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# New Insights into the Autoinhibition Mechanism of Glycogen Synthase Kinase-3β

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It has been suggested that phosphorylation at serine 9 near the N-terminus of glycogen synthase kinase-3β (GSK-3β) mimics the prephosphorylation of its substrate and, therefore, the N-terminus functions as a pseudosubstrate. The molecular basis for the pseudosubstrate's binding to the catalytic core and autoinhibition has not been fully defined. Here, we combined biochemical and computational analyses to identify the potential residues within the N-terminus and the catalytic core engaged in autoinhibition of GSK-3β. Bioinformatic analysis found Arg4, Arg6, and Ser9 in the pseudosubstrate sequence to be extremely conserved through evolution. Mutations at Arg4 and Arg6 to alanine enhanced GSK-3β kinase activity and impaired its ability to autophosphorylate at Ser9. In addition, and unlike wild-type GSK-3β, these mutants were unable to undergo autoinhibition by phosphorylated Ser9. We further show that Gln89 and Asn95, located within the catalytic core, interact with the pseudosubstrate. Mutation at these sites prevented inhibition by phosphorylated Ser9. Furthermore, the respective mutants were not inhibited by a phosphorylated pseudosubstrate peptide inhibitor. Finally, computational docking of the pseudosubstrate into the catalytic active site of the kinase suggested specific interactions between Arg6 and Asn95 and of Arg4 to Asp181 (apart from the interaction of phosphorylated serine 9 with the "phosphate binding pocket"). Altogether, our study supports a model of GSK-3–pseudosubstrate autoregulation that involves phosphorylated Ser9, Arg4, and Arg6 within the N-terminus and identified the specific contact sites within the catalytic core.



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## Introduction

Glycogen synthase kinase 3 (GSK-3) is a ubiquitously expressed and evolutionarily conserved serine threonine protein kinase. GSK-3 phosphorylates key signal transduction molecules and is a critical player in metabolism, programmed cell death, and embryonic development.[1–3] The enzyme is a promising target for drugs to treat various pathological disorders, including diabetes, neurodegenerative diseases, and psychiatric disorders.[3–7] Unlike many protein kinases, GSK-3 is constitutively active in resting cells and undergoes rapid inhibition by extracellular stimuli.[8,9] The inhibition of GSK-3 is characterized by the phosphorylation of an N-terminal serine residue (Ser9 in GSK-3β and Ser21 in GSK-3α).[8] An additional unique feature of GSK-3 is that it requires prephosphorylation of its substrates: That is, the catalytic activity is coupled to the binding of phosphorylated substrates that contain the recognition motif for GSK-3, SXXXS(p), where S(p) is a phosphorylated serine.[1,10] This raised the possibility that the phosphorylated N-terminal tail functions as a pseudosubstrate.

Protein kinases may be autoinhibited by a mechanism in which part of their polypeptide chain, termed a pseudosubstrate, occupies the active site. Pseudosubstrates are characterized by their resemblance to sequences derived from the substrate target phosphorylation sites and have been identified in cAMP-dependent protein kinase, protein kinase C, and

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Abbreviations used: GSK-3, glycogen synthase kinase-3; CREB, cAMP response element binding protein; WT, wild type.

myosin light-chain kinase.[11–15] Some experimental findings suggest that GSK-3 is autoregulated by a pseudosubstrate domain. For example, analyses of GSK-3β X-ray structure showed that a phosphate-like moiety interacted with a defined phosphate binding pocket within the catalytic domain,[16,17] presumably mimicking the interaction with the phosphorylated substrate. Furthermore, it was shown that a synthetic, phosphorylated peptide derived from the potential pseudosubstrate sequence inhibited the kinase.[16,18] The inhibition was suggested to be specific as it depended on the sequence of the phosphorylated peptide. However, the molecular basis underlying the interaction between the N-terminal pseudosubstrate region and the catalytic core of GSK-3β is not fully defined. It is most likely that sites other than the phosphorylated Ser9 are important for the pseudosubstrate binding and autoregulation.

Importantly, the N-terminus of GSK-3β also undergoes autophosphorylation at Ser9.[19–21] This suggests that the nonphosphorylated pseudosubstrate can also fold into the catalytic core to accept phosphorylation at Ser9. Autophosphorylation and autoinhibition require different binding modes of the N-terminus into the active site of the kinase. In this study, we focused on the binding mode and specificity of the N-terminus as an autoinhibitor, which is likely to resemble the binding specifics of prephosphorylated substrates.

We previously showed that Phe67, Gln89, and Asn95 located within GSK-3β catalytic core are important for substrate binding and recognition.[22] We thus speculated that one or more of these residues may serve as docking sites for the pseudosubstrate. Using molecular, biochemical, and computational analyses, we provide evidence that the pseudosubstrate associates with the active site. We demonstrate that two arginine residues near the N-terminus are critically important for the pseudosubstrate binding and autoinhibition and identify their sites of interaction within the catalytic core.

## Results and Discussion

### Features of GSK-3 pseudosubstrate region

Analyzing all publicly available sequences of the N-terminal pseudosubstrate region in GSK-3β, GSK-3α, and their orthologs in invertebrates, we found it to be well conserved and present in most GSK-3 family members (Fig. 1a). This included the simplest multicellular animals such as sponges and the unicellular choanoflagellates, the closest known relatives of multicellular animals.[30] Strikingly, Arg4 and Arg6 were absolutely conserved in all GSK-3 pseudosubstrate regions we identified. Ser9 was also highly conserved with only one protein, a GSK-3 from a hydra, where it was replaced by a threonine that can functionally replace serine (as it can be phosphorylated). The analysis further indicated that positions 3–11 of the pseudosubstrate sequence were more conserved relative to the distal positions, suggesting that these nine residues likely comprise the pseudosubstrate core of GSK-3. In addition and independently, we compared the pseudosubstrate domain of GSK-3β with the phosphorylation motif of a known GSK-3 substrate CREB (cAMP response element binding protein). The comparison aligned Ser9 in GSK-3β with the prephosphorylated position in CREB, Ser133 (Fig. 1b). This alignment showed that Arg6 and Phe10 (GSK-3β) were aligned with Arg130 and Tyr134 of CREB, respectively (Fig. 1b). The GSK-3 phosphorylation site, Ser129, in CREB was aligned with Pro5 in GSK-3β. The importance of polar/charged residues in GSK-3's substrate recognition[22] also suggested that Arg4 and Glu12, both located in proximity to the SXXXS motif (Fig. 1b), may be important sites in this regard. Altogether, our analysis prompted us to investigate the role of Arg4, Pro5, Arg6, Phe10, and Glu12 in the inhibitory capability of the pseudosubstrate.

### Arg4 and Arg6 are important sites for pseudosubstrate function

The selected sites Arg4, Pro5, Arg6, Phe10, and Glu12 were mutated to alanine (termed here R4A, P5A, R6A, F10A, and E12A, respectively). The mutant S9A in which Ser9 was mutated to alanine[23] served as a "reference" control. Wild-type (WT) GSK-3β and the various mutants were expressed in a rabbit reticulocyte lysate translation system (TNT) as shown in Fig. 2a. The translated proteins were immunoprecipitated from the reaction mixture with a specific antibody, and GSK-3 kinase assays were performed with p9CREB peptide as substrate. As shown in Fig. 2a, R4A and R6A exhibited enhanced kinase activity as compared with WT-GSK-3 (increase of 40%). This enhanced activity was comparable to that obtained with S9A. On the other hand, mutations at Pro5, Phe10, and Glu12 did not affect the kinase activity (Fig. 2a). Notably, mutagenesis of Pro5 to Ala mimicked the mammalian GSK-3α sequence; thus, it was expected that this mutation will not affect the catalytic activity. Altogether, Arg4 and Arg6 appear to regulate the catalytic activity, presumably via their ability to interact with residues within the vicinity of the catalytic site.

The N-terminus of GSK-3β can undergo autophosphorylation at Ser9.[19–21] Since this mechanism requires an interaction between the N-terminus pseudosubstrate and the catalytic core, we examined whether Arg4 and Arg6 are also involved in autophosphorylation. WT-GSK-3 and its respective mutants were subjected to *in vitro* kinase assays with or without ATP. The reactions were separated by gel electrophoresis and subjected to immunoblot analysis with GSK-3-anti-phospho (Ser9) antibody. Unlike WT-GSK-3, R4A and R6A were unable to autophosphorylate at Ser9 and behaved like the inactive mutant $KK_{85,86}MA$ (Fig. 2b). This further indicated that Arg4 and Arg6 facilitate the pseudosubstrate "folding" into the catalytic core.