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A Simple, Solid-Phase Binding Assay for the Nuclear Import Receptor Karyopherin α. Part 2: Competitive Binding

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Abstract—A qualitative assay for the evaluation of soluble ligands of the nuclear import receptor karyopherin α has been developed. The assay relies on competition with an immobilized ligand, the nuclear localization signal (NLS) from nucleoplasmin, for binding to the receptor, which is detected by an enzyme-linked colorimetric method.

Introduction

In the preceding paper,[1] we reported a colorimetric, on-bead assay for the binding of the nuclear import receptor karyopherin α (Kap α)[2–4] to immobilized peptide ligands. Here, we report its adaptation to competitive binding, wherein soluble ligands inhibit the binding of Kap α to the bead and thereby inhibit color development. Competitive binding is useful not only for the evaluation of soluble ligands, but as an essential control for specificity in the evaluation of novel ligands synthesized on the solid phase. The conditions, substrate, and controls needed for confident evaluation of soluble ligands are detailed herein.

Choice of Substrate

Two immobilized Kap α ligands from the previous study were examined: the SV40 NLS (GSTPPKKKRKV)[5] and the nucleoplasmin NLS (KRPAATKKAGQAKKKK-LD).[6] These were re-assayed in the presence of soluble SV40 NLS peptide to see if receptor binding could be inhibited through competition. Due to the high local concentration of peptide on TentaGel® (≈200 mM), a high concentration in solution was required. With immobilized SV40 NLS, inhibition was only partial (60% reduction in color) with 10 mM soluble SV40 NLS (Fig. 1, A4), from which we concluded that complete inhibition would require impractically high competitor concentrations. However, when the immobilized ligand was the NLS from nucleoplasmin, Kap α binding was almost completely (∼90%) eliminated by competitive, soluble SV40 NLS at 10 mM (Fig. 1, B4). The nucleoplasmin NLS, therefore, is preferred as a substrate for competitive binding.

Ionic Strength

At 10 mM, the highly charged SV40 NLS peptide almost doubles the ionic strength of the standard assay medium (10 mM peptide, with an assumed charge of +5 and monovalent counterions, is equivalent to 150 mM NaCl). Given that electrostatic interactions play a significant role in the binding of the NLS to Kap α,[7] it was important to control for the effect of ionic strength. Therefore, the assay with immobilized nucleoplasmin NLS was repeated with 150 mM $NH_4{\cdot}O_2CCF_3$ in place of the peptide competitor (trifluoroacetate is the counter-ion to the peptide). Little effect (<20%) was observed (Fig. 1, A4), and therefore, inhibition of receptor binding in response to soluble ligands can be attributed clearly to specific, competitive binding as opposed to salt effects. This conclusion was reinforced by varying competitor concentration while maintaining constant ionic strength. Under these conditions, color development was reduced progressively with increasing competitor concentration. (Fig. 1, row B). The percentage reduction of color versus competitor concentration was approximately 5% (0.3 mM), 50% (1 mM), 75% (3 mM), and 90% (10 mM). Finally, the weak-binding mutant, $SV40^M$ (CTPPKTKRKV),[8] proved completely ineffectual as a competitor (Fig. 1, row C). Thus, we

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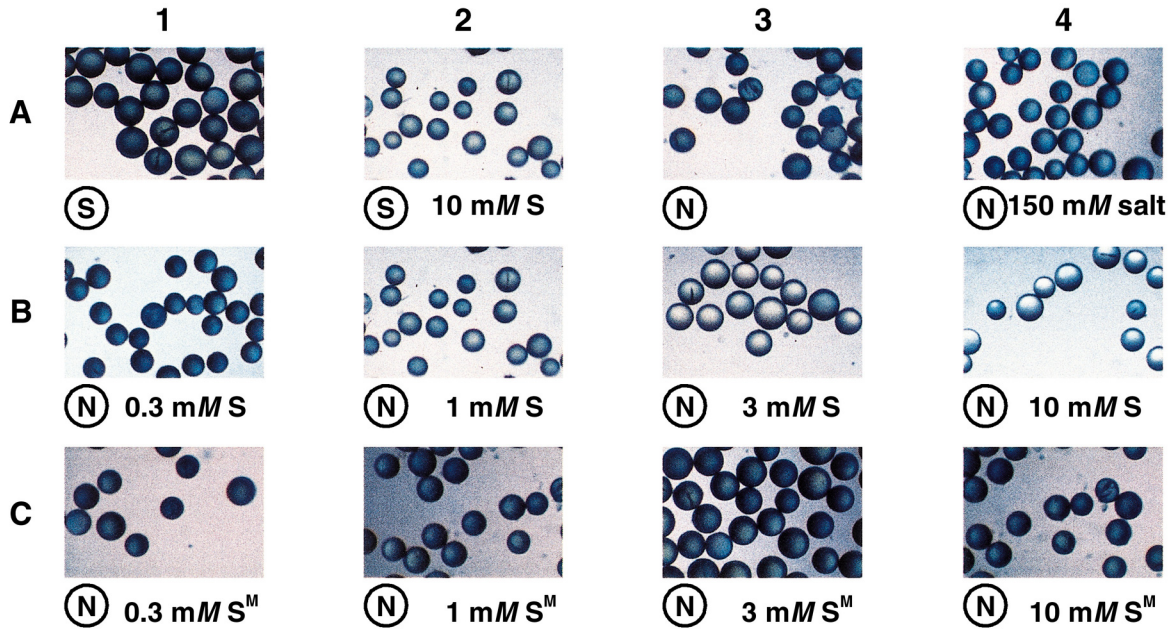

Figure 1. Circled letters denote the ligand on TentaGel®, and uncircled letters represent soluble ligands: S, SV40 NLS; S^M, $SV40^M$ NLS; N, nucleoplasmin NLS; salt, $NH_4{\cdot}O_2CCF_3$. All assays were run simultaneously and identically. A1 and A2: SV40 NLS assayed directly or with 10 mM soluble SV40. A3 and A4: Nucleoplasmin NLS assayed directly or with 150 mM salt. B and C: Competition of soluble SV40 (B) or $SV40^M$ (C) with immobilized nucleoplasmin NLS at constant *I*.

conclude that the binding assay is specific for binding to Kap α and is sensitive to the strength of both immobilized and soluble ligands.

Assay Procedure

The procedure was as described[1] except for the presence of soluble peptides, salt, or both during the incubation with GST–Kap α. In practice, competitor solutions (100 μL) of constant ionic strength at 2× the desired assay concentration were mixed with an equal volume of Kap α solution (100 nM in PBS + 0.1% Tween 20 + 2% BSA) and incubated for 1 h before being added to the beads. Peptide competitors were synthesized as described[1] on Wang[9] resin, purified by RP-HPLC (C_{18}), dissolved at 30–50 mM in H_2O, and quantitated by absorbance ($E_{205}^{1mg/mL} = 31$).[10] For the assay, the H_2O stocks were diluted to 20 mM after being adjusted to pH 7 with NaOH and buffered with 10×PBS (1/10 of final volume). To maintain constant ionic strength, $NH_4{\cdot}O_2CCF_3$ (100 mM) was included in the 20 mM $SV40^M$ solution, and solutions at 6, 2, and 0.6 mM were made by diluting the 20 mM solutions with PBS + 300 mM $NH_4{\cdot}O_2CCF_3$.

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