An ATP-dependent Activity That Releases RanGDP from NTF2*

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The small GTPase Ran functions in several critical processes in eukaryotic cells including nuclear transport, nuclear envelope formation, and spindle formation. A RanGDP-binding protein, NTF2, facilitates translocation of RanGDP through the nuclear pore complex and also acts to stabilize RanGDP against nucleotide exchange. Here, we identify a novel activity that stimulates release of GDP from Ran in the presence of NTF2. Hydrolyzable ATP enhances the GDP dissociation activity, and this enhancement is inhibited by nonhydrolyzable ATP analogues. In contrast, neither hydrolyzable ATP nor nonhydrolyzable ATP analogues affect GDP dissociation from Ran catalyzed by recombinant RCC1 or inhibition of GDP dissociation from Ran by recombinant NTF2. The ATP-dependent RanGDP dissociation activity therefore has the properties of a RanGDP dissociation inhibitor (GDI) displacement factor (RanGDF) where the GDI is NTF2. A protein phosphatase inhibitor mixture stimulates the RanGDF activity, suggesting the activity is regulated by phosphorylation. We propose that the ATP-dependent NTF2 releasing factor may have a role in the RanGDP/GTP cycle.

Ran is an abundant and evolutionarily highly conserved small GTPase of the Ras superfamily, found mainly in the nucleus of eukaryotic cells (1–3). Like other GTPases, Ran exists in both GTP- and GDP-bound states, abbreviated as RanGTP and RanGDP. These two forms of Ran interact differently with regulators and effectors (4). The intrinsic GTPase activity of Ran is very low but is greatly stimulated by a GTPase-activating protein (RanGAP) that is localized in the cytoplasm and on the cytoplasmic side of the nuclear pore complex (5–8). In contrast, RCC1, the only identified guanine nucleotide exchange factor (GEF)1 for Ran, is localized in the nucleus (1, 2, 9, 10). The compartmentalized localization of these regulators maintains a high concentration of RanGTP in the nucleus and a low concentration in the cytoplasm of interphase cells (11–14). This gradient of RanGTP concentration across the nuclear envelope is critical for the directionality of transport of many macromolecules between the nucleus and cytoplasm (15).

Unlike some small GTPases, where only the GTP-bound form has binding partners, both RanGTP and RanGDP interact with nuclear transport factors. In the context of this manuscript, the RanGDP-binding protein NTF2/p10 (16–18) is important. NTF2 exists as a homodimer and has two functions; one is to promote the import of RanGDP into the nucleus (19, 20) by facilitating diffusion through the NPC. Second, NTF2 interacts directly and specifically with RanGDP and stabilizes RanGDP against RCC1-mediated nucleotide exchange (21). The crystal structure of the NTF2-RanGDP complex (22) reveals direct contacts between the hydrophobic cavity of NTF2 and the switch II region of RanGDP.

Independently of its binding to RanGDP, NTF2 also interacts with FG repeat-containing nucleoporins (17, 23) and is found concentrated at NPCs at steady state. The interactions between NTF2 and both RanGDP and FG repeats are essential for efficient nuclear import of RanGDP (24–27). The recently solved crystal structure of the RCC1-Ran complex (28) indicates that NTF2 and RCC1 cannot bind simultaneously to RanGDP. Thus, RanGDP has to dissociate from NTF2 for RCC1-mediated nucleotide exchange to occur.

The continuous regeneration of RanGTP from RanGDP is critical for all the known functions of Ran. Recently, the activities of the known components of the Ran system were analyzed by the construction of a mathematical model (13). The model predicted that the functioning of the Ran system is particularly sensitive to two parameters. The first is the GTP/GDP ratio of the cell, in part a reflection of the fact that Ran has a significantly higher affinity for GDP than GTP (10, 13). The second is the delivery of RanGDP to the exchange factor RCC1 (13). In interphase, the latter process involves facilitation of RanGDP diffusion through the NPC by NTF2 followed by its dissociation from NTF2. Both steps are necessary before RCC1-mediated nucleotide exchange can occur. The fact that NTF2 binds RanGDP tightly enough to inhibit RCC1-mediated nucleotide exchange on Ran (21) suggested that a factor might exist that would help dissociate the RanGDP-NTF2 complex. Here, we have identified a novel activity that releases RanGDP from NTF2 in an ATP-dependent manner. We propose that this activity plays a role in the function of the RanGDP/GTP cycle.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Proteins—Wild-type Ran, RanT24N, RanQ69L, wild-type NTF2, and RCC1 were expressed in Escherichia coli BL21(DE3) and purified as described previously (21, 29). The pET 15b-W7A NTF2 mutant (27) was kindly provided by Dr. Murray Stewart. The protein, like wild-type NTF2, was expressed and purified as described previously (21). Proteins were exchanged into transport buffer (TB) (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, and Complete EDTA-free (Roche Applied Science)) using a PD10 column (Amersham Biosciences) and then concentrated

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‡ The abbreviations used are: GEF, guanine nucleotide exchange factor; RanGDI, RanGDP dissociation inhibitor; RanGDF, RanGDI displacement factor; NTF2, nuclear transport factor 2; NPC, nuclear pore complex; ATP-S, adenosine 5'-O-(thiotriphosphate); AMP-PNP, adenosine 5'-(β,γ-imino)triphosphate.
ATP-dependent RanGDF Activity

Preparation and Fractionation of Mouse Ehrlich Ascites Tumor Cells Extract—Ehrlich ascites tumor cells extract was prepared and fractionated as described previously (30). For fractionation, the clarified extract was applied to a Q-Sepharose column (Amersham Biosciences) equilibrated with lysis buffer (5 mM HEPES, pH 7.3, 10 mM potassium acetate, 2 mM magnesium acetate, 2 mM dithiothreitol, 20 μM cytochalasin B, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, leupeptin, pepstatin) and eluted sequentially with potassium acetate-free transport buffer containing 200 mM, 350 mM, and 550 mM potassium chloride. The flow-through fraction and eluted fractions (termed QFT, Q200, Q350, and Q550, respectively) were dialyzed against TB and concentrated by ultrafiltration to a uniform protein concentration (10 mg/ml). Then the Q200 fraction was dialyzed against 20 mM HEPES, pH 7.3, 20 mM sodium chloride, 1 mM magnesium acetate, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and Complete EDTA-free buffer, concentrated by Centriprep YM3 (Amicon), and applied to a HiTrap SP column equilibrated with the same buffer. Fractions (termed SP20, SP50, SP100, SP150, SP200, and SP300, respectively) were eluted stepwise in buffer containing 20, 50, 100, 150, 200, 350, and 500 mM sodium chloride.

Assay for Dissociation of [3H]GDP from Ran—[3H]GDP dissociation was assayed by a rapid filtration method as described previously (21).

Depletion of Endogenous RCC1 from Ehrlich Ascites Tumor Cells Extract—For depletion of endogenous RCC1 from the SP200, Ran24N coupled to Affi-Gel-10 (Bio-Rad) was used as described previously (29). For depletion of endogenous RCC1 from the SP200, Ran24N coupled to Affi-Gel-10 was used as described previously (29). The [3H]GDP dissociation from Ran catalyzed by RCC1 was quantified. The effects of a Q200 fraction of mouse Ehrlich ascites tumor cell extract on [3H]GDP dissociation from Ran catalyzed by RCC1 in the presence of wild-type NTF2 or W7A NTF2 was measured in the presence and absence of an ATP regeneration system. [3H]GDP dissociation from Ran catalyzed by RCC1 was quantified. B, the effects of two Q200 fractions from the Ehrlich ascites tumor cell extract were resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose filters (Schleicher & Schuell). Anti-NTF2 monoclonal antibodies (BD Transduction Laboratories), anti-histone H2B polyclonal antibodies (Santa Cruz Biotechnology), and anti-RCC1 rabbit serum were used as primary antibodies.

RESULTS

An ATP-dependent Ran GDP Dissociation Activity—The nucleotide exchange activity of Ran was assayed by measuring the dissociation of [3H]GDP from Ran-[3H]GDP. In this assay, 0.01 μM recombinant RCC1 catalytically induced the [3H]GDP dissociation from Ran (Fig. 1A) with a maximum efficiency in the presence of 0.67 μM of Ran-[3H]GDP (21). The [3H]GDP dissociation catalyzed by RCC1 was inhibited by wild-type NTF2 in a dose-dependent manner (Fig. 1A). 3.3 μM wild-type NTF2 completely inhibited the [3H]GDP dissociation from Ran under these conditions (Fig. 1A). Comparison with the endogenous situation is not straightforward. However, virtually all of the RanGDP that enters the cell nucleus, and thus might encounter RCC1, is bound to NTF2 dimers (13, 19, 20). The RanGDI activity of 1.7 ± 0.2 was measured in the presence and absence of an ATP regeneration system. [3H]GDP-Ran (0.67 μM) and wild-type NTF2 or W7A NTF2 (3.3 μM) were incubated for 5 min at 30 °C. The Q200 fraction was added and incubated for another 5 min at 30 °C. Finally, RCC1 (0.01 μM) was added, and incubation continued for 5 min at 30 °C. [3H]GDP that remained bound to Ran was measured by filter binding and scintillation counting. The data in A and B are expressed as the mean ± S.E. of [3H]GDP bound to Ran obtained from three independent experiments, relative to the intrinsic dissociation rate of GDP from Ran.

Fig. 1. An ATP-dependent Ran-GDP dissociation stimulatory activity. A, the effects of recombinant wild-type NTF2 or the W7A NTF2 mutant on [3H]GDP dissociation from Ran catalyzed by RCC1. [3H]GDP-Ran (0.67 μM) and NTF2 (0, 0.03, 0.1, 0.33, 1.0, 3.3 μM) were preincubated for 5 min at 30 °C and then further incubated in the presence of RCC1 (0.01 μM) for another 5 min at 30 °C. [3H]GDP dissociation from Ran catalyzed by RCC1 was quantified. B, the effects of two Q200 fractions from the Ehrlich ascites tumor cell extract on [3H]GDP dissociation from Ran catalyzed by RCC1 in the presence of wild-type NTF2 or W7A NTF2 was measured in the presence and absence of an ATP regeneration system. [3H]GDP-Ran (0.67 μM) and wild-type NTF2 or W7A NTF2 (3.3 μM) were incubated for 5 min at 30 °C. The Q200 fraction was added and incubated for another 5 min at 30 °C. Finally, RCC1 (0.01 μM) was added, and incubation continued for 5 min at 30 °C. [3H]GDP that remained bound to Ran was measured by filter binding and scintillation counting. The data in A and B are expressed as the mean ± S.E. of [3H]GDP bound to Ran obtained from three independent experiments, relative to the intrinsic dissociation rate of GDP from Ran.
Fig. 2. ATP does not affect the activities of recombinant RCC1 or NTF2. A, the effects of an ATP regeneration system, ATP-γS (3 mM), AMP-PNP (3 mM), or ATP (3 mM) on [3H]GDP dissociation from Ran catalyzed by recombinant RCC1 were measured. [3H]GDP-Ran (0.67 μM) was incubated with recombinant RCC1 (0, 3.3, or 6.6 nM) in the presence or absence of the different forms of ATP for 5 min at 30 °C, and [3H]GDP dissociation from Ran was measured as described in Fig. 1 legend. B, the effects of an ATP regeneration system, ATP-γS (3 mM), AMP-PNP (3 mM), or ATP (3 mM) on the inhibition of RCC1-mediated [3H]GDP dissociation from Ran by recombinant NTF2. [3H]GDP-Ran (0.67 μM) and wild-type NTF2 (1.7 μM) were incubated in the presence or absence of the different forms of ATP for 5 min at 30 °C, RCC1 (0.01 μM) was added, and incubation continued for another 5 min at 30 °C. [3H]GDP dissociation from Ran was quantified and expressed as described in the Fig. 1 legend.

ATP-dependent GDP Dissociation from Ran Requires NTF2 and Is Not Mediated by RCC1—To investigate whether endogenous RCC1 is involved in inducing the ATP-dependent [3H]GDP dissociation from Ran, we further fractionated the activity. The Q200 was applied to a HiTrap SP column, and fractions (SP20, SP50, SP100, SP150, SP200, SP350, and SP500) were eluted stepwise (see “Experimental Procedures”). By immunoblot analysis, most endogenous RCC1 and NTF2 was present in the Q200 fractions (Fig. 3A and data not shown). After separation, NTF2 predominantly eluted in the SP20 fraction (Fig. 3A), whereas RCC1 was eluted mainly in SP20, SP350, and SP500, with minor amounts present in SP200. The [3H]GDP dissociation activity of each SP fraction and its stimulation by ATP were measured in the presence of recombinant RCC1 and NTF2. The Q200 fraction was applied to a HiTrap SP column (see “Experimental Procedures”). The SP200 contained endogenous RCC1 and NTF2. Endogenous RCC1 was eluted mainly in the SP20, SP350, and SP500 fractions. The SP200 fraction also contained smaller amounts of RCC1. Endogenous NTF2 was predominantly eluted in the SP20 and histones H2A and H2B in the SP350 and SP500. B, the effects of the SP fractions on NTF2 inhibition of [3H]GDP dissociation from Ran. [3H]GDP-Ran (0.67 μM) and the W7A NTF2 mutant (3.3 μM) were incubated in the presence or absence of the different forms of ATP for 5 min at 30 °C. A fraction, as indicated, was added, and incubation continued for another 5 min at 30 °C. [3H]GDP dissociation from Ran was quantified and expressed as described in the Fig. 1 legend. C, the effects of the Q200, SP20, or SP200 fraction on [3H]GDP-Ran dissociation in the absence of added recombinant RCC1 or NTF2. The reactions were carried out and evaluated as in Fig. 1.
To more definitively rule out the participation of endogenous RanGEF in the ATP-dependent activity, we next depleted endogenous RCC1 from the SP200 using RanT24N coupled to Affi-Gel-10 beads (RanT24N-SP200). Most endogenous RCC1 (>90%) was depleted by this treatment (Fig. 4A), whereas control beads coupled to either RanQ69L or bovine serum albumin (BSA) caused a less efficient depletion of RCC1 (Fig. 4A). Using these depleted SP200 fractions, the effects of the ATP regeneration system on the \[^{3}H\]GDP dissociation in the presence of recombinant RCC1 and NTF2 were examined. As expected, total \[^{3}H\]GDP dissociation activity was reduced by RCC1 depletion, with almost no ATP-independent stimulation remaining (Fig. 4B). However, the ATP-dependent activity of the SP200 fraction (28.7% dissociation) was recovered similarly in the Q69L-depleted (20.0%), the bovine serum albumin-depleted (22.8%), and the T24N-depleted (20.0%) fractions, indicating that it was not due to endogenous RCC1.

Next we examined the effects of the ATP regeneration system on the intrinsic RanGEF activity in the Q200, SP20 and SP200 fractions in the absence of added recombinant RCC1 or NTF2. Endogenous NTF2 is present in the Q200 and SP20 fractions (see above). The ATP regeneration system enhanced \[^{3}H\]GDP dissociation in the presence of the Q200 (58% stimulation) and the SP20 (54% stimulation) but not the SP200 fraction (9% stimulation; Fig. 3C). These results, in combination with those presented above, suggest that the stimulation of \[^{3}H\]GDP dissociation from Ran by ATP depends on NTF2. In other words, ATP may regulate RanGDP release from NTF2 by a factor or factors present in the SP200 fraction. In fact, the SP200 fraction in the presence of ATP released NTF2 from RanGDP to a considerable extent (Fig. 5).

**ATP-dependent NTF2-releasing Activity Is Stimulated by Phosphatase Inhibitors**—To further clarify the mechanism of the ATP-dependent GDI displacement activity, we tested the effects of two nonhydrolyzable ATP analogues (ATPγS or AMP-PNP) on \[^{3}H\]GDP dissociation activity in the SP200 fraction. The ATP regeneration system significantly stimulated dissociation activity in the presence of NTF2 (Fig. 6). The two nonhydrolyzable ATP analogues did not affect the ATP-independent \[^{3}H\]GDP dissociation activity of the SP200. However, stimulation of the activity by ATP was inhibited (Fig. 6). These results suggest that ATP hydrolysis is essential for ATP-dependent dissociation activity.

To clarify whether the ATP-dependent \[^{3}H\]GDP dissociation activity might be regulated by phosphorylation, we examined the effects of a protein phosphatase inhibitor mixture on the activity. The phosphatase inhibitor mixture significantly stimulated the \[^{3}H\]GDP dissociation of the SP200 in the presence of NTF2 (24% stimulation; Fig. 7A). In the absence of recombinant NTF2, the phosphatase inhibitor mixture did not however stimulate \[^{3}H\]GDP dissociation (Fig. 7B). These data indicate that the ATP-dependent \[^{3}H\]GDP dissociation activity is probably regulated by phosphorylation and confirm that it requires NTF2.
DISCUSSION

NTF2 has two functions. First, it mediates the facilitated translocation of RanGDP through NPCs. Although this is not an intrinsically directional process, the activities of other Ran-interacting factors mean that this function of NTF2 acts to import RanGDP into the nucleus, in which RCC1, the RanGEF, is found (19, 20, 32). Second, NTF2 interacts directly and specifically with the GDP-bound form of Ran and inhibits RCC1-mediated GDP release from Ran (21). For this reason, we reported NTF2 as a RanGDP dissociation inhibitor or RanGDI (21). Here, we investigated the mechanism of release of NTF2 from RanGDP. The fact that in vitro NTF2 binds RanGDP tightly enough to inhibit RCC1-mediated nucleotide exchange on Ran suggested that a factor might exist that would help dissociate the RanGDP-NTF2 complex. We were able to detect such an activity in mouse Ehrlich ascites tumor cells extract. The activity functions in an ATP-dependent manner. We refer to this factor as a RanGDI (i.e., NTF2) displacement factor, or RanGDF for short, adopting the nomenclature from other small GTPase systems (e.g., Refs. 33 and 34). The ATP-dependent RanGDF activity is not specific for mouse Ehrlich ascites tumor cells, since a similar activity was detected in fractions prepared from HeLa cells (data not shown). By a combination of methods, we were able to demonstrate that the ATP-dependent activity is distinct from endogenous RCC1, and that it requires NTF2. This suggests a possible mode of action for the RanGDF, to accelerate dissociation of RanGDP from NTF2 and thus to make RanGDP more readily available for RCC1-mediated nucleotide exchange.

It has been proposed that the transfer of RanGDP from NTF2 to RCC1 might happen either at the NPC or in the nucleoplasm (35). NTF2 has been shown to bind to both RanGDP and XFG repeat-containing nucleoporins, such as yeast Nsp1p and vertebrate p62 (17, 23). Using the crystal structure of rat NTF2 in complex with RanGDP, Bayliss et al. (27) designed an NTF2 mutant, W7A, in which the affinity for XFG repeat-containing nucleoporins is reduced but which retains wild-type binding to RanGDP. It was possible that an XFG repeat-containing nucleoporin, like p62, might be the RanGDF. However, GDF activity levels measured in the presence of the W7A mutant and wild-type NTF2, either alone or in the presence of SP200 (which does not contain endogenous NTF2 but which does contain the RanGDF), were identical (Figs. 1A and 3B). In addition, we prepared recombinant p62 and assayed its effect in the filter-binding assay. p62 did not affect [3H]GDP dissociation from Ran pretreated with wild-type NTF2 (data not shown). It has also been reported (36) that the nucleoporin Nup153, which is located on the nucleoplasmic face of the NPC, is a RanGDP-binding protein. The zinc-finger region of Nup153 mediates interaction between RanGDP and Nup153 (36). We therefore prepared the zinc-finger domain of Nup153 in recombinant form. It, however, had no effect on [3H]GDP dissociation from Ran pretreated with NTF2 (data not shown). Our preliminary data therefore suggest that neither p62 nor Nup153 functions as a RanGDF.

Another candidate that interacts genetically with both Ran...
and NTF2 is MOG1. The temperature-sensitive phenotype caused by deletion of MOG1 from yeast was suppressed by over-expression of NTF2 (37). Although both Ntf2p and Mog1p are required for optimal nuclear protein import in Saccharomyces cerevisiae, over-expression of MOG1 does not rescue ntf2 mutants, indicating that the functions of the two proteins are distinct. Stewart and Baker (38) have determined the crystal structure of Mog1p to 1.9 Å resolution and have suggested that Mog1p interacts with Ran through a site similar to that bound by NTF2. We therefore investigated whether Mog1 could function as a RanGDF using recombinant mouse Mog1 protein. However, there was no detectable effect of Mog1 on the RanGDI activity of NTF2 (data not shown).

Our attempts to further purify the RanGDF have not met with success. Although further purification was sometimes achieved, this was not reproducibly the case. In addition, we failed to identify any protein bands within fractions separated by SDS-PAGE for which the presence was correlated with GDF activity. If, as our results suggest, a component of the RanGDF activity acts catalytically, as a kinase, the GDF may be present in active fractions in extremely small quantities. In any event, as summarized above, we have been unable to identify the GDF either by directed approaches or by further biochemical fractionation.

Finally, we propose some possible models for RanGDF function (Fig. 8). A RanGDF might specifically bind to NTF2 (1) or to RanGDP (2) and thereby dissociate the NTF2-RanGDP complex. In these models, RCC1 would interact with free RanGDP. There are two remaining formal possible modes of action for the activity. RanGDF might bind the NTF2-RanGDP complex and destabilize GDP binding to Ran directly (3). Alternatively, in an NTF2-dependent manner, RanGDF could interact directly with RCC1 and enhance its catalytic activity (4). Although we have not identified the ATP-dependent NTF2-releasing factor, the data presented provide the first evidence for the existence of a factor that releases RanGDP from NTF2. Our data further suggest that the function of the RanGDF is regulated by phosphorylation. Our future effort will be to continue the attempts to identify the factor and to characterize in detail its function in RanGTP-dependent processes.

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