Molecular Characterization of the Ran-binding Zinc Finger Domain of Nup153

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The nuclear pore complex is the gateway for selective traffic between the nucleus and cytoplasm. To learn how building blocks of the pore can create specific docking sites for transport receptors and regulatory factors, we have studied a zinc finger module present in multiple copies within the nuclear pores of higher eukaryotes. All four zinc fingers of human Nup153 were found to bind the small GTPase Ran with dissociation constants ranging between 5 and 40 μM. In addition a fragment of Nup153 encompassing the four tandem zinc fingers was found to bind Ran with similar affinity. NMR structural studies revealed that a representative Nup153 zinc finger adopts the same zinc ribbon structure as the previously characterized Npl4 NZF module. Ran binding was mediated by a three-amino acid motif (Leu13/Val14/Asn25) located within the two zinc coordination loops. Nup153 NZFs bound GDP and GTP forms of Ran with similar affinities, indicating that this interaction is not influenced by a nucleotidedependent conformational switch. Taken together, these studies elucidate the Ran-binding interface on Nup153 and, more broadly, provide insight into the versatility of this zinc finger binding module.

In eukaryotic cells, nucleocytoplasmic transport is a critical function for maintaining basic processes such as cell cycle regulation and gene expression. Transport across the nuclear envelope occurs through nuclear pore complexes, large macromolecular structures of ~125 MDa that are embedded in the nuclear envelope. Nuclear pore complexes are comprised of three major architectural features: 1) a central framework, which includes integral membrane proteins and resides within the plane of the membrane; 2) a cytoplasmic ring and its extended filaments; and 3) a nuclear basket, which is composed of distal and proximal rings connected by eight fibers (reviewed in Ref. 1–3). Proteomic approaches have revealed that, relative to its large size, the nuclear pore complex is composed of only a small number of nucleoporins (Nups)4 that are present in at least 8, and often 16 or 32, copies at the pore (4, 5). More recently, bioinformatics and structural analyses have further indicated that a limited repertoire of structural motifs are found within the 30 proteins that form the macromolecular pore complex (3, 6, 7).

One distinctive nucleoporin domain that has not been examined structurally is a zinc finger domain present in multiple copies in both Nup153 and Nup358/RanBP2 (referred to herein as Nup358). These two proteins localize to different sites on the pore, with Nup358 situated on the cytoplasmic filaments and Nup153 positioned on the nuclear basket. Each protein functions in nucleo-cytoplasmic transport (Refs. 8–16 and reviewed in Ref. 17) and in breakdown of the nuclear envelope at mitosis (18, 19). Nup358 takes on additional roles at the kinetochore during mitosis (20, 21).

The Nup153 and Nup358 zinc fingers are the defining members of the “RanBP2-type” zinc finger family (22). RanBP2-type zinc fingers conform to the consensus sequence pattern: W-X-C-X(2A)-C-X(3)-N-X(6)-C-X (2-3). This module is found in a functionally diverse population of proteins, with more than 1055 matches in 727 polypeptides using the ScanProsite tool. A similar consensus sequence that omits the initial tryptophan and allows only two residues between the first two cysteines defines a related polypeptide family termed Npl4 zinc fingers (NZF) (23, 24). Three-dimensional structures of several different RanBP2/NZF fingers have revealed that the module forms two orthogonal β-hairpins that chelate a single zinc atom at one end of a small barrel (24–27). The sequence conservation can be rationalized because each conserved residue performs an important structural role: the four cysteine residues coordinate zinc, the Trp residue forms the hydrophobic core of the module, and the Asn residue bridges strands 2 and 3. The small RanBP2/NZF module is a “stripped down” member of the much more complex RanBP2-type zinc finger family.

4 The abbreviations used are: Nup, nucleoporin; TFIIS, transcription factor S-II; COP1, coatomer protein; Znf, zinc finger; GST, glutathione S-transferase; TEV, tobacco etch virus; DTT, dithiothreitol; TOCSY, total correlation spectroscopy; TROSY, transverse relaxation optimized spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HSQC, heteronuclear single-quantum coherence; NZF, Npl4 zinc finger; Ub, ubiquitin; β-ME, β-mercaptoethanol; Ni-NTA, nickel-nitritriacetic acid.
larger zinc ribbon family, whose members all utilize two β-hairpins to coordinate a single zinc ion between two Cys, “knuckles.” Zinc ribbon proteins diverge significantly beyond the zinc coordination site, however, and frequently have complex structural elements inserted between the two knuckles (28).

Importantly, NZF modules can bind a wide variety of different macromolecules. For example, NZF modules in Npl4 (23), Vps36 (25), and Tab2/Tab3 (29) all bind ubiquitin (Ub) and allow these proteins to function in Ub-dependent pathways. A second NZF module in Vps36 binds Vps28 (30), bridging complexes involved in protein sorting, and the zinc finger in the transcriptional elongation factor TFIIIS binds RNA (31). A better understanding of how different NZF family members recognize their cognate binding partners will be important for defining, and ultimately predicting, ligand specificity in this large protein family.

The zinc fingers found in Nup153 and Nup358 (hereafter referred to as Nup ZnF) are distinctive in that they are found in repetitive arrays. In addition to conforming to the RanBP2-type consensus, these zinc fingers exhibit additional sequence conservation at several non-structural positions, particularly Leu13 (64% conserved), Val14 (79%), and Ala25 (79%) (numbering based on the N-terminal amino acid residues (NH2-GSPGISGGGGGILDS). Consistent with this idea, the Nup ZnF regions of Nup153 and Nup358 are important in orchestrating mitotic nuclear envelope breakdown, a type of nuclear membrane remodeling unique to higher eukaryotes. In this context, the Nup ZnFs are proposed to serve as a scaffold for recruitment of the coatomer complex COPI (18, 19). Other proteins that associate with Nup ZnF regions include the small GTPase Ran and the transport receptor exportin 1 (32–34).

Given the pivotal role that Ran plays in nucleocytoplasmic trafficking (reviewed in Refs. 3 and 35)), Ran binding by the Nup ZnF regions may be important in helping to regulate traffic through the higher eukaryotic pore. As a Ras-like GTPase, Ran fits the paradigm in which GTP and GDP binding create distinct conformational states, allowing Ran to serve as a molecular switch (36–38) (reviewed in Ref. 39). Previous studies have suggested that RanGDP, but not RanGTP, binds specifically to the Nup ZnF regions (32, 34), which is of potential interest because the roles of Ran often depend critically on its nucleotide state.

To begin to investigate how Nup ZnF modules function in nuclear pore biology, we have determined the three-dimensional structure of a nucleoporin zinc finger and characterized its Ran binding properties.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Plasmids used in this study are listed in supplemental Table SII. The expression construct for wild-type ZnF2 from human Nup153 was created by cloning annealed oligonucleotides 3 and 4 (Table SIII) into the Ndel/BamHI sites of a pGEX2T vector modified to contain a TEV cleavage site between the GST and ZnF2 polypeptides (WISP01–69). Inserts for human Nup153 zinc fingers 1, 3, and 4 were created by PCR amplification from full-length Nup153 and subcloned into WISP01–69 using Ndel/Xhol. All site-directed mutations were created using QuickChangeTM mutagenesis (Stratagene). For the Nup153 ZnF constructs L13T and V14F mutations were first introduced using oligonucleotides 9 and 10 (Table SIII), and this construct was then mutated further to alter Ala25 to Met using oligonucleotides 11 and 12 (Table SIII). Expression constructs for wild-type Npl4 NZF domain and the T13L,F14V mutant were described previously (24, 25). The latter construct was mutated further to create the T14L,F15V,M25A mutant using oligonucleotides 13 and 14 (Table SIII). The GST–Ran construct was a kind gift from Sally Kornbluth (Duke University). Site-directed mutation of this construct to create GST–RanQ69L was performed using oligonucleotides 17 and 18 (Table SII). His-tagged importin β fragments 1–462 and 45–462 in pQ6E0 (Qiagen) were a kind gift from Dirk Görlich (University of Heidelberg) (40).

**Expression and Purification of Recombinant Ran**—To produce Ran for use as the analyte in biosensor experiments, 10-liter cultures of BL21(DE3) RIL cells (Stratagene), transformed with Ran expression vectors, were grown at 37 °C in a fermenter to an OD600 of ~0.8. Isopropyl-1-thio-β-D-galactopyranoside was then added to 1 mM and the culture grown for an additional ~15 h at 22 °C. Following centrifugation, pellets from 2 liters of culture were resuspended in 100 ml of Buffer A (25 mM Tris, pH 8.0, 500 mM NaCl, 5 mM MgCl2, 5 mM β-ME, 1 mM DTT). All purification steps were performed at 4 °C. Bacteria were lysed by sonication and the lysate was centrifuged to remove insoluble debris. GST–Ran was recovered from the cleared lysate by fast protein liquid chromatography using a GSTPrep FF 16/10 affinity column (Amersham Biosciences). After washing with 4 column volumes of buffer A, protein was eluted with 20 mM glutathione in 25 mM Tris, pH 8.0, 200 mM NaCl, 5 mM MgCl2, 5 mM β-ME, and 1 mM DTT. Eluted protein was then dialyzed overnight into 25 mM Tris, pH 8.0, 50 mM NaCl, 5 mM MgCl2, 2.5 mM CaCl2, and 1 mM DTT in the presence of thrombin (Calbiochem; ~0.5 units/mg GST–Ran). Purified Ran was purified away from GST by sequential anion and cation chromatography steps. Flow-through fractions from a DEAE-Sepharose column were applied to an S-Sepharose column. A salt gradient (25 mM Tris, pH 8.0, 5 mM MgCl2, 1 mM DTT, and 50–1000 mM KCl) was used to elute protein from the S-Sepharose column, but Ran largely eluted in 50 mM KCl fractions. Material from these fractions was concentrated and residual GST and GST–Ran were removed by passage over 1.5 ml of glutathione-Sepharose. Purified Ran was loaded with nucleotide (see below), flash frozen in liquid nitrogen, and stored at ~80 °C. This procedure typically resulted in a yield of ~10 mg of Ran per 2-liter pellet.

Thrombin cleavage of GST–Ran resulted in 15 non-native N-terminal amino acid residues (NH2-GSPGISGGGGGILDS). Precise cleavage by thrombin was confirmed by matrix-assisted laser desorption ionization mass spectrometry (calculated mass, 25,635 Da; observed mass, 25,634 Da). Thrombin-cleaved GST–Ran was used for the binding experiments reported herein. A GST–Ran construct with a TEV protease cleavage site was also cloned and purified (Fig. S2 and supplementary methods) to
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produce a protein with only one non-native N-terminal amino acid residue (NH₂-S). The Nup153 ZnF binding affinities of this shorter Ran construct were similar to those of the longer thrombin-cleaved Ran protein (data not shown). Purities and concentrations of Ran preparations were analyzed by SDS-PAGE and amino acid analyses, respectively.

GST-Ran immobilized on biosensor chips was prepared slightly differently; pellets from 1-liter cultures were resuspended in 40 ml of Buffer B (10 mM NaH₂PO₄, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 5 mM β-ME) with 1/2 tablet of Complete protease inhibitor (Roche Applied Science) and sonicated. Cleared lysates were applied to a 1.5-ml bed volume of glutathione-Sepharose (Amersham Biosciences). The column was washed with 10 bed volumes of Buffer B and eluted with 20 mM glutathione in 25 mM Tris, pH 7.0, 5 mM MgCl₂, 2 mM DTT, and 10 μM ZnCl₂. Eluates were dialyzed overnight against the same buffer without glutathione and then concentrated to 1–3 ml prior to loading with nucleotide and biosensor analysis.

Expression and Purification of Recombinant Zinc Finger Domains—To prepare pure Nup153 ZnF for biosensor binding experiments, BL21(DE3) RIL cells containing the Nup153 ZnF2 expression construct were grown at 37 °C in a fermentor to an A₆₀₀ of ∼0.5. Protein expression was induced with isopropyl 1-thio-β-d-galactopyranoside (0.5 mM) and protein allowed to accumulate for an additional 4 h. Following centrifugation, a pellet from 6 liters of culture was resuspended in ZnF Buffer (10 mM NaH₂PO₄, pH 7.4, 150 mM NaCl, 5 mM β-ME, 10 μM ZnCl₂). All purification steps were performed at 4 °C. Bacteria were lysed by sonication and the lysate was centrifuged (40,000 × g for 30 min) to remove insoluble debris. GST-Nup153 ZnF2 was recovered from the cleared lysate by affinity chromatography using a GSTPrep FF 16/10 affinity column (Amersham Biosciences). Bound protein was washed with 10 column volumes of ZnF buffer and eluted with 20 mM glutathione in 50 mM Tris, pH 8.0, 5 mM β-ME, and 10 μM ZnCl₂. Protein fractions were pooled and dialyzed for 16 h into 50 mM Tris, pH 8.0, 100 mM NaCl, 10 μM ZnCl₂, 1 mM DTT in the presence of recombinant TEV protease (10 μg/ml GST-Nup153 ZnF2). The protein solution was concentrated to ~3 ml, and residual GST-Nup153 ZnF2 and GST were removed by Superdex-75 gel filtration chromatography (Amersham Biosciences) in 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM β-ME, 2 mM DTT, and 10 μM ZnCl₂. TEV protease cleavage left three non-native residues at the N terminus of ZnF2 (NH₂-GHM), but the first native amino acid was designated Val¹ to keep the numbering consistent with the NZF consensus (24).

GST-Nup153 ZnF fusion proteins were captured onto biosensor chips directly from fresh, cleared bacterial lysates. Protein expression was induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside (0.8–1 A₆₀₀, 5 h, 25 °C). Pellets from 2 ml of culture were resuspended in either B-PER lysis buffer (Pierce) or Buffer B supplemented with 10 μM ZnCl₂ (most experiments used the latter). Cells resuspended in B-PER buffer were rotated at room temperature for 10 min, followed by sonication and centrifugation. Cells resuspended in Buffer B were supplemented with lysozyme (1 mg/ml) and incubated on ice for 20 min, followed by sonication and centrifugation.

Expression and Purification of Importin β Fragments—BL21(DE3) RIL cells transformed with expression vectors for His₅-importin β (1–462) or His₅-importin β (45–462) (40) were grown at 37 °C to an A₆₀₀ of 0.8–1.0 and protein expression was induced (1 mM isopropyl 1-thio-β-d-galactopyranoside, 4 °C, 21 °C). Bacterial pellets were resuspended in 20 ml of 50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM β-ME and sonicated (all steps at 4 °C). Cleared lysates were incubated in batch with 1 ml of Ni-NTA resin (Qiagen) overnight and washed with 10 bed volumes of 1 × phosphate-buffered saline. The His-tagged proteins were eluted with 100 mM glycine, pH 3.0, and immediately neutralized.

Nucleotide Loading of Ran—Nucleotide (2 mM) and EDTA (10 mM) were added to Ran preparations and the samples were rotated at room temperature for 30 min. MgCl₂ was then added to a final concentration of 20 mM (41).

Preparation of Samples for NMR Spectroscopy—Unlabeled human ubiquitin and 15N- and 15N/13C-labeled Nup153 ZnF2 were expressed and purified as described previously (24) with the exception that the TEV protease cleavage buffer used included 5 mM sodium citrate and 5 mM β-ME in place of DTT. Purifications typically yielded 4–5 mg of pure Nup153 ZnF2 and 40 mg of ubiquitin per liter of culture. Samples for structure determinations were 1 mM labeled protein in NMR buffer (20 mM D₅-H-Tris, 50 mM NaCl, 2 mM D₅-β-ME, 10 μM ZnCl₂, pH 7.0, in 90% H₂O, 10% D₂O). Samples were degassed and flame-sealed under argon in NMR sample tubes to reduce cysteine oxidation.

NMR Data Collection and Resonance Assignments—All NMR spectra were collected at 20 °C on a Varian Inova 600 MHz spectrometer equipped with a triple-resonance 1H/13C/15N Cold-Probe and z-axis pulsed field gradient capability. Backbone resonances were assigned using a suite of triple-resonance experiments as described previously (24), employing two-dimensional versions of the HNCA (42), CBCACONH, HNCO, and HN(CA)CO (43). Side chain assignments were completed using three-dimensional H(CCO)NH-TOCSY, (H)(C(CO))NH-TOCSY (44), HCH-TOCSY, HCH-COSY, and 13N-edited TOCSY experiments. Aromatic resonances were assigned using a combination of 1H,13C-HSQC, 13C-edited NOESY experiments centered on the aromatic carbon resonances (125 ppm), a 13C-edited NOESY experiment centered on the aliphatic region, and heteronuclear correlation experiments that correlate the Cβ carbons to the C6 and Ce protons of the aromatic rings (45). Stereospecific assignments for β-methylene protons and χ¹ dihedral angle estimates were obtained using a combination of HNHB, HN(CO)HB (46), 15N-edited TOCSY, and NOESY data (47). Stereospecific assignments of side chain methyl groups and qualitative determination of χ² and χ3 dihedral propensities were obtained using long range carbon-carbon and carbon-proton couplings observed in LRCC (48) and LRCH (49) experiments. Three-dimensional 13N-edited NOESY-HSQC (50, 51) and 13C-edited NOESY-HSQC (150 ms mixing times) were used to generate distance restraints for refinement. Three-bond coupling constants (JH₁H₃,N-HA) were obtained from a three-dimensional HNHA experiment (52) and ψ dihedral restraints were derived from backbone Ha, Ca, CO, Cβ chemical shifts using TALOS (53).
All spectra were processed with FELIX 2004 (Accelrys) and referenced indirectly to 2,2-dimethyl-2-silapentanesulfonic acid (53).

Structure Determination—Backbone and side chain correlations were assigned and NOE intensities were integrated using tools in SPARKY.5 The solution structure of Nup153 ZnF2 was refined using automated NOE assignments and torsion angle dynamics as implemented in CYANA (version 2.1) (54, 55). Initial refinements used NOE data alone to define the overall fold. Once the zinc coordination geometry was established, the final refinements added restraints for zinc—sulfur distances, cysteine—Sy to Sy distances, Zn to Cβ distances (14 total), dihedral restraints (25), hydrogen bonds (12), and stereospecific assignments (20). A total of 100 randomized conformers were “folded” into three-dimensional structures by including NOE constraints iteratively using criteria defined by CYANA. The 20 structures with the lowest CYANA target function were chosen for analysis. Structures were validated using PROCHECK-NMR (56), Aqua (56), and the programs supplied at the PDB deposition site. All figures were created using PYMOL (70).

NMR Chemical Shift Mapping of the Nup153 ZnF2:Ran Interface—The RanGDP binding interface on Nup153 ZnF2 was mapped by comparing the chemical shifts in independent NMR samples of 15N-labeled Nup153 ZnF (150 μM) supplemented with 0, 0.25, 0.5, or 1.0 eq of RanGDP in titration buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 5 mM MgCl2, 10 μM ZnCl2, 5 mM β-ME, 10% 2H2O, 90% 1H2O). TROSY-15N/1H-HSQC spectra were collected for each amide pair and normalized—The RanGDP binding interface on Nup153 ZnF2

RESULTS

Individual Nup153 ZnF Modules Bind Ran—RanGDP interacts with the zinc finger regions of two nuclear pore proteins, Nup153 and Nup358 (32, 34). These Ran binding regions contain 4–8 ZnF modules connected by linkers that vary in sequence and length (28–42 amino acids, Fig. 1, A and B). Previous studies have not defined a minimal Ran binding element, although individual zinc fingers have been shown to be sufficient for COP1 association and to exert dominant negative inhibition of nuclear envelope breakdown, albeit with weaker activities than the intact tandem array of zinc fingers (19). These experiments suggested that Nup ZnFs can act as functional units, and we therefore began by testing whether individual Nup153 ZnF modules could bind specifically to RanGDP.

GST-Nup153 ZnF fusion proteins (supplemental Fig. S2) were immobilized onto biosensor chips and purified RanGDP was injected over each surface. All four Nup153 ZnF modules bound to RanGDP (Fig. 1, C and D), and the interactions were specific because RanGDP did not bind to a mutant version of the second zinc finger (ZnF2mut, Fig. 1C, inset, and described in detail in Fig. 5) or to GST alone (data not shown).

In all cases, equilibrium binding and complete dissociation were achieved within seconds (Fig. 1C and data not shown). The equilibrium binding phases of the experiment were therefore analyzed to obtain equilibrium binding constants. The data set shown in Fig. 1D yielded RanGDP dissociation constants of: 6 μM (ZnF1), 5 μM (ZnF2), 46 μM (ZnF3), and 40 μM (ZnF4). These values are in excellent agreement with measurements from several independent experiments (average values are summarized in Table 1).

Having established that a single zinc finger motif can bind to RanGDP, we next wished to determine how the native, tandem context of these ZnF modules influences Ran binding. To do so, we immobilized a GST fusion protein (4ZnF) encompassing all

5 T. D. Goddard and D. G. Kneller, University of California, San Francisco, CA.
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TABLE 1

| Zinc finger | Analyte   | Zinc finger sequence | n Backup | KD (M) |
|-------------|-----------|----------------------|----------|---------|
| ZnF1        | RanGDP    | 658-AGSSWQCDTCLLQNKVTDNKICIACQAAKL | 4        | 6 ± 2   |
| ZnF2        | RanGDP    | 722-VIGTWDDCTCLLVGKNDPEAIKCVACETTPK | 8        | 6 ± 2   |
| ZnF2-TF     | RanGDP    | 722-VIGTWDDCTTPQNKPEAIKCVACETTPK | 1        | >2000*  |
| ZnF2-TFM    | RanGDP    | 722-VIGTWDDCTTPQNKPEAIKCVACETTPK | 2        | >4000*  |
| ZnF3        | RanGDP    | 391-YGSHRCSVCOVQDPAANDEKSCVSMSEK | 3        | 39 ± 12 |
| ZnF4        | RanGDP    | 451-PQGSHDCSECLVQNKADSRKLCASESAP | 2        | 32 ± 11 |
| Npl4        | RanGDP    | 580-SAMIAQHCSTFMNNQGTHGECMLSPRT | 2        | 2385 ± 446 |
| Npl4-LVA    | RanGDP    | 580-SAMIAQHCSTFMNNQGTHGECMLSPRT | 3        | 350 ± 39 |
| ZnF2        | Ub        | 658-AGSSWQCDTCLLQNKVTDNKICIACQAAKL | 2        | >5000   |
| ZnF2-TFM    | Ub        | 722-VIGTWDDCTTPQNKPEAIKCVACETTPK | 1        | 206 ± 2*|
| Npl4        | Ub        | 580-SAMIAQHCSTFMNNQGTHGECMLSPRT | 1        | 122 ± 0.4*|
| Npl4-LVA    | Ub        | 580-SAMIAQHCSTFMNNQGTHGECMLSPRT | 2        | >10,000  |

* The order of individual zinc fingers of Nup153 within the zinc finger domain is denoted by number (e.g. ZnF1 is the first zinc finger of the domain). Zinc finger mutations have been represented by the zinc finger changed followed by the amino acids the mutations resulted in (e.g. ZnF2-TF indicates that the 2nd zinc finger has been mutated from endogenous residues (leucine, valine) to threonine, phenylalanine (TF)).

* Superscripts denote starting position of the zinc finger within the relevant protein. Bold lettering represents amino acids mutated from the wild-type sequence.

* n represents the number of individual experiments performed to test the interaction between zinc finger and analyte.

* Dissociation constants and error were determined from a statistical fit of a single binding isotherm derived from duplicate measurements at 10 different Ran or Ub concentrations (0–99 μM for ZnF2-TF, 0–709 μM for ZnF2-TFM, or 0–828 μM for Npl4).

* K_D, with error could only be obtained for one of two experiments at 5700 ± 100 μM. The second experiment was measured at >2000 μM with no measurable error.

Figure 1. Isolated Nup153 ZnF modules bind RanGDP. A, schematic representation showing the four tandem ZnFs repeats within human Nup153. B, alignment of the Nup153 ZnF modules in their native context (Nup153 amino acids 658–1002). Identical residues are highlighted in black, conserved residues in dark gray, and similar residues in light gray. The numbering used for individual zinc fingers is indicated at the top. C, sensorgrams showing RanGDP binding to immobilized Nup153 ZnF2 or the L13T,V14F,A25M triple mutant (negative control, calculated for ZnF2 and ZnF3 in this experiment). Binding is graphed as a function of the relative amount of Ran bound. The data for the 4ZnF domain fit well to a two-independent site model and the two values are measured in Biacore biosensor experiments and error determined by the standard deviation between direct comparison. Measurements of RanGDP binding to the full domain fit well to a two-independent site model, with one class of sites similar in affinity to ZnFs 1 and 2 and a second class similar to ZnFs 3 and 4 (Fig. 1E). Relative to the single zinc fingers, Ran bound to the 4ZnF fragment with a higher stoichiometry, consistent with this region containing multiple zinc finger binding sites. We therefore conclude that: 1) all four Nup153 ZnFs can bind RanGDP; 2) individual ZnF motifs can function as discrete Ran binding modules; 3) absolute RanGDP binding affinities vary with ZnF sequence, with the first two Nup153 ZnFs binding ~6-fold more tightly than the final two; and 4) when in their native, tandem context, the Nup zinc fingers bind Ran with little cooperativity or contribution from linker regions.

NMR Studies of a Nup Zinc Finger—To gain insight into the molecular basis for RanGDP binding, we determined the three-dimensional structure of the second ZnF module of human Nup153 (Nup153 ZnF2). Using standard 1H/15N/13C triple resonance heteronuclear NMR experiments, nearly complete resonance and stereo-specific assign-
ments were obtained for Nup153 ZnF2, except for the amide protons of the first two non-native N-terminal residues (GH) and the Hα of Lys23, which resides under the water resonance. As expected, the CB shifts for the coordinating cysteine residues indicated that those side chains coordinated the single zinc ion. As discussed below, two residues located on either side of the single tryptophan (Asn16 and Lys30) also exhibited notable ring current shifts.

Initial structural refinements of the zinc finger used exclusively NOE data and provided the overall domain fold in the absence of zinc coordination restraints. Torsion angle dynamics refinements within CYANA were then completed by adding additional restraints, including stereospecific Hβ proton assignments, χ1, φ, and ψ torsion angles, hydrogen bonds, and idealized zinc geometry (based on the Npl4 zinc finger (24)). The final refinement included 362 NOEs, 25 dihedral restraints, 20 stereo assignments, 14 zinc coordination-geometry restraints, and 12 hydrogen bonding restraints (see Table SI). The first four (1–4) and the final (31) residues were disordered, but the remaining residues were all well defined, and the final ensemble of NMR models was of high quality, with low average target functions, good backbone geometry and stereochemistry, and small root mean square deviations from the mean structure (0.11 Å for backbone atoms, 0.64 Å for all heavy atoms, see Fig. 2, Table SI, and Protein Data Bank code 2GQE).

Structure of Nup153 ZnF2—As shown in Fig. 2B, Nup153 ZnF2 contains four short β-strands that form two orthogonal hairpins (Thr6–Asn16 and Ile21–Lys30). The four strands are connected by a compact central loop and two short metal binding loops, termed “rubredoxin knuckles,” that include the cysteine side chains (Cys9, Cys12, Cys23, and Cys26, Figs. 2A, B, and C). The first hairpin exhibits a canonical
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FIGURE 3. Closely related Nup153 ZnF and Npl4 modules exhibit different ligand binding specificities. A, biosensor isotherms showing RanGDP binding to Nup153 ZnF2 (■) and Npl4 NZF (○). B, biosensor isotherms showing ubiquitin binding to Nup153 ZnF2 (■) and Npl4 NZF (○).

β-sheet hydrogen bonding patterns (N16O—W7H, N16H—W7O, and V14O—C9H), whereas analogous hydrogen bonds were only formed at one end of the second hairpin (K30H—I21O and C23H—T28O). The two β-hairpins surround a small hydrophobic core that consists of the Trp7 indole ring capped at either end by the Asn16 and Lys30 side chains. Asn16 and Trp7 are nearly invariant, whereas position 30 (Lys30 here) is typically a Lys or an Arg. The Trp7, Asn16, and Lys30 core residues form an extensive hydrogen bonding network that stabilizes the fold (Fig. 2C). Not unexpectedly, the backbone conformation of Nup153 ZnF2 is very similar to other RanBP/NZF modules with published structures (Fig. 2D) (24, 26, 27, 62).

Molecular Specificity in the Nup153 ZnF2-Ran Complex—Ran binding specificity was probed by comparing the Nup153 and Npl4 NZF modules. As shown in Fig. 3A, RanGDP bound Nup153 ZnF2 but not Npl4 NZF. Conversely, ubiquitin bound Npl4 NZF but not Nup153 ZnF (Fig. 3B), and the dissociation constant for the Npl4 NZF-Ub interaction agreed well with previously published results (KD = 122 ± 0.4 μM, Fig. 3B and Table 1) (25). Thus, although the zinc fingers of Nup153 and Npl4 fold into very similar structures they present specialized binding surfaces. This is consistent with the general trend, noted above, that NZF domains can bind a range of ligands: Nup153 NZFs bind Ran (32); Npl4, Rybp, and Vps36 (C-terminal) NZF bind ubiquitin (23, 25, 63); Znf265 binds RNA (26); NZF of Mdm2 binds to ribosomal proteins (64); and the N-terminal Vps36 NZF is part of an interface with Vps28 (62).

Mapping the Ran Binding Surface of Nup153 ZnF2—To understand how Nup153 ZnF2 creates Ran binding specificity within this shared architectural framework, the Ran binding surface on Nup153 ZnF2 was mapped using NMR chemical shift perturbation experiments (Fig. 4). Chemical shift changes revealed that the primary Ran binding site on Nup153 ZnF2 was centered about Cys12, Leu13, Val14, and Ala25, with smaller but still significant shifts in surrounding residues (Thr13, Trp7, Cys9, Val24, and Cys26). All but two of the Nup153 ZnF backbone amides in the complex were in fast exchange, as is typical for binding interactions with micromolar dissociation constants. The Leu13 and Val14 amides in the center of the Ran binding site were in intermediate exchange, which presumably reflects their unusually large chemical shift changes upon Ran binding.

The Nup153 ZnF2 amino acids implicated in Ran binding are clustered and exposed on one edge of the zinc coordination site (Fig. 4, B and C). The position of this interaction site is essentially identical to the ubiquitin binding site on Npl4 NZF (Fig. 4C and Ref. 24). In particular, the three amino acid positions that form the core of the Npl4 NZF ubiquitin binding site (Thr13, Phe14, and Met25) also form the core of the Nup153 ZnF2 Ran binding site (Leu13, Val14, and Ala25). Hence, in both cases the NZF fold serves to cluster residues 13, 14, and 25 into one continuous, exposed recognition surface.

To confirm the importance of the Nup153 ZnF Leu13, Val14, Ala25 motif for Ran binding, we initially tested the effects of replacing this motif with the analogous Thr13, Phe14, Met25 motif from Npl4 NZF. As expected, the triple substitution mutation abrogated RanGDP binding (Fig. 5A). Indeed, even the L13T, V14F, M25F double mutation alone reduced the RanGDP binding affinity more than 300-fold (Table 1, hZnF2-TF). To monitor the integrity of the hybrid Nup153 ZnF2-TFM, we tested this mutant for ubiquitin binding, a property known to be conferred by these amino acids within an NZF backbone (25), and found that binding affinity of Nup153 ZnF2-TFM for ubiquitin was only 2-fold weaker than wild-type Npl4 NZF (KD = 206 μM versus 122 μM; Table 1). In the converse experiment, substituting the Leu13, Val14, Ala25 motif into the Npl4 NZF scaffold enhanced RanGDP binding activity, although the binding was weak (KD = 350 μM; Fig. 5B and Table 1). We therefore conclude that, although surrounding residues can clearly modulate binding affinities, the “LVA” motif constitutes the primary determinant of Ran binding within a highly conserved NZF structural scaffold.

Interaction with Nup153 ZnFs Is Minimally Affected by the Nucleotide Bound by Ran—The zinc finger domains of Nup153 and Nup358 have previously been characterized as binding specifically to the GDP form of Ran (32, 34) and this activity has been broadly annotated in genomic data bases. With a quantitative assay in hand, we compared the relative binding affinities of RanGDP and RanGTP for Nup153 ZnF2. GST-Ran

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The structure of Nup153 ZnF2 demonstrated that Nup zinc fingers adopt the minimal RanBP2/NZF zinc ribbon fold first seen for the Npl4 NZF protein (24). As illustrated in Fig. 2D, it is now clear that RanBP2/NZF modules adopt very similar backbone conformations. However, the functional utility of the RanBP2/NZF classification has been limited by a lack of information on the specific binding interactions of the hundreds of different proteins that contain this structurally conserved module.

FIGURE 4. Mapping the Ran interface on Nup153 ZnF2. A, overlaid 1H/15N-TROSY-HSCQ spectra of 15N Nup153 ZnF2 (0.15 mM) in the presence of 0 (red), 0.25 (purple), 0.5 (cyan), and 1.0 (green) eq of RanGDP. 1H/15N pairs are labeled and those undergoing the largest shifts are indicated with arrows. Asterisks indicate resonances (Leu17 and Val14) that disappear in the presence of RanGDP because of intermediate exchange rates. B, residues with the greatest chemical shift changes upon RanGDP binding mapped onto the Nup153 ZnF2 structure. Color coding: dark blue, δ > 15 and intermediate exchange (largest shifts); light blue, 4 < δ < 8; straw, δ < 4. C, comparison of the RanGDP and Ub binding surfaces of Nup153 NZF (left) and Npl4 NZF (right). The 10 most shifted residues are colored red (largest shifts) and light pink (smaller shifts). Normalized chemical shift differences ranged from 15.1 to 3.4 for the Nup153 ZnF-Ran titration and from 7.9 to 2.01 for the Npl4 NZF-Ub titration (24).

was first loaded with either GDP or GTP and immobilized on the biosensor chip. Initial experiments employed the mutant RanQ69L, which is defective in GTP hydrolysis, to ensure the stability of the loaded nucleotide (65). Surprisingly, Nup153 ZnF2 showed only a slight binding preference for RanQ69L-GDP ($K_D = 7 \pm 1 \mu M$) over RanQ69L-GTP ($K_D = 12 \pm 1 \mu M$) (Fig. 6A and Table 2). Nup153 ZnF2 also bound with similar affinities to the GDP and GTP forms of wild-type Ran ($K_D = 8 \pm 0.4 \mu M$ and $K_D = 9 \pm 1$, respectively; Table 2). Additional experiments with ZnF2, ZnF3, and the full 4ZnF fragment immobilized and tested for binding to Ran loaded with GDP or GTP were consistent with this trend. Neither ZnF3 nor 4ZnF showed significant preference for RanGDP over RanGTP (data not shown). Also of note, similar equilibrium dissociation constants were obtained for all Nup153 ZnF2/RanGDP interactions regardless of which protein was immobilized or whether the wild-type or Q69L RanGDP proteins were used. These similarities helped to confirm the reliability of the measurements and established that the Q69L mutation did not influence Nup153 ZnF2 binding.

To confirm that our RanGTP and RanGDP preparations were loaded with the appropriate nucleotide, we took advantage of the well characterized interaction between RanGTP and importin β. Importin β binds RanGTP with very high affinity ($K_D = 140 \text{ pm}$) (66), whereas its affinity for RanGDP is much lower ($K_D = 5–10 \text{ nm}$) (67). This interaction can be recapitulated with a fragment containing the first 462 amino acids of importin β, and removal of the first 44 amino acids from this fragment eliminates Ran binding (40, 68). Interaction assays were performed by immobilizing either His-tagged importin β-(1–462) or importin β-(45–462) (negative control) on Ni-NTA resin. Immobilized importin β fragments were then incubated with purified GST-RanQ69L, previously loaded with either GDP or GTP. The appropriate nucleotide was maintained at 1 mM in each binding assay, as in the corresponding Nup153 ZnF2 biosensor assays. The resin was washed and bound proteins were eluted and analyzed by immunoblot. As expected, binding was observed only with the longer importin β-(1–462) construct and only for GTP-loaded RanQ69L (Fig. 6B). These experiments confirmed that our experimental procedures produced Ran proteins that were loaded predominantly with the expected nucleotides. Our binding experiments therefore imply that Nup153 ZnF recognition is affected only modestly, if at all, by the Ran nucleotide state.

DISCUSSION

The structure of Nup153 ZnF2 demonstrated that Nup zinc fingers adopt the minimal RanBP2/NZF zinc ribbon fold first seen for the Npl4 NZF protein (24). As illustrated in Fig. 2D, it is now clear that RanBP2/NZF modules adopt very similar backbone conformations. However, the functional utility of the RanBP2/NZF classification has been limited by a lack of information on the specific binding interactions of the hundreds of different proteins that contain this structurally conserved module.
The present study demonstrates that a subset of RanBP2/NZF modules can bind Ran, and defines a sequence motif that can be used to predict whether or not individual RanBP2/NZF fingers will bind Ran. Specifically, we have shown that three key residues: Leu14, Val15, and Ala25, form the primary Ran binding site on Nup153 ZnF2. This LVA motif is not present in most RanBP2/NZF sequences, but is highly overrepresented in the Nup ZnF subfamily (supplemental Fig. S1). The three LVA residues also exhibit significant co-variation (68% of NZF modules with LV have an Ala at position 25), suggesting that they are linked functionally. Moreover, double or triple mutations within the LVA motif abrogate Ran binding, and Ran binding activity is created when the LVA motif is transferred into a heterologous NZF module. The hybrid zinc finger (Npl4-LVA) does not bind Ran as tightly as the wild-type Nup zinc fingers, however, indicating that elements outside the LVA motif influence binding. This is also evidenced by the fact that Nup153 ZnF2 and ZnF4 bind Ran with different affinities (6 versus 32 μM), even though both contain the LVA motif. Finally, we note that conservative changes within the LVA motif can clearly be tolerated as Nup153 ZnF1 has a V14L substitution and ZnF3 has L13C and A25S substitutions.

Our data indicate that most if not all RanBP2/NZF modules that contain the LVA motif will bind Ran with micromolar affinities. This predicts RanGDP binding for at least the six of eight RanBP2/NZF repeats in human Nup358 that contain LVA motifs. This prediction is supported by preliminary biosensor experiments showing that the third ZnF module from Nup358 also binds RanGDP (KD = 51 nM). Ran and importin β were detected by immunoblot using anti-Ran (upper panels) and penta-His antibodies (lower panels), respectively.

Comparison of Ran binding to individual zinc fingers versus a fragment containing the native, tandem array of zinc fingers led us to conclude that linker regions do not contribute significantly to the interface with Ran, either by facilitating cooperative interactions or by providing additional contact sites, as was recently observed for the N-terminal Vps36 NZF in which downstream residues were found important for its interaction with Vps28 (62). Although the Nup ZnFs appear to operate as independent Ran binding sites, the presence of these arrayed sites within both Nup153 and Nup358 could, in principle, cre-
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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Cloning and purification of TEV protease-cleavable GST-Ran. A GST-Ran construct with a TEV protease cleavage site between the GST and Ran domain was created by QuickChange™ site-directed mutagenesis (Stratagene) using oligonucleotides 15 & 16 to introduce a TEV protease cleavage site into the pGEX-Ran construct (Table SII). Purification was similar to the pGEX-Ran protocol except that GST-Ran was cleaved with TEV protease (10 μg/mg GST-Ran), while dialyzing into 25 mM Tris, pH 8.0, 100 mM NaCl, 10 μM ZnCl$_2$, and 1 mM DTT at 4°C. Ran was then purified away from free GST using DEAE anion exchange chromatography using the same buffer as the thrombin protease-cleaved Ran. Flowthrough fractions were concentrated and any remaining GST was removed by passage over 1.5 ml glutathione-Sepharose. Purified, TEV protease-cleaved Ran was loaded with nucleotide, flash frozen in liquid nitrogen, and stored at -80°C (Figure S2).

Analytical GST pulldown assays. Assays were performed at room temperature. Glutathione sepharose beads (10 μl per reaction) were washed twice in PBS. For each reaction, 50 μl GST fusion protein lysates were incubated with beads in 250 μl PBS or biosensor binding buffer and washed 3 times. Bound proteins eluted in SDS sample buffer were analyzed by SDS-PAGE with Coomassie staining (Figure S2).

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Nup153 and Nup358 zinc finger alignments. The zinc fingers of Nup153 and Nup358 from fly (Drosophila melanogaster, Dm), chicken (Gallus gallus, Gg), and human (Homo sapiens, Hs) are aligned. Identical residues are highlighted in black, conserved residues in dark grey, and similar residues in light grey. Residues at positions 13, 14, and 25 are denoted by asterisks.

Figure S2. Production and purification of GST- Nup153 ZnF and Ran proteins. (A) GST and GST-Nup153 ZnF fusion proteins were captured from lysates on glutathione Sepharose, eluted in SDS sample buffer and analyzed by SDS-PAGE and Coomassie blue staining. The fusion protein ZnF2-TFM refers to mutations made to the 2$^{nd}$ zinc finger of Nup153 (L13T, V14F, and A25M). In parallel, the same lysates were used to capture GST-fusion proteins onto biosensor chips. (B) SDS-PAGE and Coomassie blue staining analyses of purified recombinant Ran proteins prepared by the thrombin- or TEV protease-cleavage methods and used in biosensor binding experiments.
Table SI. Structure statistics for NUP153-ZnF2

|                        | <TAD>          |
|------------------------|---------------|
| NOE distance restraints \( \text{(Å)} \) | 362           |
| Sequential (|\( i-j \)|=1)          | 225           |
| Medium range (2≤|\( i-j \)|≤5)       | 42            |
| Long range (|\( i-j \)|>5)        | 95            |
| Zinc coordination restraints | 14           |
| Dihedral restraints     | 20            |
| CYANA Target function \( \text{(Å}^4 \) | 0.14±0.004   |
| Residual distance restraint violations |                      |
| Number of violations ≥ 0.1 Å | 0             |
| Maximum violation (Å)   | 0.10          |
| Residual dihedral restraint violations |                    |
| Number of violations ≥ 1° | 0             |
| Maximum violation (°)   | 0.023         |
| Van der Waals violations |                                |
| Number ≥ 0.1 Å          | 0±0           |
| Maximum violation (Å)   | 0.08          |
| Ramachandran statistics |                                |
| Favored                 | 67.8%         |
| Allowed                 | 31.1%         |
| Generously allowed      | 1.1%          |
| Disallowed              | 0.0%          |
| RMS deviations to the average coordinates \( \text{(Å)} \) |                        |
| Backbone                | 0.11±0.03     |
| Heavy atoms             | 0.64±0.06     |

\( ^a \)\(<\text{TAD}>\) is the ensemble of 20 lowest-penalty structures calculated using CYANA.

\( ^b \)Only meaningful and non-redundant restraints as determined by the CYANA CALIBA function.

\( ^c \)backbone torsion angle statistics include all residues in the entire zinc-finger domain.

\( ^d \)RMS deviations are calculated for the ordered residues (5-30) of the final structure ensemble.
| Plasmid Name   | Description               | Source          |
|---------------|---------------------------|-----------------|
| WISP06-1      | GST-h153-ZnF1             | this study      |
| WISP06-2      | GST-h153-ZnF2             | this study      |
| WISP06-3      | GST-h153-ZnF3             | this study      |
| WISP06-7      | GST-h153-ZnF4             | this study      |
| WISP06-4      | GST-h153-ZnF2 (TF)        | this study      |
| WISP06-5      | GST-h153-ZnF2(TFM)        | this study      |
| WISP03-27     | GST-Npl4-NZF              | ref. 25         |
| WISP03-29     | GST-Npl4-NZF(LV)          | ref. 25         |
| WISP06-6      | GST-Npl4-NZF(LVA)         | this study      |
| pGEX-Ran      | GST-wtRan                 | Kornbluth lab   |
| pGEX-TEV-Ran  | GST-TEV-wtRan             | this study      |
| pQE-RanQ69L   | GST-RanQ69L               | this study      |
| pQE-lmp71 1-462 | 6-His-lmp71-462         | Gehrlich lab    |
| pQE-lmp7 45-462 | 6-His-lmp745-462      | Gehrlich lab    |
| pGEX-4hZnF    | GST-h153-ZnF domain       | ref. 20         |
| Number | Oligo name   | Sequence                                                                 |
|--------|--------------|--------------------------------------------------------------------------|
| 1      | ZnF1f        | 5’ GGCCATATGGCTGGTGCTATCATGG 3’                                         |
| 2      | ZnF1r        | 5’ TTCTCGAGGATCCCTACAATTTTTGTGCTGC 3’                                   |
| 3      | ZnF2f        | 5’ TATGTTGATCGGCACTGG GTGATTGCGATACCTGCTGGTGCGAGAACGC CCGAAGCGATCAAATGCGTGAGCGGAAACCCCGAAGCGTACCA 3’ |
| 4      | ZnF2r        | 5’ GATCTACGTTTTGCG66GTTTTCGACGCACGATTTGATGCTCCGATGTGCTACCCAGTGCGATACCA 3’ |
| 5      | ZnF3f        | 5’ GGCCATATGCCCATGTGGTAGACCTGCTGGTGCGAGAACGC CCGAAGCGATCAAATGCGTGAGCGGAAACCCCGAAGCGTACCA 3’ |
| 6      | ZnF3r        | 5’ TTCTCGAGGATCCCTACAATTTTTGTGCTGC 3’                                   |
| 7      | ZnF4f        | 5’ GGCCATATGCCCATGTGGTAGACCTGCTGGTGCGAGAACGC CCGAAGCGATCAAATGCGTGAGCGGAAACCCCGAAGCGTACCA 3’ |
| 8      | ZnF4r        | 5’ TTCTCGAGGATCCCTACAATTTTTGTGCTGC 3’                                   |
| 9      | ZnF2-TFf     | 5’ GGGAATTCGAGATTCCTGCACGCTTCAGAACAAACCCGGAAGCG 3’                      |
| 10     | ZnF2-TFr     | 5’ GGGAATTCGAGATTCCTGCACGCTTCAGAACAAACCCGGAAGCG 3’                      |
| 11     | ZnF2-TFMf    | 5’ CGGAAGCGATCAAATGCGTGATGCGAAACCCGGAAGCG 3’                           |
| 12     | ZnF2-TFMr    | 5’ CGGAAGCGATCAAATGCGTGATGCGAAACCCGGAAGCG 3’                           |
| 13     | Npl4-LVAf    | 5’ GGCCATATGCCCATGTGGTAGACCTGCTGGTGCGAGAACGC CCGAAGCGATCAAATGCGTGAGCGGAAACCCCGAAGCGTACCA 3’ |
| 14     | Npl4-LVAr    | 5’ CGGAAGCGATCAAATGCGTGATGCGAAACCCGGAAGCG 3’                           |
| 15     | GST-Ran (TEV)f | 5’ GGGAATTCGAGATTCCTGCACGCTTCAGAACAAACCCGGAAGCG 3’  |
| 16     | GST-Ran (TEV)r | 5’ GGGAATTCGAGATTCCTGCACGCTTCAGAACAAACCCGGAAGCG 3’  |
| 17     | GST-RanQ69Lf | 5’ GGGAATTCGAGATTCCTGCACGCTTCAGAACAAACCCGGAAGCG 3’  |
| 18     | GST-RanQ69Lr | 5’ GGGAATTCGAGATTCCTGCACGCTTCAGAACAAACCCGGAAGCG 3’  |
Figure S1
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