Discovering Novel Interactions at the Nuclear Pore Complex Using Bead Halo

A RAPID METHOD FOR DETECTING MOLECULAR INTERACTIONS OF HIGH AND LOW AFFINITY AT EQUILIBRIUM

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A highly sensitive, equilibrium-based binding assay termed "Bead Halo" was used here to identify and characterize interactions involving components of the nucleocytoplasmic transport machinery in eukaryotes. Bead Halo uncovered novel interactions between the importin Kap95 and the nucleoporins (nups) Nic96, Pom34, Gle1, Ndc1, Nup84, and Seh1, which likely occur during nuclear pore complex biogenesis. Bead Halo was also used to characterize the molecular determinants for binding between Kap95 and the family of nups that feature multiple phenylalanine-glycine motifs (FG nups). Binding was sensitive to the number of FG motifs present and to amino acid (AA) residues immediately flanking the FG motifs. Also, binding was reduced but not abolished when phenylalanine residues in all FG motifs were replaced by tyrosine or tryptophan. These results suggest flexibility in the binding pockets of Kap95 and synergism in binding FG motifs. The hypothesis that Nup53 and Nup59 bind directly to membranes through a C-terminal amphipathic alpha helix and to DNA via an RNA recognition motif domain was also tested and validated using Bead Halo. The results support a role for these nups in nuclear pore membrane biogenesis and in gene expression. Finally, Bead Halo detected binding of the nups Gle1, Nup60, and Nsp1 to phospholipid bilayers. This may reflect the known interaction between Gle1 and phosphoinositides and suggests similar interactions for Nup60 and Nsp1. As the Bead Halo assay detected molecular interactions in cell lysates, as well as between purified components, it can be adapted for large-scale proteomic studies using automated robotics and microscopy. Molecular & Cellular Proteomics 7:121–131, 2008.

In the context of the cellular milieu, protein interactions of high and low affinity are key for the survival of organisms. Current proteomic studies aim to uncover all such interactions with the ultimate goal of reconstructing, understanding and predicting all cellular behavior at a system-wide level. Despite the great advances in cellular proteomics, our ability to detect protein interactions of low affinity, in particular, is limited. Current proteomic analyses using biochemical tools, such as Protein A-tag (also termed ZZ-tag; TAP tag) pull-downs or co-immunoprecipitations, are limited to only detecting high affinity interactions. This is because the necessary wash steps that remove nonspecific interactors (i.e. from the agarose or magnetic beads used as solid support during the isolations) also remove specific low affinity interactions. It is estimated that pull-down methods can identify protein interactions stronger than the $K_d \sim 5 \mu M$ range (1, 2). The sensitivity can be improved through extended incubation times followed by rapid collection and washing of the beads, but nonspecific interactions also increase (3). To detect protein interactions of low affinity (beyond the $K_d \sim 5 \mu M$ range), we recently reported the development of an assay termed Bead Halo, which can detect molecular interactions of high and low affinity in real time at equilibrium (2). Here, we expand the repertoire of interactions tested using this novel technique to include protein–DNA and protein–phospholipid interactions. The nucleocytoplasmic transport machinery of yeast was used as the experimental landscape.

The yeast nucleocytoplasmic transport machinery relies on nucleoporins to form the nuclear pore complex, karyopherins (kaps)¹, to ferry cargo across the nuclear pore complex (NPC) and the RanGTPase system to load or unload cargos from kaps (supplemental Fig. 1) (reviewed in Ref. 4). The NPC forms and maintains the sole aqueous conduit between the cytoplasm and nucleoplasm of cells and gates all macromolecular transport between these two compartments. It is composed of ~33 nups, and some (the FG nups) contain large, natively unfolded domains with multiple FG repeats (42). The FG nups function as binding sites for kaps during their stochastic translocation across the NPC (5), and as the structural elements of a permeability barrier that prevents entry of large (>30 kDa) non-karyophilic particles into the nucleus (2, 6–9).

¹ The abbreviations used are: kaps, karyopherins; AA, amino acid; CFP, cyan fluorescent protein; GTP, guanosine triphosphate; MBP, maltose-binding protein; NLS, nuclear localization signal; NPC, nuclear pore complex; nups, nucleoporins; POMs, pore membrane nucleoporins; RRM, RNA-recognition motif; WT, wild-type; YFP, yellow fluorescent protein.
Experimental Procedures

Recombinant Proteins—Recombinant nups were expressed as GST fusions in the Escherichia coli expression vector pGEX-2TK (GE Healthcare) and purified as described (10, 11). GST-Nup116 (AA 348–458)-6xHIS was modified by site-directed mutagenesis to generate F–A mutations of some or all phenylalanine residues in FG motifs. The DNA encoding other mutants of Nup116 or Nup100 were synthesized de novo (GenScript) and cloned into pGEX-2TK. The GST-Nup53ΔC mutant lacks the C-terminal 15 AAs and was created by deletion mutagenesis of pGEX-2TK NUP53. All yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP)-fusions were initially purified as GST fusions, and the GST was later removed by thrombin cleavage when indicated (GST Handbook, GE Healthcare). The fluorescent fusion proteins were purified by gel filtration in a FPLC Superdex-200 column pre-equilibrated in binding buffer (20 mM HEPES pH 6.8, 150 mM KCl, 2 mM Mg(OAc)$_2$, 1 mM DTT, 0.1% Tween-20). Protein in the eluates was concentrated to 0.4–1 mg/ml using a Centricon-10 (Millipore).

Preparation of Sepharose Beads Coated with GST Fusions—Purified GST fusions were loaded onto glutathione–Sepharose beads (GE Healthcare) at a concentration of 1–10 μg per ml of packed beads, as indicated. GST fusions in crude E. coli extracts could also be used if the extracts were titrated in advance to ascertain the amount of GST fusion protein per unit volume of crude extract (10). Beads loaded with GST fusion from crude extracts were washed 8 times with 5 μl binding buffer and was washed 5–7 containing 0.1 mM ATP. After the final wash, an equal volume of binding buffer was added to create a 50% slurry of “loaded” beads. Typically, 10–20 μl of 50% bead slurry was mixed directly with 0.75 ml binding buffer, and centrifuged at 20,000 $\times g$ for 10 min before use in the Bead Halo assay. The free Cy5 dye used as a control was prepared by quenching a Cy5 mono NHS ester (GE Healthcare) with hydroxylamine. A 10 μM stock solution of Cy5 was prepared in binding buffer.

The Bead Halo Assay—For experiments involving all purified proteins, an aliquot of GST fusion-coated beads (0.75 μl portion of a 50% slurry) was mixed with 0.5 μl of EBHN 4 × stock buffer (40 mM EDTA, 2% 1,6-hexanediol, 40 mg/ml bovine serum albumin, 500 mM NaCl) and 0.75 μl of purified soluble fluorescent protein, to obtain a 2 μl sample suitable for imaging on a microscope slide.

The EBHN buffer supplement controls the stringency of the assay, with the final working solution containing 10 mg/ml bovine serum albumin (as a blocking and crowding agent), 10 μM EDTA (to prevent divalent cation-dependent interactions), 240 mM salt in total (to disrupt weak ionic interactions), and 0.5% 1,6-hexanediol (to disrupt weak hydrophobic interactions). For experiments involving cleared yeast lysate, 0.5 μl of a 50% bead slurry was mixed with 0.5 μl EBHN 4 × stock buffer and 1 μl soluble yeast lysate. For experiments involving purified fluorescent liposomes, a 0.75 μl portion of a 50% bead slurry was mixed directly with 0.75 μl of a liposome solution. For experiments involving purified fluorescent liposomes, 0.75 μl of the 50% slurry was mixed with 0.5 μl EBHN 4 × stock buffer and 0.75 μl 10 μM DNA or free Cy5 dye. In all cases, beads were imaged under a Nikon fluorescence microscope with a 20 × air objective using fluorophore-specific filters and a 2 × binned CCD for image acquisition. Image exposure settings were adjusted for each sample to obtain maximum signal intensity without saturation.

Affinity-capture Experiments with Nsp1—A portion of the Saccharomyces cerevisiae Nsp1 gene encoding AA 377–471 was cloned into vector pGEX-2TK, expressed as GST fusion protein in BL21 Codon Plus E. coli (Novagen), and purified as described (10). Mutants of Nsp1 (AA 377–471), where every instance of phenylalanine was replaced with tyrosine (F→Y) or with serine (F→S), were synthesized de novo (GenScript) and prepared the same way as the wild-type (WT) protein. For the affinity capture experiments, 5 μg of each GST-Nsp1 (WT or mutants) was immobilized onto 10 μl of glutathione–Sepharose beads. The beads were incubated with ~4 mg of yeast cytosol in a total volume of 1 ml for 2 h at 4 °C in the absence or presence of 12 μg RanGTP (His-Gsp1G71L loaded with GTP). Beads were recovered and washed twice with binding buffer. Bound proteins were extracted with 2% SDS, resolved by SDS-PAGE, transferred to PVDF membrane, and analyzed by Western blotting using specific rabbit polyclonal antibodies.

Results

The Bead Halo Assay—Agarose microbeads are widely used for biochemical chromatography. One popular use is as a solid phase support for the capture (immobilization) of affinity-tagged fusion proteins (such as GST, 6xHIS, Protein A) from cell extracts. Such pull-down experiments typically involve a binding phase, a washing phase to remove unbound proteins and nonspecific interactions, and an analysis phase, which follows protein elution or extraction from the beads. In all cases, the technique precludes detection of specific low affinity interactions because they are lost during the wash steps, due to their generally fast off rates. To overcome this limitation, we recently developed a nonquantitative method termed Bead Halo, which can detect both high and low affinity interactions. Bead Halo detects low affinity interactions because it monitors the samples in real time under equilibrium conditions.
binding conditions without the need for wash steps. The assay is based on the simple principle of observing by microscopy a soluble, fluorescently labeled macromolecule binding to the surface of microbeads, onto which a second macromolecule had been pre-immobilized via an affinity tag (Fig. 1). The visual appearance of the surface-bound, fluorescently labeled macromolecules is as a bright circle or halo around microscopic cross-sections of beads, which reflects the location on the bead surface where the affinity-tagged protein is immobilized.

The Bead Halo assay detects interactions over a wide range of affinities (Fig. 2). The sensitivity is determined by the concentration of the immobilized fusion protein. Depending on the molar ratio between the bead-immobilized “receptor” and the soluble fluorescent “ligand,” high affinity interactions are typically manifested as the complete titration of the ligand out of solution onto the bead surface. Low affinity interactions are observed as a fluorescent halo around beads with surrounding fluorescence in the solution. To illustrate, we reconstituted a known high affinity interaction ($K_d = 0.22 \mu M$) between the Nup100 FG domain and Kap95 (1), and compared it to a known low affinity interaction ($K_d > 5 \mu M$) between the Nup100 and Nup116 FG domains (2). The FG domain of Nup100 (AA 1–640) was immobilized as a GST-tagged fusion protein on glutathione–Sepharose beads at a concentration of 1, 3, 5, or 10 $\mu g/\mu l$ beads, and was subsequently mixed with Kap95-YFP (a fusion between the importin Kap95 and the Yellow Fluorescent Protein) or CFP-Nup116 (AA 165–716, containing 34 FG repeats), or CFP-Nup116 (AA 348–458, containing 10 FG repeats) in EHBN. The mixtures were transferred to micro-wells on a microscope slide and viewed directly (within minutes) with a $20 \times$ air objective under a fluorescence microscope fitted with CFP- or YFP-compatible fluorescence filters. An aliquot of the immobilized protein was resolved by SDS-PAGE, stained with Coomassie blue, and shown on the left. Sepharose beads are 50–150 $\mu m$ in diameter. The fluorescent halos around beads indicate positive interactions in a qualitative manner. The affinities shown were measured or estimated previously (1, 2).

Nup100 (AA 1–640) was immobilized as a GST-tagged fusion protein on glutathione–Sepharose beads at a concentration of 1, 3, 5, or 10 $\mu g/\mu l$ beads, and was subsequently mixed with Kap95-YFP (a fusion between the importin Kap95 and the Yellow Fluorescent Protein) or with CFP-Nup116 AA 165–716 (a fusion between the CFP and the FG domain of Nup116) or with CFP-Nup116 AA 348–458 (a similar fusion, but with a smaller portion of the Nup116 FG domain containing fewer FG motifs, as indicated in Fig. 2A). As negative controls, we used GST and CFP-maltose binding protein (MBP). GST-coated beads did not capture the soluble fluorescent proteins tested (Fig. 2B, top row), and CFP-MBP did not bind to beads coated with the GST fusions used (Fig. 2B, left column). For the high affinity interaction between the GST-Nup100 FG domain and Kap95-YFP ($K_d = 0.22 \mu M$) (Fig. 2B, second column), bright fluorescent halos were observed around the beads and no fluorescence was observed free in solution, indicating that all of the Kap95-YFP was captured by the nup-coated beads. For the low affinity interaction between the

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**Fig. 1.** Bead Halo: a fluorescence-based method for detecting molecular interactions in real time on the surface of microbeads. Derivatized Sepharose microbeads coated with an affinity-tagged protein (e.g. a GST fusion or a HIS-tag fusion) are mixed with soluble fluorescent macromolecules (proteins, membranes, or nucleic acids) in purified form or in a cell extract. The mixtures are then spotted on a microscope slide and are visualized under a fluorescence microscope using a $20 \times$ objective. A positive interaction is visible as a halo of fluorescence around the beads, and a lack of interaction is observed as dark beads with an opaque appearance surrounded by diffuse fluorescence in the background. The concentration of immobilized protein on the bead surface determines the detection sensitivity of the assay, and the composition of the binding buffer establishes the detection specificity of the assay (see “Experimental Procedures”).

**Fig. 2.** The detection sensitivity of Bead Halo is established by the concentration of the immobilized fusion protein. A, the diagrams on top depict full-length nucleoporins, the fragments used, and the location of the FG motifs. B, GST and GST-Nup100 (AA 1–640, the FG domain) were immobilized onto glutathione–Sepharose beads at the concentrations indicated on the left. Amounts of the GST (0.75 $\mu l$ of a 50% slurry) were subsequently mixed with 0.1–0.2 $\mu g$ of purified CFP-MBP, Kap95-YFP, CFP-Nup116 (AA 165–716, containing 34 FG repeats), or CFP-Nup116 (AA 348–458, containing 10 FG repeats) in EHBN. The mixtures were transferred to micro-wells on a microscope slide and viewed directly (within minutes) with a $20 \times$ air objective under a fluorescence microscope fitted with CFP- or YFP-compatible fluorescence filters. An aliquot of the immobilized protein was resolved by SDS-PAGE, stained with Coomassie blue, and shown on the left. Sepharose beads are 50–150 $\mu m$ in diameter. The fluorescent halos around beads indicate positive interactions in a qualitative manner. The affinities shown were measured or estimated previously (1, 2).
Nup100 and Nup116 FG domains \(K_D > 5 \mu M\) (2), fluorescence was observed in the solution and as a halo around the beads, indicating that some but not all of the CFP-Nup116 was captured by the nup-coated beads (Fig. 2B, right columns). We had characterized this low affinity interaction previously, and several other of the same type, and concluded that some (but not all) FG nups contain “cohesive” FG domains that interact via hydrophobic FG motifs to form a meshwork of filaments at the NPC center, which regulates the diffusion of macromolecules across the nuclear envelope (2). The \textit{in vitro} data shown here demonstrate that the low affinity interaction between the cohesive FG domains is sensitive to the length of the FG domain and/or to the number of FG motifs. This is similar to what was observed \textit{in vivo} (2). Specifically, the Nup100 FG domain bound better to a 552 AA FG domain of Nup116 (AA 165–716; containing 34 FG motifs) than to a smaller 111 AA fragment (Nup116 AA 348–458; containing 10 FG motifs). Likely, the combined avidity of interactions between multiple FG motifs strengthens the FG domain interactions.

Discovery of Novel kap–nup Interactions—Although kaps are best known for their ability to bind nuclear import or export signals on cargos, and FG motifs on FG nups, their interaction with other nucleoporins such as the pore membrane nups (POMs) or the non-FG nups (supplemental Fig. 1) may also be physiologically relevant and could explain some outstanding issues in nucleocytoplasmic transport and/or NPC biogenesis. For example, a direct binding interaction between Kap95 and non-FG nups would explain 1) the Kap95-dependent recruitment of non-FG nups (e.g. Nic96) into new or preexisting NPCs (14) and 2) the importin \(\beta\)-regulated assembly of the Nup107 complex (a non-FG nup oligomer) unto NPCs after mitosis (15, 16). The Kap95 protein is the yeast homolog of the vertebrate importin \(\beta\) and the Nup84 complex is the yeast homolog of the vertebrate Nup107 complex (supplemental Fig. 1). Despite these functional connections, a direct binding interaction between Kap95 (importin \(\beta\)) and these nups has not been documented.

To test if Kap95 binds directly to non-FG nups, we coated beads with various GST–non-FG nups and monitored their ability to capture soluble Kap95-YFP using Bead Halo (Fig. 3). Of the 13 non-FG nups (or large soluble fragments thereof) tested, six bound to Kap95-YFP directly (namely Nic96, Nup84, Seh1, Pom34, Gle1, and Ndc1) and seven did not (namely Nup82, Nup85, Gle2, Nup157, Nup120, Nup192, and Nup170). In control experiments, GST and CFP-MBP were completely inert (as before), and GST–Kap60 bound to all of the available Kap95–YFP, as expected for this high affinity interaction \(K_D = 0.15 \text{ nM}\) (17). These results demonstrate a direct interaction between Kap95 and non-FG nups, and between Kap95 and POMs, and suggest a direct role for Kap95 in NPC biogenesis.

\textbf{The Molecular Determinants in FG nups Recognized by Kap95—}The binding between kaps and FG domains of nups is well documented (examples in (10)), but important details regarding their interaction remain unanswered. For example, it is known that kaps bind directly to phenylalanine (F) side chains in GLFG and FXFG motifs (18, 19), but it is unknown whether the adjacent leucine residues in GLFG motifs play a role in binding, or more generally, whether any aromatic AA such as tyrosine or tryptophan can substitute for the phenylalanine residue. It is also unclear how many FG motifs are needed for effective kap–nup binding interactions. Lastly, it is known that Kap95/importin \(\beta\) binds FG motifs at various locations on its surface (18–21), but it remains unclear whether the binding is simultaneous and/or cooperative, since only short peptides with one FG motif were generally used in those studies. Native FG domains are typically 150–700 AA long and contain 5–40 FG motifs each (22). Here, we provide answers to some of these questions using Bead Halo.

The contribution of specific AA residues in GLFG motifs to Kap95 binding was analyzed using point mutants in the Nup100 (AA 300–400) and/or Nup116 (AA 348–458) FG domains (Figs. 2A and 4A), where every instance of a target AA was replaced by another residue. For example, in the Nup116 F>A and F>W mutants every phenylalanine in FG motifs was substituted by alanine or tryptophan, respectively. As a positive control, beads coated with the WT FG domains captured Kap95–YFP efficiently, as evidenced by the fluorescent halo around beads and by the absence of fluorescence in the surrounding solution (Fig. 4). Also as expected for the negative control, none of the immobilized GST fusions captured the inert CFP-MBP fusion (see (2) and data not shown).

Mutation of every phenylalanine to alanine abolished bind-
ing of the FG domains to Kap95 (Fig. 4A). This was expected, given the prominent role of the phenylalanine residue in mediating GLFG motif–Kap95 interactions (19). More interestingly, mutation of every phenylalanine to tryptophan or tyrosine resulted in a reduction of Kap95 binding to the FG domains, but in general, these mutants were still able to bind Kap95 effectively, though at a lower affinity (Fig. 4A). Thus, it appears that any of the three AAs with aromatic side chains (Phe, Tyr, or Trp) can be accommodated in the Kap95 binding pockets.

Mutation of all leucine residues to alanine in the GLFG motifs of Nup100 and Nup116 weakened the interaction with Kap95 but did not abolish the binding (Fig. 4A). In essence, the L>A mutation converted the GLFG motifs into degenerate XXFG motifs, which are less hydrophobic than GLFG motifs but appear to be sufficient for kap binding. In contrast, the same L>A mutation completely abolished the interaction between GLFG-rich domains of nups (2). Yeast FG nups contain a variety of evolutionarily conserved FG motifs such as FXFG, GLFG, SAFG, PSFG, SFG, and NXFG (22). Given the low hydrophobicity of the latter four (which are less hydrophobic than the more abundant GLFG and FXFG motifs), it seems likely that they participate only in kap–nup interactions, rather than mediating FG domain–FG domain interactions.

Mutation of every glutamine to serine (Q>S) in the inter-FG motif sequences of the FG domains resulted in only a minor reduction in Kap95 binding (Fig. 4A). These AA substitutions maintained the polarity of the inter-FG motif regions but significantly altered the primary structure of the nup given the preponderance of glutamine residues (18 for a 100 AA segment). A different glutamine-substituted mutant of the Nup116 FG domain (Q>A), which significantly altered the primary structure and the polarity of the nup, showed a unique pattern of binding to Kap95-YFP. Instead of yielding the more typical fluorescent halo around the bead surface, the Nup116 Q>A FG domain appeared to bind Kap95-YFP within the bead, rather than on the surface. It is unclear why this happens reproducibly with a very small subset of GST fusions, but it should be noted as an alternate readout for the Bead Halo assay. Possibly, the structural geometry of some GST fusions facilitates access to internal binding sites in beads, which can be accessed by the fluorescent ligand.

Controlling the Detection Sensitivity of Bead Halo—Given the pronounced difference in Kap95 binding observed between the WT and F>A mutant FG domains, we sought to test whether Kap95 binding to FG domains is roughly proportional to the number of FG motifs present. Although the Bead Halo assay is qualitative in nature, it can be sensitized to detect

![Fig. 4. Identifying molecular determinants in FG domains involved in Kap95 binding (A) AA sequences for the nup fragments used are shown. Specific mutants of Nup116 (AA 348–458) or Nup100 (AA 300–400) were obtained by site-directed mutagenesis or by de novo synthesis. For each FG domain mutant, every instance of a target AA (e.g. phenylalanine) was substituted by a different AA (as in the 10F>A mutant, for example). The wild-type (WT) and mutant FG domain fragments were purified from E. coli as GST fusions and were loaded separately onto glutathione–Sepharose at 10 μg/μl beads. An aliquot of the immobilized protein was resolved by SDS-PAGE, stained with Coomassie blue, and is shown to the left of the panels. Beads were mixed with purified CFP-MBP or Kap95-YFP in EHB buffer, and the mixtures were examined under a fluorescence microscope as before (B). Mutants of the Nup116 FG domain fragment (AA 348–458) displaying different numbers of FG motifs (indicated by tick marks on the nup diagrams) were immobilized on Sepharose beads at the indicated concentration. After mixing with Kap95-YFP, the mixtures were examined directly under a fluorescence microscope as before.](image-url)
even subtle changes in affinity between the interacting pair of proteins (as shown in Fig. 2). In the course of generating the 10F→A Nup116 mutant, partial mutants were also generated, which contained different numbers of intact FG motifs (see diagrams in Fig. 4B). We immobilized these FG domains separately as GST fusions on beads at three different concentrations (1 μg/μl, 3 μg/μl, or 5 μg/μl) and tested their ability to capture Kap95-YFP in the Bead Halo assay. Indeed, the number of FG motifs correlated well with the strength of Kap95 binding (Fig. 4B). For example, reducing the FG motif content from 10 to 6 caused a pronounced reduction in Kap95 binding. Further elimination of phenylalanines reduced binding gradually, until no binding was detected when only two or fewer FG motifs remained intact. Thus, three or more FG motifs may be necessary for a detectable association between Kap95 and FG domains. However, if one considers the fact that GST fusions are dimeric (which effectively doubles the local number of FG repeats present), then it may be that six or more FG motifs are necessary for a detectable association of Kap95 with FG domains.

A recent report used Protein A pull-downs to show that the FXFG-rich domain of yeast Nsp1 can capture kaps from mammalian cell extracts, and that F→Y and F→S mutants thereof (where all FXFG motifs were mutated to YXYG or SXSG motifs) could not (23). This conclusion appears to differ from our result shown above for the Nup116 F→Y mutant, but the experiments were performed with different FG domains (the Nsp1 FXFG domain versus the Nup116 GLFG domain) and different protein interaction assays (a standard Protein A pull down assay, rather than the more sensitive Bead Halo assay; see introduction). To resolve this issue, we tested binding of Kap95 to WT, F→Y and F→S Nsp1 FG domains in the Bead Halo assay and in a GST-pull down assay (Fig. 5), which has a similar sensitivity to Protein A pull-downs. Nsp1 contains a long ~600 AA FG domain with a highly repetitive central FXFG repeat region. For the experiment here, a 95 AA portion of the FG domain (AA 377–471) was used (Fig. 5A). First, we reproduced the Nsp1-1K binding results reported previously (23) using our GST-pull down technique (10). Beads were coated with recombinant Nsp1 FG domains and were incubated with soluble yeast extracts to allow capture of endogenous kaps by the bead-immobilized nups. Once the nonspecific associations (and the specific low affinity associations) were lost due to washes of the beads, the bound proteins were extracted from the beads, resolved by SDS-PAGE, and the presence of kaps in the eluates was probed by Western blotting with specific antibodies. As expected, the WT Nsp1 FG domain effectively captured importins such as Kap95, Kap121, and Kap123 from yeast lysates (Fig. 5B); and as observed previously for most importins (10), the binding was diminished by the presence of RanGTP. Just as reported for the Protein A pull-down experiments (with Nsp1 and mammalian cell extracts (23)), our Nsp1 FG domain mutants (F→Y and F→S) were also incapable of capturing and/or retaining importins from yeast cytosol in pull-down assays (Fig. 5B). However, in the Bead Halo assay, the F→Y Nsp1 mutant (but not the F→S mutant) was clearly observed interacting with Kap95-YFP at equilibrium, albeit at a reduced affinity in comparison to WT (Fig. 5C). Thus, we suggest that the residual ability of the F→Y Nsp1 mutant to bind kaps is the reason why Nsp1 F→Y mutant yeast were viable and F→S yeast were not viable in a sensitized strain (23).
integral part of cell biology and of nuclear pore complex biogenesis, we modified the Bead Halo assay to detect such interactions. In principle, any molecular interaction could be examined in the Bead Halo assay as long as one of the components is immobilized on beads and the other is fluorescently labeled and soluble. In this case, GST-nup fusions were used as the immobilized component and fluorescently labeled liposomes were used as the soluble component. Texas Red-labeled liposomes with a phospholipid content typical of endogenous yeast microsomal membranes (12, 13) were incubated with GST, GST-kaps, or GST-nups (Fig. 6). Of the ~33 yeast nups, three (Pom34, Pom152, and Ndc1) have easily recognizable transmembrane domains, one (Gle1) is a known phosphoinositide binding protein (24, 25), and others may contain unrecognized lipophilic domains that interact with membrane monotonically or bind phospholipid head groups. For example, Nup53 and Nup59 contain a predicted C-terminal amphipathic alpha-helix (26) with similarity to the N terminus of Sar1 (supplemental Fig. 2). Sar1 binds to liposomes via its N terminus and can bend membranes (via monotopic insertion of its N terminus into the lipid bilayer) during COPII vesicle budding (13). Interestingly, overexpression of Nup53 in yeast causes proliferation of intracellular membranes in a C terminus-dependent manner (26). In mammalian cells, the Nup53 homologue is also tightly associated with the nuclear membrane (27).

To test if yeast Nup53 interacts directly with phospholipids in liposomes, we mixed GST-Nup53 coated beads with soluble fluorescent liposomes in the Bead Halo assay. Fluorescent halos were detected around the beads, indicating direct binding (Fig. 6). By contrast, beads coated with GST or with a GST-Nup53 mutant lacking the C-terminal 15 residues (GST-Nup53ΔC) failed to capture the fluorescent liposomes. We conclude that Nup53 binds membrane phospholipids directly in a manner that requires its predicted C-terminal amphipathic alpha-helix. Nup59, the yeast homolog of Nup53, also contains a putative amphipathic C-terminal helix and also bound directly to the liposomes (Fig. 6). When 24 additional nups and 2 kaps were tested for their ability to bind liposomes, we observed that only Gle1, Nup60, and Nsp1 had the ability to bind liposomes (Fig. 6). Gle1 binds directly to phospholipid head groups (24, 25), and Nup60 and Nsp1 may have similar activities.

Another class of vital cellular interaction is the binding of proteins to nucleic acids. The Bead Halo assay was adapted to detect such interactions using GST fusions as the bead-immobilized component and fluorescently labeled DNA as the soluble component. In a positive control, we reconstituted the binding of Prp20 to DNA. Prp20 is a guanine-nucleotide exchange factor for Ran and has a known DNA-binding activity (28). When GST-Prp20 coated beads were mixed with a soluble Cy5-labeled oligonucleotide, binding was observed as fluorescent halos around the beads (Fig. 7). These halos were much brighter than the controls, which showed very minimal or no background binding to the uncoupled Cy5 fluorescent dye. Most GST-nups or GST-kaps tested also showed no interaction with the single-stranded DNA probe (Fig. 7 and data not shown) except for Nup53 and its homolog Nup59. These two nups had been predicted to contain nucleic-acid binding pockets in RNA Recognition Motif (RRM) domains based on the crystal structure of their vertebrate homolog, Nup35 (29). This prediction was confirmed here.
A Modified Bead Halo Assay for Proteomics Studies—As demonstrated above, the Bead Halo assay is useful for detecting molecular interactions between bead-immobilized proteins and purified (fluorescent) binding partners (e.g. protein, liposomes or DNA). Next, we asked if the soluble fluorescent component could be provided as part of a crude, heterogeneous mixture of biomolecules such as those present in whole cell extracts. For that test we used soluble yeast extracts prepared from cells expressing GFP-labeled proteins. Yeast expressing either GFP, cNLS-GFP (a GFP fusion with a classic nuclear localization signal), or Kap95-GFP were grown to log phase, harvested, and lysed with glass microbeads. A medium speed (20,000 × g) supernatant of the lysate was then mixed with bead-immobilized GST, GST-Kap60, or GST-nups and the mixtures were visualized under a fluorescence microscope as before. The asterisks indicate positive interactions.

**DISCUSSION**

In this study, we identified and characterized interactions among components of the nucleocytoplasmic transport machinery to demonstrate the usefulness of Bead Halo for a wide range of applications. We identified novel interactions between the importin Kap95 and the non-FG nups Nic96, Nup84, Seh1, Gle1, Pom34, and Ndc1 (Fig. 3). These non-FG nups are part of the structural framework of the NPC ring scaffold (30) and do not appear to interact with Kap95 while it is in transit across NPCs (Patel and Rexach, data not shown). Instead, Kap95 may bind to these non-FG nups to mediate (as an importin) or regulate (as a chaperone) their delivery into NPCs. This is suggested by recent studies that implicate Kap95/importin β in the control of NPC assembly (14–16). Indeed, Kap95 is required for the proper localization of newly synthesized Nic96 to the nuclear envelope in vivo (14), but the exact mechanism underlying this relationship was not described. Our results suggest that Kap95 bind directly to Nic96 to facilitate its delivery (as a cargo) to NPCs.
Two other proteins required for NPC assembly, Nup84 and Seh1, were also observed to bind Kap95 directly (Fig. 3). These two non-FG nups form part of a larger, so-called Nup84 complex (31) whose homolog in vertebrate cells (termed the Nup107–Nup170 complex) is required for NPC biogenesis (32). As vertebrate importin β/Kap95 serves as a negative regulator of NPC assembly (15, 16), it has been suggested that the Nup107 complex is a direct target of importin β inhibition prior to NPC assembly. Our results, demonstrating direct binding between Kap95 and individual components of the yeast Nup84 complex (Nup84 and Seh1), provide tangible evidence in support of that hypothesis.

The cytosolic domain of two transmembrane nups Pom34 (AA 159–299) and Ndc1 (AA 255–510) also bound to Kap95 directly in the Bead Halo assay (Fig. 3). This interaction may facilitate the targeting of these POMs to NPCs in a manner similar to the importin β/Kap95-dependent nuclear import of inner-nuclear membrane proteins, which translocate across the NPC while tethered to the ER membrane (33). In the case of the POMs, their tether to Kap95 would need to be broken during transit at the NPC, rather than after passage across the NPC. Finally, we observed that Kap95 binds to Gle1 (Fig. 3), a nucleoporin that activates Dbp5 at NPCs to mediate mRNA export (24, 25). Presumably, Kap95 may deliver Gle1 to NPCs during its biogenesis, or may facilitate the nucleocyttoplasmic transport of Gle1 (34).

The molecular determinants underlying the well known interaction between Kap95 and FG nups were dissected here using Bead Halo (Figs. 4 and 5). We found that Phe residues in FG motifs are absolutely necessary for Kap95 binding because their substitution for Ala or Ser abolished binding. By contrast, their substitution with other aromatic AAs (Tyr or Trp) slightly reduced, but did not abolish, the binding. A scan of the yeast FG nups looking for naturally occurring WG or YG motifs identified a very small number in Nup116, Nup100, Nup145, Nup57, and Nup1, although not necessarily in their natively unfolded regions. Thus, it appears that Phe residues were selected during FG nup evolution, possibly because they exhibit the ideal shape and degree of hydrophobicity needed to support the reversible FG domain interactions that gate passive diffusion across the NPC (2). For the GLFG-rich nups, we also found that Leu residues in GLFG motifs are involved in Kap95 binding. Although Leu side chains have not been observed inside the hydrophobic binding grooves of Kap95, they may enhance the stability of the kap–nup interaction by shielding Phe from the solvent, as predicted (19). We also found that within a given nup FG domain, an increasing number of FG repeats (from 0 to 10) correlated well with stronger Kap95 binding. However, when larger FG domains that contain dozens of FG motifs were used, there was no longer a correlation between Kap95 binding affinity and the number of FG motifs present (1). Thus, the affinity of Kap95 for a particular FG domain may reach a maximum level when all of the putative binding pockets in Kap95 (potentially ≥10 (21)) have been occupied. Alternatively, factors such as the type of FG repeat present (SAFGXPSFG, GLFG, or FXFG), the spacing between FG motifs, and/or the composition of AA sequences between FG motifs may also be critical for determining the strength of individual FG nup–kap interactions. Finally, the numerous glutamine residues in the inter-FG motif sequences of yeast GLFG-nups are likely not involved in kap-binding because their substitution for Ala or Ser did not affect binding significantly. This result is consistent with the high AA substitution rate in between FG motifs (22).

A recent report demonstrated that the FXFG-rich domain of yeast Nsp1 can capture mammalian kaps from HeLa extracts, but a corresponding F->Y mutant version cannot (23). This negative result was used to conclude that the FG domain of Nsp1 can be vital to some strains of mutant yeast due to a function other than its ability to bind kaps. The alternative function proposed (i.e. that the Nsp1 FG domain forms homopolymers or hydrogels at the NPC that restrict passage of non-karyophilic proteins into the nucleus (23)), is at odds with our results from three different in vivo tests, which demonstrated that the intact FG domain of Nsp1 cannot form polymers under physiological conditions in vivo (2), or even in vitro in physiological buffers such as HEPES-, Tris-, or phosphate-buffered saline (supplemented with DTT and Tween-20; data not shown). Based on the interaction between Kap95 and the Nsp1 F->Y mutant detected here (Fig. 5), we speculate that the sensitized yeast strains used in the previous Nsp1 mutant study survived due to a reduced, but not abolished, ability of Nsp1 to interact with kaps (as shown here), rather than to a loss in Nsp1 FG domain interactions as proposed (23). Notably, we detected binding of Kap95 to the WT and F->Y mutant versions of a small ~100 AA portion of the larger ~600 AA Nsp1 FG domain (Fig. 5). As the degree of Kap95 binding to nups seems roughly proportional to the number of FG motifs present (Fig. 4B), it is expected that a F->Y mutant version of the larger Nsp1 FG domain would bind kaps even better.

Beyond protein–protein interactions, the Bead Halo assay also detected protein–phospholipid and protein–DNA interactions involving Nup53 and Nup59 (Figs. 6 and 7). Previously, it was shown that overproduction of Nup53 in yeast causes proliferation of intranuclear membranes in a manner that requires its C terminus (26). Here we showed that Nup53 and Nup59 bind membranes directly (Fig. 6), and demonstrated that the putative C-terminal amphipathic alpha helix of Nup53 (supplemental Fig. 2) is necessary for its membrane association. Based on the similarity to the N-terminal amphipathic alpha helix of Sar1 (supplemental Fig. 2), we suggest that the C termini of Nup53 and Nup59 can insert into membranes monotonically to promote or maintain membrane curvature at the nuclear pore membrane. Nup53 and Nup59 also bound DNA directly (Fig. 7). These nups were predicted to contain nucleic-acid binding pockets based on the crystal structure of the RRM domain of a vertebrate homolog, Nup35 (29); here
we confirmed that prediction. The RRM is a widely ubiquitous protein domain that is used for nucleic-acid binding (RNA or DNA) or for protein–protein interactions (35). The nucleic-acid-binding properties of Nup53 and Nup59 may be involved in gene expression (silencing, activation, or mRNA export) at the NPC (36).

We also detected interactions between the nups Gle1, Nup60, and Nsp1 with phospholipid bilayers using Bead Halo. Since Gle1 is known to bind phosphoinositide (IP6) head groups directly (24, 25), it may be that Nup60 and Nsp1 have similar activities. Further work will be needed to clarify whether these nups bind directly to the phospholipid head groups, or interact monotonically with the hydrophobic layer of membranes as predicted for Nup53 and Nup59.

The Bead Halo assay (Fig. 1) may be useful in large-scale proteomic studies because it can detect a wide range of interactions (e.g. protein–protein, protein–phospholipid, protein–DNA) with high sensitivity (Fig. 2) and can be easily optimized for high throughput screening. The salient advantage is the rapid detection of high and low affinity interactions in real time under equilibrium binding conditions. However, the assay requires affinity-tagged and fluorescently tagged components, which require preparation. It is not always easy to produce purified soluble fluorescent proteins, but it is often the case that proteins can be stably expressed in vivo as GFP fusions. Since extracts from these cells can become a source of fluorescent protein for the Bead Halo assay (as in Fig. 8), this demonstration opens a new dimension in proteomic studies, where extracts from different cell lines can be screened directly in high throughput against a selection of bead-immobilized probes. For instance, ~75% of yeast proteins have been tagged endogenously with GFP (37). Preparing small-scale extracts from such strains in a 96-well format may allow for the rapid preparation of thousands of GFP-fusions in crude cell lysates. Testing these extracts in the Bead Halo assay could be optimized for high-throughput screening using robotic handling and microscopy. Libraries of yeast containing affinity-tagged proteins are also available in which a large percentage of the yeast proteome has been tagged with GST (38), TAP (39), FLAG (40), or 6xHIS-HA-ZZ (41). In principle, the corresponding Sepharose microbeads could be loaded (to high density) with affinity-tagged fusions captured directly from yeast extracts, washed extensively, and mixed with either purified fluorescent proteins or with GFP fusions in crude extracts. Such high-throughput Bead Halo assays would expand the current landscape of known high affinity protein interactions, and could unveil a new landscape of low affinity interactions (the low affinity “interactome”) due to its wider range of detection sensitivity over standard high-throughput pull-down based methods (38–41).

Lastly, in cases where Bead Halo facilitates the discovery of novel interactions (such as between nups and phospholipids or between Kap95 and non-FG nups, as shown here) additional genetic, cell biological, and biochemical analyses will be necessary to establish the mechanism and physiological significance of each interaction.

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