Fluorescence Resonance Energy Transfer Biosensors That Detect Ran Conformational Changes and a Ran·GDP-Importin-β-RanBP1 Complex in Vitro and in Intact Cells*

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The Ran GTPase plays a central role in nucleocytoplasmic transport. Association of Ran-GTP with transport carriers (karyopherins) triggers the loading/unloading of export or import cargo, respectively. The C-terminal tail of Ran-GTP is deployed in an extended conformation when associated with a Ran binding domain or importins. To monitor tail orientation, a Ran-GFP fusion was labeled with the fluorophore Alexa546. Fluorescence resonance energy transfer (FRET) occurs efficiently between the green fluorescent protein (GFP) and Alexa546 for Ran-GDP and Ran-GTP, suggesting that the tail is tethered in both states. However, Ran-GTP complexes with importin-β, RanBP1, and Crm1 all show reduced FRET consistent with tail extension. Displacement of the C-terminal tail of Ran by karyopherins may be a general mechanism to facilitate RanBP1 binding. A Ran-GDP-RanBP1-importin-β complex also displayed a low FRET signal. To detect this complex in vivo, a bipartite biosensor consisting of Ran-Alexa546 plus GST-GFP-RanBP1, was co-injected into the cytoplasm of cells. The Ran redistributed predominately to the nucleus, and RanBP1 remained cytoplasmic. Nonetheless, a robust cytoplasmic FRET signal was detectable, which suggests that a significant fraction of cytoplasmic Ran-GDP may exist in a ternary complex with RanBP1 and importins.

The flux of macromolecules between the nucleus and cytoplasm of a fibroblast has been estimated to be in excess of 10 million/min (1). This huge traffic flows through about 3,000 nuclear pore complexes (NPCs) that are plugged into the double membrane that encomasses the nucleus (2–5). The Ran GTPase controls the overall vectoriality of much of this traffic. Ran-GDP is converted to the GTP-bound state by an exchange factor, RanGAP (also called RCC1), which is localized in the nucleus and binds to histones. Ran-GTP is converted back to the GDP-bound form by a GTPase-activating protein, RanGAP, which is localized to the cytoplasm and NPCs. The asymmetric distribution of these factors generates a steep Ran-GTP gradient across the nuclear pores. Soluble transport carriers (karyopherins) recognize and bind to specific signal sequences in cargo destined for translocation through the pores. The carriers can interact with nucleoporins (proteins that compose the nuclear pore complex) and move through the pores by a process of facilitated diffusion. The function of Ran-GTP in nucleocytoplasmic transport is to disassemble import complexes in the nucleus by displacing cargo from the importin carrier and to drive assembly of export complexes by co-operatively binding to the exportin carrier together with cargo. For both importins and exportins, a carrier-Ran-GTP complex is ultimately delivered to the cytoplasm, where RanGAP, in co-operation with another factor called Ran-binding protein 1 (RanBP1),1 triggers hydrolysis of the GTP and release of Ran-GDP from the carrier. The Ran-GDP then cycles back into the nucleus as a complex with another factor called NTF2, where the RanGAP catalyzes nucleotide exchange to generate Ran-GTP, which dissociates from the NTF2 to complete the cycle (2–5).

Ran-GDP has an extremely low affinity for the cargo carriers and so is not re-imported in association with them (6). Nor does Ran-GDP detectably associate with RanBP1. Nonetheless, a stable ternary complex can be formed in vitro between Ran-GDP, importin-β, and RanBP1 (6, 7). The function of this complex in nucleocytoplasmic transport, if any, remains unknown. As an added complexity, there are cofactors that participate in the transport complexes. RanBP3, for example, associates with the exportin Crm1 and increases the affinity of the carrier for both Ran-GTP and for export cargo (8, 9). Similar cofactors may exist for importins.

Our understanding of the mechanics and regulation of this Ran-dependent transport cycle requires structural knowledge of all the factors that interact with Ran and knowledge of the dynamics that govern their interactions. A number of crystal structures have been solved: (i) Ran-GDP alone and in a complex with nuclear transport factor 2 (NTF2) and (ii) Ran in the GTP-bound conformation in complexes with a Ran binding domain (RanBD) of RanBP2, importin-β, and karyopherinβ2 (10–14). These structures show that the orientation of the C-terminal tail of Ran is profoundly altered by complex formation with the RanBD or the transport carriers.

Why does the C terminus of Ran move? Although the structure of uncomplexed Ran-GTP has not been solved, experiments using antibodies against the acidic C-terminal tail suggest that the tail is tethered to the body of the protein but is released when Ran-GTP binds importin-β (15). Release of the tail is predicted to facilitate RanBP1 binding, because the tail wraps around and enforces the RanBD (16). This increased on-rate may increase the efficiency of GTP hydrolysis by Ran-GTP. However, RanBP1 and RanBP3 may be a general mechanism to facilitate this process, because RanBP1 and RanBP3 have similar conformations of the C-terminal tail, which is predicted to facilitate RanBP1 binding, because the tail wraps around and enforces the RanBD (16). This increased on-rate may increase the efficiency of GTP hydrolysis by Ran-GTP.

1 The abbreviations used are: RanBP1, Ran-binding protein 1; GFP, green fluorescent protein; RGA, Ran-GFP-Alexa546; RA, Ran-Al exa546; NES, nuclear export signal; FRET, fluorescence resonance energy transfer; RG, Ran-GFP; GST, glutathione S-transferase; BHK, baby hamster kidney cells; RanBD, Ran binding domain.

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GAP. However, there have been no direct tests of this model, and the orientation of the C-terminal tail is not known for free Ran-GTP, nor for Ran-GTP bound in a complex with exportins such as Crm1, nor for the ternary complex of Ran-GDP-importin-β-RanBP1.

As an approach to address these issues, we created a sensor for the C-terminal tail conformation of Ran, using fluorescence resonance energy transfer (FRET) (17). The sensor consists of a fusion of green fluorescent protein (GFP) to the C terminus of Ran, coupled with chemical modification to provide a FRET acceptor on Cys-112 of Ran. This sensor shows high FRET efficiency in the GDP-bound state and low FRET efficiency when bound to RanBP1 or importin-β, consistent with the known changes in orientation of the tail. We have used this sensor to demonstrate that the C terminus does not re-orient when Ran is converted to the GTP-bound state but does move away from the body of the protein in complexes of Ran-GDP with RanBP1 and importin-β, or of Ran-GTP with Crm1 and export cargo. A second, two-component, FRET sensor was created to visualize complexed Ran and RanBP1 in vivo and consists of Ran labeled with Alexa546 and RanBP1 fused to the C terminus of GFP. When injected into cells, this bipartite sensor displays a cytoplasmic FRET signal consistent with the presence of a ternary Ran-GDP-RanBP1-importin complex. These data provide the first evidence for such a complex in intact cells and suggest that current models of nuclear transport will need to be adjusted to account for its existence.

MATERIALS AND METHODS

Plasmids—The gene encoding GFP was cloned into the RanHI/BglII sites of the pQ690 plasmid (Qiagen), which adds a His6 tag at the C terminus of GFP. The Ran open reading frame was cloned into the Escherichia coli expression system, and affinity-purified to equilibrate. Microinjected cells were visualized on a Nikon Eclipse TE200 inverted microscope with a ×100 water immersion lens (NA 1.2). Images were captured with a Hamamatsu Orca CCD camera controlled by Openlab 3.0.1 software. Signals were visualized using the following Chroma filter/dichroic sets: F480–540/22×, S500/22M, 475DCPL; F45–555/28×, S617/73M, 475DCPL; and F480–540/20×, S617/73M, 475DCPL (where F480 = fluorescence emission of Alexa546 with direct excitation of the dye at its absorption peak). Direct excitation of Alexa546 fluorophores, and bleed-through fluorescence from GFP using the F480 filter set, were calculated from control injections and subtracted from the F480 images using the Image Calculator and Math functions of ImageJ software. The resulting FRET images were then colorized using the Fire LUT of ImageJ.

To calculate predicted FRET signals in the nuclear and cytoplasmic compartments of the cell, fractional concentrations for each Ran species, computed using the steady-state Virtual Cell model (1), were multiplied by the measured FGA/FGG ratios for each species from the in vitro fluorometric assays. The products, which represent the relative FRET efficiencies, were then summed for each compartment to give the overall fractional Ran concentrations and FRET signals for each compartment. These FRET signals were corrected for the different Ran concentrations in each compartment to give normalized nuclear and cytoplasmic FRET values. The ratio of these two FRET values (the theoretical N/C FRET ratio) was then calculated. If there were no differences in the relative FRET signals in the nucleus versus the cytoplasm, this quotient would equal 1.0. A value of <1.0 indicates that the fraction of Ran complexes (i.e., of Ran conformers with an extended C-terminal tail) in the cytoplasm is less than in the nucleus. An experimental N/C FRET ratio was obtained from images of the cells microinjected with RGA by dividing the N/C ratio of the FRET image by the N/C ratio of the Ran (FAA) image.

RESULTS AND DISCUSSION

To create a FRET sensor for the conformation of the C terminus of Ran, we needed to attach a fluorescent donor to the C terminus itself, and a suitable acceptor chromophore elsewhere on the protein. GFP is an excellent FRET donor for the synthetic fluorophore Alexa546 (24). The R, value (the distance at which the FRET efficiency is 50%) for this pair is calculated to be 51 Å, which is roughly twice the mean diameter of Ran-GDP. In contrast to other small GTPases, the C terminus of Ran is not post-translationally modified by prenylation. The C-terminal region is also exposed, or predicted to be exposed, in all complexes of Ran with Ran-binding proteins for which structures have been solved to date, including those with NTF2, RanBP1, importin-β, and Kap92 (2). We therefore expected that a C-terminal fusion between Ran and GFP would not disrupt any essential biological function of Ran. To attach the acceptor to this fusion protein, we used Alexa546 maleimide. There are no exposed Cys residues on the surface of GFP that would react with this maleimide, and when GFP was injected into the cytoplasm of about 50 cells. Cells were then incubated at 37 °C (33.5 or 39.5 °C for tsBN2) for 30 min to allow injected proteins to equilibrate. Microinjected cells were visualized on a Nikon Eclipse inverted microscope, and the fluorescence of the Alexa546 fluorophore, and bleed-through fluorescence from GFP using the F480 filter set, were calculated from control injections and subtracted from the F480 images using the Image Calculator and Math functions of ImageJ software. The resulting FRET images were then colorized using the Fire LUT of ImageJ.
Ran would change dramatically between these different states (Fig. 1A).

When Ran-GFP (named RG) was excited at 467 nm, it displayed a single emission peak at 508 nm (Fig. 1B). Ran labeled with Alexa546 (named RA) showed negligible emission both at 508 and 568 nm (the emission peak for the Alexa fluorophore) when excited at 467 nm but did emit strongly at 568 nm when excited directly at 556 nm. However, when RG was labeled with Alexa546 to create the sensor (named RGA), it produced a substantial FRET signal when excited at 467 nm, with emission peaks at both 508 and 568 nm (Fig. 1B). At equal molar protein concentrations, the GFP emission of RGA at 508 nm was lower than that of RG, as predicted for a FRET donor.

RGA was able to bind both 32P-GTP and 32P-GDP (data not shown), suggesting that it is functional. To test function more rigorously and to determine whether the FRET response would be sensitive to conformational changes in the Ran protein, we incubated RGA with excess importin-β or GST-RanBD (from RanBP1). These proteins are known to form complexes with Ran that deploy the C terminus in an extended conformation (11, 14). Both of these proteins caused an increase in emission of the donor GFP at 508 nm and a decrease in the emission of the acceptor at 568 nm, consistent with a drop in FRET efficiency caused by movement of the GFP away from the Alexa (Fig. 2A). To determine the affinity of the RanBD for RGA, we titrated increasing concentrations of GST-RanBD into the RGA solution and quantified the change in the acceptor/donor fluorescence ratio. This change was corrected for the stoichiometry of modification by Alexa546 and converted to fractional binding and then plotted against the calculated free GST-RanBD (Fig. 2B). The data were then fitted by an equation for binding to a single site on RGA, giving a calculated dissociation constant of ~1.5 nM. This is similar to the estimated $K_d$ for GST-RanBP1 binding to Ran as determined using plasmon resonance (25). Taken together, the data suggest that the RGA sensor is fully active and can bind a known factor with an affinity comparable with that for unmodified Ran-GTP.

To determine whether conversion of free RGA between the GDP- and GTP-bound states induces a change in FRET efficiency, RGA was incubated with RanGEF (RCC1) plus GTP or with RanGAP plus GDP. As shown in Fig. 2C, the FRET signals ($F_{GTA}/F_{GFG}$ fluorescence ratio) from RGA subjected to these two treatments were identical. This result suggests that, as had been predicted, the C-terminal acidic tail is not released by conversion of Ran to the GTP-bound state, although the C-terminal loop must move out to accommodate changes in the Switch I region (11).

When NTF2 was added to the RGA-GDP, a small but reproducible increase in FRET was observed (Fig. 2C). This effect may be because of conformational constraints on the orientation of the GFP, because there is no difference in the distances between Cys-112 and the (truncated) C terminus of free Ran-GDP and the Ran-NTF2 complex as determined from the crystal structures (10, 12). Addition of importin-β alone, the GST-RanBD alone, or of the two factors together to RGA-GTP, all caused substantial drops in FRET of increasing magnitude (Fig. 2C). These effects are consistent with the known structures of the complexes. The C terminus of Ran bound to importin-β or Kap22 is believed to be displaced from the body of Ran but is not constrained by binding to the karyopherins (13); whereas in a complex with a RanBD, the C terminus is wrapped around the RanBD and is held rigidly at a considerable distance from the body of the Ran protein (11). We can use the FRET efficiency to estimate an average distance between the donor (GFP) and the acceptor (Alexa546) fluorophores, based on the assumption that the dipole moments of GFP and Alexa are oriented randomly with respect to one another ($\kappa = 0.67$) (24). The calculated average distance between donor and acceptor fluorophores increases from ~45 to 50 Å upon binding.
Conformational Changes in Ran Detected by FRET

An exportin and its cofactor deploy the C-terminal tail of Ran-GTP. The average $F_{\text{GFP}}/F_{\text{GFA}}$ ratios of the indicated protein combinations were obtained as described in the legend to Fig. 2. Crm1 was added to a final concentration of 0.5 μM, NES peptide was 1.0 μM, and RanBP3 was 0.5 μM.

Importin-β, to 54 Å on binding the RanBD, and to 57 Å on binding both importin-β and the RanBD. In the crystal structure of Ran-GDP, the distance from Cys-112 to a residue near the C terminus (Thr-207) is 31.5 Å, and in the crystal structure of the Ran-RanBD complex this distance is increased to about 43 Å. Therefore, the magnitude of the conformational change induced by the RanBD, as determined by FRET, is similar to that determined from the known crystal structures. This concordance shows that the RGA sensor responds appropriately to changes in the position of the C-terminal tail relative to Cys-112. Note that the extreme C terminus of Ran (amino acids 208–216) of the complexes with importin-β and Kap2β was not visible in the crystal structures, presumably because its conformation is unconstrained (13, 14).

To determine whether exportins also induce release of the C terminus of Ran, we measured the FRET of RGA-GTP in the presence of Crm1 and a peptide that corresponds to the nuclear export signal of MVM NS2 protein (22). The NES peptide alone had no significant effect on the emission of the RGA. Crm1 alone caused a small decrease, consistent with the known low affinity of Crm1 for Ran-GTP in the absence of cargo. When added together, however, the Crm1/NES caused a substantial drop in the $F_{\text{GFA}}/F_{\text{GFP}}$ fluorescence ratio, suggesting that in the export-competent complex the C terminus of Ran is released (Fig. 3). The export cofactor, RanBP3, binds Ran-GTP with very low affinity (26) but can form a complex with Ran-GTP and Crm1. RanBP3 increases the affinity of the Crm1-GTP complex.

The spectrum was reanalyzed after the addition of 1 μM importin-β (open circles) and 1 μM GST-RBD (solid lines). The emission peak of the donor fluorophore, GFP ($F_{\text{GFP}}$), is 508 nm and the acceptor fluorophore, Alexa546 ($F_{\text{GFA}}$), is at 568 nm. $B$, binding curve for GST-RanBP1 binding domain (RBD) to RGA. Changes in $F_{\text{GFA}}/F_{\text{GFP}}$ emission peak ratios of 10 nM RGA-GTP were measured over a range of GST-RBD concentrations. These values were converted to fractional binding, assuming a molar 1:1 stoichiometry of the RGA-GST-RBD complex. Free GST-RBD was calculated (total – bound). A hyperbolic curve was fitted to the data using Kaleidograph curve fitting software, assuming a single binding site. The $K_d$ was calculated to be 1.46 nM. $C$, emission spectra of RGA-GDP, RGA-GTP, RGA-GDP+NTF2 (1.0 μM), RGA-GTP+importin-β (1.0 μM), RGA-GTP+GST-RBD (1.0 μM), and RGA-GTP+importin-β+GST-RBD (1.0 μM each) were measured independently at least three times. RGA was at 0.05 μM. Shown are the average ± S.D. for the observed acceptor/donor emission ratios, $F_{\text{GFA}}/F_{\text{GFP}}$. 

![Fig. 2. Conformational changes in the RGA sensor detected by FRET.](image-url)
for export cargo (8, 9). As shown in Fig. 3, RanBP3 causes a greater drop in FRET efficiency, in the presence of Crm1, than does the NES. These data suggest that RanBP3 may behave in this complex like a classical RanBD, by embracing the deployed C terminus of Ran and holding it in a rigid, extended conformation.

Taken together, these data support the general concept that all karyopherins, whether importins or exportins, release the C-terminal tail of Ran from its “docked” position on the body of the Ran protein and that RanBDs stabilize this extended conformation. One purpose for the release of the C terminus by karyopherins may be to increase the on-rate for RanBD binding, as has been proposed by Villa Bralslavsky et al. (16). The RanBD functions as a co-activator of RanGAP, so this increased on-rate would be expected to increase the efficiency of GTP hydrolysis by RanGAP, and hence increase the efficiency with which the transport cycle is terminated in the cytoplasm. Whether there are other functions for the C terminus (e.g., in translocation through the nuclear pore complex) remains to be established.

We also asked whether it is possible to use this RGA sensor to directly demonstrate the existence of a Ran-GTP gradient across the nuclear envelope of a living cell. The levels of each of the major forms of Ran in the cytoplasm and nucleus have been computed using a Virtual Cell model of nucleocytoplasmic transport, and the calculated values match well with those that have been accessible to experimental determination (1). Because from the present study we know the relative $F_{CG}^{FA}/F_{CG}^{FGG}$ fluorescence ratios for the major forms of RGA likely to be present in the cell, we can sum the contribution of each individual species to the overall $F_{CG}^{FA}/F_{CG}^{FGG}$ ratio in the cytoplasmic and nuclear compartments (e.g., nuclear FRET = $\sum$(fractional distribution of species $i$) $\times i F_{CG}^{FA}/F_{CG}^{FGG}$) (Fig. 4A). This computation gives a theoretical N/C FRET ratio of $0.64$. This ratio is $<1$. Correction for the differing concentrations of Ran in the two compartments provides a FRET value that reflects the proportion of Ran conformers in the “extended-tail” state. This FRET difference between the nucleus and cytoplasm is in principle detectable by fluorescence microscopy, using the appropriate filter sets. We therefore microinjected RGA into the cytoplasm of BHK21 cells, as described under “Materials and Methods.” We found that the FRET signal for RGA was predominately nucleoplasmic and, unexpectedly, the N/C ratio for the FRET signal was generally only $4\%$ lower than the N/C ratio for the Ran ($F_{CG}^{FA}$) distribution itself (experimental N/C FRET ratio = $0.96$) (Fig. 4, A and B). This result deviated substantially from our theoretical N/C FRET ratio and suggested that either the nuclear Ran-GTP is largely free rather than complexed to karyopherins or that cytoplasmic Ran exists in a complex that releases the C terminus. One known cytoplasmic Ran complex is Ran-GDP-NTF2, but NTF2 does not cause release of the C terminus. However, another potential complex with this property is Ran-GDP-importin-$\beta$-RanBP1. This interesting complex has been characterized in vitro, but its potential function in nuclear import is not understood, and it has not been shown to exist in vivo (7, 27). Therefore, we asked if the C terminus of Ran is displaced in the ternary nuclear and cytoplasmic FRET and $F_{CG}^{FA}$ images of $\sim25$ cells microinjected with RGA. Representative images are shown in panel B. B, RGA sensor was injected into the cytoplasm of BHK21 cells at a concentration of 25 $\mu$m. After 30 min, images were captured with the three filter sets ($F_{CG}^{FA}$, $F_{CG}^{FA}$, $F_{CG}^{FGG}$) and direct excitation and bleed-through fluorescence were subtracted (FRET), C, mean $F_{CG}^{FA}/F_{CG}^{FGG}$ ratios for Ran-GDP, Ran-GDP+importin-$\beta$, Ran-GDP+GST-RBD, and Ran-GDP+importin-$\beta$+GST-RBD were obtained as described in the legend to Fig 2. RGA was 0.05 $\mu$m, and other proteins were present at 1.0 $\mu$m.
complex of RGA-GDP with importin-β and a RanBD. Ran-GDP
does not form a complex with either of these factors alone, so it
is not clear what conformation it would adopt when bound to
both of them simultaneously. As shown in Fig. 4C, importin-β
alone had no effect on FRET efficiency when the RGA was in
the GDP-bound state, and the RanBD caused only a very small
decrease (likely because of its high affinity for any residual
RGA-GTP); however, addition of both factors caused a drop of
almost 50% in the F^AA/F^GG fluorescence ratio, corresponding
to a 7 Å shift in the C-terminal tail away from Cys-112. Therefore,
the conformational change in Ran-GDP induced by formation of
this ternary complex is similar to that induced in Ran-GTP
binding to either importin-β or a RanBD and could account for
the reduced FRET in the cytoplasm of microinjected cells.

To test whether Ran-GDP forms a complex with importin-β
and RanBP1 in vitro, we developed a different, bipartite Ran-
FRET sensor. RA was mixed with GST-GFP-RanBP1 (named
G-BP1). Full-length RanBP1, as opposed to RanBD, was used
in this cell-based assay to ensure its proper localization.
RanBD, which lacks an essential nuclear export signal, redistributes
to the nucleus upon microinjection in the cytoplasm
and has been found to be toxic to cells (28). When tested in
vitro, this RA/G-BP1 sensor was functional. In the GTP-bound
state it caused an increased F^AA/F^GG fluorescence ratio when mixed with G-BP1 (Fig. 5) but, in the GDP-bound form, it only
caus ed an increase in F^AA/F^GG ratio when both G-BP1 and
importin-β were added together (Fig. 5).

After establishing that this bipartite sensor was functional
in vitro, we co-injected an equimolar mix of RA and G-BP1 into
the cytoplasm of BHK21 cells and allowed the proteins to
equilibrate. As expected, the RA redistributed primarily to
the nucleus and the G-BP1 remained cytoplasmic (Fig. 6, panels a
and c). RA alone and G-BP1 alone were similarly microinjected
to establish the contributions of direct excitation (Fig. 6, panel f)
and bleed-through fluorescence to the observed signals (Fig.
6, panel f). Images were taken with the three filter sets, and
background subtractions were made as described under “Materials and
Methods.” The arrows in panels a–d indicate a nuclear injection.

FIG. 5. The RA/G-BP1 sensor is functional in vitro. Emission
spectrum of 100 nM G-BP1 (solid line) was obtained using an excitation
wavelength of 467 nm. The spectrum was rescanned after the addition
of 0.1 μM RA-GTP (dashes), RA-GDP (open circles), or RA-
GDP + importin-β (filled diamonds).

FIG. 6. The bipartite sensor yields a cytoplasmic FRET signal.
The cytoplasm of BHK21 cells was microinjected with RA + G-BP1 (panels a–d), RA (panels e–h), G-BP1 (panels i–l), RA + GST-GFP (panels m–p), and RA + G-BP1 + RanGAP (panels q–t), and incubated 30 min at
37 °C. Images were captured on live cells using the three filter sets,
and background subtractions were made as described under “Materials and
Methods.” The arrows in panels a–d indicate a nuclear injection.

FIG. 7. Decreased RanGEF causes an increase in the cytoplasmic
RAG/BP1 FRET signal. A, tsBN2 cells were grown at 33.5 °C.
One dish of cells was treated with cycloheximide and shifted to the
nonpermissive temperature (39.5 °C). Both sets of cells were co-injected
with RA + G-BP1 and incubated at the indicated temperature for 30
min. Images were captured as described under “Materials and
Methods.” B, cytoplasmic ratio of the FRET to F^AA signals was calculated
from >15 cells for each condition.

Contrary to the result expected if it were caused by
unhydrolyzed cytoplasmic Ran-GTP in a complex with RanBP1
and importin-β. As a further test for the existence of a cyto-
plasmic Ran-GDP-importin-RanBP1 complex, we used tsBN2
cells that contain a thermolabile allele of the RanGEF (23, 29).
Loss of RanGEF at the nonpermissive temperature for tsBN2
cells would be predicted to reduce the Ran-GTP concentration
and increase cytoplasmic Ran-GDP (1, 30). Therefore, if the
FRET signal were generated by a RA-GTP complex, it should
decrease, but if it were generated by a RA-GDP complex, it
should increase. When we compared the FRET and Ran (FAA) signals of microinjected tsBN2 cells that were grown at 33.5 °C, we found that the shifted cells had a ~68% higher cytoplasmic FRET signal than cells grown at the permissive temperature (Fig. 7, A and B). This result argues strongly that the RA/G-BP1 biosensor is detecting a ternary cytoplasmic complex similar to that created by Ran-GDP-importin-β-RanBP1.

Taken together, these results suggest that the release of the acidic C terminus of Ran is diagnostic of a complex with a karyopherin and/or RanBD. They also indicate the existence of a cytoplasmic ternary complex of Ran-GDP with karyopherins and RanBP1. This complex has not been taken into account in current models of nucleocytoplasmic transport, even though it has been characterized extensively in vitro, and may be general to members of the importin family (31). The biological function of such a complex remains to be determined. It would presumably reduce the concentration of free Ran-GDP in the cytoplasm and hence reduce the rate of formation of a Ran-GDP-N/C ratio. This difference implies that there are features of the model, or components, that need to be adjusted or added. One potential adjustment is that the Ran-GDP-importin-RanBP1 complex should be able to traverse the nuclear pore complex and may participate in cargo import. The careful analysis of more sophisticated transport models will be necessary to resolve this issue.

A conformational change in Ran is strongly suggested by the shift in fluorescence of the FAA probe with temperature (Fig. 7, A and B). The careful analysis of probes that detect different Ran biosensors in which cyan and yellow versions of importin-α (YIC) or Yrb1p (YRC) are used would be necessary to resolve this issue. While this work was in progress, Kalab et al. (32) developed different Ran biosensors in which cyan and yellow versions of GFP were attached to either the RanBD of the yeast RanBP1 homolog, Yrb1p (YRC) or to the N-terminal fragment of importin-α (YIC). These probes successfully detected a Ran-GTP gradient in frog egg extracts to which sperm chromatin had been added and a high nuclear/cytoplasmic Ran-GTP gradient. We surmise that the YRC probe cannot form a ternary complex with Ran-GDP and importins, perhaps because of steric hindrance caused by a C-terminal CFP fusion, or that some other factor present in frog extracts prevents the formation of this complex with YRC, which would otherwise suffer from the same disadvantage as the RGA sensor described here. Together, these studies demonstrate that several types of FRET biosensors for Ran can be created that address different aspects of the functions of this GTPase.

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