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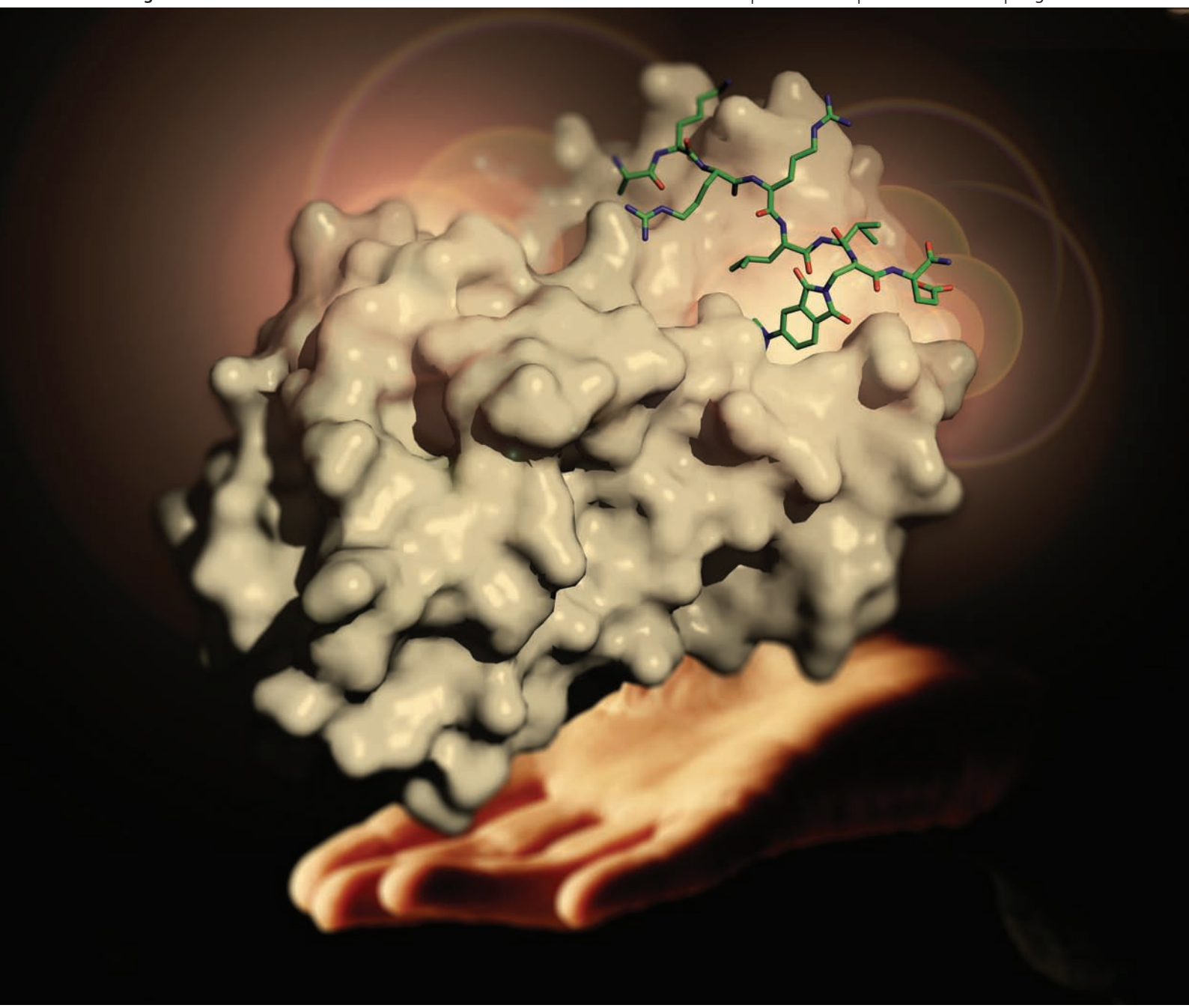

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COMMUNICATION

Rational design of a cyclin A fluorescent peptide sensor†

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We report the design and development of a fluorescent sensor specifically designed to target cyclin A, a protein that plays a key role in the regulation of the cell cycle. Computational studies provide a molecular picture that explains the observed emission increase, suggesting that the 4-DMAP fluorophore in the peptide is protected from the bulk solvent when inserted into the hydrophobic binding groove of cyclin A.

Eukaryotic cells proliferate under control of a series of strictly coordinated molecular mechanisms, which are primarily regulated by heterodimeric enzymes formed by the association of catalytic cyclin-dependent kinases (CDKs), and activating cyclin units.[1] It has been demonstrated that alterations in the regulation of such complexes can lead to aberrant cell proliferation,[2] and therefore they represent preeminent targets for the development of anticancer agents.[3] In addition to the direct inhibition of the CDK catalytic site,[4] there is a growing interest in the design of molecules targeting the cyclin binding groove (CBG) that mediates substrate recruitment,[5] and exploiting these CBG-binding molecules for the development of fluorescent sensors of cyclin A.[6] Herein we demonstrate that CBG-targeting peptides equipped with an environment-sensitive fluorophore manifest their binding to cyclin A through a significant emission increase, and thus afford a new strategy for detecting cyclin proteins. We also demonstrate that the resulting cyclin A biosensors can be used in competitive titrations to assay CDK inhibitors.

Environment-sensitive fluorophores such as 7-nitrobenzo-2-oxa-1,3-diazole (NBD),[7] or 4-dimethylaminophthalimide (4-DMAP),[8] are a special class of molecules that display spectroscopic properties dependent on the physicochemical characteristics of their suroundings,[9] generally exhibiting low quantum yield in aqueous solution, but becoming highly fluorescent in non-polar solvents, or when bound to hydrophobic sites in proteins. This sensitivity to the changes in their vicinity has been exploited for the development of peptide probes,[10] in which they are often assembled as derivatives of 2,3-diaminopropionic acid (Dap)[11] (Fig. 1).

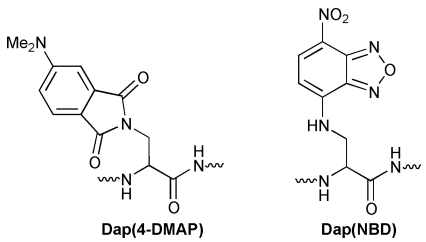

Fig. 1 Structures of the two fluorogenic amino acid residues assembled in the solid phase Dap(4-DMAP), and Dap(NBD). See the ESI† for detailed on-resin synthesis of the peptide probes.

The cyclin A groove is partly defined by a set of highly conserved hydrophobic amino acids that make multiple van der Waals contacts with the peptide substrates bound in partially extended conformations.[12] We realized that such a hydrophobic environment in the CBG could be exploited to induce a fluorescence emission increase of an environment-sensitive fluorophore incorporated at the appropriate position of a cyclin binding peptide, provided that the structural determinants that promote high affinity binding are not disrupted with the introduction of the exogenous fluorophore, and that the fluorophore is effectively shielded from the solvent upon binding to the cyclin A groove.

The peptides used in this study are based on peptide libraries that defined the consensus sequence for specific cyclin A binding,[14,15] R*x*L*hh′*, where *x* is any amino acid, and *h* and *h′* are hydrophobic residues such as Ile or Phe. We screened a set of peptides that included the sensing fluorophores within slightly different sequence contexts (see Fig. 2). Peptides were synthesized as 4-DMAP (**a** series) or NBD (**b** series) derivatives. Thus peptides **1** and **2** included the fluorogenic residues at position 4, which is known to act as a relatively passive linker between the hydrophobic resides flanking it. In peptides **3** to **5**, the fluorogenic residue replaces the aromatic Phe[5] that makes extensive hydrophobic interactions with the bottom of the CBG (see Fig. 2), and also include the consensus Ile (**5**), a flexible Gly residue (**4**), or a polar Asp (**3**) at the linker position. Finally, the 4-DMAP fluorophore was incorporated at the N-terminus of the optimized cyclin binding

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† Electronic supplementary information (ESI) available: Peptide synthesis and characterization. Fluorescence spectroscopy methods. Cyclin A expression and purification. Internalization and *in vivo* studies of peptide **5a**. See DOI: 10.1039/c1ob06009k

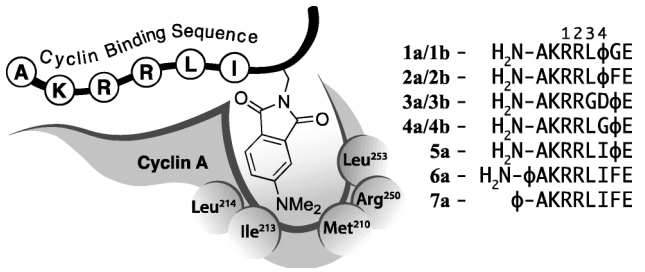

Fig. 2 Left: Schematic representation of a peptide interacting with the CBG while inserting the 4-DMAP fluorophore into the pocket formed by a cluster of hydrophobic residues (Met[210], Ile[213], Leu[214] in helix α1, Leu[253] and methylenes of Arg[250] in helix α3).[12,13] Right: Peptide sequences used in this study. **1a–7a**: ϕ = Dap(4-DMAP); **1b–4b**: ϕ = Dap(NBD).

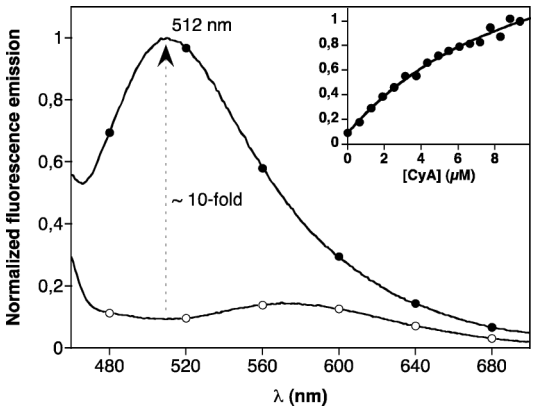

Fig. 3 Fluorescence spectra of peptide **5a** in 10 mM HEPES pH 7.6, 100 mM NaCl buffer: emission before (○), and after the addition of 6 equivalents of cyclin A (●). Inset showing the normalized emission of **5a** at 512 nm with increasing amounts of cyclin A, and the best fit to a 1 : 1 binding model used to calculate the binding constant.[19]

motif, either in a Dap side-chain (peptide **6a**), or directly attached to the N-terminal amine of the final Ala residue (peptide **7a**).

Detailed synthetic procedures are given in the ESI.† In short, peptides were assembled following standard Fmoc solid phase peptide synthesis protocols,[16] and the fluorophores were introduced into the peptide scaffold after completion of the backbone synthesis, while the peptides were still attached to the resin. The 4-DMAP fluorophore was linked to the peptide in a two-step procedure involving coupling with the anhydride precursor, and then activation with HBTU/HOBt to promote phthalimide ring closing (see ESI†).[17] In all cases, except **7a**, an orthogonal (alloc) protection scheme was used to allow the selective modification of the Dap side chain.

Steady-state fluorescence showed that all the peptides were weakly emissive in buffer upon irradiation at their corresponding excitation wavelength (λ_{exc} 4-DMAP = 395 nm; λ_{exc} NBD = 478 nm), and that cyclin A was essentially non fluorescent under these conditions. Unfortunately, incubation of 1.5 μM solutions of each peptide with cyclin A showed that all the NBD, as well as most of the 4-DMAP peptides were unresponsive to the presence of the protein, displaying the same emission spectra in its presence as they did in buffer (see ESI†). In contrast with these results, peptides **4a** (AKRRLGϕE) and **5a** (AKRRLIϕE), featuring the 4-DMAP fluorophore at position 5, displayed a significant emission increase, as well as blue-shifted emission, upon interacting with cyclin A (Fig. 3). These effects are consistent with the burial of the 4-DMAP fluorophore in the hydrophobic patch of the CBG and its shielding from the bulk solvent.[9,18]

Fluorescence titrations showed that peptide **5a**, featuring the Ile[4] linker, binds cyclin A with a better binding constant (K_D ~ 8.4 μM) than peptide **4a** (K_D ~ 18.1 μM). This is in line with the observation that peptides with hydrophobic residues in that position show better inhibitory properties. Interestingly, in addition to increased binding affinity, peptide **5a** also displayed a larger emission increase upon binding to cyclin A (over ten-fold increase upon saturation with cyclin A). Selectivity of **5a** for cyclin A was demonstrated by incubation with other proteins like BSA, histone H1 or IgG, which did not induce any significant increase in the emission intensity of the fluorophore (see ESI†). In addition to cyclin A detection, comparison of the Stokes shift of the complexed **5a** probe with the shifts observed in solvents of different polarities allowed us to calculate an apparent dielectric constant of ~3.8 for the hydrophobic pocket of the CBG. This value is in agreement with the apparent dielectric constants found for other hydrophobic binding sites, and the values commonly used in computational studies of proteins.[20] Preliminary studies with HeLa cells suggest that peptide **5a** is not internalized effectively, but does not appear to have a toxic effect on the cells, even after prolonged incubation at high concentrations (see ESI†).

Molecular modeling of the cyclin A/**5a** complex was performed as detailed in the ESI.† Briefly, the complex was built by *in silico* mutation of the crystal structure of cyclin/CDK in complex with the RRLIF peptide inhibitor (PDB code 1OKV), followed by energy minimizations with Macromodel.[21] The initial complex was further relaxed by a 1 ns length restrained MD, followed by a 5 ns production phase with NAMD,[22] and the OPLS forcefield,[23] which was considered for further analysis. The first remarkable observation was the stability of the interactions between cyclin and the peptide **5a**, with an RMSD with respect to the average structure of 1.2 Å for the non-hydrogen ligand atoms. A mean number of 6 simultaneous hydrogen bonds between the peptide and cyclin was achieved, mainly with residues Asp[216], Trp[217], Gln[254], Asp[283], Ile[281] (main chain) and Tyr[280] in the CBG. Importantly, the fluorogenic residue (4-DMAP) remains substantially buried in a hydrophobic binding pocket constituted by residues Ile[213], Leu[214] and Met[210] of the CBG, and further supported by internal contacts of 4-DMAP with residue Leu[3] in the peptide, which binds close to Trp[217] (see Fig. 4).

A similar analysis was performed for peptide **4a**, which was also shown to be an effective sensor upon binding to cyclin, despite its somehow diminished emission due to the single aminoacid substitution at position 4 (Ile in **5a**, Gly in **4a**). According to the MD simulations, peptide **4a** adopts a slightly modified binding pose where the flexible Gly[4] allows an internal salt bridge between Arg[2] and Glu[6] in the peptide. This conformation results in a shifted binding mode of Leu[3] and 4-DMAP to the CBG. In addition, the internal interaction of Arg[2] and Glu[6] is also responsible for a diminished number of 4 average hydrogen bonds of **4a** with the CBG. However, the solvent accessible surface area of the fluorophore residue, as measured along the MD trajectories, does not show any significant difference between both peptide complexes with cyclin A (data not shown).

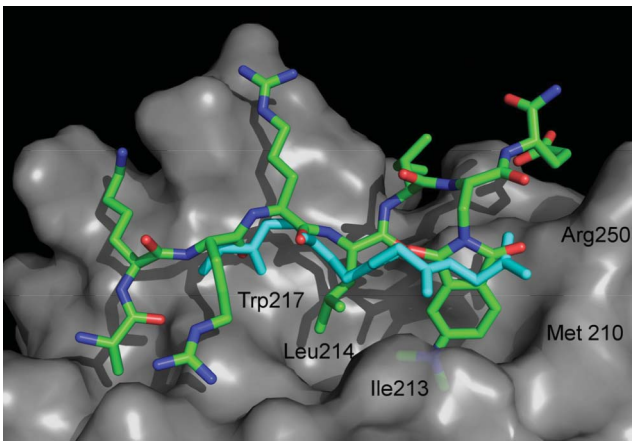

Fig. 4 Binding orientation of peptide **5a** (green sticks) into the CBG (gray surface), as observed from an equilibrated snapshot of the MD trajectory. It can be appreciated that the 4-DMAP residue is buried into the hydrophobic CBG, in agreement with the crystal structure of the peptide used as starting point for these studies (main chain shown with blue sticks).

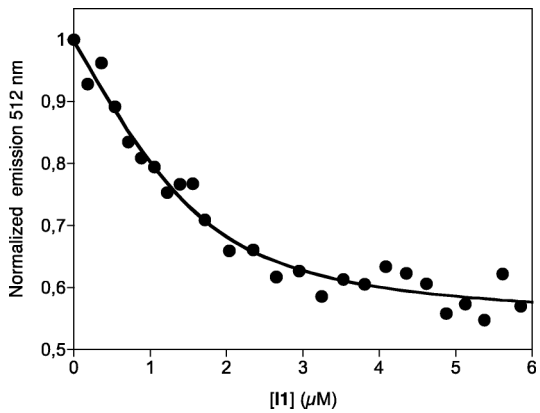

Fig. 5 Competition titration of cyclin A (1.5 μM) and the environment-sensitive probe **5a** (1.5 μM in 10 mM HEPES pH 7.6, 100 mM NaCl buffer) with increasing concentrations of the peptide inhibitor **I1** (●). The curve represents the best fit to a 1 : 1 competition model (see ESI† for details).

We finally sought to demonstrate the use of these newly developed fluorescent probes as tools for the identification of cyclin A inhibitors. We therefore synthesized a set of short peptides with known CDK2-cyclin A inhibitory properties,[14a] and performed competition titrations of each of these against the 4-DMAP peptide probe **5a**. Fig. 5 below shows the fluorescence displacement assay of probe **5a** with inhibitor **I1**. Data fitting to a standard competition model,[24] allowed us to reproduce the relative kinase inhibition properties of the peptides, observing better binding constants for those peptides that display lower IC_{50} values. Thus, while peptide **I1** (HAKRRLFG, IC_{50} = 0.67 μM) displayed *in vitro* a good binding constant (K_D ~ 0.31 μM), peptides **I2** (SAKRRLFG, IC_{50} = 0.73 μM) and **I3** (HSKRRLFG, IC_{50} = 1.4 μM) showed weaker binding affinities for cyclin A (K_D ~ 0.73 and 4.6 μM, respectively) (see ESI† for details).

Conclusions

We have developed a new peptide fluorescent sensor that specifically interacts with the substrate recruiting groove of cyclin A. Detailed molecular dynamics calculations explain the observed changes in fluorescence emission in relation with the shielding of the 4-*N*,*N*-dimethylaminophthalimide fluorophore from the bulk water, and suggest that the nature of the residue at position 4 can modulate the binding mode of the peptides. However, for the two active peptides **4a** and **5a**, equipped with Gly and Ile, the binding mode is shown to be equally susceptible to bury the 4-DMAP into the CBG. We also demonstrated that this probe can be used to easily identify cyclin A inhibitors through competition titrations. Future efforts aim at improving the poor cell uptake observed in preliminary experiments with HeLa cells, increasing the binding affinity, and developing the competition assay as a high-throughput screening method to identify novel cyclin A binders.

Acknowledgements

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