Mutations within the Ran/TC4 GTPase
EFFECTS ON REGULATORY FACTOR INTERACTIONS AND SUBCELLULAR LOCALIZATION*

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Ran, a member of the Ras superfamily of GTPases, is predominantly localized in the nucleus and is a necessary component in the active transport of proteins through nuclear pores. Disruption of Ran function affects the regulation of mitosis, DNA synthesis, and RNA processing and export. To explore the mechanisms of Ran function, mutants of the Ran GTPase were characterized, several of which are capable of dominating interfering with nuclear protein import. Unlike wild-type Ran, the putative gain-of-function mutant (G19V Ran) was not sensitive to the exchange factor, RCC1. In addition the G19V Ran and effector domain mutants (L43E and E46G Ran) were not sensitive to the GTPase-activating protein, Fug1. Epitope-tagged G19V Ran and L43E Ran isolated from transfected BHK21 cells were each about 50% GTP-bound, whereas the wild-type and a C-terminal deletion mutant (Δ-DE Ran) were primarily cytosolic. Similar results were observed when permeabilized BHK21 cells were incubated with extracts of COS cells expressing the mutants. Thus mutants of these and other similar proteins has revealed a 135-amino acid residue Ran-binding domain (RanBD), which is necessary and sufficient for binding to Ran (13). In addition, Ran can associate, through a different type of domain, with the β-subunit of the nuclear cargo receptor, β-karyopherin/β-importin/p97 (14). The interaction of Ran with β-karyopherin is not inhibited by the RanBD, suggesting that Ran may interact with a complex of nuclear transport proteins including a RanBP and β-karyopherin (15–17).

The Ran GTPase has been shown to be necessary for the in vitro nuclear import and export of synthetic karyophiles possessing nuclear localization signals (NLSs) (for review, see Refs. 14 and 18). Recently, our laboratory demonstrated that several mutants of Ran can dominantly interfere with the dexamethasone-stimulated nuclear import of glucocorticoid receptors expressed in BHK21 cells (19). A putative gain-of-function mutant (G19V Ran), a loss-of-function mutant (T24N Ran), and a C-terminal deletion mutant (Δ-DE Ran) all inhibited protein nuclear import. However, an effector domain mutant, L43E Ran, had no effect on import, yet was markedly toxic to cell growth. Ran, therefore, possesses an essential function, dominantly inhibited by the L43E Ran mutant, that is independent of nuclear protein import. To understand the molecular mechanisms underlying these cell biological effects, it was essential to characterize the Ran mutants biochemically. The present study describes the subcellular distribution of the mutants and their interactions with known regulatory factors.

EXPERIMENTAL PROCEDURES

Creation of Mutations within Ran—Mutations within selected codons were achieved by the polymerase chain reaction using pUC19-TC4 DNA as the template (a gift from P. D'Eustachio, New York University, New York, NY) and mismatched primers for amplification. Products were

1 The abbreviations used are: RCC1, regulator of chromosome condensation 1; RanGAP, Ran GTPase-activating protein; RanBP, Ran-binding protein; RanBD, Ran-binding domain; Δ-DE Ran, C-terminally deleted Ran; BSA, bovine serum albumin; HSA, human serum albumin; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-[N-morpholino]propanesulfonic acid, NLS, nuclear localization signal; TBS-T, Tris-buffered saline plus 0.1% Tween 20; GAP, GTPase-activating protein.

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subcloned into the bacterial expression vector, pGEX2T, and the mammalian expression vector, pCH3. The C-terminal deletion mutant, Δ-DE Ran, was created as described previously (9).

To generate the GST-Ran fusion proteins, DH5α Escherichia coli transformed with pGEX2T-Ran were grown to A600 = 1, and then induced with 1 mM isopropyl-1-thio-galactopyranoside for 16 h at 23°C. GST fusion proteins were purified from cell lysates using a glutathione-Sepharose matrix (Pharmacia Biotech Inc.) and concentrated to 1 mg/ml using a Centricron-10 concentrator (Amicon). Protein concentrations were determined by a Bradford protein assay. Proteindegradation during preparation was monitored by SDS-polyacrylamide gel electrophoresis and Coomassie staining.

GAP Assays—The plasmid encoding the murine homolog of human RanGAP1, Fig1, was supplied by J. DeGregori (NCI, National Institutes of Health) (6). GST-Fug1 was expressed and purified as described above. GST hydrolysis stimulated by GST-Fug1 was measured as described previously (21). Briefly, 0.5 µg of GST-Ran was loaded with [α-32P]GDP at a final concentration of 21.2 pmol/µl (9) and 10-fold diluted with GAP buffer (40 mM Tris-HCl, pH 8.0, 8 mM MgCl2, 80 µM BSA, and 2 mM GTP) in a final volume of 150 µl at 23°C. RanGAP activity was supplied by the addition of GST-Fug1 at a final concentration of 21.2 pmol/µl, 21.2 pmol/µl GST was used as a negative control. Fractions (25 µl) were removed at specified times and filtered as described above. Nucleotides were released from the filters and separated by thin layer chromatography (22). Quantitation was performed using a Bio-Rad GS-250 PhosphorImager.

Nucleotide Release Assays—Release of GDP from Ran was measured as described previously (21). Briefly, 2 µg of Ran (thrombin cleaved to remove GST) was loaded with [α-32P]GDP (3000 Ci/nmol, DuPont NEN) then diluted 10-fold in buffer containing 50 mM MOPS, pH 7.1, 2 mM GDP, 1 mM dithiothreitol, 10 mM MgCl2, and 0.1 mg/ml BSA in a final volume of 50 µl. RCC1 (gift from T. Nishimoto, Kyushu University, Fukuoka, Japan) was expressed in DH5α E. coli and added to the assay as a crude cytosolic fraction (1:10 dilution). Bacterial lysate not containing RCC1 was added at 20% of the final volume. The condition “No ATP” included the removal of ATP and the addition of GAP buffer (40 mM Tris-HCl, pH 8.0, 8 mM MgCl2, 80 µM BSA, and 2 mM GTP) as described previously (9), except that the free nucleotide was removed prior to use by applying the sample to a Centrisep G-25 spin column (Princeton Separations).

Binding of β-karyopherin/β-importin and RanBP1 by Ran mutants was measured by incubating 1 µg of GST-Ran expression vector with the anti-HA1 antibody, 12CA5, and added to the assay as a crude cytosolic fraction (1:10 dilution). Bacterial lysate not containing RCC1 was added at 20% of the final volume. The condition “No ATP” included the removal of ATP and the addition of GAP buffer (40 mM Tris-HCl, pH 8.0, 8 mM MgCl2, 80 µM BSA, and 2 mM GTP) as described previously (9), except that the free nucleotide was removed prior to use by applying the sample to a Centrisep G-25 spin column (Princeton Separations).

RESULTS

Effects of Mutations within Ran on Interactions with RCC1 and RanGAP—Ran mutations were examined that can dominantly interfere with nuclear transport and/or cell growth in an in vivo assay (19). The G19V and T24N mutations of Ran were based on analogous residues that affect either the guanylate nucleotide state or interactions with regulatory proteins (25). These mutants of Ran exhibit dominant effects on cell cycle progression and nuclear functions (26–28). The L43ERan and E469G Ran “effector domain” mutants are analogous to mutants at residue Val-55 and Asp-58 of Rab3A. Mutation at Val-55 of Rab3A disrupts interaction with all regulatory proteins and downstream effectors, whereas the Asp-58 mutant retains sensitivity to Rab3-GRF and a downstream effector, Rabphilin (22, 29, 30).

To determine the ability of these Ran mutants to interact with RanGAP, Ran proteins loaded with [α-32P]GTP were in-
The presence of Mg$^{2+}$ release stimulated by RCC1 (Fig. 2) and also was insensitive to GDP. G19V Ran mutant exhibited minimal GTPase activity even in the presence of Fug1 (Fig. 1) and also was insensitive to GDP. As shown in Fig. 2, potently inhibits RCC1-catalyzed nucleotide exchange on Ran interaction (31), and, in fluorescence measurements, T24N Ran (data not shown) was not detectable by overlay or by precipitation from solution using GSH-Sepharose (Fig. 3, A and C).

**GTP-GDP Ratios of Cells Expressing Ran and Mutants of Ran**—Given the dramatic effects of several of the Ran mutants on cell growth and nuclear protein import (19), it was important to determine whether the mutants are able to bind and hydrolyze nucleotide when expressed in intact cells. BHK21 cells therefore transfected with pCH3-Ran plasmids to express HA1-tagged proteins. Transfected cells were incubated with $^{[32P]}$orthophosphate then harvested and immunoprecipitated with the anti-HA1 antibody, 12CA5. Labeled nucleotides associated with the HA1 Ran proteins were then separated by thin layer chromatography. The extracted wild-type HA1-Ran was 99% GDP-bound, whereas significant proportions of the HA1-G19V Ran and HA1-L43E Ran mutants (57% and 49%, respectively) were in the GDP-bound state (Fig. 4).

**Effect of Ran Mutations on Interaction with RanBPs**—To determine if the mutations in Ran described above are important for the interaction of Ran with RanBPs, GST-Ran and GST-Ran mutants were loaded with $[^32P]$GTP and $0.5 \mu g$ of radiolabeled GST-Ran was incubated in a final volume of 150 $\mu l$. At the indicated times, samples were filter-bound and washed. Nucleotides were released from the filter by incubation in buffer containing 1% SDS and 25 mM EDTA. Separation of GTP and GDP was accomplished by thin layer chromatography, and values were quantitated directly using a Bio-Rad phosphoimaging system.

**GTP-GDP Ratios of Cells Expressing Ran and Mutants of Ran**—Given the dramatic effects of several of the Ran mutants on cell growth and nuclear protein import (19), it was important to determine whether the mutants are able to bind and hydrolyze nucleotide when expressed in intact cells. BHK21 cells were therefore transfected with pCH3-Ran plasmids to express HA1-tagged proteins. Transfected cells were incubated with $[^32P]$orthophosphate then harvested and immunoprecipitated with the anti-HA1 antibody, 12CA5. Labeled nucleotides associated with the HA1 Ran proteins were then separated by thin layer chromatography. The extracted wild-type HA1-Ran was 99% GDP-bound, whereas significant proportions of the HA1-G19V Ran and HA1-L43E Ran mutants (57% and 49%, respectively) were in the GDP-bound state (Fig. 4).

One cannot conclude from these experiments that wild-type Ran is predominantly GDP-bound within the intact cells, because significant hydrolysis of Ran-GTP occurs during the immunoprecipitation. For example, when GST-Ran loaded with $[^32P]$GTP was incubated with BHK21 cell extract under conditions and for a time equal to that required for immunoprecipitation, 90% of the GTP bound to the Ran was converted to GDP. However, the results do indicate that both HA1-G19V Ran and HA1-L43E Ran are predominantly associated with GTP in intact cells. This conclusion is supported by our previous observation that transiently expressed HA1-G19V Ran co-immunoprecipitates as a stable complex with endogenous RanBP1 (13).

**Localization of Mutant HA1-Ran Proteins by Immunofluorescence**—To determine the effect of mutations within Ran on the subcellular localization of the protein, HA1-tagged Ran pro-
peptides were transiently expressed in BHK21 cells and detected with the anti-HA1 antibody, 12CA5 (Fig. 5). Wild-type HA1-Ran was primarily nuclear, a location identical to that of endogenous Ran (32). This result indicates that the HA1 tag does not interfere with expression or targeting of the protein. In other studies (19), expression of wild-type HA1-Ran was not toxic and did not interfere with nuclear protein import.

HA1-G19V Ran, HA1-E46G Ran (data not shown), and HA1-L43E Ran proteins were all localized predominantly at the nuclear envelope, suggesting that mutants locked in the GDP-bound state bind tightly to proteins at the nuclear pore. The HA1-T24N Ran mutant was predominantly nuclear, but was also concentrated at the nuclear envelope. The C-terminal deletion, HA1-Δ-DE Ran, exhibited a diffusely cytoplasmic distribution in most cells.

**ATP-dependent in Vitro Import of HA1-Ran into the Nucleus—**Using an in vitro nuclear import assay, Moroiianu and Blobel (33) have shown that a synthetic NLS-containing substrate and recombinant Ran can accumulate in the nucleus of permeabilized cells in an ATP-dependent and wheat germ agglutinin-sensitive manner. We used a similar assay to examine the import of COS cells expressing HA1-Ran stimulated nuclear import of both the nuclear import substrate, NLS-HSA, and HA1-Ran into the nucleus of digitonin-permeabilized BHK21 cells (Fig. 6). Import occurred within 15 min at 30 °C and was dependent on the presence of ATP. The HA1-Ran used in these studies was likely to be primarily in the GDP-bound state (Fig. 4). Import of both HA1-Ran and NLS-HSA was inhibited both by wheat germ agglutinin and by chilling at 0 °C (data not shown), indicating a facilitated active transport mechanism. HA1-Ran formed a ring at the nuclear envelope and was diffuse within the nucleus. Import of the NLS-HSA did not require the presence of HA1-Ran in the extract, indicating that the amount of endogenous Ran in the COS cell digitonin extracts was sufficient to support nuclear import. In other experiments, we observed that Ran import was not ATP-dependent or wheat germ agglutinin-sensitive in assays where a GST fusion of Ran was employed, although GST-Ran import was inhibited by incubating at 4 °C (data not shown). GST fusion proteins of Ran mutants may not, therefore, be appropriate for studies of Ran function in nuclear import.

**Effects of Ran Mutations on Their in Vitro Nuclear Accumulation—**HA1-tagged Ran mutants, isolated from transfected COS cells, were tested for their ability to enter the nucleus using the in vitro nuclear import assay. As shown in Fig. 7A, the HA1-G19V and HA1-T24N Ran were able to accumulate in the nucleus to a similar extent as wild-type Ran but with a more pronounced ring at the nuclear envelope. HA1-L43E Ran and HA1-E46G Ran (data not shown) bound to the nuclear envelope with little nuclear accumulation. HA1-Δ-DE Ran, bound only weakly to the nuclear envelope and did not enter the nucleus. The diminished HA1-Δ-DE Ran signal did not result from low protein expression as is shown by an immunoblot of the COS extracts used in the import assays (Fig. 7B).

In other studies, when GST fusion proteins were tested in these assays, all of the mutants entered the nucleus even in the absence of ATP. Additionally, the transient expression of Ran as a fusion protein with the *Aquoria* green fluorescent protein (which at 27 kDa is of similar size to GST) in BHK21 cells also led to nuclear accumulation irrespective of the mutant form of Ran used (data not shown). These observations suggest that the presence of a large tag on Ran may interfere with the normal regulation of Ran import.

**DISCUSSION**

These results demonstrate that the interaction of Ran with effectors and regulatory proteins is sensitive to mutations in distinct regions of the GTPase structure. They demonstrate also that the subcellular distribution of Ran is determined by the nucleotide state of the protein and by its ability to bind specific RanBPs. Mutants that cannot cycle between the GDP-
and GTP-bound states accumulate at the nuclear envelope and support a model for Ran function in which nuclear protein import and shuttling between the nuclear and cytoplasmic compartments is coupled to the GTP-GDP cycle. This model is reminiscent of that for the shuttling of Rab proteins such as Sec4p between the cytosol and vesicle membranes (34).

The biochemical characterization of Ran mutants reveals differences between the interactions of Ran with regulatory factors versus those of other GTPases such as Ras or Rab3A. Unlike G12V Ras, which remains sensitive to Ras exchange factors (35), G19VRan was insensitive to RCC1-catalyzed guanine nucleotide exchange. Nonetheless, G19V Ran isolated from transfected cells possessed a much higher GTP:GDP ratio than did wild-type Ran, suggesting that G19V Ran is an activating mutation. This result is important in light of the fact that G19V Ran is a dominant interfering mutant of nuclear protein import (19). The ability of G19V Ran to accumulate in the GTP-bound state despite insensitivity to RCC1 may be a consequence of the high GTP:GDP ratio in the cell, and of the low endogenous GTPase activity of the G19V mutant.

The analysis of the dominant-negative mutation of Ran (T24N) supports and confirms results from other laboratories (28). Like T17N Ras, T24N Ran binds nucleotide poorly and likely for this reason is unable to interact stably with either class of RanBPs.

The effector regions of Ran are not yet defined. Effector domain mutants of other small GTPases, such as Ras and Rab3A, have been valuable in distinguishing the contributions of multiple regulators and downstream effectors to their cellular functions. Given the overall similarity between the structures of Ras and Ran (36, 37), the amino acid residues of Ran corresponding to the Switch 1 domain of Ras are likely to be involved in effector interactions. Mutation of Ran at residue 43 (L43E Ran) is analogous to Ile-36 effector domain mutants of Ras (25) and the V55E mutants in Rab3A, while the E46G mutation is analogous to a D58V mutation in the Rab3A effector domain, described previously (29). The Ran L43E Ran mutant was insensitive to GTP hydrolysis stimulated by recombinant Fug1, the murine homolog of RanGAP (Fig. 1) (6). Unlike the Rab3A V55E, however, L43E Ran was sensitive to nucleotide exchange stimulated by RCC1, and a significant fraction of this mutant was GTP-bound when isolated from intact cells.
The biochemical properties of both L43E Ran and E46G Ran were similar to the D58V mutation in Rab3A, suggesting that residues necessary for interactions at the Ran effector domain differ slightly in function from those at the Rab3A effector domain. The L43E Ran mutation also disrupted binding to RanBPs and β-karyopherin. This defect may account for the inability of L43E Ran to dominantly interfere with nuclear protein import despite the fact that it is trapped in the GTP-bound state within the cell (19).

Together, these results suggest the existence of multiple effector domains on Ran, a situation analogous to that of the Rac/Rho GTPase family (38). Association of Ran with RanBDs appears to be mediated, at least in part, through a region adjacent to and probably including β2 effector region of Ran. The region on Ran that interacts with β-karyopherin must, however, be distinct from the RanBD binding site; this region is exposed either by removal of the C terminus of Ran or by association of GTP-Ran with a RanBD (15). The surface of Ran that interacts with the RanBD must also be distinct from that for RCC1 binding, because these two factors can also form a ternary complex with Ran (21, 27, 39), and the Leu-43 muta-
ation that interferes with RanBD binding does not reduce the
sensitivity of Ran to RCC1-catalyzed exchange. Finally, Ran-
BDs must also be capable of forming ternary complexes with
Ran and RanGAP because RanBDs act as co-stimulators of
GTP hydrolysis on Ran in the presence of RanGAP (13, 40, 41).

The subcellular locations of the Ran mutants discussed in
this report differ in significant aspects from those described by
others. For example, Ren et al. (26) found that a G19V/Q69L
mutant of Ran exhibited a predominantly nuclear localization
when overexpressed in 293 Tag cells, and in budding yeast a
GST fusion of G21V GSP1p (the yeast homolog of Ran; Ref. 1)
was also nuclear (42). Our results indicate that G19V Ran
associates with the nuclear envelope when expressed as an
HA1-tagged protein in BHK21 cells; however, some nuclear
accumulation is evident for G19V in an in vitro assay. The
cause for the nuclear pore localization in transfected cells may
thus be due to the inability of G19V Ran to exit the nucleus.
G19V Ran is predominantly GTP-bound in cells, so it is likely
that the binding at the nuclear envelope is due to interaction
with the RanBDs of RanBP2, a component of the nuclear pore
complex (11, 12). Our observations are supported by in vitro
analysis of the interaction of Ran bound to a non-hydrolyzable
analog of GTP with a nuclear pore complex (43).

Wild-type HA1-Ran is nuclear, as is endogenous Ran (3),
demonstrating that the abnormal localization of the G19V mu-
tant is not mediated by the epitope tag. The level of heterolo-
guously expressed HA1-Ran proteins is only 1.7-fold higher than
the level of endogenous Ran in transfected BHK21 cells (19).
Therefore the cells are not burdened by massive overexpression
(>10-fold over endogenous levels) as occurs in 293 Tag cells
when expression is driven from autonomously replicating plas-
mids (26). For these reasons, it is likely that the observed
subcellular distributions of the HA1-tagged Ran mutants ident-
ify physiologically meaningful states of the Ran nuclear trans-
port cycle. On the other hand, the attachment of large epitopes
such as GST or green fluorescent protein to Ran results in
ATP-independent nuclear accumulation of Ran in vitro and in
the nuclear accumulation of Ran in transfected cells, irrespec-
tive of the type of Ran mutant employed. These observations
suggest that such large fusion proteins are inappropriate for
studies of the subcellular localization of Ran.

The localization of the HA1-tagged L43E and E46G Ran
mutants were similar to that of G19V Ran. Although a large
fraction of L43E Ran in the intact cell is likely to be GTP-
bound, we could not detect a stable interaction of this mutant
with RanBP1 in the overlay assay. It remains unclear at pres-
ent, therefore, whether L43E or E46G Ran associates with
RanBP2 in the nuclear pore, or becomes trapped in the pore by
interaction with other components of the transport machinery.
Because little L43E or E46G Ran enters the nucleus during in
vitro import, it is possible that, unlike G19V, L43E Ran cannot
enter the nucleus. The location of L43E Ran in the nuclear pore
was unexpected as it shows no dominant interference in
cellular protein import (19). Possibly weak interaction with
RanBP2 allows association of L43E Ran with the nuclear pore
yet reduces its inhibitory potency.

Contrary to expectations, the T24N Ran mutant was also
distributed partially at the nuclear membrane, although with
significant accumulation in the nucleoplasm. Because T24N
Ran does not interact in vitro with RanBP2, which is located at
the nuclear pore, other components of the nuclear pore may
exist that can interact with Ran in the GDP-bound state. One
candidate protein for the T24N Ran-binding protein is p10-
NTP2 (43, 44). The recent demonstration that Ran in the GDP-
bound state can form a complex with p10, NLS receptor, and
nucleoporin is consistent with this idea (17). T24N Ran can
potently inhibit nuclear protein import in vivo (19), an effect
that may be a consequence of the interaction of T24N Ran with
this complex.

Deletion of the C terminus of Ran resulted in a predomi-
antly cytosolic distribution of the protein. Ren et al. (26) have
reported previously that Δ-DE Ran, when expressed in 293 Tag
cells, is nuclear. We believe that this difference, as with the
G19V Ran results, may be a consequence of the very high levels
of heterologous protein expression in the 293 Tag cells. The
localization of the Δ-DE Ran is consistent with our recent discovery
that this mutant interacts efficiently with the cytoplasmic pro-
tein β-karyopherin/importin, a component of the NLS recep-
tor (15). Such an interaction may account for the dominant
inhibition of nuclear protein import by Δ-DE Ran in intact cells
(19).

Taken together, these results argue that Ran can interact
with nuclear pore complexes that are in different states, and
which may represent different steps in the nuclear transport
mechanism. This hypothesis is consistent with the model de-
scribed recently by Nehrbass and Blobel (17), in which tran-
sient complexes assemble and disassemble within the nuclear
pore in a process coupled to the Ran GTP/GDP cycle. Ran-GDP
forms a complex with p10, α- and β-karyopherin/importin,
nuclear cargo, and nucleoporins. We predict that the T24N Ran
mutant may stabilize a Ran-GTP-β-karyopherin-
RanBP2 complex and block further cycling. The possibility
that the different Ran mutants described above can trap the
nuclear pore complex machinery at different steps in the trans-
port process suggests that they may provide useful tools to test
the validity of models for Ran-mediated functions in intact cells.

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