{410}$.

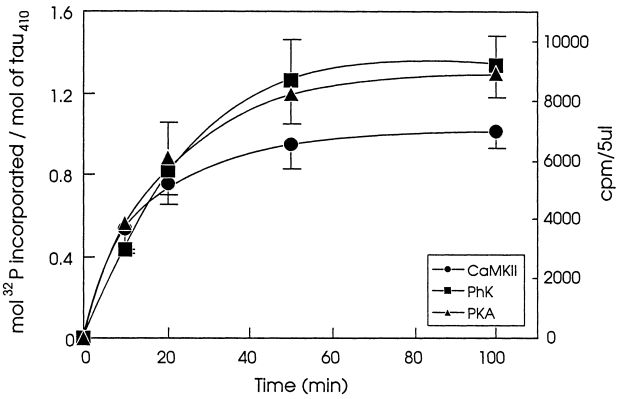

Fig. 2. Phosphorylation of tau_{410} by different kinases. Tau_{410} was incubated either with CaMKII (500 μg/ml), PhK (500 μg/ml) or PKA (10 μg/ml). Aliquots of the reaction mixtures were removed at different times and γ-^{32}P incorporation was quantitated. Controls without tau were subtracted from all values.

3.3. Phosphorylation of tau at Ser-262

The specific phosphorylation of tau at Ser-262 was determined by using two different antibodies. The monoclonal antibody 12E8, which binds to tau phosphorylated at Ser-262 and/or Ser-356 [25], and the polyclonal antibody AbSer-262 which binds to tau only when it is unphosphorylated at Ser-262 [26].

To evaluate which kinase is kinetically most favorable to phosphorylate Ser-262 and/or Ser-356, we carried out the time kinetics of phosphorylation of these sites by the different kinases and quantitated the phosphorylation by radioimmunodot-blot developed with these two antibodies (Fig. 3). Fig. 3A shows the plot of time-dependent phosphorylation of tau_{410}, developed by 12E8 antibody. The increase of immunoreactivity in the samples treated with CaMKII was several fold higher than the samples treated with the other two kinases during 20 min. Western blots of tau_{410} incubated at different times with each kinase also showed a clear increase of immunoreactivity for 12E8 antibody in the samples treated by CaMKII (Fig. 4). Quantitation of the same samples by immunodot-blots developed with AbSer-262 antibody showed a higher decrease of immunoreactivity for the samples treated with CaMKII than the samples treated with PKA or PhK (Fig. 3B). Thus, like 12E8 antibody, the AbSer-262 antibody also showed that Ser-262 is a several fold more preferable site for phosphorylation by CaMKII than PKA or PhK.

3.4. Contribution of CaMKII in the phosphorylation of tau in brain

To evaluate quantitatively the role of CaMKII in phosphorylation of tau at Ser-262 in brain tissue, we investigated (i) the phosphorylation of tau_{410} in vitro using rat brain extract as a source of all kinases in the presence or absence of KN-62, a specific inhibitor of CaMKII; and (ii) the phosphorylation of tau at Ser-262 in the presence or absence of KN-62 (inhibitor) or bradykinin (activator) using brain slices kept metabolically active in oxygenated aCSF.

Tau_{410} incubated with rat brain extract for 10 min at 32°C under phosphorylation conditions containing [γ-^{32}P]ATP showed only 18% inhibition in the presence of 10 μM KN-62 as compared with the absence of this inhibitor used as a control (100%). However, the specific phosphorylation of tau at Ser-262/356 during the 10 min of incubation as determined

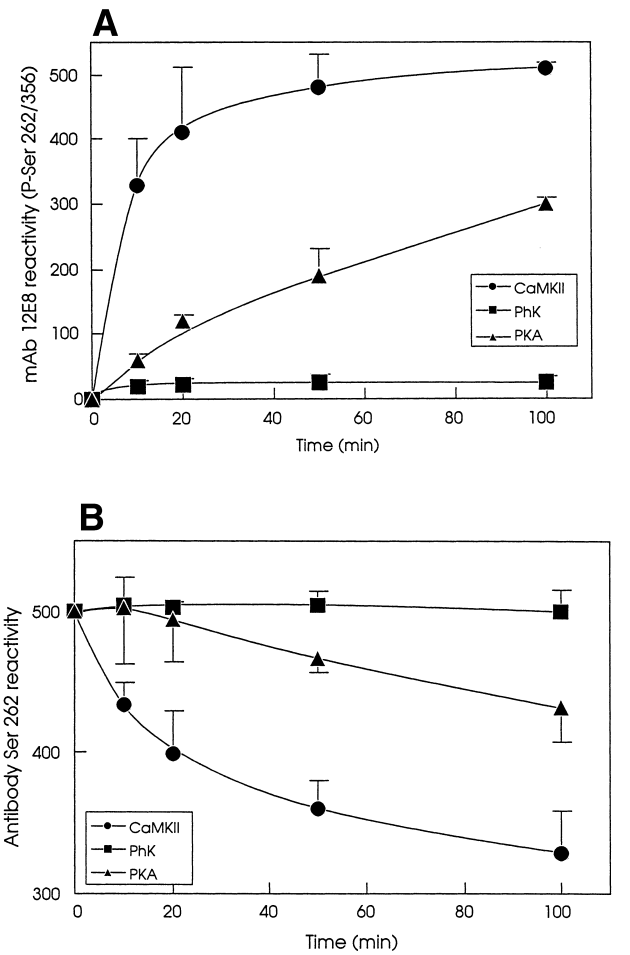

Fig. 3. A: Kinetics of phosphorylation of Ser-262/356 by different kinases. Radioimmunodot-blot for tau phosphorylated separately by CaMKII (●), PhK (■) or PKA (▲), developed by 12E8 antibody were quantified by phosphorimaging analysis, the background for 0 time was subtracted from all values. B: Kinetics of phosphorylation of Ser-262 by different kinases. Radioimmunodot-blots for tau phosphorylated separately by CaMKII (●), PhK (■) or PKA (▲) developed by AbSer-262 were quantified by phosphorimaging analysis, the value for 0 time was expressed as 500 arbitrary units.

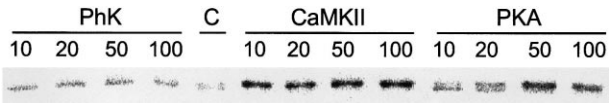

Fig. 4. Western blots showing 12E8 immunoreactivity of tau after phosphorylation by several kinases. Tau was incubated at 30°C in the presence of PhK, CaMKII, PKA and without enzyme (C). Aliquots of the reaction mixtures were removed at different times (10, 20, 50 and 100 min), subjected to SDS-PAGE, transferred to Immobilon membrane and the blots developed by 12E8 antibody.

by radioimmunoblot with 12E8 antibody was inhibited by 45% in the presence of KN-62. These data suggested that almost half of phosphorylation of tau at Ser-262 by brain kinases is caused by CaMKII.

In brain slices incubated in aCSF in the presence of 1 μM bradykinin to stimulate the brain CaMKII activity the 12E8 activity/unit tau (Tau-1 dephosphorylated) increased by 60% as compared to the control (no bradykinin) in 1 h, 120% in 2 h and then remained constant until 4 h. When the phosphorylation was studied in the presence of KN-62, the 12E8 activity per unit of tau (Tau-1 dephosphorylated) decreased by 35% in 4 h in the brain slices. Thus, the studies in the brain slices, a model of in vivo studies, confirmed that CaMKII is a major Ser-262 kinase which is responsible for at least one third of total Ser-262 phosphorylation in tau.

4. Discussion

Identification of the protein kinases that may phosphorylate tau at Ser-262 is important since this site, the only one in the tubulin binding repeat domains, is believed to play a critical role in the binding of tau to tubulin, and consequently the stimulation of assembly and the maintenance of microtubule structure. The present study is the first (i) to compare the ability of PhK to phosphorylate Ser-262 with the ability of CaMKII and PKA to phosphorylate this site in unphosphorylated recombinant tau; and (ii) to show quantitatively the contribution of CaMKII in the phosphorylation of tau at Ser-262 in mammalian brain. Using two different phospho-dependent antibodies we have found that over a 20 min period tau phosphorylated with any of these three kinases at similar stoichiometry of phosphate incorporated per mol of tau, CaMKII could incorporate ~4-fold more phosphate than PKA and ~10-fold more than PhK in Ser-262. These differences in the phosphorylation levels persist over 100 min of the reaction, though are reduced between CaMKII and PKA due to the non-plateauing of the PKA-induced phosphorylation, and increased between CaMKII and PhK due to the earlier plateauing of the PhK-induced phosphorylation. On the other hand even when the total phosphorylation in tau by PhK was similar or higher than CaMKII, the specific phosphate incorporation at Ser-262, as determined by 12E8 or AbSer-262 antibodies, was very low.

In a previous study phosphorylation of Ser-262 was reported in tau in which 2.1 mol of phosphate per mol of the protein was incorporated over a period of 6 h [21]. However, the phosphorylation and dephosphorylation are relatively rapid events and are in a dynamic equilibrium in the cell. Thus, it is of considerable importance that when identifying protein kinases that might be involved in phosphorylating a protein the phosphorylation reaction in vitro be carried out over minutes and not hours. Over long periods of time kinetically unfavorable sites become non-specifically phosphorylated. Thus, it is likely that the phosphorylation of Ser-262 by PhK in the previous study represented low levels of phosphorylation continued over a period of 6 h [21]. In the present study we employed the amount of kinases that incorporated 1 mol of phosphate/mol of tau during 20 min. The substrate used in the previous study [21] was tau_{441} (τ4L) whereas we employed tau_{410} (τ3L). Singh et al. [34,35] have shown that when two N-terminal inserts are present in tau structures (tau_{410}), then three repeat tau (tau_{410}) is phosphorylated to a higher extent than four repeat tau (tau_{441}) by CaMKII and C-kinase, but other kinases (CK1 and CK3) phosphorylated to the same extent both tau isoforms. No studies have shown the different affinities of the three or four repeat tau toward PhK. It is possible that PhK has more affinity for Ser-262 in four than three repeat tau.

Employing KN-62, a specific inhibitor of CaMKII activity, we found in studies using brain extract (in vitro) and as well as in studies in brain slices (in vivo model) that this protein kinase is responsible for at least one third of total Ser-262 phosphorylation of tau in rat brain. Stimulation of CaMKII by bradykinin in rat brain slices demonstrated that the phosphorylation of tau at Ser-262 is more than doubled (120%). Thus, CaMKII is likely to be a major tau Ser-262 kinase in brain. $MARK^{110}$ has been reported to in vitro phosphorylate tau Ser-262 as a preferred substrate [9]. However, to date, the contribution of this kinase in the phosphorylation of tau Ser-262 in mammalian brain has not been shown.

In conclusion, the present study shows (i) that of the three kinases examined, CaMKII is most effective in the phosphorylation of tau at Ser-262; and (ii) that this enzyme is a major tau Ser-262 kinase in mammalian brain. Previously, we have observed that the phosphorylation of Ser-262 is catalyzed by CaMKII ≫ C-kinase ≫ GSK-3 ~ A-kinase ≫ CK-1 [20]. The abnormally hyperphosphorylated tau in Alzheimer disease is phosphorylated at Ser-262 and phosphorylation at this site inhibits tau's biological activity in binding to microtubules. The present study suggests that CaMKII deserves serious consideration as one of the kinases involved in the disease.

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