CK1δ-peptides modulating metabolism of APP

Subjects: Neurosciences

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Alzheimer's disease (AD) is the major cause of dementia, and affected individuals suffer from severe cognitive, mental, and functional impairment. Histologically, AD brains are basically characterized by the presence of amyloid plaques and neurofibrillary tangles. Previous reports demonstrated that protein kinase **CK1δ influences the metabolism of amyloid precursor protein (APP)** by inducing the generation of amyloid-β (Aβ), finally contributing to the formation of amyloid plaques and neuronal cell death. **CK1δ as a promising therapeutic target** and suggested an innovative strategy for the treatment of AD based on peptide therapeutics specifically modulating the interaction between CK1δ and APP. Initially, **CK1δ-derived peptides** manipulating the interactions between CK1δ and APP695 were identified by interaction and phosphorylation analysis *in vitro*. Selected peptides subsequently proved their potential to penetrate cells without inducing cytotoxic effects. Finally, for at least two of the tested CK1δ-derived peptides, a reduction in Aβ levels and amyloid plaque formation could be successfully demonstrated in a complex cell culture model for AD. Consequently, the presented results provide new insights into the interactions of CK1δ and APP695 while also serving as a promising starting point for further development of **novel and highly innovative pharmacological tools for the treatment of AD**.

Keywords: Alzheimer's disease ; AD ; casein kinase 1 ; CK1 ; amyloid precursor protein ; APP ; therapeutic peptide ; protein-peptide-interaction ; amyloid-β ; Aβ

1. Background

Globally, approximately 50 million people are affected by dementia and due to a dramatically rising population and an increasing life expectancy, the number of people affected by dementia is expected to increase to 152 million people by 2050. With around two thirds of all cases, Alzheimer's disease (AD) is the most common form of dementia [1]. AD is a progressive neurodegenerative disease characterized by irreversible morphological and biochemical changes in certain brain areas, in particular in the cortex and the hippocampus, which is linked to loss of cognitive function (reviewed in [2][3]). From a histological point of view, AD is mainly characterized by three pathological hallmarks including neuronal loss, extracellular amyloid plaques consisting of aggregated amyloid-β (Aβ) peptides, as well as the intracellular formation of neurofibrillary tangles composed of a hyperphosphorylated form of the microtubule-associated protein tau [2][3][4][5]. According to the amyloid cascade hypothesis, deficiencies in the processes related to production, accumulation, and disposal of Aβ are the primary cause of AD [6]. It is also believed that Aβ supports an increase in tau-phosphorylation and initiates multiple pathways leading to neuronal cell death, since Aβ oligomers show neurotoxic effects including the disruption of Ca^{2+} homeostasis, the induction of oxidative stress, excitotoxicity, inflammation, and apoptosis [2][6][7].

Aβ peptides are generated by proteolytic cleavage of the amyloid precursor protein (APP), a type-I single transmembrane protein [8][9]. APP splice variants can be found in many different tissues, whereas variant APP695 is predominantly expressed in neurons in the central nervous system. Although the physiologic function of APP remains elusive, an involvement in cell adhesion, regulation of cell–cell or cell–matrix interactions, or neuronal differentiation is under discussion [7][8][10][11]. Proteolytic cleavage of APP can either be non-amyloidogenic, resulting in generation of the neuroprotective product soluble APPα as a result of α-secretase cleavage, or amyloidogenic, leading to the formation of Aβ monomers due to cleavage mediated by β- and γ-secretase. These Aβ monomers can further aggregate into oligomers, protofibrils, and fibrils, consequently resulting in the characteristic accumulation of amyloid plaques in brain parenchyma of AD patients [10][11][12].

Increasing evidence suggests that protein phosphorylation plays a key role in mediating AD-associated pathological events. APP is phosphorylated by protein kinases at several sites, which likely affect Aβ production, for example, by modulating generation of the toxic peptide $Aβ_{1–42}$ by β-secretase-mediated cleavage [8][13]. Several phosphorylation sites have been identified on APP that can be associated with AD. Eight potential phosphorylation sites were found in the APP cytoplasmic domain, seven of which were phosphorylated in AD brains [14]. Threonine residue 668 (T668) is phosphorylated *in vivo* by protein kinases glycogen synthase kinase-3β (GSK3β), stress-activated protein kinase 1b

(SAPK1b)/c-Jun N-terminal kinase 3 (JNK3), cell division cycle 2 (Cdc2), and cyclin-dependent kinase 5 (Cdk5), and phospho-T668-APP was found upregulated in the hippocampus of AD brains. Inhibition of T668 phosphorylation significantly reduced Aβ production, highlighting the important role of APP phosphorylation for the Aβ-associated pathogenesis of AD [8][14]. Further evidence suggests that CK1 protein kinases (formerly termed casein kinase 1) can be involved in AD pathogenesis. The highly conserved isoforms of the CK1 family are essentially involved in the regulation of numerous cellular processes, including growth, proliferation, and differentiation as well as apoptotic processes. Alterations in CK1 expression and/or activity as well as mutations in the coding sequence of CK1 isoforms can be associated with neurological disorders and tumorigenesis [15][16]. In hippocampal regions of AD brains, CK1δ mRNA and protein levels were found upregulated by 24.4- and 33-fold, respectively [17][18]. Furthermore, CK1ε is proposed to be involved in APP processing at the γ-secretase level, since CK1ε likely regulates a component of the γ-secretase complex. Consequently, overexpression of CK1ε resulted in increased Aβ production in cells stably expressing APP695. By treating these cells with a CK1ε-specific inhibitor, this increase in Aβ production could be reduced [19]. Finally, in silico analysis revealed the presence of several potential CK1-targeted consensus motifs in the intracellular regions of APP, and phosphorylation of the ectodomain E1 of APP by CK1-like ectoprotein kinases has already been demonstrated *in vitro* and in cell culture [20] [21]. These findings suggest that members of the CK1 family play a crucial role in AD neuropathology and highlight CK1 as a promising target for the development of new therapeutics for the treatment of neurodegenerative diseases.

Although CK1-specific small molecule inhibitors (SMIs) have already been shown to reduce Aβ production, they usually inhibit the activity of certain CK1 isoforms in general. Thereby, these inhibitors induce certain side effects due to inhibition of CK1 isoforms in essential cellular processes and in healthy tissue. To circumvent this, alternatives to SMIs focusing on intervention in CK1 activity at the level of kinase–substrate interactions are of special interest. Therefore, in the present study, we aimed at characterizing CK1δ-derived peptides that specifically block the interaction of CK1 isoform δ and APP695 without directly affecting kinase activity. A CK1δ-derived peptide library was first tested for interactions with APP695 protein fragments and, subsequently, competitive inhibition of CK1δ-mediated phosphorylation of APP695 fragments by interacting peptides was analyzed. Finally, cell entry of selected peptides and peptide-mediated effects on Aβ production and formation of amyloid plaques was investigated in an established cell culture model for AD. In summary, our results contribute to the detailed characterization of the interactions of CK1δ and APP695 and highlight a new therapeutic option for AD.

2. Results and Discussion

ELISA-based interaction analysis was performed to define the dominant motifs involved in mediating the interaction of CK1δ with APP695 and identified most significant binding to APP695 protein fragments N-APP, E2, and APP-C for a set of seven peptides (δ-41, δ-101, δ-111, δ-241, δ-281, δ-311, and δ-371). The interaction potential of these peptides as well as the potential to competitively block the interaction of CK1δ with APP695 has been further analyzed by *in vitro* phosphorylation experiments. Among the set of seven CK1δ-derived peptides identified in ELISA, only three peptides were able to significantly reduce phosphorylation of N-APP or E2. However, these peptide-mediated effects are not as substantial as those being induced by SMIs and failed to reduce phosphorylation by more than 41% (as observed for peptide δ-311 and phosphorylation of E2). With IC_{50} values within the submicromolar range, most current CK1-specific SMIs would show more potent effects on CK1δ than was observed for the CK1δ-derived peptides at the selected concentration [15]. Nevertheless, the major advantage of the tested peptides was the highly substrate-selective inhibition of N-APP and E2 phosphorylation, since no inhibition of CK1δ-mediated phosphorylation of α-casein could be observed for most peptides. This superior selectivity of CK1δ-derived peptides has already been pointed out in a previous study analyzing the inhibition of CK1δ-mediated phosphorylation of α-tubulin [22].

For the C-terminally located APP695 fragment APP-C, containing the transmembrane domain (TMD) as well as the intracellular domains (AICD) of APP695, only weak phosphorylation by CK1δ could be observed. Although APP695 in general can be phosphorylated by CK1δ and ectoprotein-CK1 during biosynthesis and trafficking, its TMD is buried within the phospholipid bilayer and can probably not be accessed by protein kinases [11][20]. Additionally, when working with recombinant proteins, the highly hydrophobic TMD is likely masked by hydrophobic molecules, which need to be included in the purification procedure to facilitate isolation of soluble protein but might also block *in vitro* phosphorylation by CK1 [23]. Either way, CK1-mediated phosphorylation of the TMD-related proportion of APP695 appears not to be relevant, as can be concluded from the performed biochemical analysis. Vice versa, these results demonstrate that major sites for CK1δ-mediated phosphorylation are located within the N-APP and E2 fragments of APP695. This finding is further confirmed by a ScanSite-based search for phosphorylation motifs, demonstrating that the majority of putative CK1-targeted phosphorylation sites are located in the acidic domain within amino acids 266 to 295 (data not shown) as well as by a previous report localizing phosphorylation sites targeted by CK1 within amino acids 181 to 224 of APP695 [20][24].

The respective sequences of CK1δ-derived peptides interacting with APP695 are generally expected to derive from the protein surface of CK1δ, thereby resembling actual kinase–substrate interaction motifs. However, while most of the identified interacting peptide sequences are localized on the CK1δ protein surface, peptide δ-241 especially occupies only a rather small surface area while still demonstrating remarkable binding to N-APP and APP-C. Consequently, the size of the corresponding surface area of each peptide on CK1δ does not necessarily correlate with the results of ELISA, indicating that the binding affinity of each peptide in general is more important for its specific interaction with the tested APP695 fragments. Furthermore, peptide sequences presenting robust interaction with APP695 fragments may also be located within domains distant to the active site of the kinase. This is supported by the fact that binding motifs for CK1 might be different to CK1-targeted phosphorylation motifs, which also can be distant to each other within substrate proteins [25].

In addition to the above-mentioned issues, the assumption that isolated CK1δ-derived peptides statically remain in shape as present in the whole protein structure of CK1δ might be generally misleading. While the native tertiary structure as well as the stability of the full-length protein is based on the sum of interactions determined by its constituting amino acids (e.g., hydrophobic effects, polarities, charges, stabilizing disulphide bridges, etc.), the structure and shape of isolated peptides is rather flexible and thus might not closely reproduce the actual interaction interface of both full-length proteins as present *in vivo* [26]. Prior to the performed peptide–protein docking simulations, the secondary structures of all analyzed peptides have been predicted by the PSIPRED algorithm [27]. However, only for peptides δ-41 (docking to E2), δ-101 (docking to N-APP/E1 and E2), and δ-241 (docking to N-APP/E1), the structural features presented by the corresponding sequence in full-length CK1δ could also be reproduced by the isolated peptide sequence in the best-ranked docking simulations. In most cases, the structural features of full-length CK1δ are not represented by the isolated peptides when docking to N-APP/E1 or E2. As a further limitation, all docking simulations had to be performed using structural data, which were available for fragments of APP695 only. Even though these models suggest that not all of the tested CK1δ-derived peptides resemble the situation presented by full-length CK1δ and APP695 *in vivo*, the superior evaluation of these simulations (as indicated by the obtained parameters used for ranking) still clearly supports our *in vitro* data demonstrating significant peptide–protein interaction with and competitive inhibition of the CK1δ-mediated phosphorylation of APP695 fragments.

Although all presented *in vitro* data was generated by using artificial fragments of APP695, obtained results were successfully complemented by the effects observed in subsequently performed cell culture experiments. Prior to investigation of therapeutic effects on AD-like phenotypes, peptide-induced cytotoxic effects and cell entry of CK1δ-derived peptides were tested using differentiated hNPCs. Consistent with the highly substrate-selective effects of CK1δ-derived peptides detected by *in vitro* kinase reactions, no cytotoxic effects on differentiated hNPCs could be observed. This finding indicates that no general inhibition of CK1δ kinase activity was induced by treatment with the selected CK1δ-derived peptides. In general, cell entry into differentiated hNPCs could be observed for most but not all tested peptides. The plasma membrane represents the final barrier through which therapeutic agents must penetrate to enter the cell, and the permeability of hydrophilic, small-molecular drugs is usually poor. Because uptake mechanisms like simple or facilitated diffusion, active transport, or even endocytosis are often insufficient, external delivery systems such as cell-penetrating peptides (CPPs), antibodies, or liposomes might be used to improve delivery and cell uptake of peptides [28][29][30]. However, at least for peptides δ-111, δ-281, and δ-371 used in our experimental approach, extensive cell entry into differentiated hNPCs could be observed without applying any further modifications on peptide sequence or delivery.

Finally, therapeutic effects of CK1δ-derived peptides were demonstrated using the Alzheimer's-in-a-dish model, which can be used to recapitulate AD-like phenotypes including Aβ and tau pathology based on a 3D human neural cell culture system [31][32]. In order to establish this system, an immortalized hNPC cell line is transduced with lentiviral vectors encoding mutated APP695 (Swedish and London mutation) and mutated PSEN1 (ΔE9), which are both associated with familial and early-onset forms of AD. Transduced hNPCs expressing high levels of mutant APP695 and PSEN1 are then seeded in 3D culture and differentiated by growth-factor deprivation. This 3D culture system is superior to conventional 2D systems as it closely mimics the *in vivo* environment and supports neuronal differentiation as well as the formation of neuronal networks. Using this cell culture model, extracellular aggregation of Aβ can be detected after six weeks of differentiation, whereas robust tau phosphorylation in neurites and cell bodies as well as tau pathology in general can be observed after 10 to 14 weeks (summarized in [31][32]).

Because extracellular Aβ aggregates in the selected cell culture model become obvious after six weeks of differentiation, the established 3D cultures were treated for six weeks with selected CK1δ-derived peptides as well as a β-secretase-specific SMI serving as control. For peptide δ-311, moderate levels of cell entry were detected, and, subsequently, superior effects on the generation of Aβ and amyloid plaque formation could be demonstrated. Interestingly however, reduction in Aβ levels were not necessarily correlated with the results of the cell entry analysis. Although cell penetration

was most obvious for peptides δ-111, δ-281, and δ-371, Aβ levels were significantly reduced after treatment with δ-41, δ-101, and δ-311. Since peptides in general are prone to rapid degradation and no peptide-specific staining could be observed for peptides δ-41 and δ-101 although both peptides demonstrate significant effects on APP metabolism, these peptides might be well taken up into the cells but are then quickly metabolized [33]. Alternatively, the biotin tag of these peptides could be masked by binding to APP, resulting in reduced or even absent staining with TRITC-labeled streptavidin. In the case of δ-41, the peptide-mediated effects on Aβ levels and amyloid plaque formation were rather low, whereas the effects of δ-101 were sufficient to induce a substantial reduction in Aβ levels and plaque formation. However, Aβ levels were only reduced by a maximum of 13% as observed for peptide δ-101. We assumed that the effects on Aβ levels could be potentiated by combined treatment with two or more CK1δ-derived peptides, since each peptide potentially occupies a different interaction site for CK1δ on the APP695 protein. Therefore, effects mediated by concurrent treatment with different CK1δ-derived peptides (e.g., a combination of δ-101 and δ-311) could result in more significant additive or synergistic effects.

In summary, CK1δ-derived peptide δ-101 represents an especially potent therapeutically active peptide, which displayed superior binding to APP695 protein fragments, and has the unique potential to preserve its native and stable conformation (compared to full-length CK1δ) when interacting with APP. Together with other promising candidate peptides like δ-311, it might serve as a starting point for the development of future strategies for the treatment of AD.

References

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