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Crystallographic and Biochemical Analysis of the Ran-binding Zinc Finger Domain

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The nuclear pore complex (NPC) resides in circular openings within the nuclear envelope and serves as the sole conduit to facilitate nucleocytoplasmic transport in eukaryotes. The asymmetric distribution of the small G protein Ran across the nuclear envelope regulates directionality of protein transport. Ran interacts with the NPC of metazoa *via* two asymmetrically localized components, Nup153 at the nuclear face and Nup358 at the cytoplasmic face. Both nucleoporins contain a stretch of distinct, Ran-binding zinc finger domains. Here, we present six crystal structures of Nup153-zinc fingers in complex with Ran and a 1.48 Å crystal structure of RanGDP. Crystal engineering allowed us to obtain well diffracting crystals so that all ZnF–Ran complex structures are refined to high resolution. Each of the four zinc finger modules of Nup153 binds one Ran molecule in apparently non-allosteric fashion. The affinity is measurably higher for RanGDP than for RanGTP and varies modestly between the individual zinc fingers. By microcalorimetric and mutational analysis, we determined that one specific hydrogen bond accounts for most of the differences in the binding affinity of individual zinc fingers. Genomic analysis reveals that only in animals do NPCs contain Ran-binding zinc fingers. We speculate that these organisms evolved a mechanism to maintain a high local concentration of Ran at the vicinity of the NPC, using this zinc finger domain as a sink.

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Introduction

Nucleocytoplasmic transport is controlled and facilitated by protein assemblies termed nuclear pore complexes (NPCs), which reside in circular openings of the nuclear envelope where inner and outer nuclear membranes are fused. The NPC is a large macromolecular assembly with a calculated mass of ~50 MDa.[1] On the basis of electron microscopy (EM) studies of *Xenopus laevis* oocytes and *Saccharomyces cerevisiae*, the general shape and structure of the pore is conserved across eukaryotes.[2,3] These EM studies define the pore as a ring embedded in the nuclear envelope, exhibiting 8-fold rotational symmetry around a central axis and imperfect 2-fold symmetry between the cytoplasmic and nucleoplasmic faces. Despite its size, the NPC is composed of only ~30 proteins, or nucleoporins (Nups), arranged in a few biochemically defined subcomplexes that assemble the entire structure in a modular fashion.[4] As the single constitutive barrier to regulate permeability, the NPC transports a wide range of substrates across the double membrane of the nuclear envelope.[5–7] Active transport through the NPC is mediated by nuclear transport receptors (NTRs), also called karyopherins or importins/exportins.[8,9]

The small G protein Ran is the master regulator of NTR-mediated, nucleo-cytoplasmic protein transport.[10] Ran selectively promotes binding or release of import or export cargos to NTRs by means of a chemical gradient. Ran binds mostly GDP in the cytoplasm, and mostly GTP in the nucleus. The GTPase-activating protein RanGAP, localized to the cytoplasmic face of the NPC, and the chromatin-bound GTP-exchange factor RCC1, together promote this asymmetry by modulating nucleotide hydrolysis and exchange, respectively. The established gradient provides directionality to protein transport. In the nucleus, RanGTP releases import cargo from NTRs by competitive binding. RanGTP is recycled back to the

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Abbreviations used: NPC, nuclear pore complex; EM, electron microscopy; Nup, nucleoporin; NTR, nuclear transport receptor; ZnF, zinc finger.

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cytoplasm *via* a trimeric complex formed with NTRs and export cargo. At the cytoplasmic face of the NPC, RanGTP interacts with RanGAP to hydrolyze GTP and disrupt the trimeric NTR-mediated export complex. NTF2 (nuclear transport factor 2) recycles RanGDP back into the nucleus.[11]

Nup153 and Nup358 (RanBP2) are large, metazoa-specific nucleoporins with multiple roles.[12–16] Both interact with Ran through a zinc finger cassette composed of several individual zinc finger (ZnF) motifs.[17,18] Nup153 is predominantly localized to the nuclear face of the NPC, although recent studies suggest the three major domains of Nup153 are localized to different regions of the NPC.[19–22] The N-terminal portion contains a pore-targeting region and an RNA-binding domain.[23–27] The ZnF cassette is comprised of multiple zinc fingers and defines the center of Nup153. The C-terminal region of Nup153 harbors ~30 phenylalanine-glycine (FG-) repeats, unstructured motifs found in several Nups lining the inner channel of the NPC that are responsible for NTR interaction.[28–31] Nup358 has several characterized domains, including a cyclophilin homology domain, a SUMO ligase domain, a structural leucine-rich region, previously characterized Ran-binding domains (RanBDs), and a cassette containing multiple ZnFs.[12,15,32–34] The ZnFs of Nup153 and Nup358 are representative of the RanBP2-type ZnF family, recognized by the conserved sequence pattern W-X-C-X(2,4)-C-X(3)-N-X(6)-C-X(2)-C (Fig. 1).[35] RanBP2-type zinc fingers fold into a structure composed of two β-hairpin strands that sandwich a zinc ion coordinated with four cysteine residues.[36] The RanBP2-type ZnF structure is distinct from other ZnFs; however, it defines only a common scaffold, not a common function. Ran binding has been reported for Nup153 and Nup358 ZnFs and not for the other structural homologs.[37,38] It is unclear what exact role these Ran-binding ZnFs have at the NPC, nor is it conclusively analyzed whether they bind Ran in a nucleotide-dependent or a nucleotide-independent manner.[17,18,37,39]

We have determined the crystal structures of all four ZnFs of Nup153 in complex with RanGDP. Our structural data suggests that all ZnF modules preferentially bind to RanGDP rather than RanGTP, supported by mutational and microcalorimetric data. While the primary sequence of the ZnF in the cassette is conserved, the number of ZnFs varies among species. Our data supports a largely uncooperative model for binding of Ran to the individual ZnF modules within Nup153 or Nup358, explaining why the exact number of consecutive ZnFs is not conserved. Although we detect differences between ZnF binding to RanGDP *versus* RanGTP, they are moderate and may not be of functional consequence. We propose that the ZnFs within Nup153 and Nup358 are used primarily to create a Ran sink and thereby increase the local concentration of Ran at both the nucleoplasmic and cytoplasmic face of the NPC.

Results and Discussion

Crystallographic analysis of Nup153-ZnF•RanGDP complexes

All protein constructs used in this study were from *Rattus norvegicus*, except Nup358, which was from *Homo sapiens*. The central region of Nup153 (residues 658 – 885) contains four zinc fingers; ZnF1 (residues 658 – 686), ZnF2 (residues 723 – 750), ZnF3 (residues 790 - 817), and ZnF4 (residues 848 - 885). The individual ZnF domains were cloned and recombinantly expressed as glutathione-*S*-transferase fusion proteins in *Escherichia coli*. In addition to individual ZnF domains, the tandem pairs of ZnF1 and ZnF2 (ZnF12, residues 658 - 750) as well as ZnF3 and ZnF4 (ZnF34, residues 790 - 885) were examined (see Supplementary Data Table S1 for all protein constructs used in this study). Full-length Ran was cloned and expressed as a His-tagged fusion protein in *E. coli*. RanGDP was separated from RanGTP using ion-exchange chromatography and the nucleotide-loaded state was validated with HPLC. Our RanGDP structure is solved at 1.48 Å resolution with two molecules in the asymmetric unit. Molecule A is well ordered with all residues in both switch regions defined. In molecule B, however, a portion of the switch II region (residues 69 – 74) is disordered. In molecule A, a van der Waals interaction between Phe77 and the neighboring molecule B orders switch II. Without this packing interaction and in the absence of the gamma-phosphate from GTP switch II is flexible.

Diffraction-quality crystals of a Nup153-ZnF in complex with RanGDP were initially obtained for ZnF2, ZnF4, ZnF12, and ZnF34, but only the ZnF2 complex crystals diffracted satisfactorily. Well diffracting crystals of all other complexes were obtained after introducing a structure-based, surface point mutation in Ran, F35S, to stabilize a crystal

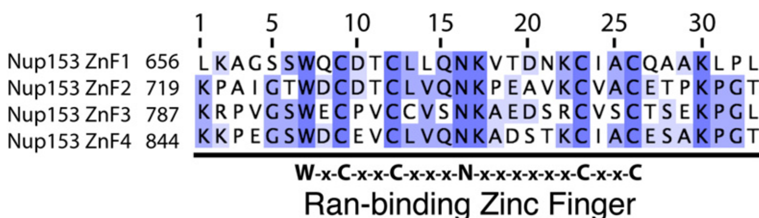

Fig. 1. Alignment of the four Ran-binding zinc fingers of Nup153 from *R. norvegicus*. The numbering used for individual zinc fingers is listed above. Identical residues are highlighted in dark blue, with decreasing levels of conservation highlighted in lighter shades of blue. Below is the consensus sequence for the RanBP2 family of zinc finger proteins.

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