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A Simple, Solid-Phase Binding Assay for the Nuclear Import Receptor Karyopherin α. Part 1: Direct Binding

Michael D. Connolly, Seung Bum Park, Brian M. Reedy and Robert F. Standaert*

Department of Chemistry, Texas A&M University, PO Box 30012, College Station, TX 77842-3012, USA

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Abstract—The nuclear import receptor karyopherin α recognizes nuclear localization signals (NLSs), peptides that direct the transport of proteins into the nucleus. A simple, colorimetric assay has been developed to facilitate the identification and comparison of karyopherin ligands by direct and competitive binding using NLSs immobilized on the solid phase (TentaGel® resin).

Introduction

Proteins are imported into the nucleus in an active process initiated by the binding of the import substrate to the nuclear import receptor karyopherin α (Kap α, also called importin α).[1–3] Exquisite temporal control of import is exercised over many key regulatory and signalling proteins, including transcription factors[4] and mitogen activated protein kinases (MAPKs).[5] Nuclear protein import is therefore an area of active research with significant potential for therapeutic intervention.[6]

Import substrates are recognized by Kap α through a short peptide motif, termed the nuclear localization signal (NLS). NLSs are a heterogeneous group of peptides (Table 1), consisting minimally of a hexapeptide in which 3–5 residues are basic (K or R). The prototype signal from the SV40 large T antigen has the sequence PKKKRKV (Entry 1, Table 1). A second class, referred to as bipartite, is typified by the NLS from *Xenopus laevis* nucleoplasmin (Entry 5, Table 1). These signals have two interdependent basic clusters, neither competent as a signal by itself, separated by a spacer of about 10 residues. The X-ray crystal structure of a fragment of yeast karyopherin α[7] revealed two binding sites, 25 Å apart, for the SV40 NLS peptide SPKKKRKV. The larger site bound the hexapeptide KKKRKV specifically (P1–P6), and the smaller site, bound only a KK dipeptide fragment (P1′–P2′). Bipartite NLSs were proposed to need both sites for high affinity binding.

As part of our effort to develop novel ligands for Kap α, which might be useful as specific agonists or antagonists of import and as tools for understanding molecular recognition, we required a rapid and reliable screen for binding to the receptor. No quantitative determination of binding affinity for karyopherin to any ligand has been made, and there were no simple means of evaluating novel ligands. Several groups have used qualitative means to assess the interaction. Affinity chromatography, using peptide ligands cross-linked to agarose, has been used to compare affinities of several peptides for the receptor, and conversely to assess the selectivities of different receptor isoforms.[14,15] Jans's group has developed an ELISA using NLS–β-galactosidase fusions that provides quantitative relative affinities for peptide NLSs.[16] Because we are developing synthetic, non-peptide karyopherin ligands, we turned our efforts toward an assay performed directly on synthesis resin. We report here a simple, colorimetric assay for karyopherin ligands immobilized on TentaGel® resin that, while qualitative, clearly differentiates ligands by affinity within the range of biological activity. The assay, illustrated in Scheme 1, is based on detection of biotinylated Kap α with alkaline-phosphatase linked streptavidin and the color reagent 5-bromo-4-chloroindolyl 3-phosphate (BCIP).[17,18]

Peptide Substrates

Three NLS-related peptides were targeted for study: the SV40 T antigen NLS (GSTPPKKKRKV), a K→T

*Corresponding author. Tel.: +1-979-862-1405; fax: +1-979-845-9452; e-mail: standaert@mail.chem.tamu.edu; www.chem.tamu.edu/faculty/standaert

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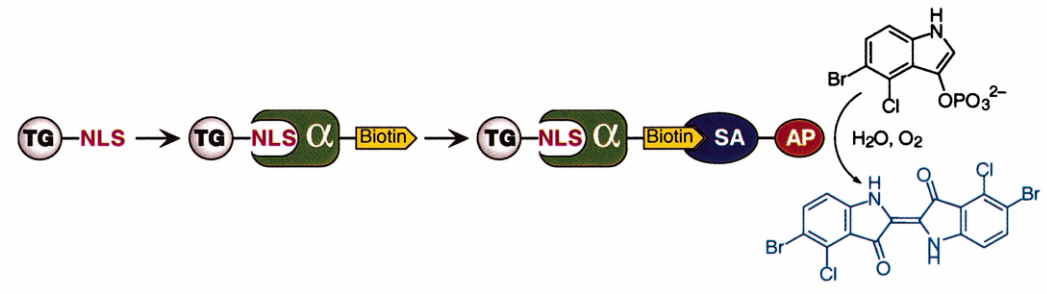
Table 1. Representative nuclear localization signals

Entry	Sequence[a]	Source	References
1	PKKKRKV	SV40 large T antigen	8,9
2	PKTKRKV	Mutant SV40 (weak activity)	10
2	PAAKRVKL	Human c-myc	11
3	GKKKYKLKH	HIV-1 matrix protein	12
5	KRPAATKKAGQAKKKKLD	*X. laevis* nucleoplasmin	13

[a]Probable P1–P6 and, for the bipartite NLS, P1′–P2′ residues are underlined.



Scheme 1.

mutant of this NLS (SV40^{M}: GSTPPKTKRKV) and the bipartite nucleoplasmin NLS (KRPAATKKAGQAKKKKLD). The prototype SV40 NLS, with five basic residues, has strong signal activity in cells from many species. The mutant has weak but observable signal activity[19] and marks the low end of the useful affinity range. Finally, the prototype bipartite NLS from nucleoplasmin was chosen to represent this interesting class of signal. The peptides were synthesized as described below on TentaGel® resin (80–100 μm bead size, 0.3 mmol/g loading) with the hydroxymethyl benzoic acid linker, which allows on-bead deprotection of acid-labile side-chain protecting groups without cleavage.

Assay Implementation

Two methods that have gained favor for the sensitive detection of protein binding in on-bead assays are fluorescence and enzyme-linked colorimetry.[20,21] We attempted the former with rhodamine-labeled GST–Kap α, but could not get a strong signal with clean background. We therefore turned to an enzyme-linked method using alkaline phosphatase, which has been recognized as an enzyme of choice in on-bead assays.[21] We first examined an antibody-based protocol for detecting GST–Kap α, using anti-GST antibody with alkaline phosphatase-conjugated secondary antibody. This approach was successful but cumbersome, and to streamline it, we turned to a more direct linking protocol using biotin–streptavidin.

Results

As a procedural starting point, we took the protocol employed by Liang et al.[22] for the identification of carbohydrate ligands of a plant lectin. It was found that the reported wash and blocking conditions produced a clean background on unmodified TentaGel® (Fig. 1, Panel 4). However, three key variables needed optimization: Kap α concentration, incubation time of Kap α with the beads, and color reagent. With an incubation time of 3 h, the optimal Kap α concentration was found to be 50 nM. Doubling the concentration to 100 nM did not increase signal, and increasing it to 500 nM caused significant background staining, even with unmodified TentaGel® (not shown). At 50 nM, the amount of protein (10 pmol/well) is ≈0.05% of the amount of immobilized peptide (≈20 nmol/well); this finding is consistent with

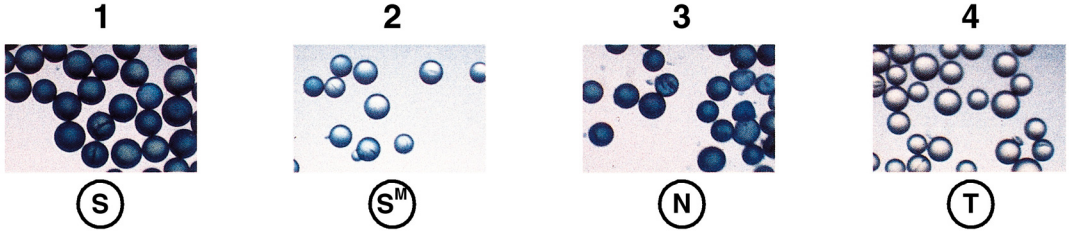


Figure 1. Assay of native NLS peptides. Circled letters denote the ligand on TentaGel®: S, SV40 NLS; S^{M}, SV40^{M} NLS; N, nucleoplasmin NLS; T, TentaGel® (unmodified). All assays were run simultaneously and subjected to identical development times. Photomicrographs were taken with Ektachrome tungsten-balance slide film, from which the images were scanned directly at the photolab and are reproduced without enhancement or modification.

surface-only binding of the protein, as is expected with TentaGel®, where protein binding capacity is typically 0.1–1% of total loading.[23] Optimal incubation times were found to depend on the immobilized ligand, and to provide insight into the relative binding affinity of different ligands. With SV40, regarded as a very strong NLS, some color developed with as little as 5 min of incubation, and maximum intensity was attained within 20 min. In contrast, nucleoplasmin and $SV40^M$ required a 60-min incubation to reach maximum intensity.

As shown in Figure 1, the final color was also indicative of the relative binding strength of the NLS: most intense for SV40 (Panel A), approximately 80% as intense for nucleoplasmin (Panel B),[24] and faint (approximately 5% as intense) but distinguishable from background for $SV40^M$ (Panel C). Comparison of two alternative color reagents, BCIP alone or in combination with nitro blue tetrazolium (NBT),[25] revealed that the former was superior. As has been found with other systems,[21,22] NBT rendered the assay too sensitive; both SV40 NLS and $SV40^M$ gave dark-blue colors with only 5 min of development. Thus, an important virtue, the ability to distinguish among Kap α ligands of varying strength, was lost.

Conclusion

The assay described herein will be a valuable tool in the discovery of novel karyopherin ligands, and has already proved fruitful in this capacity in our laboratory. We have applied the assay only to uniformly sized pools of identical beads and found it to be very reliable. Due to the assay's sensitivity to protein:ligand stoichiometry, care is urged in its application to mixed pools from combinatorial synthesis.

Assay Procedure

Definitions and materials

PBS is 150 mM NaCl, 10 mM NaH_2PO_4 (pH 7.2, NaOH); TBS is 150 mM NaCl, 20 mM Tris (pH 7.4, HCl); suffixed T denotes 0.05% Tween 20, and suffixed B_n denotes n% bovine serum albumin; APB is 100 mM NaCl, 100 mM Tris, 2 mM $MgCl_2$ (pH 9.2, HCl); stop buffer is 40 mM EDTA (pH 7.4, NaOH). Kap α, as a fusion with GST, was expressed in *Escherichia coli* as described.[3] Biotinylation with biotinamidocaproate *N*-hyroxy-3-sulfosuccinimide ester was performed with the ImmunoProbe kit from Sigma according to the manufacturer's instructions to provide a biotin/protein ration of 2.5. Alkaline phosphatase-conjugated ExtrAvidin® (AP–SA), BCIP, and NBT were obtained from Sigma. Protein concentrations were determined spectrophotometrically, taking $\epsilon_{280} = 89{,}100\ M^{-1}\ cm^{-1}$ for GST–Kap α.

Procedure

TentaGel® beads were dispersed at 1 mg/mL in PBS/glycerol; the glycerol concentration (30% for unmodified TentaGel®, 40% for the peptides on TentaGel®) was adjusted empirically to give the beads neutral buoyancy so they do not settle. Uniform aliquoting among multiple wells, which is critical for consistent results, is then easily effected with a pipettor. Thus, 100 μL (0.1 mg) portions of bead suspension were distributed in a 96-well plate with a 0.45 μm membrane filter bottom (Millipore No. MAHV N45), and the liquid was removed by placing the plate on a vacuum manifold. In the steps that follow, all incubations and washes were performed with gentle agitation on a platform shaker. The beads were blocked by incubation with PBS-TB_3 for 30 min followed by washing with 3×200 μL of PBS-TB_3 (3 min). Biotinylated Kap α (50 nM in PBS-TB_1, 200 μL) was added, and the beads were incubated for 30 min (SV40) or 60 min (nucleoplasmin and $SV40^M$). The beads were washed with 3×200 μL of TBS-TB_1 and incubated for 15 min with 200 μL of AP-SA (75 ng/mL in APB-TB_1). After washing (3×200 μL of APB), the beads were suspended in 200 μL of APB and transferred to standard polystyrene microtiter plates. The APB was exchanged for APB containing BCIP (0.15 mg/mL), and after 30 min, the reaction was stopped by two exchanges of the medium with stop buffer.

Peptide Synthesis

C-terminal residues were attached with the Mitsunobu reaction,[26] and extension was performed manually using conventional methods[27] employing N_α-F_{moc} protection with side-chain protection as follows: Boc (K), Pmc (R), *t*-Bu (S, T, and D), and Tr (Q). Coupling was effected with PyBOP[28]/HOBT/*i*-Pr_2NEt in *N*-methylpyrrolidinone (NMP). Side-chain deprotection was effected with TFA/H_2O/1,2-ethanedithiol (95:2.5:2.5 v/v) for 2 h, followed by washes with TFA (1×), NMP (3×), and CH_2Cl_2 (3×); the identity of the products was established by HPLC and MALDI-TOF MS analyses of cleaved (NaOH) peptides.

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