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Novel terbium-sensitizing peptide substrates for cyclin-dependent kinase 5 (CDK5) and their demonstration in luminescence kinase assays

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Novel time-resolved terbium luminescence assays were developed for CDK5 and CDK2 by designing synthetic substrates which incorporate phospho-inducible terbium sensitizing motifs with kinase substrate consensus sequences. A substrate designed for CDK5 showed no phosphorylation by CDK2, opening the possibility for CDK5-specific assay development for selective drug discovery.

Since its discovery over 30 years ago,[1] cyclin-dependent kinase 5 (CDK5), a proline-directed serine/threonine protein kinase, has continually garnered attention largely due to its role in Alzheimer's disease (AD)[2] and other neurological disorders.[3] More recently, CDK5 has also been implicated in cancer.[4] Although CDK5 shares a close sequence homology with other cyclin-dependent kinases (CDKs),[5–7] its activity is predominantly relegated to post-mitotic neurons where it is activated not by cyclins, but by the regulatory protein p35.[8] p35-regulated CDK5 activity is essential for neuronal development, synaptic function, and cognitive processes such as learning and memory.[3] Within cells, physiological CDK5/p35 activity is constrained to the periphery near the cellular membrane[9] to which p35 is anchored by an N-terminal myristoyl moiety.[2,10] However, upon exposure to neurotoxic signals, a cellular influx of Ca^{2+} activates proteolytic calpain to cleave p35, thereby releasing protein p25 from the membrane-bound p10.[11] In addition to enabling CDK5 to access more potential substrates with its acquired cellular mobility, p25 has a half-life 10-fold longer than p35. This allows for the more stable and prolonged activation of CDK5 by p25, resulting in the dysregulation of CDK5 activity observed in neurodegenerative disorders.[2,12] Dysregulated CDK5/p25 activity contributes to all three histopathological hallmarks of AD: extracellular deposition of β-amyloid (Aβ) plaques, formation of neurofibrillary tangles (NFT), and neuron death.[3] Such hallmarks are the consequences of AD, but much less is known about the molecular interactions leading to this disease. The dysregulation of CDK5, a tau kinase, results in tau hyperphosphorylation and subsequent NFT formation.[13,14] Aberrant CDK5/p25 activity has also been shown to induce Aβ accumulation through the upregulation of amyloid precursor protein (APP)[15] and by activating beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) both through direct phosphorylation,[16] and *via* the STAT3 pathway.[17] CDK5/p25 triggers p53[18] and JNK3[19] pathways in addition to other apoptotic consequences by initiating nuclear envelope dispersion, DNA damage, mitochondrial dysfunction, while feeding forward additional oxidative stress and Ca^{2+} overload.[3,20] Additional tools are needed to clearly understand the role aberrant CDK5 activity plays in AD and each of its hallmarks, and the effect CDK5 inhibition has on slowing and reversing AD progression.

Although hyperactive CDK5 has been clearly implicated in AD,[13,21] inhibitors which target CDK5 have yet to be approved for clinical treatment.[22] This is largely due to the lack of selectivity of classified CDK5 inhibitors over other closely related CDKs such as CDK2, CDK5's closest homolog, without incurring harmful, off-target effects.[23] Just as no inhibitors for CDK5 have been approved, assays which specifically detect CDK5 activity among closely related kinases are limited, which makes screening inefficient. By developing synthetic peptide substrates capable of distinguishing the activities of these closely related kinases, we aim to provide improved assays as tools that could be used for selective kinase inhibitor drug discovery.

In recent years, the Parker Lab has developed tyrosine kinase assays featuring synthetic peptide substrates designed by combining kinase recognition sequences[24] with various detection techniques including antibody-free time-resolved terbium (Tb) luminescence.[25] Like homogeneous time-resolved fluorescence (HTRF) assays, these assays are nonradioactive and well-suited for high-throughput inhibitor screening; however, the advantage is they do not require specific antibodies which are costly and often unavailable for phosphosites of interest, restricting such HTRF assays to the use of generic instead of kinase-specific substrates.[26]

The present work describes the creation of novel peptide substrates that can be used for *in vitro* assays capable of detecting CDK5 activity over its closest relative CDK2 (62% sequence

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	-8	-7	-6	-5	-4	-3	-2	-1	0	1	2	3	4
4L-CDK5tide	L	L	L	L	K	H	H	K	S	P	K	H	R
Tb1-CDK5tide	D	K	D	A	D	H	W	K	S	P	K	H	R
Tb2-CDK5tide	D	K	D	A	D	H	H	W	S	P	K	H	R
4L-CDK2tide	L	L	L	L	H	H	H	R	S	P	R	K	R
Tb1-CDK2tide	D	K	D	A	D	H	W	R	S	P	R	K	R
Tb2-CDK2tide	D	K	D	A	D	H	H	W	S	P	R	K	R

Fig. 1 Substrates designed to assay CDK5 and CDK2. Residues DKDAD and W (orange) located N-terminal of S (phosphorylation site, position 0) are incorporated for phospho-inducible terbium chelation and as a UV acceptor, respectively.[28] These residues are substituted into previously reported substrate consensus sequences of CDK5[27] and CDK2[38] (residues in blue). Peptides 4L-CDK5tide and 4L-CDK2tide are comprised of the corresponding kinase consensus sequence with N-terminal leucine residues (black) to ensure column retention for LC–MS assay detection.

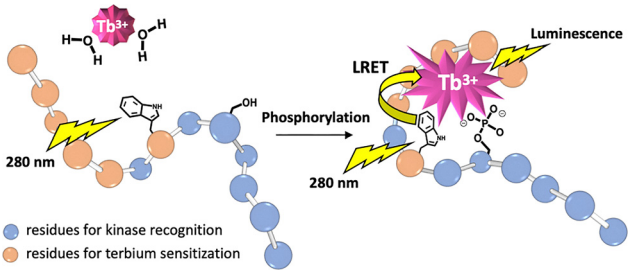

Fig. 2 Time-resolved terbium luminescence assay featuring a synthetic peptide designed by merging a serine kinase consensus substrate sequence (blue residues) with a phospho-inducible terbium sensitization motif (orange residues). Upon kinase phosphorylation, the peptide chelates and sensitizes terbium. Excitation of a nearby positioned tryptophan residue (donor) at 280 nm and subsequent energy transfer to chelated terbium (acceptor) allow for luminescence.

homology).[5] This was accomplished by designing peptides that feature a previously reported consensus sequence for CDK5 (KHHKSPKHR)[27] and implementing these peptides in kinase reactions, with either LC-MS or time-resolved terbium luminescence detection. To ensure C18 column retention for the LC–MS assay, four leucine residues were added on the N-terminus forming 4L-CDK5tide (Fig. 1). For the higher throughput terbium-based readout, versions of the substrate with a phospho-inducible Tb-chelating/sensitizing motif[28] were also designed with the CDK5 substrate sequence. This enables an antibody- and fluorophore-free CDK5 assay featuring synthetic substrates that directly luminesce upon phosphorylation. Specifically, this was achieved by merging the CDK5 consensus sequence with each of two Tb-sensitizing motifs reported by the Zondlo Lab, DKDADXWXS (Tb1) and DKDADXXWS (Tb2), in which X denotes any residue. Re-engineered from the EF-Hand Ca^{2+}-binding domain[28–31] and Imperiali's lanthanide binding tag,[32] these motifs when phosphorylated (D*KD*AD*XW*XpS* and D*KD*AD*XX*WpS*) coordinate Tb^{3+} *via* the phosphate group of phosphoserine, three aspartate sidechains and the carbonyl oxygen of position 7 (residues in contact with metal are indicated with an asterisk). By this design, excitation of tryptophan at 280 nm and energy transfer to a terbium ion (Tb^{3+}), coordinated nearby, leads to sensitized Tb luminescence (Fig. 2). This resulted in the novel CDK5 substrates Tb1-CDK5tide and Tb2-CDK5tide, respectively (Fig. 1).

Since non-phosphorylated substrate[28] and ATP[33] both interact with Tb^{3+}, it was important to establish conditions that minimized background and maximized phosphorylated peptide signal. Initial studies were performed in which Tb^{3+} (Fig. S13) and ATP (Fig. S14) concentrations were varied to achieve an optimal difference in signal between phosphorylated and non-phosphorylated Tb1-CDK5tide. While some background is observed for the non-phosphorylated substrate, calibration experiments with synthetic phosphopeptide standards of the Tb-sensitizing peptides demonstrated luminescence proportional to the amount of phosphopeptide present (Figs. S15, S17 and S19). Of the Tb-sensitizing peptides applied, Tb1-CDK5tide displayed the greatest dynamic range between 100% phosphorylated *vs.* 100% non-phosphorylated in each of the calibration experiments.

To show kinase activity with these substrates, CDK5/p25 kinase reactions were performed with 4L-CDK5tide, monitored with LC–MS, and Tb1-CDK5tide and Tb2-CDK5tide, detected *via* luminescence. We confirmed that the peptides were indeed CDK5 substrates, and phosphorylation was easily monitored and quantified (Fig. 3(A), solid symbols). Of the two substrates designed for Tb luminescence, Tb1-CDK5tide and Tb2-CDK5tide were phosphorylated at nearly the same rate, which suggests that the placement of a bulky tryptophan residue near the phosphorylation site was not a major issue, and the activity was more affected by replacing basic residues typically favoured by CDK5. Substrates Tb1-CDK5tide and Tb2-CDK5tide were phosphorylated less rapidly by CDK5 than 4L-CDK5tide, which was not unexpected since residues of the CDK5 consensus sequence were replaced at specific positions to enable Tb coordination and sensitization. Nonetheless, phosphorylation of the Tb-CDK5tides was still efficient enough to be useful in assays, especially considering the advantage that luminescence readouts can be performed in less than 30 seconds per sample (*vs.* the ~20–30 min per sample for LC–MS). As a measure of assay robustness, we calculated Z' scores[34] from calibration experiments using combinations of synthetic phosphopeptide and non-phosphorylated peptide for Tb1-CDK5tide and Tb2-CDK5tide. Z' scores of 0.5–1 are typically indicative of an excellent assay, and 0–0.5 are viewed as marginally effective. Our calculated Z' scores were well above 0.8 for samples with greater than 50% phosphopeptide; above 0.7 with 25% phosphopeptide; and just above 0.5 (an average of 0.55 for Tb1-CDK5tide and 0.53 for Tb2-CDK5tide) with 10% phosphopeptide (Fig. S21). As Z' scores of 0.5–1 are indicative of an excellent assay, 10% phosphopeptide of 10 μM total peptide detected would suggest a detection limit under these assay conditions of just below 1 μM phosphopeptide.

Next, we tested whether these substrates were selective for CDK5 over CDK2. CDKs are known to share many similarities in structure[7] and substrate preferences.[6,35] Since CDK2 is structurally very similar to CDK5,[36,37] and reported substrate consensus sequences of CDK5[27] and CDK2[38] are also very similar,[39,40] we were surprised to find that all three CDK5tides

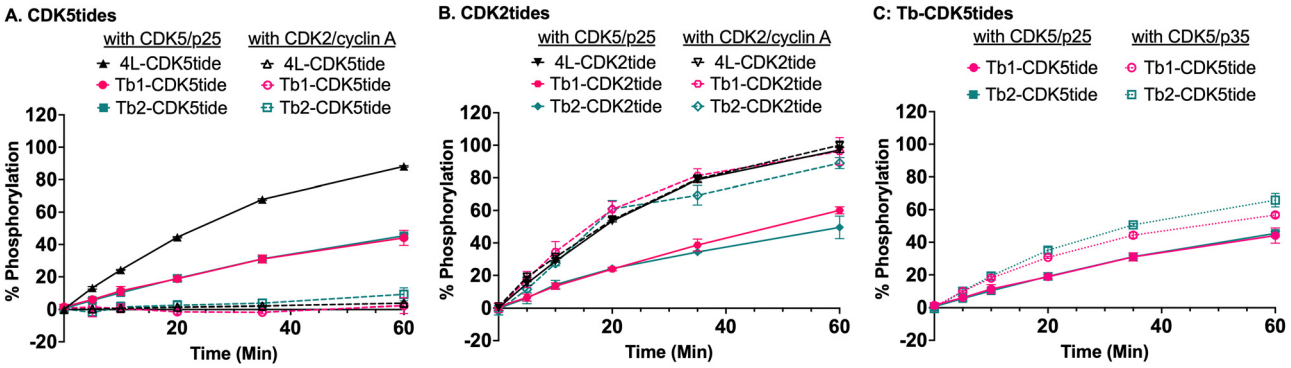

Fig. 3 Reaction progress in percent phosphorylation of (A) peptide substrates (CDK5tides) designed for CDK5 assayed with CDK5/p25 (solid symbols) and CDK2/cyclin A (open symbols); (B) peptide substrates (CDK2tides) designed for CDK2 assayed with CDK5/p25 (solid symbols) and CDK2/cyclin A (open symbols); and (C) Tb1-CDK5tide and Tb2-CDK5tide assayed with CDK5/p25 (solid symbols) and CDK5/p35 (dotted symbols). All reactions were performed in triplicate at 25 °C using 25 μM substrate with 10 nM kinase/regulatory protein in kinase reaction buffer (25 mM HEPES, 50 μM ATP, 10 mM $MgCl_2$, 0.2 mg mL^{-1} BSA, 0.2 mM DTT, pH 7.5). Reactions using 4L-CDKtides were monitored by LC–MS. Reactions using substrates Tb-CDKtides were monitored with time-resolved terbium luminescence assays.

showed minimal to no product formation in the presence of CDK2/cyclin A (Fig. 3(A), open symbols). To confirm the activity of the recombinant CDK2/cyclin A used in our experiments, we applied a similar substrate design strategy to create a set of CDK2tides based on the CDK2 consensus sequence (Fig. 1).[38]

When treated with CDK2/cyclin A, each of the peptides 4L-CDK2tide, Tb1-CDK2tide and Tb2-CDK2tide were readily phosphorylated showing that our CDK2/cyclin A complex was indeed active (Fig. 3(B) and Fig. S20). However, unlike the CDK5tide substrates, which were phosphorylated by CDK5 (Fig. 3(A)) and not by CDK2 (Fig. S20), the CDK2tide substrates were not selective and were phosphorylated by both CDK2 and CDK5 (Fig. 3(B)). Moreover, CDK2/cyclin A phosphorylation of the CDK2tides was not impacted by the incorporation of the Tb-sensitizing motifs (Fig. 3(B) and Fig. S20), whereas CDK5/p25 still seemed to prefer substrates (4L-CDK2tide) lacking the motif (Fig. 3(B) and Fig. S16). Also, while the Tb motifs affected CDK5/p25 substrate phosphorylation, CDK5/p25 phosphorylated 4L-CDK5tide at a similar rate as 4L-CDK2tide, and Tb-CDK5tides at a rate similar to Tb-CDK2tides (Fig. 3(A), (B) and Fig. S16). The main side chain difference in the core substrate sequences between CDK5tides and CDK2tides is H (for CDK5) *vs.* K (for CDK2) at position +3 (Fig. 1). Overall, these experiments showed that CDK5/p25 tolerated both H and K at position +3 equally well, whereas CDK2/cyclin A only tolerated K in that position.

Encouraged by the ability of designed CDK5tides to selectively assay CDK5/p25 over its closest homolog CDK2, we were curious to test them with CDK5/p35, as substrate selectivity of CDKs is often directed by the interacting regulatory protein.[6,7,35] When assayed with CDK5/p35, a slightly higher rate of phosphorylation was observed for both Tb1-CDK5tide and Tb2-CDK5tide than in the CDK5/p25 assay performed under the same conditions (Fig. 3(C)). This was not completely unexpected since the consensus substrate sequence for CDK5 was obtained using CDK5/p35.[27] Thus, the difference in rate of phosphorylation seen here between CDK5 when regulated by p35 *vs.* p25 could be due to either a shift in substrate preference (and relative activity on this particular substrate) guided by the regulatory protein or an intrinsic difference in activity between the two versions.

To better understand the selectivity of the CDK5 substrates, we examined the previously published crystal structure[36] of CDK2/cyclin A3 complexed with the substrate HHASPRK (Fig. 4(A)) derived from the CDK2 substrate consensus sequence.[38] Although CDK2 is the closest homolog of CDK5 with many similarities in sequence, structure, substrate preferences, and inhibitor profiles,[22,23] they also have several key differences. In addition to not being involved in the cell cycle or regulated by a cyclin like CDK2, the mechanism of activation for CDK5 is different.[41,42] CDK2 has a dual mechanism of activation[43,44] that requires both the binding of a regulatory cyclin such as cyclin A or cyclin E[45] and the phosphorylation of a threonine residue (T160) on its activation T-loop.[44,46] The presence of phosphorylated T160 (pT160) on the activation loop of CDK2 drives a strong preference for lysine at position +3 of the substrate,[36] as demonstrated by the published crystal structure that revealed the formation of a hydrogen bond between pT160 of active CDK2 and the +3-lysine residue of the substrate (Fig. 4(A)). Further, it has also been previously shown that substitution of the +3-lysine with alanine greatly reduced substrate phosphorylation by fully active CDK2.[47] In the present work, the lack of phosphorylation of the CDK5tide substrates by CDK2 is most likely due to the placement of histidine instead of lysine in position +3 (Fig. 1). This suggests that CDK2 does not simply prefer a basic residue in the +3-substrate position, but that this residue should also possess a sidechain long enough to form a favourable interaction with pT160.

CDK5, on the other hand, only requires regulatory protein binding to become active. Although CDK5 possesses a serine residue at position 159 (S159), analogous to T160 in CDK2, it does not require phosphorylation for CDK5 to become active.[48] Furthermore, structural studies suggest that phosphorylation of S159 is sterically disfavoured and substitution of S159 with

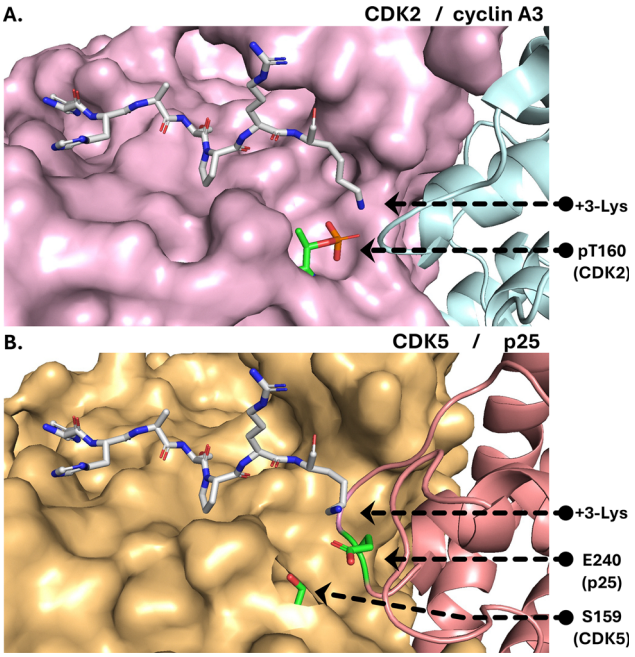

Fig. 4 (A) Crystal structure of substrate HHASPRK in the binding cleft of CDK2/cyclin A3 (PDB:1QMZ)[36] and (B) Cartoon of HHASPRK from PDB:1QMZ (with +3-lysine rotamer) added and aligned with crystal structure of CDK5/p25 (PDB: 1H4L).[37] Labels added highlighting interaction of +3-lysine with pT160 of CDK2 (A) and E240 of p25 (B). Figures generated in Pymol from published structural models.

glutamate (as an analogue of phosphoserine) impaired p35 binding and greatly reduced activity.[37] In terms of structure, p35 and p25 form more contacts with CDK5 than cyclin A does with CDK2, thus the mere binding of the regulatory protein is enough to tether the CDK5 activation loop forming an extended conformation resembling that of CDK2 dually activated by phosphorylation and cyclin binding (Fig. 4(B)).[36,37,41] In addition to forming more contacts than cyclin A with the respective kinase, p35/p25 has also been shown to interact directly with the substrate. Tarricone *et al.* showed that a glutamate residue located in position 240 (E240) of p35/p25 imparts substrate selectivity for basic residues at position +3 in a manner similar to pT160 of CDK2 (Fig. 4(B)).[37] Upon substitution of E240 with alanine and glutamine, activity was greatly decreased and the ability to distinguish between lysine and alanine in the +3-substrate position was lost. This suggests that E240 provides an interaction between the substrate's +3 position and the active kinase complex that is analogous to the +3 K/pT160 interaction for CDK2/cyclin A. CDK5 maintains a strong preference for a basic residue located in substrate position +3, but does not require the longer side chain length to reach its interaction partner, which helps explain its ability to phosphorylate both CDK5tides (+3-histidine) and CDK2tides (+3-lysine) in our experiments, in contrast to CDK2.

Conclusions

In this work, a set of synthetic peptide substrates were developed that can be used to specifically assay CDK5 over its close homolog CDK2. This set includes 4L-CDK5tide designed for LC–MS detection from the CDK5 substrate consensus sequence, as well as substrates Tb1-CDK5tide and Tb2-CDK5tide, which merge Tb-sensitizing motifs with the consensus sequence. Incorporation of Tb-sensitizing motifs allows for antibody-free, time-resolved luminescence proportional to phosphopeptide produced that will be compatible with high-throughput assay detection. These substrates could be used for multiplexed dual screening of CDK5 and CDK2 *in vitro*. Eventually we aim to apply these substrates in cell-based assays, however as Tb^{3+} is known to complex with ATP and is coordinated by calcium-binding protein domains such as the EF-Hand motif (from which the Tb-sensitizing peptides are designed), assay background and signal quenching can present issues. In some cases, pre-chelated complexes of Tb^{3+} with other ligands have been successful in facilitating detection even in the presence of complex biological solutions.[49] We are currently developing strategies to leverage peptide enrichment with the substrates reported here, to circumvent signal interference caused by such biomolecules in cell-based assays.

Author contributions

This project was conceptualized by Jason L. Heier and Laurie L. Parker. Methodology was provided by Jason L. Heier and Laurie L. Parker. Investigation and validation were carried out by Jason L. Heier and Dylan J. Boselli. Formal analysis and data curation were performed by Jason L. Heier and Dylan J. Boselli. Visualization was provided by Jason L. Heier and Laurie L. Parker. This manuscript was written by Jason L. Heier and edited by Dylan J. Boselli and Laurie L. Parker. Laurie L. Parker supervised and authorized this project and acquired funding supporting this research.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been submitted in the form of SI. Supplementary information: Includes experimental and analytical methods, peptide characterization, and collected and analysed data. See DOI: **https://doi.org/10.1039/d5cb00189g**.

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