Dissecting the Interactions between NTF2, RanGDP, and the Nucleoporin XFXFG Repeats*

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We have used a range of complementary biochemical and biophysical methods to investigate the interactions between nuclear transport factor 2 (NTF2), the Ras family GTPase Ran, and XFXFG nucleoporin repeats that are crucial for nuclear trafficking. Microcalorimetry, microtiter plate binding, and fluorescence quenching in solution are all consistent with the binding constant for the NTF2-RanGDP interaction being in the 100 nM range, whereas the interaction between NTF2 and XFXFG repeat-containing nucleoporins such as Nsp1p is in the 1 μM range. Although the accumulation of NTF2 at the nuclear envelope is enhanced by RanGDP, we show that Ran binding does not alter the affinity of NTF2 for nucleoporins nor does the binding of nucleoporins alter that Ran binding does not alter the affinity of NTF2 for RanGDP. These results indicate that, instead, Ran increases the binding of NTF2 to nucleoporins by another mechanism, most probably by Ran itself binding to nucleoporins and NTF2 binding to this nuclear pore-associated Ran.

The Ras family GTPase Ran is a key component of the nuclear trafficking machinery and is also important in cell cycle progression (1, 2). In nuclear protein import, for example, Ran is thought to regulate crucial interactions between cargo and carrier molecules in both the cytoplasm and nucleus. Thus, cargo proteins carrying nuclear localization signals bind to carrier molecules of the importin-β family in the cytoplasm and are then translocated into the nucleus through nuclear pore complexes, after which the carrier-cargo complex is dissociated by RanGTP. The carrier is then recycled to the cytoplasm bound to RanGTP, where RanGTPase-activating protein generates RanGDP, which dissociates from the carrier freeing it to interact with another cargo molecule and so undergo a further import cycle (3). The cycling of carriers between nucleus and cytoplasm and their binding and release of cargo molecules in the appropriate compartment is thus intimately tied to Ran nucleotide state, with RanGDP being generated in the cytoplasm by RanGTPase-activating protein and Ran being recharged with GTP in the nucleus by its guanine nucleotide exchange factor RCC1 (1). RanGTP is being continuously exported from the nucleus and must be recycled back to the nucleus for exchange. HeLa cells contain 7 μM Ran, being mainly nuclear (4). The nuclear import of RanGDP is mediated by NTF2, a small dimeric protein that binds both RanGDP and to a number of nuclear pore proteins (nucleoporins) that terminally S-tagged canine Ran was obtained by subcloning wild-type canine Ran into a series of core XFXFG motifs joined by variable hydrophilic linkers (5–8).

Although the interactions between RanGDP, NTF2, and XFXFG nucleoporins are essential for mediating the nuclear import of RanGDP, there is little quantitative information on the strength of these interactions or how they are modulated and orchestrated. We have therefore used a range of biochemical methods to assess these interactions and here show the strength of the interaction between NTF2-Ran to be in the 100 nM range, whereas that between NTF2 and the XFXFG repeats of a representative nucleoporin Nsp1p is in the micromolar range. We also show that the presence of Ran does not affect the strength of the interaction between NTF2 and the Nsp1 XFXFG repeats; conversely, the presence of Nsp1 does not affect the interaction between Ran and NTF2. The results obtained on purified proteins contrast with observations on permeabilized cells, where RanGDP increased the accumulation of NTF2 at the nuclear envelope (14). However, this effect is not seen with R76E-Ran, which does not bind NTF2 (16), indicating that accumulation of NTF2 at the nuclear envelope is mediated through a direct Ran-NTF2 interaction. The different models accounting for these results are evaluated.

**EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Rat NTF2, canine RanGDP, and the 18 XFXFG repeat-containing fragment of nucleoporin Nsp1p (18R-Nsp1) were expressed and purified as described (7, 17, 14). N-terminally S-tagged canine Ran was obtained by subcloning wild-type canine Ran DNA in pET30a and was expressed after induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h in 2YT-kanamycin medium and purified using a slight modification of the protocol used to purify wild-type canine Ran, i.e. a 0–100 mM linear NaCl gradient to elute it from the DE-52 column.

Microtiter Plate Binding Assay—NTF2 (0.7 μM) in PBS was aliquoted onto a 96-well plate (Falcon 3911) and incubated for 2 h with gentle rocking. After blocking with PBS-I-block (0.2%), S-tagged Ran diluted in PBS containing 0.2% I-Block was added to the wells at a range of concentrations (0–8 μM) and incubated for either 2 h at room temper-

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1 The abbreviations used are: NTF2, nuclear transport factor 2; NPC, nuclear pore complex; NE, nuclear envelope; 18R-Nsp1, NSP1 protein construct containing 18 XFXFG repeats; NB-DCl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; NB, 4-nitrobenzo-2-oxa-1,3-diazole; PBS, phosphate-buffered saline.

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NBD Labeling of NTF2—NTF2 dialyzed against PBS buffer containing 2 mM MgCl₂ was incubated with a 10-fold excess of NBD-Cl over NTF2 in PBS, 20 mM Tris, pH 8.0, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA for 30 min at room temperature and the absorbance read at 405 nm. To determine binding constants, the data were fitted by nonlinear least-squares analysis using the program Scientist (MicroMath Inc., Salt Lake City, UT).

RESULTS

Kₐ for the NTF2-RanGDP Interaction.—We used three complementary methods to determine the Kₐ for the NTF2-RanGDP interaction. Using microtiter plates coated with 0.7 µM NTF2, S-tagged RanGDP showed a Kₐ of 240 ± 30 nM (Fig. 1A), whereas plates coated with the NTF2 E42K mutant (which does not bind RanGDP (Ref. 8)) did not bind RanGDP (data not shown). We also used microcalorimetry and fluorescence to assess the strength of the NTF2-Ran interaction in solution. Microcalorimetry gave a Kₐ of 150 ± 30 nM (Fig. 1C). Using NTF2 fluorescently labeled on cysteine residue with NBD-Cl (an environment- and conformation-sensitive probe (Ref. 20)), the decrease in fluorescence at 506 nm on addition of Gsp1p (the yeast Ran homologue) was concentration-dependent and gave a Kₐ of 75 ± 40 nM (Fig. 1B). However, canine RanGDP did not produce any change in fluorescence, even though NBD-NTF2 was qualitatively equivalent to wild-type NTF2 in terms of its binding to Ran and Nsp1 using column chromatography or bead-binding assays (data not shown). The crystal structure of the Ran-NTF2 complex (21) shows that the 3 cysteines of NTF2 are located on a face of the molecule opposite to the Ran binding site, and so it is unlikely that the NBD and Ran/Gsp1 interact directly. It is possible that binding of NTF2 to Gsp1p produces a slight conformation change reflected by the change in fluorescence emission of the attached NBD group. However, in the case of canine RanGDP, the binding on NTF2 must be slightly different, so that this change in conformation does not occur. Because of the sensitivity of NBD probes to small changes in environment, a faint difference in the way canine Ran and its yeast homologue Gsp1p bind to NTD2 could certainly account for the effect observed. Unfortunately, neither the crystal structure of the Gsp1p nor its complex with NTF2 have been yet solved. None of the binding isotherms obtained gave any indication of cooperativity between the two Ran binding sites on an NTF2 dimer molecule; the curves were all fitted to a single isotherm with no allosteric curvature shape. This would imply that each binding site for Ran on an NTF2 chain behaves independently. Additionally, the stoichiometry of binding deduced from the binding between NBD-NTF2 and Gsp1p (Fig. 1B) was approximately 1 Gsp1p molecule bound per NTF2 dimer chain.

RanGDP Binding Does Not Alter the Affinity of Nsp1 XFXFG Repeats for NTF2.—We used intrinsic tryptophan fluorescence to determine the Kₐ for the interaction between NTF2 and a bacterially expressed construct containing 18 XFXFG repeats from yeast nucleoporin Nsp1p (18-RNS1).
Binding isotherms (Fig. 3A) showed no cooperativity, indicating that the XFXFG repeat-binding site on each chain of the NTF2 dimer behaved independently. We therefore, as a first approximation, analyzed the data assuming that all 18 XFXFG repeats in the Nsp1p construct and the two chains in the NTF2 molecule were able to interact independently. A $K_d$ value of 1.4 ± 0.21 μM for the whole Nsp1 protein or 25 μM (based on the concentration of individual XFXFG motifs) was obtained (Fig. 3A). As illustrated in Fig. 3B, when the titration was repeated in the presence of a 2-fold molar excess of RanGDP over NTF2 (when 92% of the NTF2 would be complexed with Ran) we obtained a $K_d$ of 1.2 ± 0.18 μM for the whole Nsp1 protein or 22 μM (based on individual XFXFG repeats), indistinguishable from that obtained in the absence of RanGDP. Therefore, NTF2 free in solution or in complex with Ran has a similar affinity for the XFXFG repeat region of Nsp1p. Using bead-binding assays or fluorescence, Bayliss et al. (15) obtained $K_d$ values for the XFXFG-NTF2 interaction of 2–3 μM based on whole 18 repeat Nsp1 constructs, which is equivalent to a $K_d$ of 35–50 μM based on individual repeats and clearly similar to the results we obtained here.

Addition of the Nap1p XFXFG repeats to NTF2 produced an increase in fluorescence when added to free NTF2 and a decrease in fluorescence when added to NTF2 in the presence of RanGDP (Fig. 3, A and B). This suggests that Ran binding may have altered the local environment of the XFXFG repeat binding site on NTF2. X-ray crystallography (21) has indicated that, although RanGDP binding causes a small rigid-body rotation of the two chains in the NTF2 dimer, the overall conformation of each chain in the complex is similar to that observed in uncomplexed NTF2 (22). Therefore, the change in tryptophan environment resulting from XFXFG repeat binding is due to the proximity of the bound Ran rather than Ran binding producing a substantial conformational change in NTF2. Indeed, such an explanation would also be consistent with the negligible change in NTF2’s affinity for XFXFG repeats observed in the presence of RanGDP. Addition of the 18R-Nsp1p repeats to RanGDP did not produce any change in fluorescence when tested under the same conditions as for free NTF2 (data not shown) indicating that this effect was not due to the XFXFG repeats altering the Ran intrinsic fluorescence.

**The Presence of Nap1p XFXFG Repeats Does Not Change the Affinity of NTF2 for Ran**—To establish whether the nucleoporin XFXFG repeats would alter the interaction between NTF2 and RanGDP, we determined the value for the $K_d$ between Gsp1p and NTF2 in the presence of an excess of 18R-Nsp1 (10 times the $K_d$). As before, we then followed the decrease in fluorescence at 506 nm upon titration of NBD-NTF2 with Gsp1p. In this assay, we are detecting only the binding of RanGDP to NTF2, since we are monitoring the change in fluorescence in the presence of 18R-Nsp1p was indistinguishable from that obtained for NTF2 and Gsp1p alone (Fig. 2). Thus, NTF2 free in solution or bound to Nap1p has the same affinity for RanGDP.

**Total NTF2 Concentration in Rat Liver Homogenates and HeLa Cells**—To place the $K_d$ values obtained for the interaction between NTF2 and Ran in cellular context, we determined the total cellular concentration of NTF2 using isotope dilution. Ran is present at 7 μM in HeLa cells, of which 80% is thought to be nuclear (4). For NTF2, we found a total concentration of 2 μM both in rat liver homogenates and HeLa cells. NTF2 is located primarily at the nuclear rim (10, 11) and concentrated there by about 120-fold compared with total NTF2 (9), which correlates with the determined cytosolic concentration of NTF2 of 0.3 μM (6). Calculations based on these concentrations and the 100 nM $K_d$ for the NTF2-RanGDP interaction indicate that the NTF2-Ran complex will be found mainly localized at the nuclear envelope (Table I).

**Effect of Ran or R76E-Ran on the Accumulation of NTF2 at the Nuclear Envelope**—RanGDP enhances accumulation of NTF2 at the nuclear envelope (14), raising the possibility of a cooperative interaction between NTF2, RanGDP, and a NPC constituent. Our results on purified proteins show that Ran does not have any cooperative effect on the binding of NTF2 to 18R-Nsp1, since the $K_d$ obtained for the interaction between NTF2 and 18R-Nsp1 was the same, whether NTF2 was complexed with RanGDP or not (see Fig. 3). To assess whether the Ran-induced increase in NTF2 accumulation at the nuclear envelope required a direct interaction between Ran and NTF2, we used the R76E Ran mutant, which does not interact with NTF2 although it retains the ability to bind nucleoporin XFXFG repeats and other components of the nuclear trafficking machinery (16). Unlike wild-type Ran, addition of the R76E mutant did not increase the accumulation of fluorescently labeled NTF2 at the nuclear envelope (Fig. 4), demonstrating
that direct interaction between Ran and NTF2 is necessary to obtain the enhanced accumulation of NTF2 at the nuclear envelope.

**DISCUSSION**

The results using three separate methods were broadly comparable giving a $K_d$ value in the order of 100 nM (Fig. 1) for the RanGDP-NTF2 interaction. Although the microtiter plate assay gave a slightly weaker interaction (by 2- or 3-fold), this difference was probably not significant as the standard deviations obtained with this assay were quite large. It was therefore reassuring that two different assays (microplate and solution fluorescence quenching) gave comparable results. Similar $K_d$ values were obtained for canine Ran and the yeast homologue Gsp1, whereas addition of sequences at the Ran N-terminus such as the S and His tags decreased the affinity of RanGDP for NTF2 by a factor not more than 2. The on-rate association between NTF2 and Ran GDP would be expected to be in the range $10^7$ to $10^8$ s$^{-1}$ M$^{-1}$ (23), and so a 100 nM $K_d$ would imply an off-rate of the order of 1–10/s, which is somewhat slower than the rate of nuclear trafficking, which is thought to be of the order of 10–100/s. Thus, the binding constant for the NTF2-RanGDP complex is sufficiently strong to ensure that the complex remains intact during nuclear import but sufficiently weak for it to dissociate on a time scale rapid relative to nucleotide exchange once in the nucleus. Moreover, combined with the likely concentrations of Ran and NTF2, a $K_d$ in the 100 nM range indicated that, whereas RanGDP would be expected to be only partially complexed in the cytoplasm and nucleus, at the nuclear envelope (primarily in nuclear pore complexes) virtually all the RanGDP present

**TABLE I**

|          | Cytosol | Nuclear envelope | Nucleus |
|----------|---------|-----------------|---------|
| NTF2     | 0.3 μM (6) | 20 μM$^{f}$ | 0.6 μM$^{e}$ |
| RanGDP   | 1.3 μM$^{f}$ | 1.3 μM$^{f}$ | 0.5 μM$^{f}$ |
| Fraction NTF2 bound (%) | 91 | 6.5 | 59 |
| Fraction NTF2 free (%) | 9 | 97.5 | 41 |
| Fraction RanGDP bound (%) | 21 | 99 | 71 |
| Fraction RanGDP free (%) | 79 | 1 | 29 |

$^{a}$ The calculated concentration of NTF2 at the NE is a minimal approximation considering total cell concentration: 2 μM (this study), a 10 times accumulation factor of NTF2 at the NE (9), and the negligible volume occupied by the NE.

$^{b}$ Approximation from Ref. 10, and our data (not shown).

$^{c}$ Approximation was made from the total known cell content being 7 μM (4), of which 80% being nuclear, from that considered that 90% of the Ran in the nucleus would be in the GTP-bound state and 90% of the Ran in the cytosol would be in the GDP-bound state.

$^{d}$ Considered the same concentration as the one found in the cytosol. Calculations for the fractions bound were derived from the equation: fraction NTF2 bound = $\frac{N_0 + R_e + K_d - \sqrt{(N_0 + R_e + K_d)^2 - 4 \times N_0 \times R_e}}{2 \times N_0}$ in the model NTF2 + Ran-GDP $\rightarrow$ Ran-GDP/NTF2, with $N_0$ being total NTF2 and $R_e$ being total Ran.
should be complexed with NTF2 (Table I). Two implications for nuclear import can be drawn from those results. First, the stable nature of the complex between RanGDP and NTF2 (off-rate of the order of 1–10/s) would mean that the complex probably stays intact during trafficking. Second, the higher local concentration of NTF2 at the NE implies that 99% of the Ran present at the NE would be found in such a complex; hence, even if dissociation of RanGDP from the NTF2-Ran backbone resulting from the RanGDP binding on the NTF2 complex would occur, the reassociation would occur quickly. Those conclusions are consistent with a model for the nuclear import of Ran that would cross the NE in complex with NTF2 (Fig. 6).

The affinity of NTF2 for the Nsp1 repeats is weaker than that between NTF2 and Ran and was of the order of 25 μM based on the concentration of individual XFXFG repeats (Fig. 3). However, this lower affinity does not preclude the possibility of this interaction to occur in vivo at the NPC, since the XFXFG repeats are present in high copies number on several nucleoporins at the NE and therefore their local concentration is expected to be high, probably of the order of 50 mM (15). The off-rate for the NTF2/Nsp1 interaction has been estimated to be in the order of 500–5000/s for an individual repeat (15), implying that this interaction would be more transient than that between NTF2 and RanGDP. This would allow rapid attachment and detachment of NTF2 to the nucleoporins. In the context of the more stable interaction between NTF2 and Ran, a 25 μM affinity of NTF2 for nucleoporin XFXFG repeat repeats would also enable rapid attachment and detachment of the RanGDP-NTF2 complex to the nucleoporins during trafficking. Such a mechanism would be consistent with the results showing the crucial importance of a direct interaction with NTF2 for the import of RanGDP (13, 14).

Our results showed no synergy between the interactions involving RanGDP, NTF2, and Nsp1. RanGDP had no effect on the affinity of NTF2-Nsp1 interaction, nor did the presence of Nsp1 have an effect on the Ran-NTF2 interaction (Figs. 2 and 3). The crystal structure of NTF2 either free (22) or in complex with RanGDP (21) are certainly consistent with those results, since there is no major conformational changes in the protein backbone resulting from the RanGDP binding on the NTF2 molecule.

Previous work using permeabilized cells had shown that Ran increased the accumulation of NTF2 at the nuclear envelope implying a co-operativity between Ran, NTF2, and a constituent of the NPCs (14). However, our in vitro results with purified proteins do not support such a co-operative role for RanGDP in the NTF2-Nsp1 interaction (Fig. 3). How then can account for the increased nuclear envelope binding of NTF2 induced by Ran? Certainly, our results (Fig. 4) with R76E-Ran that does not bind to NTF2 (16) indicated that a direct interaction between NTF2 and Ran was required for this effect. Therefore, as illustrated in Fig. 5, either NTF2 is interacting with another type of nucleoporin in a Ran-sensitive manner or NTF2 is binding to RanGDP that is itself bound to the NPC. Thus, in model A, NTF2 binds a non-XFXFG-repeat nucleoporin with higher affinity when NTF2 is bound to RanGDP (Fig. 5A). Although results obtained with an NTF2 mutant (W7A-NTF2) with reduced affinity for XFXFG repeat-containing nucleoporins are consistent with this hypothesis (15), it is...
along the central channel of the pore bordered with several nucleoporins. The off-rates between the different complexes are in the range of 10–100/s, whereas there would be a rapid attachment/detachment to the nucleoporin. Local concentrations of Ran, NTF2, and nucleoporins favor the formation of the Ran-NTF2 complex at the nucleoporin is expected to be equivalent to the one for the NTF2/XFG repeat interaction. Such differences in stability are consistently observed (15). Our results show that Ran does not change the affinity of the NTF2-RanGDP complex and that the exchange of GTP for GDP on Ran by the exchange factor RCC1 will not affect the affinity of NTF2 for RanGDP. The off-rates for the Ran/NTF2 and NTF2/XFG repeat binary complexes are in favor of a “hopping” of the complex from one nucleoporin to the next along the central channel of the pore bordered with several XFG repeats containing nucleoporins (27). Once delivered in the nucleus, the exchange of GTP for GDP on Ran by the exchange factor RCC1 will favor the disruption of the Ran/NTF2 complex.

In summary, the affinity constant for the Ran/NTF2 complex and the NTF2/XFG repeat-containing nucleoporins such as Nsp1p complex are 100 nM and 1 μM, respectively. Upon RanGDP binding, there is no alteration for the affinity of NTF2 for nucleoporins. Conversely, upon Nsp1p binding there is no alteration for the affinity of NTF2 for RanGDP. In permeabilized cells, an enhanced accumulation of NTF2 at the NE proceeds through a direct Ran-NTF2 interaction indicating most probably that Ran itself ensures the binding of NTF2 to nucleoporins via a nuclear pore-associated Ran.

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