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A Photoregulated Ligand for the Nuclear Import Receptor Karyopherin α

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Abstract—The ability to orchestrate the transport of proteins between nucleus and cytoplasm provides cells with a powerful regulatory mechanism. Selective translocation between these compartments is often used to propagate cellular signals, and it is an intimate part of the processes that control cell division, viral replication, and other cellular events. Therefore, precise experimental control over protein localization, through the agency of light, would provide a powerful tool for the study and manipulation of these events. To this end, a prototype photoregulated nuclear localization signal (NLS) was derived from a native NLS. A library of 30 mutants of the bipartite NLS from *Xenopus laevis* nucleoplasmin containing a novel, photoisomerizable amino acid was prepared by parallel, solid-phase synthesis and screened in vitro for binding to the nuclear import receptor karyopherin α, which mediates the nuclear import of cellular proteins. A single peptide was identified in which the *cis* and *trans* photoisomers bind the receptor differentially. The strategy used to obtain this peptide is systematic and empirical; therefore, it is potentially applicable to any peptide-receptor system.

Introduction

Temporally controlled nuclear protein transport is a cardinal element of cellular regulation: which proteins enter the nucleus, and when, is critical for the regulation of cell division, signal transduction, gene expression, viral replication, and other cellular processes. The nuclear envelope is the firewall for cellular signals; signal carriers are most often proteins which cannot traverse the firewall passively but must enter the nucleus under active transport. Examples of signal carriers include mitogen-activated protein kinases (MAPKs), transcription factors, cyclins, and tumor suppressors, e.g., p53.[1–8] One illustrative, well-studied example is the transcription factor NF-κB, which is sequestered in the cytoplasm in complex with an inhibitory protein (IκB).[9] Signal-induced phosphorylation of IκB results in disruption of the NF-κB/IκB complex, nuclear translocation of NF-κB, and transcription of NF-κB dependent genes.

Our goal is to bring nuclear protein traffic, which is exquisitely orchestrated in the cell, under the precise control of the experimentalist through the agency of light. Realization of this goal will provide new tools for studying and controlling the transport process, as well as dependent processes such as gene transcription. For instance, the function of an individual transcription factor could be extricated from the complex set of signals converging on a cell.

In this report, we describe a first step toward this goal through the identification of a peptide that binds the nuclear import receptor subunit karyopherin α (Kap α) in a wavelength-dependent fashion in vitro, thereby serving as the prototype for a photoregulated NLS. This peptide was identified through scanning mutagenesis of a native NLS with a novel photoisomerizable amino acid (mp-Abc, Scheme 1), the *cis* and *trans* geometric isomers of which can be interconverted by light. A library of 30 mutants was prepared by solid-phase synthesis and screened via an on-bead receptor-binding assay. One peptide, derived by substitution of two wild-type residues with mp-Abc (number 30 in Table 1), displayed clearly distinct receptor affinities between its *cis* and *trans* photoisomers. Only the *cis* form binds, and for this reason, we term it PKL-1c (photoregulated karyopherin ligand 1, *cis* active).

The NLS is a peptide motif that directs the transport of proteins into the nucleus by binding to Kap α (also called importin α or PTAC58).[10–12] It is heterogeneous

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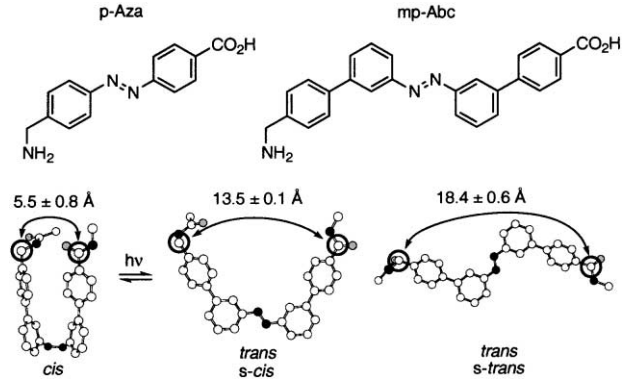
but consists minimally of a cluster of 6 amino acids containing 3–5 lysine or arginine residues.[13,14] The prototype NLS from the SV40 large T antigen has the sequence PKKKRKV.[15] A second class of NLS, termed bipartite, consists of two interdependent basic domains separated by a linker of 10–12 variable amino acids. The prototype bipartite NLS, which formed the subject of this investigation, is from *Xenopus laevis* nucleoplasmin and has the sequence KRPAATKKAGQAKKKKLD (basic clusters underscored).[16] The first basic domain consists of two residues, the second consists of 3–5 residues, and neither cluster is functional by itself. Import of proteins into the nucleus through the nuclear pore complex is an active, energy-consuming process.[17] In addition to the 60-kDa α subunit, which binds the import substrates via the NLS, karyopherin has a 90-kDa β subunit which mediates interactions with the nuclear pore complex and accessory proteins.[18–24]



Scheme 1. (Top) Structures of p-Aza and mp-Abc. (Bottom) Conformational change upon photoisomerization of mp-Abc. Coloring is as follows: carbon, white; nitrogen, black; oxygen, gray. The representative conformations of mp-Abc shown are the lowest-energy structures of each type (*cis*, *trans*/s-*cis*, or *trans*/s-*trans*) found in a Monte Carlo conformational search, and the distances shown are the energy-weighted average ± standard deviation for all structures of each type. Details of the computations are in the Experimental.

Table 1. Coupling summary for parallel synthesis[a,b]

Residue→ K R P A A T K K A G *[a] A T K K A G Q A K K K K L D Peptide↓
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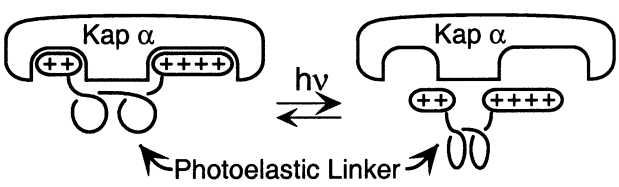
[a]* = mp-Abc.

[b]Each column represents one of the 25 steps in the parallel synthesis, the direction of which was C to N, i.e., right to left. A diamond indicates that the reactor for the corresponding peptide was present during the coupling, and the sequence of each peptide can thus be read (left to right) by noting the residue corresponding to each diamond in its row.

Results

Pursuit of light-mediated control of nuclear protein transport begins with the pursuit of a molecule that binds Kap α in a light-dependent fashion. A simple strategy toward this goal was suggested by the X-ray crystal structure of a fragment of yeast Kap α complexed with an SV40 NLS nonapeptide.[25] This structure revealed two binding sites, a larger one occupied by six ordered residues (KKKRKV, P1–P6) and a smaller one occupied by two ordered residues (KK, P2–P1). The sites were oriented head to tail and separated by 25 Å, which is equivalent to a fully extended octapeptide. From this information, it was inferred that the bipartite NLS chelates the receptor. More recently, the crystal structures of yeast[26] and murine[27] Kap α fragments in complex with the bipartite nucleoplasmin NLS were also solved. As the structures were solved after our identification of PKL-1c, they did not influence our experiments, but they do lead to useful insight into the possible mode of binding of PKL-1c to the receptor (see discussion).

We reasoned that if the length of the linker in a bipartite NLS could be toggled reversibly by light about the 25 Å threshold, the peptide's affinity for the receptor would likewise be toggled because chelation would be possible in the long form but not in the short form (Scheme 2). Thus, we sought a photoelastic linker that would expand and contract in response to light. The viability of targeting the linker of the bipartite NLS was reinforced by experiments showing that the linker is very tolerant of mutations. The nucleoplasmin NLS maintains activity when subjected to numerous point mutations, lengthened by 4–12 residues,[16] or replaced by PA_{20}.[28] However, shortening the linker by four residues

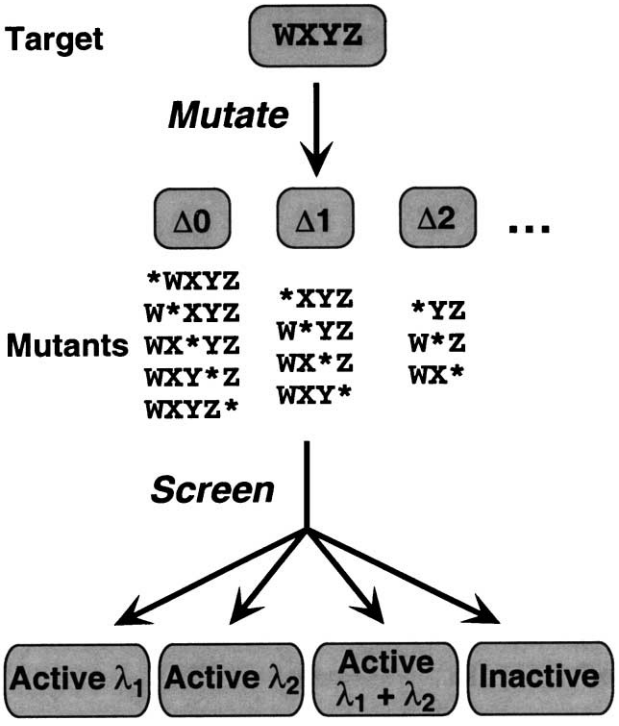


Scheme 2. Strategy for photochemical control of the binding of an NLS to Kap α. The circled + signs denote the basic clusters recognized specifically by the receptor, and the coil depicts the mutation-tolerant linker modified by the presence of a photoisomerizable amino acid to expand and contract in response to light.

(AGQA) eliminated its activity,[16] consistent with the need for at least eight residues to span 25 Å.

We next sought to identify a suitable photoelastic element for installation within the linker; amino acids were the obvious choice, and in the literature, we identified p-Aza (Scheme 1), an azobenzene-containing amino acid introduced by Chmielewski in 1994.[29,30] Azobenzenes are widely employed as photochemical switches due to the ease with which their *cis* and *trans* isomers can be interconverted by light. Isomerization of the thermodynamically favored *trans* form to the *cis* form, induced by exposure to near-UV light (ca. 350 nm), is accompanied by pronounced geometric change, most significantly for our purposes a shortening of the chromophore.

Molecular modeling was used to estimate the change in length of p-Aza that occurs upon photoisomerization. Using the *N*-acetamide/*C*-methylamide derivative as a model for p-Aza in the context of the peptide, we performed a MonteCarlo conformational search for both *cis* and *trans* isomers, resulting in the identification of 18 *cis* and 12 *trans* conformers from 1000 random starting geometries. Within each set, the span of the amino acid (separation of the arene substituents *para* to the azo linkage) was essentially constant at 6.2 Å for *cis* and 12.0 Å for *trans*, from which we conclude that *trans*→*cis* isomerization of p-Aza shortens its span by 6 Å, equivalent to the removal of two standard residues.

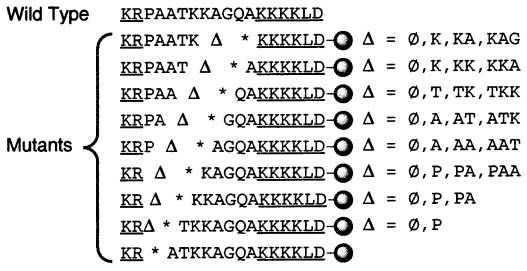


Scheme 3. General strategy for obtaining photoregulated mutants of biologically active peptides. A target sequence is subjected to systematic mutagenesis with a one or more photoisomerizable amino acids (∗) and screened for photodependent biological activity.

To amplify the photoelastic behavior of p-Aza, we designed a novel amino acid, termed mp-Abc, based on azobiphenyl (Scheme 1). An analogous MonteCarlo simulation was performed on this amino acid, resulting in the identification of 268 *cis* conformers and 192 *trans* conformers. Analysis of the results revealed that the low-energy *cis* conformers of mp-Abc favor a compact shape like that shown in Scheme 1 with spans averaging 5.5 Å (shorter than p-Aza and equivalent to a conventional dipeptide). Although longer *cis* conformers (up to 16.9 Å) were observed, these had significantly higher energy; no conformer longer than 6.6 Å was found within 4.1 kcal/mol of the minimum energy structure, the threshold of 4.1 kcal/mol corresponding to a factor of 10^{-3} in stability at 25 °C. Thus, the longer *cis* structures do not make a meaningful contribution to the population, a conclusion born out in subsequent calculations using water and chloroform solvation models.

The 192 *trans* conformers of mp-Abc fall into two distinct families, s-*cis* (96) and s-*trans* (96), having spans of 13.5 and 18.4 Å, respectively. Considering the longer distance to be a more meaningful measure of the *trans* isomer's reach, we concluded that *trans*→*cis* isomerization of mp-Abc shortens its span by about 13 Å, equivalent to removal of about 4 conventional residues. Thus, mp-Abc was predicted to have superior photoelasticity to p-Aza, and it formed the centerpiece of our strategy for creating a photoswitchable linker for the bipartite nucleoplasmin NLS. It was synthesized with *N*-Fmoc protection, shown to be compatible with standard Fmoc peptide synthesis protocols, and to display typical azobenzene photochemical behavior within model peptides; this work, as well as the results of extensive molecular modeling of p-Aza and several Abc isomers, are the subject of a forthcoming publication.[31]

After the selection of mp-Abc as the photoelastic element, the two remaining issues were location (where to put it) and deletion (which native amino acids to sacrifice in order to accomodate its intrusion). Because the structure of the linker was unknown at the time, we elected an empirical approach of systematic, scanning mutagenesis in which the reagent mp-Abc would be substituted into all possible positions within the linker. This approach, illustrated for a generic case in Scheme 3, is directly analogous to scanning alanine mutagenesis of native proteins,[32] through which critical and non-critical



Scheme 4. Implementation of the general strategy on the linker of the bipartite NLS from *X. laevis* nucleoplasm. The indicated set of 30 mutants were prepared in this study. Shaded spheres denote TentaGel resin, ∗ is mp-Abc, Δ denotes a set of nested deletions, and ø represents the absence of any residue. Constant regions are underscored.

residues of a polypeptide can be identified. For the specific case of the linker in the bipartite nucleoplasm NLS, length is a critical parameter, and we therefore elected to delete 2–5 native residues to accomodate the intrusion of mp-Abc; this range reflects consideration of the length of the longest *trans* conformations of mp-Abc (18 Å, equivalent to a fully extended pentapeptide) and the shorter *cis* conformations (5 Å, equivalent to a dipeptide).

The library we prepared thus contained 30 peptides (Scheme 4 and Table 1). It was constructed by solid-phase peptide synthesis using TentaGel resin with the acid-stable 4-hydroxymethylbenzoic acid (HMBA) linker and standard Fmoc protocols.[33] Parallel synthesis allowed the entire library of 30 mutants to be constructed with 25 peptide coupling reactions (see Table 1); use of MicroKan® reactors (330 μL internal volume, loaded with 15 mg/5 μmol of resin) afforded ample quantities of each peptide for analysis while maintaining an economy of materials (<0.5 mmol of mp-Abc was needed for the whole library). Deprotection of the side chain protecting groups without cleavage from the resin afforded a solid-phase library of mutants in which each member was represented as a homogeneous pool of beads of known sequence.

The peptides were screened for binding to Kap α using a colorimetric, on-bead assay we developed.[34,35] As we desired a peptide that bound Kap α in a light-dependent manner, the assay was performed in parallel on beads that had been thermally equilibrated ($>95\%$ *trans*) and on beads that had been irradiated with 366-nm light (approximately 80% *cis*).[31]

From this screen, a single candidate (PKL-1c) was identified that met the desired criterion of photosensitive binding. The peptide, having the sequence KR[mp-Abc]ATKKAGQAKKKKLD (substitution of mp-Abc for the first two residues of the linker, Pro-Ala), bound slightly weaker than the wild type nucleoplasmin NLS in the *cis* form but had no detectable binding in the *trans* form (Fig. 1). Specificity of the interaction was confirmed through competition with a soluble peptide containing the SV40 NLS; the soluble peptide at 10 mM completely prevented color development.

HPLC analysis of the peptide cleaved from the resin showed it to be 75% pure (typical for a crude peptide of its length) with one impurity of 10% and no others exceeding 5% (Fig. 2, top). The identity of the peptide was confirmed by MALDI-TOF mass spectrometry (Fig. 2, bottom), which showed a dominant parent ion peak (m/z calcd for $[M+H]^+ = 2188.29$; found = 2187.97) with only small peaks attributable to fragmentation or impurities. Mass spectral data carry added weight in this instance because the mass of PKL-1c is unique within the library, and all findings have been verified with indepently synthesized PKL-1c.

Discussion

PKL-1c is the prototype for a reversibly photoregulated NLS, and an assessment of its capacity to function in cells is underway. Light is an ideal agent for manipulating physiological systems because it can be delivered rapidly, in precise quantity, and non-invasively to cells. Accordingly, photochemistry has already provided powerful tools for the study of physiological systems, most notably through the use of 'caged' derivatives of biologically active molecules such as cyclic nucleotides, neurotransmitters, and more recently, proteins.[36–40] These compounds often feature *o*-nitrobenzyl or related protecting groups which can be rapidly and irreversibly cleaved by light to release the active principal.

The present work is conceptually different from uncaging in that it is based on fully reversible photochemistry. Despite the obvious appeal of reversibility, there is only one system outside nature where reversible photochemistry has been used to manipulate the response of a cell. In 1971, Erlanger introduced the compound 'Bis Q'[41] (Scheme 5) which, like mp-Abc, is a derivative of azobenzene and undergoes facile *cis*→*trans* photoisomerization. The *trans* isomer of Bis Q, but not the *cis* isomer, is an effective AChR agonist, i.e., a neurotransmitter. This property, along with the rapid, reversible, and clean photoisomerization, was exploited by Lester and coworkers in a series of elegant experiments on the kinetics of AChR activation using Bis Q and some related compounds.[42,43] By using flashes of light to control the isomer distribution, they were able to control the effective neurotransmitter concentration.

This pioneering work amply demonstrated the virtues of reversible photochemistry, but to our knowledge, no new applications of reversible photochemical reactions in vivo have been introduced subsequently, which is surprising given the number of applications in vitro to biomolecules.[44–46] A number of small molecules, most containing the azobenzene unit, have been prepared as photoregulated modulators of enzyme activity.[45,47,48] Photoregulated enzymes have also been prepared through random side-chain modification with reagents based on azobenzene or other photoisomerizable groups;[49–51] more recently, *p*-phenylazo-phenylalanine (Pap, Scheme 5) was incorporated into the backbone of phospholipase A_2 by semi-synthesis,[52] and into streptavidin by cell-free, in-vitro translation.[53]

Less work has been done with biologically active peptides, although there have been noteworthy successes. For example, several modifications of the pore-forming peptide antibiotic gramicidin A have been made to render its ion conductance in synthetic bilayers photosensitive.[54–58] We have found only two cases involving photoregulation of peptide–protein association. In the earliest example, Harada et al. raised an antibody against a Pap-containing peptide which bound only the *trans* photoisomer.[59] More recently, two groups substituted Pap into selected positions of the 15-residue ribonuclease S peptide and found that the Thr^4→Pap and Asp^{14}→Pap mutants displayed photoregulatable ribonuclease activity upon complementation with the S protein.[60,61]

Caging of biologically active molecules is a clear and general strategy for controlling physiological response

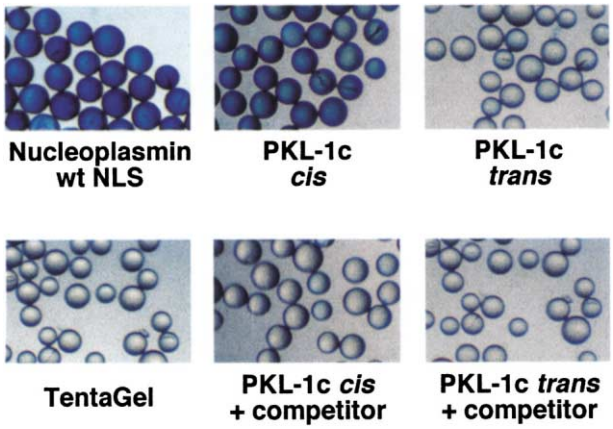


Figure 1. Binding of Kap α to peptides. Deprotected peptides on TentaGel® resin were incubated with 50 nM biotinylated GST-Kap α in phosphate buffered saline at pH 7.4, with or without an added competitor (10 mM SV40 T antigen NLS peptide GSTPPKKKRKV). Protein binding was detected by alkaline-phosphatase-linked streptavidin and the color reagent 5-bromo-4-chloroindolyl 3-phosphate (BCIP) as described;[34,35] binding of the receptor is indicated by a blue color. Prior to assay, the beads were incubated at 37 °C for 24 h to equilibrate PKL-1c to the *trans*; the *cis* form was produced by irradiation at 366 nm (30 min with a 4-W handheld TLC lamp) immediately prior to the addition of biotinylated GST-Kap α.

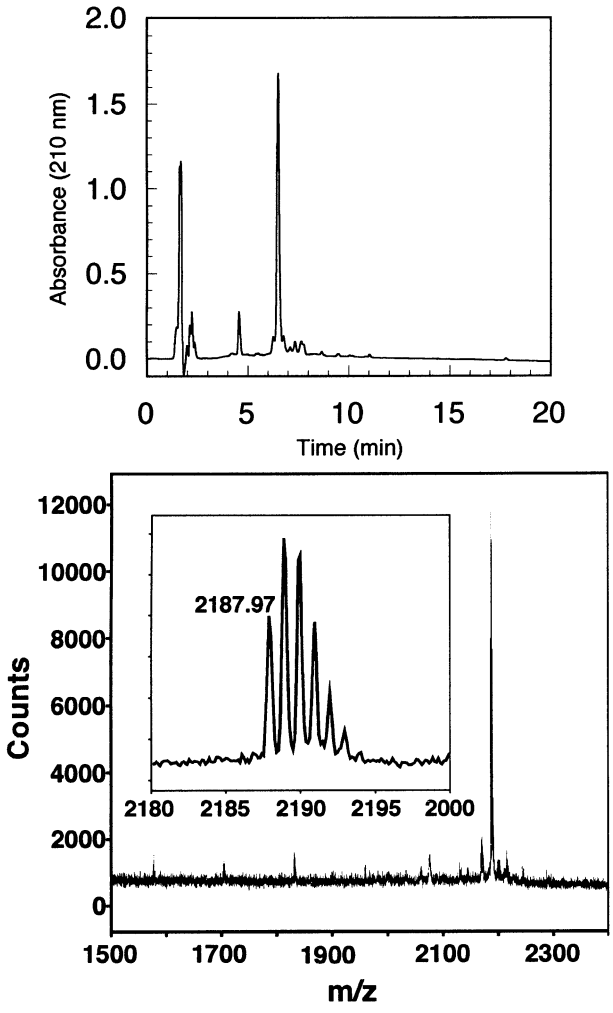


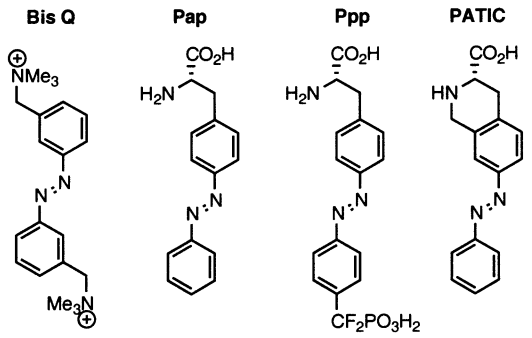
Figure 2. Reversed-phase HPLC chromatogram (top) and MALDI-TOF mass spectrum (bottom) of crude PKL-1c.

through an irreversible process; however, as the examples mentioned illustrate, there is no general strategy for developing reversible processes that mediate physiological response, in particular for generating photoregulated peptide ligands for proteins. The Pap-binding antibody of Harada et al. was obtained by the opposite tactic (generating a protein to match a peptide). For gramicidin S, the tactics applied are target specific, relying on the unique properties of the peptide. In the case of the ribonuclease S peptide, both groups targeted only selected residues for mutagenesis.

The empirical approach employed in the present work, scanning mutagenesis of a native peptide with a photoisomerizable amino acid, is an extension of the selective mutagenic approach employed by Woolley and Hamachi with the ribonuclease S peptide. Our approach, which is distinguished by being systematic and exhaustive, has been made feasible by advances in parallel, small-scale peptide synthesis, coupled with a rapid receptor-binding assay. Together, these allowed us to prepare and evaluate a large number of candidates economically with respect to both materials and time. Because the approach is systematic, it can be applied to any peptide-receptor system for which a suitable screen exists without any knowledge of the three-dimensional structure of either partner, nor any other special insight. As the discovery of PKL-1c illustrates, however, the approach can be guided and focused by structural information. Thus, it offers one potentially general approach to the problem of developing photoregulated peptide-receptor systems. It has three key requirements: one or more photoisomerizable amino acids, an efficient synthetic strategy, and a rapid screen.

With respect to the first issue, the current arsenal of photoisomerizable amino acids (Schemes 1 and 5) includes Pap,[62,63] p-Aza (the inspiration for mp-Abc), mp-Abc and several isomers of it,[31] PATIC (a conformationally constrained azobenzene-based amino acid),[64] and three phospho-amino acid analogues typified by Ppp;[65] this collection will no doubt grow in the future. With respect to the second issue, synthesis, peptide chemistry is very efficient and reliable, and technical innovations (e.g., microreactors) allow for simple, parallel synthesis of defined libraries of peptides. For this work, synthesis of the 30-peptide library required about 3 weeks and <0.5 mmol of Fmoc-mp-Abc; thus, synthesis is not rate-limiting.

Screening, the most variable and implementation-dependent aspect, proved the most difficult and time-consuming stage in the present work but may prove simpler in other systems. Karyopherin is particularly difficult in this regard in that it is auto-inhibited and no absolute binding determinations have been made for any karyopherin ligand, let alone a photoregulated one. Although an ELISA producing relative binding affinities has been developed by Jans and coworkers,[66] it is not readily adapted to synthetic molecules. For our work, we employed an on-bead assay that is qualitative but readily distinguishes strong and weak NLS peptides. A significant problem with screening azobenzene-based



Scheme 5. Structures of Bis Q and photoisomerizable amino acids.

compounds on beads is that photoisomerization is incomplete; in model peptides, mp-Abc can be converted to >98% *trans* by thermal equilibration, but only 80–85% *cis* by irradiation at 366 nm.[31] Thus, analysis of *cis* isomers is complicated by the ever-present background of *trans* isomer, a problem that becomes more pronounced as the length of the assay increases and partial equilibration occurs. We are actively pursuing improvements of our present assay.

Among the findings of this investigation, two observations are particularly striking. First, the discovery of a successful candidate in a small library bodes well for the potential generality of our strategy of systematic, scanning mutagenesis. Secondly, the discovery of a *cis*-active peptide runs contrary to expectation and is purely fortuitous. Our chelation model predicted the longer, *trans* form of the peptide should be active, but the opposite was found. The activity of the *cis* form can perhaps be understood by considering the recently-solved crystal structure of yeast and murine Kap α fragments in complex with the wild-type nucleoplasmin NLS. These structures show the linker in a largely extended conformation with two turns. One of these turns forms around the Pro-Ala dipeptide replaced by mp-Abc in PKL-1c.

Our initial analysis of the conformation of mp-Abc focused simply on its length, and our conclusion for the *cis* form was that it is approximately as long as a conventional dipeptide. In light of the discovery of PKL-1c, we undertook further analysis to explore the possibility that the *cis* form of mp-Abc could favorably adopt a conformation similar to that of the Pro-Ala unit in the nucleoplasmin NLS. To that end, a set of 379 energy-minimized mp-Abc conformers generated through a MonteCarlo search was compared to the conformation of Pro-Ala as observed in the X-ray crystal structure of the *X. laevis* nucleoplasmin NLS bound to Kap α.[26] The specific point of comparison was overlap of the two bond vectors indicated in Fig. 3 (left), which dictate the path of the polypeptide backbone as it enters and leaves the Pro-Ala unit.

Of the 379 conformers, 11 had root mean square deviations (RMSD) of ≤0.5 Å for the four atoms defining the two bond vectors, and another 47 had RMSDs between 0.5 and 1.0 Å; the average RMSD (weighted for conformational energy described in the Experimental) was 1.7 Å. From this exercise, we conclude that many mp-Abc conformers could be substituted for Pro-Ala within the nucleoplasmin NLS with only minimal perturbation of the backbone. Figure 3 (right) shows the best-match mp-Abc conformer superposed onto the Pro-Ala unit. It must be stressed that Figure 3 is purely for illustration and is not meant to imply that the conformation of mp-Abc shown is adopted in PKL-1c when it binds Kap α (it most likely is not). A detailed understanding of the interaction between PKL-1c and Kap α must await experimental structure determination.

Another question raised by our results is why no *trans*-active candidates were found. Indeed, many members of the library bind Kap α in the *trans* form, but also appear to bind in the *cis* form. We believe that in most cases, this is due to the background of *trans* isomer (15–20% at first and growing during the assay) in the photogenerated *cis* form. In other words, *cis*-active peptides are more easily and clearly detected than *trans*-active peptides. We believe that with better and faster assay methods, more candidates will emerge from this library, including *trans*-active peptides.

Finally, it is noteworthy that the NLS is a transferrable motif: an NLS, taken from one protein and attached to another either by genetic fusion or chemical cross-linking, will direct the other into the nucleus. Therefore, a single photoregulated NLS should be applicable to many proteins. With recent advances, it is now possible to ligate synthetic molecules site-specifically to essentially any protein that can be expressed in *Escherichia coli*.[67] As the conjugates can be introduced easily into cells, it

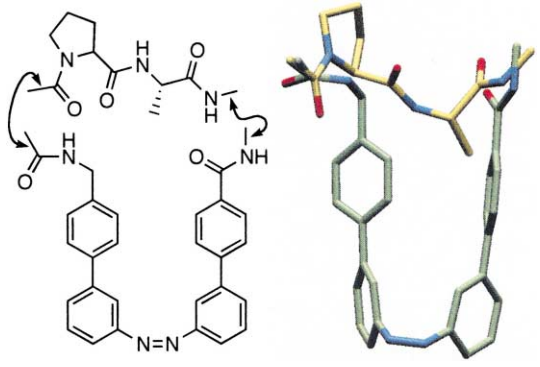


Figure 3. Comparison of mp-Abc and a prolyl-alanyl moeity. (Left) Structural diagram; the arrows indicate the two bonds used in the three-dimensional superposition at right. (Right) Three-dimensional superposition. The conformation of the prolyl-alanyl dipeptide is exactly as found in the X-ray crystal structure of the nucleoplasmin NLS-yeast Kap α complex.[26] The conformation of mp-Abc (as its *N*-acetamide, *C*-methylamide) is one obtained from a Monte Carlo conformational search using a water solvation model as described in the Experimental. All 379 energy-minimized conformers found in this search were compared to the prolyl-alanyl unit on the basis of least-squares superposition of the four atoms defining the terminal bond vectors (marked by arrows at left). Among the eleven structures having RMSD ≤0.5 Å, the one shown (RMSD = 0.41 Å), which has an energy of 2.6 kcal/mol above the minimum, had the best overlap of the adjacent amide and carbonyl bonds.

should be possible to realize the full potential of the photoregulated NLS for experimental control of cellular signaling.

Conclusion

Through use of a systematic, empirical strategy, we have identified a novel, photoisomerizable ligand (PKL-1c) for the nuclear import receptor karyopherin α. PKL-1c is one of very few molecules, and the first peptide, to display photoregulated binding to a biological receptor. The identification of PKL-1c provides the first illustration of the use of parallel synthesis and screening of photoisomerizable molecules to identify a molecule possessing a desired and photoregulated property. The strategy employed is general and could potentially be applied to any peptide–protein pair. In the present case, the greatest difficulty was encountered in the binding assay; improved methodologies in this area promise to increase the applicability of the system greatly, and quantitative assays would accelerate ligand optimization. With regard to PKL-1c itself, the next step is develop it as a tool for the functional control of protein localization in cells, which is a task of considerable magnitude. Recently, we have seen promising early signs, but considerable obstacles remain between this goal and its realization.

Experimental

General. The amino acid mp-Abc was synthesized with *N*-Fmoc protection by a route to be described in a forthcoming publication.[31] In the interim, details will be provided upon request. All other reagents were obtained from commercial suppliers and were used without further purification. NovaSyn® TG HMBA resin was purchased from Novabiochem (San Diego, CA), Fmoc amino acids from Novabiochem or Advanced ChemTech (Louisville, KY), and anhydrous *N*-methylpyrrolidinone (NMP) and DIEA from Advanced ChemTech. Diethyl azodicarboxylate (DEAD), triphenylphosphine, TFA, and 1-hydroxybenzotriazole (HOBt) were purchased from Aldrich. Acetonitrile for gradient analysis was from EM Science (AX0142–1). Other solvents and reagents were of reagent grade from commercial suppliers. MicroKans® were purchased from Irori (La Jolla, CA). 96-Well plates with a 0.45 μm membrane filter bottom (MAHV N45) and Ultrafree-MC® centrifugal filtration units (UFC3BTK00) were purchased from Millipore.

Molecular modeling. Molecular mechanics calculations were performed using MacroModel[68] version 6.0 and the supplied MM2* forcefield.[69] p-Aza and mp-Abc were modeled as their N-terminal acetamide/C-terminal methylamide derivatives to simulate the context of a polypeptide. An extensive set of molecular mechanics calculations on pAza and several Abc isomers will be reported in a forthcoming publication.[31]

To ascertain the effective length of mp-Abc, a MonteCarlo conformational sampling was performed using 1000 randomly generated starting geometries each for the *cis* and *trans* isomers, followed by energy minimization to convergence (≤ 0.01 kJ/Å gradient) and elimination of structures having energies >50 kJ/mol above the lowest-energy structure. For each set of related conformers (*cis*, *trans*/s-*cis*, and *trans*/s-*trans*), the average distance between the atoms indicated in Scheme 1 was calculated after energy-weighting the distance in each individual conformation to reflect its contribution to the population of n conformers at 25 °C. A conformer's weight w is given by $w = c^{*}\exp(-\Delta E/RT)$, where ΔE is the difference in energy between the conformer and the lowest-energy conformer in the population, and where c is a normalization constant such that $\Sigma w = n$. The average distance is then given by $R_{avg} = \Sigma R w/\Sigma w$, and the standard deviation is given by $s_R = \{[n(\Sigma R^2 w) - (\Sigma r w)^2)]/[n(n\text{-}1)]\}^{1/2}$.[70]

For comparison with the Pro-Ala dipeptide, the Monte Carlo simulation for *cis* mp-Abc was repeated with the GB/SA water solvation model[71] applied during energy minimization to more closely approximate the behavior of mp-Abc in aqueous solution. This search resulted in the identification of 379 unique conformers, all having energies within 20 kJ/mol of the most stable one.

Procedure for loading first amino acid on the solid support. The first amino acid was loaded with the Mitsunobu reaction.[72] NovaSyn TG HMBA resin (2.0 g, 0.6 mmol) was suspended in 100 mL of anhydrous THF containing triphenylphosphine (787 mg, 3 mmol) and Fmoc-Asp(O*t*-Bu)-OH (1.24 g, 3 mmol). DEAD (523 mg, 3 mmol) was added slowly to the reaction mixture over 20 min. The reaction mixture was gently stirred at room temperature for 24 h, after which the resin was washed well with THF and dichloromethane (DCM). Extent of loading was determined by quantitative Fmoc analysis as follows. An aliquot of ≈10 mg of beads was deprotected with 1 mL of 20% (v/v) piperidine/NMP for 15 min with intermittent mixing. The absorbance at 301 nm was determined after 50-fold dilution with NMP, and the concentration of dibenzofulvene was calculated using $\varepsilon_{301} = 7800\ M^{-1}\ cm^{-1}$.

Overview of library construction. The 30-peptide library was prepared using a total of 25 peptide coupling steps as indicated in Table 1. Steps 1–14 were performed starting with 1 mmol of resin in a single, conventional shaker flask (a medium-porosity fritted funnel modified with a Teflon stopcock in its stem and a screw thread at its top) with mechanical shaking. Starting after Step 6, approximately 0.11 mmol was removed after each coupling to provide equal amounts of the 9 fully protected intermediates KKKKLD-•, AKKKKLD-•,... ATKKAGQAKKKLD-• (the • represents the synthesis resin). Steps 15–25 were performed using 30 MicroKan reactors, each of which was permanently numbered with a hot wire and loaded initially with 15 mg of resin bearing one of the nine intermediates. The number of MicroKans for each intermediate (one to four) can easily be inferred from Table 1. Reactions with Micro-

Kans were performed in round-bottom flasks with magnetic stirring. After liquids were introduced, the flask (sized according to the number of MicroKans used in the step) was placed under vacuum until all entrapped air had been removed, vented to nitrogen at 1 atm, and stoppered. For rinsing, bulk liquids were removed by filtration, and each MicroKan was drained individually by applying suction to the bottom of the can (using a bored out rubber septum as a vacuum seal) while adding generous amounts of rinse solvent from a wash bottle.

Peptide synthesis. N_α-Fmoc protection and the following side-chain protecting groups were used: Boc (K), Pmc (R), *t*-Bu (T and D), and Tr (Q). Peptide coupling cycles for standard amino acids were performed as follows. The resin was mixed with 2.5 equiv of *N*-Fmoc-protected amino acid, 2.5 equiv of PyBOP,[73] 2.5 equiv of HOBt, and 5 equiv of DIEA in NMP. The reaction mixture was shaken for 1.5 h (flasks) or stirred for 3 h (MicroKans) at room temperature, then washed 6× with NMP. For mp-Abc, less monomer was used (1.5 equiv of Fmoc-mp-Abc, 1.5 equiv of HOBt, 1.5 equiv of PyBOP, and 3 equiv of DIEA), and the reaction time was lengthened to 4 h. Fmoc deprotection was carried out by a 30-min treatment with 20% piperidine/NMP, followed by washing 6× with NMP. For the first six steps, completion of each reaction was verified with the Kaiser ninhydrin test.[74] Reactions in MicroKans were not monitored during library construction; instead, a control peptide (the wild-type nucleoplasmin NLS) was synthesized simultaneously using the same reagents. The control was synthesized both in a MicroKan with the library and in a separate shaker flask. Completion of each coupling in the shaker flask was determined by the Kaiser test or, for proline, the chloranil test.[75] The crude control peptides from the MicroKan and from the shaker flask gave essentially identical HPLC chromatograms (data not shown).

Preparation for on-bead binding assay. A small amount of resin was transferred from the MicroKan to an Ultrafree-MC centrifugal filtration device. The side chain protecting groups were removed by treating the resin with a cocktail of 95% TFA, 2.5% water, and 2.5% anisole for 2 h at room temperature. The deprotection cocktail was filtered off, and the beads were washed once with neat TFA, 3× each with NMP, MeOH, H_2O, and finally phosphate buffered saline (PBS; 10mM sodium phosphate, 150 mM sodium chloride, pH 7.4). For assay, the beads were transferred to the 96-well filter-bottom plate. Equilibration to the the *trans* isomer was effected by incubating the beads in PBS for $\geq$24 h in the dark at 37 °C. Conversion to the *cis* isomer was effected by irradiating the beads in PBS with 366-nm light from a 4-W handheld lamp (Ultraviolet Products, San Gabriel, CA) for 30 min.

On-bead colorimetric binding assay. The assay was performed as described[34,35] using recombinant murine karyopherin α_2 (PTAC58) as its glutathione-*S*-transferase (GST) fusion.[12] For mp-Abc-containing peptides, the assay was run in the dark (a windowless room with lights out). Subdued, indirect illumination was used only where strictly necessary (a red photographic safelight is suggested).

Cleavage of peptide from the solid support. Ice-cold, 1-M aqueous sodium hydroxide (20 mL per gram of resin) was added to the resin. The mixture was allowed to stand for 15 min at room temperature and was then acidified with 10% aqueous AcOH (20 mL per gram of resin), filtered, adjusted to pH 7, and lyophilized.

HPLC. Reversed-phase HPLC was performed with a Waters Symmetry C_{18} column (4.6×150 mm, 5 μm particle size) eluted with a 20-min gradient of 10–40% B, where solvent A is 0.1% v/v TFA in water and solvent B is 0.08% v/v TFA in acetonitrile. Prior to analysis, mp-Abc-containing peptides were thermally equilibrated to eliminate peaks arising from the *cis* photoisomer. Detection was by absorbance at 210 or 290 nm (the latter is isosbestic for the *cis* and *trans* forms of mp-Abc).

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