Unraveling Phosphorylation of the Splicing Factor SC35 by the Dual Signaling Kinase PKC-Θ in Human T-Cells

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Protein phosphorylation mediates a wide range of cellular and physiological processes including transcription, cellular growth, and immune responses. Understanding the substrate specificity of kinase phosphorylation is critical for mapping the signaling pathways controlled by a particular protein kinase.

Introduction

Our protein kinase of interest, protein kinase C-theta (PKC-θ), is implicated in the regulation of T-cell immune responses. We have shown that PKC-θ has a dual role as a cytosolic and nuclear signaling kinase in both T-cells and in cancer, particularly the cancer stem cells implicated in recurrence and metastasis. Given PKC-θ's complex regulatory role, we asked whether PKC-θ also regulates alternative splicing, since alternative splicing is important for T-cell regulation. Many immunologically relevant genes undergo alternative splicing, and the splicing factor regulators have, at least until recently, largely been ignored. The alternative splicing factor SC35 (also known as SRSF2) is of particular interest in T-cell biology and is a well-characterized splicing factor that belongs to the serine/arginine-rich (SR) protein family, an important class of splicing regulators.

Materials & Methods

Treating a sample set of synthesized proteins corresponding to a target of interest with a kinase of interest is an effective way to identify potential phosphorylation substrates. We wanted to identify potential phosphorylation sites within SC35 to profile exactly where PKC-θ phosphorylates SC35.

Active recombinant PKC-θ was provided to JPT Peptide Technologies (Berlin, Germany) for **Kinase Specificity Profiling** on peptide microarrays. Unmodified SC35 peptides were synthesized in a stepwise manner on a cellulose membrane corresponding to the entire overlapping SC35 protein sequence (**Figure 1**). To analyze PKC-θ's ability to phosphorylate SC35 peptides, a JPT peptide microarray was constructed by spotting the synthesized SC35 peptides onto the microarray (a planar glass slide) in triplicate, with each triplicate representing an SC35 peptide (**Figure 1**). Each spot was chemoselectively immobilized on the glass plate and was well defined to allow reliable spot identification of the corresponding sequence (**Figure 1**).

Peptides or appropriate controls were then incubated with PKC-θ kinase solution in the presence of γ-33P-ATP prior to high-resolution phosphorimaging (**Figure 1**). An identical assay was carried out in which PKC-θ kinase solution was incubated with a kinase phosphorylation inhibitor. Both experiments were then compared to establish the peptides phosphorylated by PKC-θ. GenepixPro 7.2 and ArrayPro 4.0 spot recognition software packages were used for data analysis. Peptide constructs that displayed a normalized mean signal equal to or greater than two SDs above the mean were considered likely to be positive for phosphorylation events. Excel, R, and Python were used to determine the statistical significance of sequences and phosphorylation events.

By efficiently creating a series of overlapping peptide fragments from the protein of interest, this method can be used to powerfully and elegantly identify small phosphorylated regions within the protein target of interest and thus contain target residues of interest. These resides can subsequently be identified by amino acid sequence analysis for phosphorylation motifs and directly validated by mutational analysis.

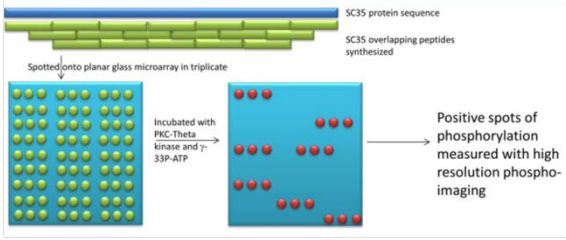

Figure 1: A series of overlapping peptides corresponding to the SC35 amino acid sequence were synthesized, spotted in triplicate onto a glass planar microarray and incubated with the PKC-Theta kinase and γ-^{33}P-ATP. Spots positive for phosphorylation which have incorporate the γ -^{33}P-ATP are then detected by high resolution phospho-imaging

Results

Of the 51 peptide constructs tested, 14 were positive for phosphorylation events. SC35 contains two long stretches of RS repeats and an RRM domain. Sequence analysis of these 14 peptides showed that all but two of the positive peptides localized to the long tract dipeptide RS domains. In contrast, peptides 5 and 9 localized to the RRM domain at the N-terminal. The peptide with the strongest phosphorylation signal (peptide 1) was located in the RS domain (171AA–186AA). We also examined the relationship between phosphorylated peptides and recently identified PKC-θ motifs.

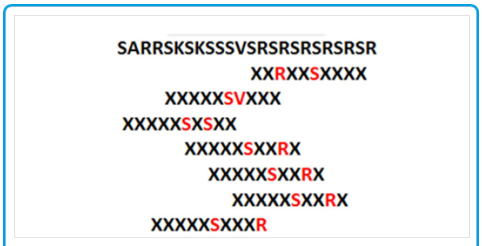

Figure 2: The PKC-Theta motifs associated (Hutti et al., 2004) with the highest scoring phosphorylation events are depicted in the graphic above along with the associated amino acid sequence (modified from a figure by McCuaig, R., 2015).

The strongest peptide signals (peptides 1, 2, and 3) contained seven PKC-θ phosphorylation motifs. Furthermore, the other 11 phosphorylation-positive peptides also contained regions corresponding to putative PKC-θ phosphorylation motifs.

Kinase Specificity Profiling

A summary of the top PKC-θ phosphorylation motifs is depicted in **Figure 2** for the top 3 peptide signals. Overall, our data demonstrate that PKC-θ directly phosphorylates SC35 in both the RMM and RS regulatory domains (**Figure 3**).

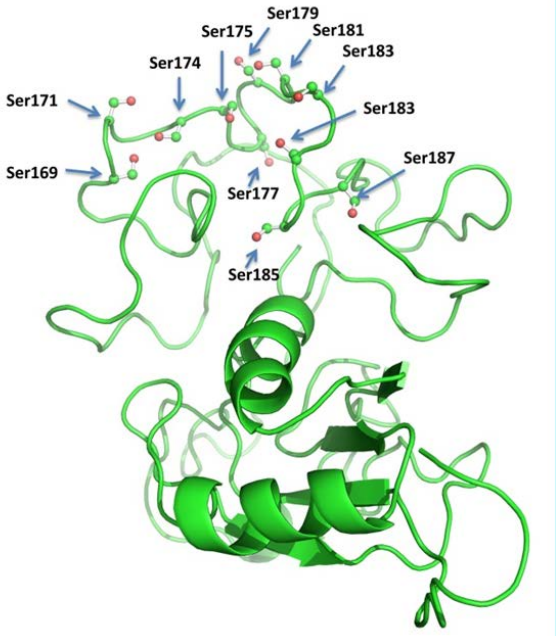

Figure 3: The top 3 peptide phosphorylation hits for PKC-Theta occurred within the RS domain of SC35. Depicted above is a protein schematic of SC35 showing the RS domain at the top of the schematic with the serine's which correspond to known PKC-Theta phosphorylation motifs and fall within the 3 peptide sequences labelled within the protein structure.

Discussion & Conclusions

The peptide microarray platform is a high fidelity and reliable method for investigating critical areas of protein regulation by post-translational modifications such as phosphorylation. It provides a method to identify - with a high degree of confidence - putative phosphorylation sites by specific kinases of interest such as PKC-θ, which can subsequently be experimentally validated. This pipeline is illustrated by this experiment, which shows that PKC-θ phosphorylates SC35 regions critical to its function including those that control nuclear localization, substrate specificity, and protein-protein interactions. Mutation of potential phospho-epitopes within the defined target region can then be performed to identify the exact residues that are phosphorylated, providing insights into normal and aberrant protein function.

References

1. "PKC-Theta is a novel SC35 splicing factor regulator in response to T cell activation"
   McCuaig et al., Front Immunol. (2015)
2. "A rapid method for determining protein kinase phosphorylation specificity."
   Hutti et al., Nature Methods (2004)

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Professor Sudha Rao obtained her BSC (Hons.) degree at Keele University, UK. She received a prestigious Aventis Fellowship and obtained her PhD from the University of London, Kings College in 2000. During this period, she subsequently joined Aventis and as a Senior Scientist was involved in developing therapeutics strategies in the arena of asthma & COPD. In 2003 Rao was subsequently appointed Team Leader of the Immune Regulation & Nuclear Dynamics Group at JCSMR, ANU. She moved to the University of Canberra in 2010 and currently is a Professor in Molecular & Cellular Biology.
Professor Rao has extensive experience in transcriptional biology and genomic technologies that spans both pharmaceutical and academic settings. The primary focus of her research group has been to unravel complex epigenetic-signatures in transcription programs in the context of the immune system, as well as to understand the deregulatory mechanisms operating in cancer settings.

The Company

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