Characterization of a Novel Activated Ran GTPase Mutant and Its Ability to Induce Cellular Transformation

Shawn K. Milano, Woojin Kwon, Ryan Pereira, Marc A. Antonyak, and Richard A. Cerione

From the Department of Molecular Medicine, and the Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853

Background: Ran is overexpressed in human cancers.

Results: Here, we describe a novel activating mutant of Ran and how it up-regulates the expression of the matricellular protein SMOC-2 and induces oncogenic transformation.

Conclusion: These findings identify a novel mechanism by which Ran transforms cells.

Significance: The results of this study highlight the potential role played by Ran in human cancer.

Ran (Ras-related nuclear) protein, a member of the Ras superfamily of GTPases, is best known for its roles in nucleocytoplasmic transport, mitotic spindle fiber assembly, and nuclear envelope formation. Recently, we have shown that the overexpression of Ran in fibroblasts induces cellular transformation and tumor formation in mice (Ly, T. K., Wang, J., Pereira, R., Rojas, K. S., Peng, X., Feng, Q., Cerione, R. A., and Wilson, K. F. (2010) J. Biol. Chem. 285, 5815–5826). Here, we describe a novel activated Ran mutant, Ran(K152A), which is capable of an increased rate of GDP-GTP exchange and an accelerated GTP binding/GTP hydrolytic cycle compared with wild-type Ran. We show that its expression in NIH-3T3 fibroblasts induces anchorage-independent growth and stimulates cell invasion, as well as activates signaling pathways that lead to extracellular regulated kinase (ERK) activity. Furthermore, Ran(K152A) expression in the human mammary SKBR3 adenocarcinoma cell line gives rise to an enhanced transformed phenotype and causes a robust stimulation of both ERK and the N-terminal c-Jun kinase (JNK). Microarray analysis reveals that the expression of the gene encoding SMOC-2 (secreted modular calcium-binding protein-2), which has been shown to synergize with different growth factors, is increased by at least 50-fold in cells stably expressing Ran(K152A) compared with cells expressing control vector. Knocking down SMOC-2 expression greatly reduces the ability of Ran(K152A) to stimulate anchorage-independent growth in NIH-3T3 cells and in SKBR3 cells and also inhibits cell invasion in fibroblasts. Collectively, our findings highlight a novel connection between the hyper-activation of the small GTPase Ran and the matricellular protein SMOC-2 that has important consequences for oncogenic transformation.

Ran is a monomeric small GTPase belonging to the Ras-related superfamily of GTPases. Its GTP binding/GTP hydrolytic cycle is regulated by the guanine nucleotide exchange factor (GEF), RCC1 (regulator of chromosome condensation), and the GTPase-accelerating protein, Ran GAP (1–3). Ran traditionally functions in nucleocytoplasmic transport, mitotic spindle assembly, and post-mitotic nuclear envelope formation (4–7). However, recent studies have suggested roles for this GTPase in cell growth regulation and malignant transformation. In particular, Ran has been reported to be overexpressed in a variety of tumor types and human cancer cell lines (8–10), as well as to potentially serve as a biomarker for ovarian cancer (11). The overexpression of Ran was also shown to stimulate anchorage-independent growth in a non-invasive mammary cell line and to function as a novel effector of osteopontin-mediated malignant transformation (12). Our laboratory recently reported that an activated form of Ran, Ran(F35A), shows accelerated growth in low serum and supports anchorage-independent growth in normal fibroblasts (13). Moreover, fibroblasts expressing Ran(F35A) promoted tumor formation when injected into nude mice. However, the mechanisms by which Ran contributes to cellular transformation and tumorigenesis are still not clear, thus prompting us to examine in more detail how the hyperactivation of this small GTPase impacts cell growth.

A Ran GDP/GTP gradient between the nucleus and the cytoplasm is reported to drive nucleocytoplasmic transport (14). In interphase cells, the Ran GEF, RCC1, localizes to the nucleus where Ran is activated as a result of GDP-GTP exchange (1, 2). Ran GAP, however, is cytoplasmic and hydrolyzes Ran GTP to the GDP-bound state in the cytosol (3, 15). The distinct cellular location of RCC1 and Ran GAP generates a gradient for activated Ran molecules (highest in the nucleus) and therefore allows a direct coupling between the activation-deactivation cycle of Ran and nucleocytoplasmic transport. Given that the GTP binding/GTP hydrolytic cycle of Ran is linked to proper nucleocytoplasmic transport in cells, as well as to mitogenic signaling activities, we felt that it would be especially interesting to examine how mutants of Ran that are capable of an accelerated cycling between the GDP- and GTP-bound states influ-

1 To whom correspondence should be addressed: Dept. of Molecular Medicine, Cornell University, Ithaca, NY 14853-6401. Tel: 607-253-3888; Fax: 607-253-3659; E-mail: rac1@cornell.edu.

2 The abbreviations used are: GEF, guanine nucleotide exchange factor; CS, calf serum; Mant-GDP, 2′-(or 3′)-O-(N-methyl-anthraniloyl)guanosine 5′-diphosphate; EGFR, EGF receptor.
Activated Ran Induces Oncogenic Transformation

ence cell growth. These mutants, unlike Ran(F35A), would exhibit an accelerated exchange of GDP for GTP, compared with wild-type Ran, while retaining GTP hydrolytic activity, such that they would not be irreversibly active and therefore should not block nucleocytoplasmic transport nor exhibit cellular toxicity. Indeed, a mutant of Cdc42, Cdc42(F28L), which was generated in our laboratory and exhibited constitutive nucleotide exchange (16), has allowed us to avoid the toxic effects caused by GTP hydrolysis-defective mutants of this GTPase (16) and has proven to be extremely valuable in identifying roles for Cdc42 in cell growth regulation (17–19).

Thus, in the present study, we set out to examine the effects of Ran mutants capable of accelerated GDP-GTP exchange on cell growth in both normal fibroblasts and in a human mammary adenocarcinoma cell line. In NIH-3T3 fibroblasts, we show that a novel activated Ran mutant, Ran(K152A), is able to effectively induce anchorage-independent growth and trigger signaling pathways that lead to ERK activation. Furthermore, we demonstrate that Ran(K152A) stimulates the invasive activity of these cells. We then go on to show that Ran(K152A), when stably expressed in the human mammary SKBR3 adenocarcinoma cell line, enhances the ability of these breast cancer cells to grow in soft agar and causes a robust stimulation of both ERK and JNK. Microarray analysis identified the SMOC-2 protein as a potential modulator of Ran(K152A)-promoted cell growth. SMOC-2 belongs to the BM-40 family of modular matricellular proteins, which are secreted factors that mediate signaling between the extracellular matrix and the surface of cells through direct interactions with receptors or by synergizing with growth factors (20). SMOC-2 has been reported to potentiate angiogenesis (21) by interacting with growth factors such as FGF and VEGF, as well as to help promote integrin-dependent signals that regulate cell cycle progression (22). We show that the gene expression of SMOC-2 is increased by at least 50-fold in cells stably expressing Ran(K152A) compared with cells expressing control vector. Moreover, knocking down SMOC-2 expression greatly reduces the ability of Ran(K152A) to stimulate anchorage-independent growth and cell invasion. Overall, these findings highlight a previously unappreciated role for Ran in cellular growth regulation that is dependent on the matricellular protein SMOC-2.

EXPERIMENTAL PROCEDURES

Materials—Mant-GDP was obtained from Invitrogen and [γ-32P]GTP was purchased from PerkinElmer Life Science. The HA antibody was obtained from Covance, whereas the phos- pho-ERK and phospho-JNK antibodies were from Cell Signaling. The vinculin antibody was obtained from Sigma.

Plasmids—The wild-type Ran cDNA was cloned into either the pET-28a(+) vector (Novagen) or the HA-tagged pcDNA3.1 vector (Invitrogen). The QuickChange site-directed mutagenesis kit (Stratagene) was used to generate the Ran(F35A), Ran(D65I), Ran(D125E), Ran(K152A), and Ran(K152Q) mutants. The wild-type SMOC-2 cDNA was cloned into the Myc-tagged pcDNA3.1 vector, and the SMOC-2 siRNA-insensitive mutant was generated using the QuickChange site-directed mutagenesis kit. The primers designed for generating this mutant were as follows: 5’-GCC CGC GCA GAA GTT TAG CGC ACT CAC GTT CTT GAG-3’ (forward primer) and 5’-CTC AAC AAC GTG AGT GCC GTA AAC TTC TTC GCG GCC GGC-3’ (reverse primer).

Expression and Purification of Recombinant Ran—The Ran-pET-28a(+) plasmid was transformed into BL21(DE3) bacterial cells. The cell were grown in LB broth containing 0.05 mg/ml kanamycin and 0.5 mM isopropyl β-D-1-thiogalactopyranoside for ~6 h at 37 °C, harvested by centrifugation, and then lysed by freeze thaw and sonication in buffer A (30 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM MgCl2, 0.3 mM tris(2-carboxyethyl)phosphine hydrochloride, 0.5 mM PMSF, 0.01 mg/ml leupeptin, and 0.01 mg/ml aprotinin). The lysates were centrifuged at 30,000 rpm for 45 min, and then the supernatants were collected, treated with 1 mM GTP at room temperature for 30 min, and loaded onto Talon resin columns that had been pre-equilibrated with buffer A. The columns were washed with 10 bed volumes of buffer A, and the samples were eluted in buffer A containing 300 mM imidazole. The samples were concentrated using centrifugal filters to ~500 μl and loaded onto a Superdex 200 gel filtration column pre-equilibrated with 30 mM Tris-HCl, pH 7.4, 5 mM MgCl2, and 0.3 mM tris(2-carboxyethyl)phosphine hydrochloride (buffer B). Peak fractions were pooled, concentrated, and stored at −80 °C until needed. Protein purity was >95% based on SDS-PAGE and Coomassie Blue staining of the recombinant Ran proteins, with typical yields of 5–10 mg of purified Ran per liter of culture.

Nucleotide Exchange Assays—Recombinant His-tagged Ran proteins were initially preloaded with Mant-GDP by combining 100 μM Ran, 2 mM Mant-GDP, and 10 mM EDTA in 100 μl of HMN buffer (20 mM Hepes, pH 7.5, 5 mM MgCl2, 100 mM NaCl) in 1.5-m1 tubes. The sample tubes were wrapped in foil and rotated for 30 min at room temperature. MgCl2 (40 mM) was then added to each tube, and the samples were passed over a PD10 column (Amersham Biosciences) equilibrated with HMN buffer. After 2.5 ml of HMN buffer flowed through the columns, fractions were collected in 500-μl aliquots for the next 2.5 ml of buffer. Protein was detected by Bradford reagent, and peak aliquots were pooled, concentrated, and stored at 4 °C. Mant-GDP dissociation from wild-type and mutant Ran proteins was determined on a Molecular Devices SPECTRAMax GEMINI XS microplate spectrofluorometer using Magellan software (version 2, Tecan). For each assay, Mant-GDP-bound Ran (4 μM) was incubated with 2 mM GTP in HMN buffer at a final volume of 300 μl in a 96-well plate. The excitation wavelength was 360 nm, whereas the emission wavelength was 465 nm. Both band widths were set at 35 nm. Fluorescence from each well was read at 10-min intervals. The final end point for Mant-GDP dissociation from Ran was determined by adding recombinant GST–RCC1 (100 nM) to the assay incubation. The rate of dissociation of Mant-GDP from Ran was determined by performing a nonlinear fit to a single exponential decay equation.

Cell Culture—NIH-3T3 cells were maintained in DMEM medium containing 10% calf serum (CS), whereas SKBR3 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS). To generate stable cells lines, NIH-3T3 or SKBR3 cells were transfected with the HA-pcDNA3.1 vector alone or with the same vector containing one of the Ran con-
structs using Lipofectamine. The NIH-3T3 cells were then maintained in DMEM supplemented with 10% CS and 1.3 mg/ml G418, whereas the transfected SKBR3 cells were maintained in RPMI 1640 supplemented with 10% FBS and 750 µg/ml G418. After 10–14 days, G418-resistant colonies were selected and subcultured in DMEM supplemented with 10% CS and 1 mg/ml G418 for NIH-3T3 cells, or RPMI 1640 supplemented with 10% FBS and 300 µg/ml G418 for SKBR3 cells. In some cases, NIH-3T3 cells were transfected transiently with plasmids encoding wild-type Ran or the different Ran mutants using Lipofectamine.

**Ran Cellular Localization**—NIH-3T3 or SKBR3 cells transiently expressing either HA-tagged wild-type Ran, Ran(D125E), or Ran(K152A) were fixed with 3.7% formaldehyde and permeabilized with PBS containing 0.1% Triton X-100. The cells were then blocked in PBS containing 5% bovine serum albumin. Following blocking, the cells were incubated with the indicated primary antibodies for 2 h, rinsed with PBS, and then incubated with Oregon green-conjugated secondary antibody (Molecular Probes) for an additional hour. The cells were then washed, mounted, and visualized using either the 40× or 63× objectives on a Zeiss Axioskop fluorescent microscope. Images were processed using IPLAB (Spectra Services).

**Anchorage-independent Growth**—To determine the ability of the different Ran-expressing NIH-3T3 cell lines to grow in soft agar, 8 × 10^3 cells were mixed with DMEM supplemented with 10% CS and 0.3% agarose and plated on a solidified layer containing DMEM supplemented with 0.6% agarose and 10% CS. The cells were fed weekly by adding 1 ml of DMEM supplemented with 10% CS and 0.3% agarose. After 10–12 days, colonies >50 µm were scored under a microscope. A similar procedure was followed to assess the ability of the SKBR3 cells stably expressing the indicated Ran constructs, with the exception of substituting RPMI 1640 and FBS for DMEM and CS, respectively.

**Cell Invasion Assays**—NIH-3T3 cells stably expressing the HA-pcDNA3.1 vector alone or the HA-tagged Ran(K152A) mutant, maintained in DMEM containing 10% CS, were seeded in serum-free DMEM at 2 × 10^4 cells/well in the upper chamber of a BD Biosciences Biocoat Matrigel invasion chamber. The invasion chambers were placed into the wells of a 24-well plate, and DMEM medium containing either no serum or 10% CS was added to the wells of the plate containing the chambers. The cultures were maintained in a humidified tissue incubator (37 °C, 5% CO_2) for 1 day. The cells that accumulated on the lower side of the filter were fixed with methanol, stained with Giemsa stain, and then scored.

**Ran-induced Activation of Mitogenic Signaling Proteins**—NIH-3T3 or SKBR3 cells stably expressing the HA-pcDNA3.1 vector alone, or one of the various HA-tagged Ran constructs, were lysed in 25 mM Tris, pH 7, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM NaVO_4, and 1 mM β-glycerol phosphate. Insoluble materials were removed by centrifugation at 13,000 rpm for 10 min at 4 °C, and the supernatants were subjected to SDS-PAGE and Western blot analysis using phospho-ERK and phospho-JNK antibodies.

The blots were reprobed with a vinculin antibody as a loading control.

**Microarray Analysis**—Gene expression studies were performed on serum-starved NIH-3T3 cells stably expressing Ran(K152A) and NIH-3T3 cells stably expressing the HA-pcDNA3.1 vector alone using the Affymetrix GeneChip Mouse Genome 430 Array (version 2.0). Total RNA was extracted from each of the cell lines. Samples were collected from three separate plates for each experimental condition resulting in a total of six RNA samples used for whole genome gene expression analysis. Each sample was submitted to the Cornell Microarray Core facility, where RNA quantitation, quality assessment, cRNA synthesis and labeling, as well as array hybridization and scanning were performed. Gene expression data for a total of 45,101 probes were obtained.

The mean of three replicate measurements was calculated for each experimental condition: average_k152a and average_vector. Probes that had less than a 2-fold change when comparing cells expressing Ran(K152A) versus vector alone (log_2(average_k152a/average_vector) ≤ 1.0) were excluded. In addition, low intensity probes with fluorescent intensity values <20 in at least one of a total of six observations were excluded. As a result, a total of 4,000 probes that passed the fold change, and low intensity filters were used for further analysis.

Average linkage hierarchical clustering of genes was performed using Cluster software (version 3.0) (40). Clustered trees and gene expression heat maps were viewed using Java Treeview software (41).

**RT-PCR**—Total RNA was extracted using the RNeasy kit (Qiagen) from cells that were serum-starved for 12–15 h. Reverse transcriptase (RT) reactions were performed with SuperScript III enzyme (Invitrogen), using oligo(dT) nucleotides as a primer. The RT reactions were then subjected to PCR using primer sets to amplify SMOCC-2 (mouse); 5'-ATG TTC CAG TAT GAC TCC ACT GAG CAA GT-3' (forward) and 5'-GCA TTG CAC TGG CTG GGA CTG GTA GA-3' (reverse). To exclude the possibility of amplifying contaminating genomic DNA in our RNA preparations, RT reactions lacking the SuperScript III enzyme were subjected to PCR using the primer set for GAPDH (glyceraldehyde 3-phosphate dehydrogenase); 5'-ATG TTC CAG TAT GAC TCC ACT CAC G-3' (forward) and 5'-GAA GAC ACC AGT AGA CTC CAC GAC A-3' (reverse). For semi-quantitative RT-PCR, the RT reactions were subjected to real-time PCR using Power SYBER Green PCR Master Mix and the 7500 Fast Real-Time PCR system (Applied Biosystems). Each reaction was performed in triplicate, and the amount of target RNA was normalized relative to the amount of GAPDH mRNA.

**SMOC-2 Knockdown**—The knockdown of SMOCC-2 in cells was performed using Stealth siRNAs designed against the SMOCC-2 transcript (Invitrogen). The sequences of the three SMOCC-2-specific siRNAs that were used include the following: SMOCC-2 siRNA 1, 5'-CAG AAG TTC TCA GCG CTC ACG TTC T-3'; SMOCC-2 siRNA 2, 5'-AGG TGT GTG GCT GAG AAG TAT A-3'; and SMOCC-2 siRNA 3, 5'-GAA TGC AAT GAT GAC GCC ACC TAC A-3'. As a control, Stealth siRNA negative control with medium GC content was used. The siRNAs were transfected transiently into NIH-3T3 or...
SKBR3 cells using Lipofectamine 2000 (Invitrogen), and the relative knockdown efficiency was determined using RT-PCR.

RESULTS

Design and Characterization of Novel Activated Mutants of Ran GTPase—Our laboratory has generated activated forms of the small GTPase, Cdc42, which are able to cycle rapidly between the GDP- and GTP-bound states, through their ability to undergo constitutive GDP-GTP exchange while retaining the capability of hydrolyzing GTP. One such Cdc42 mutant, Cdc42(F28L), was shown to cause cellular transformation in NIH-3T3 cells and to induce tumor formation in immunocompromised mice (16, 19). More recently, we have reported that a Ran(F35A) mutant, when expressed in cells, is capable of trig-
gerating mitogenic signaling pathways leading to cellular trans-
formation (13). This mutant appears to be activated in cells, although it does not exhibit constitutive nucleotide exchange
activity in vitro, and so the mechanism responsible for its ability to send transforming signals is not clear. Given that the ability of Ran to cycle between its GDP- and GTP-bound states is linked to proper nucleocytoplasmic transport in cells, we wanted to examine mutants of Ran capable of accelerated GDP-
GTP exchange, within the context of cell growth control and cellular transformation.

Activated mutants of Ran were designed by taking advantage of the existing x-ray crystal structures for this and other small
GTPases (23–25). The nucleotide-binding pocket from the x-ray structure of GDP-bound Ran was the primary template used in creating the Ran mutants (24, 26, 27). Both structural and biochemical data have implicated three residues at the Ran nucleotide binding site as strong candidates for mutation (Fig. 1A). The first, Asp-125, is part of the conserved NKXD motif that is found in virtually all G-proteins and is involved in binding to the guanine ring of GDP or GTP (28, 29). Our laboratory has shown that mutating the corresponding aspartic acid in Cdc42 to an asparagine residue yielded a mutant, Cdc42(D118N), capable of constitutive GDP-GTP exchange (28). Given that Asp-125 makes a critical contact with GDP based on the x-ray crystal structure of wild-type Ran, it seemed reason-
able to expect that mutation of Asp-125 could give rise to acceler-
ated nucleotide exchange. The second residue, Lys-152, is in posi-
tion to interact with the ribose moiety of GDP or GTP. Thus, we
suspected that mutating this residue would eliminate these inter-
actions and thereby enhance intrinsic GDP-GTP exchange (28).
Given that Asp-125 makes a critical contact with GDP based on the x-ray crystal structure of wild-type Ran, it seemed reason-
able to expect that mutation of Asp-125 could give rise to acceler-
ated nucleotide exchange. The second residue, Lys-152, is in posi-
tion to interact with the ribose moiety of GDP or GTP. Thus, we
suspected that mutating this residue would eliminate these inter-
actions and thereby enhance intrinsic GDP-GTP exchange (28).
}

FIGURE 1. The biochemical characterization of activated Ran mutants. A, schematic drawing of the nucleotide-binding pocket of Ran based on the x-ray crystal structure of Ran bound to GDP. The indicated residues were mutated to generate activated Ran mutants. B, each purified Ran mutant (15 μg) was resolved by SDS-PAGE, followed by staining with Coomassie Blue. C, Mant-GDP dissociation from wild-type Ran and the Ran mutants. Ran proteins (5 μM) preloaded with Mant-GDP were used in the assay. GTP was added in a 200-fold excess over protein (1 mM GTP) to initiate nucleotide exchange that was recorded using a spectrofluorometer. D, relative rates of nucleotide exchange comparing wild-type Ran to the Ran mutants. Nucleotide exchange data obtained in A were replotted as bar graphs using Excel. The error bars represent S.D.
Activated Ran Induces Oncogenic Transformation

mutated forms of Ran were expressed in bacteria and purified in sufficient amounts for biochemical characterization (Fig. 1B). Fig. 1C compares the rates of dissociation of the fluorescent GDP analog Mant-GDP from wild-type Ran and the different Ran mutants, as an outcome of Mant-GDP/GTP exchange. When compared with wild-type Ran and the Ran(F35A) mutant, the D125E, K152Q, and K152A mutants each showed enhanced rates of GDP dissociation (Fig. 1, C and D). These mutants, unlike wild-type Ran, were also constitutively activated when transiently expressed in NIH-3T3 cells, as indicated in assays using histidine-tagged β-importin to pull down GTP-bound Ran from cellular lysates (13). An example comparing the activities of the rapidly exchanging Ran(K152Q) mutant versus the constitutively active, GTP hydrolysis-defective Ran(Q69L) mutant, and wild-type Ran in cells, is shown in supplemental Fig. S1. Ran(D65I) had very weak affinity for GDP or GTP and showed no detectable GTP hydrolysis under standard assay conditions (data not shown). The D125E, K152A, and K152Q mutants were each capable of binding GTP like wild-type Ran, and all hydrolyzed GTP when assayed in the presence of the Ran GAP FUG1 (supplemental Fig. S2). Ran(F35A) was unable to hydrolyze GTP under these assay conditions but instead was entirely dependent upon RanBP1 working together with Ran GAP for detectable GTP hydrolytic activity (13). This made the F35A mutant unique among the Ran GTPases examined and may help to explain its enhanced activity in cells (13). Overall, these data show that the newly designed Ran mutants D125E, K152A, and K152Q are capable of enhanced nucleotide exchange while maintaining GTP hydrolytic activity.

Novel Activated Ran Mutant Stimulates Cell Transformation—We next examined the cellular location of the activated, HA-tagged Ran mutants, both in NIH-3T3 (normal fibroblasts) and SKBR3 (human mammary adenocarcinoma) cells. We chose to examine Ran in SKBR3 cells because it was reported previously to significantly increase cell proliferation when transfected into the non-invasive rat Rama 37 mammary cell line (12). Wild-type Ran has been shown to localize predominantly in the nucleus where it is activated by the GEF, RCC1 (14, 30). Immunofluorescence analysis performed on the NIH-3T3 cell lines (Fig. 2A) and the SKBR3 cell lines (Fig. 2B) transiently expressing the activated Ran mutants showed that each of the mutants localized to the nucleus, similar to the wild-type Ran protein.

We next examined the abilities of the transiently expressed, HA-tagged Ran mutants to transform NIH-3T3 cells. We started by assaying the anchorage-independent growth of the various transfectants, as this serves as a hallmark of cellular transformation. Interestingly, the highly active Ran(D125E) mutant had little ability to transform cells when expressed in fibroblasts (data not shown). We suspect that this is due to the fact that the excessive activation of Ran can have an inhibitory effect on nucleocytoplasmic transport. Similar effects are observed with GTP hydrolysis-defective Ran mutants, which essentially are irreversibly active (31–33). Therefore, we turned our attention to the Ran(K152A) mutant that has a more modest capability for undergoing GDP-GTP exchange compared with Ran(D125E), while still exhibiting an accelerated nucleotide exchange reaction relative to wild-type Ran. Fig. 3A shows that NIH-3T3 cells transiently expressing HA-Ran(K152A) were capable of exhibiting anchorage-independent growth to a comparable extent to the oncogenic Ras(G12V) mutant.

Fig. 3B compares the abilities of the transiently expressed wild-type Ran and Ran(K152A) to stimulate ERK and JNK activity, as read-out by Western blotting with phosphospecific antibodies. Although cells expressing either the vector alone or wild-type Ran showed little activation of ERK or JNK, comparable to cells that were serum-starved, cells expressing Ran(K152A) showed a markedly enhanced activation of ERK and a modest (typically ~2-fold) stimulation of JNK activity.

We then examined the ability of the activated Ran(K152A) mutant to stimulate the invasive capability of NIH-3T3 cells. Fig. 3C shows that fibroblasts stably expressing the K152A mutant migrated through a Matrigel barrier at significantly greater rates than vector control cells.

Given earlier suggestions providing a link between Ran and mammary cancer, and in particular, the finding that overexpressed wild-type Ran can transform a noninvasive rat mammary cell line (12), it was of interest to see whether the Ran(K152A) mutant might be capable of enhancing the trans-
formed phenotypes of the human mammary adenocarcinoma cell line SKBR3. We generated SKBR3 cell lines stably expressing HA-tagged forms of Ran(K152A), wild-type Ran, and the pcDNA3.1 vector alone. Fig. 4A shows that a Ran(K152A)-expressing clone not only formed larger colonies in soft agar, compared with breast cancer cells expressing wild-type Ran but also gave rise to a greater number of colonies. The Ran(K152A) mutant also causes a robust stimulation of both ERK and JNK activities in SKBR3 cells when compared with vector alone (Fig. 4B).

Gene Expression Microarray Analysis of NIH-3T3 Cells Expressing Activated Ran(K152A) Mutant—To gain additional insights into how the activated Ran(K152A) mutant stimulates mitogenic signaling activities and transforms cells, we undertook a microarray analysis of the RNA messages whose levels were significantly influenced in response to Ran activation. We generated gene expression data from NIH-3T3 cells to minimize potential ambiguity arising from aberrant gene expression that might accompany the transformed phenotypes of cancer cells such as the SKBR3 cell line. The microarray profile of NIH-3T3 cells stably expressing Ran(K152A) was compared against NIH-3T3 cells stably expressing the pcDNA3.1 vector alone (control). The Affymetrix GeneChip Mouse Genome 430 Array (version 2.0) used for the experiment allowed for the analysis of >39,000 transcripts on a single array (Fig. 5A). Total RNA extracted from NIH-3T3 cells stably expressing either the Ran(K152A) mutant or NIH-3T3 cells stably expressing the pcDNA3.1 vector alone was submitted to the Cornell Microarray Core facility, where the array hybridization and scanning as well as array data acquisition and normalization were performed.
After the data were filtered and sorted based on the fold change for gene expression for cells stably expressing the Ran(K152A) mutant versus the vector alone control (Fig. 5A), we classified the messages whose expression levels were up-regulated or down-regulated in response to activated Ran (supplemental Table 1). Fig. 5B lists three proteins whose transcripts were up-regulated by >3-fold in cells expressing Ran(K152A) compared with control cells. Also listed are Ran, as well as Ran binding and Ran regulatory proteins whose messages were increased modestly in cells expressing Ran(K152A). Thus far, we have not found any obvious link between two of the proteins, namely transglutaminase 2 and connective tissue growth factor, and Ran function. However, one protein that did ultimately show such a connection (see below), SMOC-2, exhibited a striking 60-fold increase in its expression (Fig. 5B) in cells expressing Ran(K152A). The microarray data were validated using real-time PCR (Fig. 6A), which showed an ~50-fold increase in SMOC-2 gene expression in cells stably expressing Ran(K152A). SMOC-2 belongs to the BM-40 family of modular matricellular proteins that are secreted and participate in wound repair and angiogenesis as well as interact with growth factors to promote cycle progression, cell attachment, and migration (21, 22, 34, 35). Therefore, given these functions and the fact that SMOC-2 is markedly up-regulated in Ran(K152A)-expressing cells, we took a closer look at its possible role in Ran-dependent transformation.

**Effects of SMOC-2 on Ran(K152A) Cellular Transformation**
To examine whether SMOC-2 is necessary for the ability of the Ran(K152A) mutant to promote cellular transformation, we knocked down SMOC-2 in the background of NIH-3T3 cells stably expressing Ran(K152A) (Fig. 5A) and examined the effects on both anchorage-independent cell growth (Fig. 6B) and cellular invasiveness (Fig. 6C). Indeed, knocking down SMOC-2 markedly compromised the ability of Ran(K152A) to induce anchorage-independent growth and colony formation in soft agar (Fig. 6B), while introducing an siRNA-insensitive form of SMOC-2 into these cells (supplemental Fig. S3) reversed the block (Fig. 6D). Furthermore, knocking down SMOC-2 significantly reduced the ability of Ran(K152A) to promote their invasion (Fig. 6C). Given that we had previously shown that EGF message levels were increased in cells expressing the activated Ran(F35A) mutant and that EGFR signaling was a necessary component of cellular transformation by Ran(F35A) (13), we examined whether inhibiting EGFR tyrosine kinase activity with AG1478 blocked the up-regulation of SMOC-2 expression in Ran(K152A). However, the data in Fig. 6A shows that this was not the case. Although AG1478 treatment caused at least a 50% reduction in the ability of Ran(K152A) to induce anchorage-independent growth, it had very little effect in cells where SMOC-2 was already knocked down (Fig. 6B). Interestingly, the ability of Ran(K152A) to enhance ERK activation was lost when SMOC-2 expression was knocked down (Fig. 6E). Thus, these data collectively suggest that SMOC-2 and the EGFR may be working together in promoting Ran-dependent transformation but that SMOC-2 gene expression levels are not dependent on EGFR-signaling.
SMOC-2 also appears to play an important role in the ability of activated Ran to enhance the transformed characteristics of SKBR3 cells. Knockdowns of SMOC-2 markedly impacted the ability of these cancer cells to show anchorage-independent growth (Fig. 7A), whereas adding back an siRNA-insensitive form of SMOC-2 restored colony formation (Fig. 7B). In fact, colony formation in soft-agar was even reduced below the levels observed in vector-control SKBR3 cells, suggesting that SMOC-2 has a fundamentally important role in the transformed phenotypes exhibited by these cancer cells. Furthermore, the ability of Ran(K152A) to enhance ERK and JNK activation was strongly affected when SMOC-2 expression was knocked down (Fig. 7, C and D).

**DISCUSSION**

The small GTPase Ran plays well established roles in nuclear processes, including the regulation of nucleocytoplasmic transport and the formation of the mitotic spindle (15, 36). However, recently, we reported that serum, as well as specific growth factors such as heregulin, which binds to ErbB3 and ErbB4 and activates ErbB2 tyrosine kinase activity, increase the pool of GTP-bound Ran in cells (13). The ability of growth factors to promote the activation of Ran implies that this GTPase plays a role in the control of cell growth. Indeed, we showed that an activated Ran mutant (Ran(F35A)), when expressed in NIH-3T3 cells, was able to induce their transformation, as read-out by stimulating growth under low serum conditions and colony formation in soft agar as well as tumor formation in nude mice (13). Collectively, these findings may then be related to reports suggesting a connection between Ran and cancer progression. In particular, Ran has been suggested to serve as an indicator of the degree of tumor invasiveness in ovarian cancer, such that its high expression appears to be correlated with poor patient survival (11). Ran has also been reported to be overexpressed in colon, gastric, lung, and pancreatic tumor tissues, and the knockdown of Ran expression was shown to reduce the survival of different cancer cell lines (10, 37).

This raises an obvious question, namely how might Ran be functioning to play roles in mitogenic signaling and, when aberrantly regulated, in cancer progression? A potential clue comes from our earlier work with the Ran(F35A) mutant, as those studies led us to propose a working model where the excessive activation of Ran could result in the accelerated nucleocytoplasmic transport of transcripts encoding growth factors, and consequently, the increased translation of these transcripts into protein (13). In fact, we found that cells expressing the Ran(F35A) mutant exhibited increased levels of EGF and enhanced EGFR signaling to Ras and PI3K (13), presumably as an outcome of the autocrine stimulation of this mitogenic path-
way by EGF. However, one of the primary difficulties in interpreting these findings is the lack of clarity regarding why the Ran(F35A) mutant shows a greater degree of activation in cells compared with wild-type Ran, as our in vitro biochemical analysis indicates that this mutant does not exhibit an increased ability to undergo spontaneous GDP-GTP exchange. A possible explanation for the increased levels of cellular GTP-bound Ran(F35A) is its poor ability to hydrolyze GTP in response to Ran GAP alone, suggesting that it might exist in the GTP-bound state for a longer lifetime relative to wild-type Ran. However, this does not easily fit with a role for Ran in stimulating nucleocytoplasmic transport, as that cycle should be coupled to the GTP binding/GTP hydrolytic cycle of Ran and thereby require that Ran exhibit normal GTP hydrolysis. Thus, how the Ran(F35A) mutant induces cellular transformation, although provocative, particularly when considered in light of the recent findings that connect Ran to cancer progression, still remains unanswered.

To obtain additional insights into how Ran activation might be linked to cellular transformation and cancer, we set out to use a strategy involving Ran mutants that are capable of accelerated GDP-GTP exchange compared with wild-type Ran, while still maintaining GTP hydrolytic activity. We have used this strategy to examine the transforming capabilities of Rho GTPases, and particularly Cdc42 (16, 19) because GTP hydrolysis-defective forms of this protein are often toxic to cells.
wise, GTP hydrolysis-defective forms of Ran have been shown to block cell cycle progression, most likely as an outcome of inhibiting nucleocytoplasmic transport (31–33). Our expectation was that Ran mutants capable of undergoing a more rapid nucleotide exchange reaction, at least compared with the wild-type protein, would have the potential to accelerate the nucleocytoplasmic transport cycle and therefore contribute to mitogenic signaling by stimulating RNA processing, along the lines that we have previously suggested for the role of Ran in cell growth control (13). Indeed, we found that the Ran(K152A) mutant that exhibits a faster intrinsic rate of GDP-GTP exchange compared with wild-type Ran, while still showing GTP-hydrolytic capability, was very effective at inducing cellular transformation. Interestingly, we found that another Ran mutant, Ran(D125E), which showed a significantly faster rate of intrinsic GDP-GTP exchange compared with the Ran(K152A) mutant, was ineffective at transforming fibroblasts. We suspect that this is due to an excessive percentage of the D125E mutant being in the GTP-bound state at any particular time in the cell. This would interfere with normal nucleocytoplasmic transport, probably by tying up β-importin and thereby counteract any advantage provided by its ability to spontaneously exchange GDP for GTP.

Microarray analyses were performed on cells transformed by the Ran(K152A) mutant and compared with similar analyses for control (non-transformed) cells to examine whether the expression of specific genes were being stimulated significantly, as this could help shed light on important pathways operating downstream from activated Ran. One particularly intriguing protein whose expression was markedly increased in fibroblasts transformed by the Ran(K152A) mutant was SMOC-2. The SMOC-2 protein is often referred to as a matricellular protein (34). These are secreted extracellular matrix proteins that influence cell-matrix interactions and have been suggested to be associated with tissue remodeling, morphogenesis, and vascular growth (21, 22, 35). SMOC-2 has been reported to work together with other growth factors, including VEGF and FGF, to initiate signaling pathways downstream from their receptors (38, 39). Although it was not immediately obvious that SMOC-2 expression would be under Ran control, the fact that it showed a >50-fold increase in message levels in cells expressing the Ran(K152A) mutant compared with control cells, made us curious to see whether it might be important for cellular transformation. This in fact turned out to be the case, as knockdowns of SMOC-2 expression significantly reduced the ability of the activated Ran mutant to stimulate the anchorage-inde-

---

**FIGURE 7.** SMOC-2 is important for Ran promoted cellular transformation in human mammary SKBR3 adenocarcinoma cells. A, SKBR3 cells stably expressing the vector alone, Ran(K152A), or Ran(K152A) and SMOC-2 siRNAs (denoted as Kd 1, 2, or 3) were subjected to anchorage-independent growth (soft agar) assays. The experiments were performed three times, and the resulting colonies that formed in each experiment were counted, averaged, and graphed. The error bars represent S.D. B, SKBR3 cells stably expressing the vector alone, Ran(K152A), and SMOC-2 siRNA, or Ran(K152A), SMOC-2 siRNA, and an siRNA-insensitive form of SMOC-2 (SMOC-2 mutant) were subjected to anchorage-independent growth (soft agar) assays. The experiments were performed three times, and the resulting colonies that formed in each experiment were counted, and the data were averaged and graphed as a percent of the control Ran(K152A). The error bars represent S.D. C and D, serum-starved SKBR3 cells stably expressing the vector alone, Ran(K152A), or Ran(K152A) and SMOC-2 siRNAs were lysed. As a positive control, an additional plate of serum-starved SKBR3 cells overexpressing the vector alone was stimulated with medium containing serum for 10 min prior to being lysed. The cell extracts were analyzed by Western blot analysis using phospho-ERK, phospho-JNK, HA, and vinculin antibodies.
dependent growth and invasiveness of fibroblasts, as well as the transformed characteristics of the human breast cancer cell line SKBR3. Importantly, an siRNA-insensitive form of SMOC-2 was capable of restoring the anchorage-independent cellular growth in both of the cell lines depleted of SMOC-2 (due to the siRNA knockdown). Moreover, we have found that the stable expression of SMOC-2 in NIH-3T3 cells confers transformed characteristics upon these cells, as indicated by their ability to show colony formation in soft-agar (supplemental Fig. S4).

We suspect that the ability of Ran to stimulate the RNA processing and translation of growth factors similar to EGF, thereby resulting in enhanced mitogenic signaling activities, is responsible for triggering the necessary signal(s) to up-regulate SMOC-2 expression. Although one obvious candidate for a growth factor that signals the up-regulation of SMOC-2 was EGF itself, we have not found that EGFR signaling is directly linked to the regulation of SMOC-2 expression. An intriguing possibility is that activated Ran might be increasing the translation of those growth factors that have been shown to work together with SMOC-2, such that the up-regulation of SMOC-2 would be the outcome of a positive feedback response to a particular growth factor-signaling event. Efforts are now underway to test this idea.

In closing, to our knowledge, this is the first demonstration that SMOC-2 is important for and can actually induce cellular transformation in cell culture and that a signaling connection exists between the activation of the Ran GTPase and this matrix-cellular protein. These findings highlight what might be a key feature of those cancers where the up-regulation of Ran expression has been linked to the aggressiveness of the disease.

Acknowledgments—We thank Cindy Westmiller for help in the preparation of this manuscript. We also thank Ausras Milano for help with the microarray analysis.

REFERENCES

1. Macara, I. G. (2001) Transport into and out of the nucleus. Microbiol. Mol. Biol. Rev. 65, 570–594
2. Bischoff, F. R., and Ponstingl, H. (1991) Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature 354, 80–82
3. Bischoff, F. R., Kreber, H., Kempf, T., Hermes, L., and Ponstingl, H. (1995) Human Ran GTPase-activating protein Ran GAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. Proc. Natl. Acad. Sci. U.S.A. 92, 1749–1753
4. Joseph, J. (2006) Ran at a glance. J. Cell Sci. 119, 3481–3484
5. Quimby, B. B., and Dasso, M. (2003) The small GTPase Ran: Interpreting the signs. Curr. Opin. Cell Biol. 15, 338–344
6. Sato, M., and Toda, T. (2010) Space shuttling in the cell: Nucleocytoplasmic transport and microtubule organization during the cell cycle. Nucleus 1, 231–236
7. Matsusaka, Y., and Stewart, M. (2004) Structural basis for the assembly of a nuclear export complex. Nature 432, 872–877
8. Ahe, B., Kamai, T., Shirakata, H., Oyama, T., Arai, K., and Yoshida, K. (2008) High expression of Ran GTPase is associated with local invasion and metastasis of human clear cell renal cell carcinoma. Int. J. Cancer 122, 2391–2397
9. Rensen, W. M., Mangiacasale, R., Criciarello, M., and Lavia, P. (2008) The GTPase Ran: Regulation of cell life and potential roles in cell transformation. Front. Biosci. 13, 4097–4121
10. Azuma, K., Sasada, T., Takedatsu, H., Shimura, H., Koga, M., Maeda, Y., Yao, A., Hirai, T., Takabayashi, A., Shichijo, S., and Itoh, K. (2004) Ran, a small GTPase gene, encodes cytotoxic T lymphocyte (CTL) epitopes capable of inducing HLA-A33-restricted and tumor-reactive CTLs in cancer patients. Clin. Cancer Res. 10, 6695–6702
11. Ouellet, V., Guyot, M. C., Le Page, C., Filali-Mouhim, A., Lussier, C., Tonin, P. N., Provencen, D. M., and Mes-Masson, A. M. (2006) Tissue array analysis of expression microarray candidates identifies markers associated with tumor grade and outcome in serious epithelial ovarian cancer. Int. J. Cancer 119, 599–607
12. Kurisetty, V. V., Johnston, P. G., Johnston, N., Erwin, P., Crowe, P., Fernig, D. G., Campbell, F. C., Anderson, I. P., Rudland, P. S., and El-Tanani, M. K. (2008) Ran GTPase is an effector of the invasive/metastatic phenotype induced by osteopontin. Oncogene 27, 7139–7149
13. Ly, T. K., Wang, J., Pereira, R., Rojas, K. S., Feng, Q., Cerione, R. A., and Wilson, K. F. (2010) Activation of the Ran GTPase is subject to growth factor regulation and can give rise to cellular transformation. J. Biol. Chem. 285, 5815–5826
14. Görlich, D., Panté, N., Kutay, U., Aebi, U., and Bischoff, F. R. (1996) Identification of different roles for RanGD and RanGTP in nuclear protein import. EMBO J. 15, 5584–5594
15. Stewart, M. (2007) Molecular mechanism of the nuclear protein import cycle. Nat. Rev. Mol. Cell Biol. 8, 195–208
16. Liu, S., Bagrodia, S., Cerione, R., and Moran, D. (1997) A novel Cdc42Hs mutant induces cellular transformation. Curr. Biol. 7, 794–797
17. Wang, J. B., Wu, W. J., and Cerione, R. A. (2005) Cdc42 and Rac cooperate to mediate cellular transformation by intersectin-L. J. Biol. Chem. 280, 22883–22891
18. Lin, Q., Fuji, R. N., Yang, W., and Cerione, R. A. (2003) RhoGDI is required for Cdc42-mediated cellular transformation. Curr. Biol. 13, 1469–1479
19. Lin, R., Cerione, R. A., and Moran, D. (1999) Specific contributions of the small GTPases Rho, Rac, and Cdc42 to Dbl transformation. J. Biol. Chem. 274, 23633–23641
20. Vannanhe, C., Götsling, S., Paulson, M., Maurer, P., and Hartmann, U. (2003) Characterization of SMOC-2, a modular extracellular calcium-binding protein. Biochem. J. 373, 805–814
21. Rocnik, E. F., Liu, P., Sato, K., Walsh, K., and Vaziri, C. (2006) The novel SPARC family member SMOC-2 potentiates angiogenic growth factor activity. J. Biol. Chem. 281, 22855–22864
22. Pai, E. F., Krebsch, W., Petsko, G. A., Goody, R. S., Krengel, U., and Wittinghofer, A. (1999) RAN GTPase is an effector of the invasive/metastatic phenotype of GTP hydrolysis. EMBO J. 9, 2351–2359
23. Scheffzek, K., Klebe, C., Fritz-Wolf, K., Kabsch, W., and Wittinghofer, A. (1995) Crystal structure of the nuclear Ras-related protein Ran in its GDP-bound form. Nature 374, 378–381
24. Pai, E. F., Krebsch, W., Holmes, K. C., John, J., and Wittinghofer, A. (1989) Structure of the guanine-nucleotide-binding domain of the Ha-ras oncogene product p21 in the triphosphate conformation. Nature 341, 209–214
25. Partridge, J. R., and Schwartz, T. U. (2009) Crystallographic and biochemical analysis of the Ran-binding zinc finger domain. J. Mol. Biol. 391, 375–389
26. Stewart, M., Kent, H. M., and McCoy, A. J. (1998) The structure of the Q69L mutant of GDP-Ran shows a major conformational change in the switch II loop that accounts for its failure to bind nuclear transport factor 2 (NTF2). J. Mol. Biol. 284, 1517–1527
27. Tu, S. S., Wu, W. J., Yang, W., Noblant, P., Hahn, K., and Cerione, R. A. (2002) Antiapoptotic Cdc42 mutants are potent activators of cellular transformation. Biochemistry 41, 12350–12358
28. Cool, R. H., Schmidt, G., Lenzen, C. U., Prinz, H., Vogt, D., and Wittinghofer, A. (1999) The Ras mutant D119N is both dominant negative and activated. Mol. Cell. Biol. 19, 6297–6305
29. Ren, M., Drivas, G., D’Eustachio, P., and Rush, M. G. (1993) Ran/TC4: A small GTP-binding protein that regulates DNA synthesis. J. Cell Biol. 120, 313–323
30. Clarke, P. R., Klebe, C., Wittinghofer, A., and Karsenti, E. (1995) Regulation

Activated Ran Induces Oncogenic Transformation

JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 287 • NUMBER 30 JULY 20, 2012 24965
Activated Ran Induces Oncogenic Transformation

32. Dasso, M., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1994) A mutant form of the Ran/TC4 protein disrupts nuclear function in Xenopus laevis egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation. EMBO J. 13, 5732–5744

33. Ren, M., Coutavas, E., D’Eustachio, P., and Rush, M. G. (1994) Effects of mutant Ran/TC4 proteins on cell cycle progression. Mol. Cell. Biol. 14, 4216–4224

34. Bornstein, P., and Sage, E. H. (2002) Matricellular proteins: Extracellular modulators of cell function. Curr. Opin. Cell Biol. 14, 608–616

35. Maier, S., Paulsson, M., and Hartmann, U. (2008) The widely expressed extracellular matrix protein SMOC-2 promotes keratinocyte attachment and migration. Exp. Cell Res. 314, 2477–2487

36. Clarke, P. R., and Zhang, C. (2008) Spatial and temporal coordination of mitosis by Ran GTPase. Nat. Rev. Mol. Cell Biol. 9, 464–477

37. Morgan-Lappe, S. E., Tucker, L.A., Huang, X., Zhang, Q., Sarthy, A. V., Zakula, D., Vernetti, L., Schurdak, M., Wang, J., and Fesik, S. W. (2007) Identification of Ras-related nuclear protein, targeting protein for Xenopus kinesin-like protein 2, and stearoyl-CoA desaturase 1 as promising cancer targets from an RNAi-based screen. Cancer Res. 67, 4390–4398

38. Kupprion, C., Motamed, K., and Sage, E. H. (1998) SPARC (BM-40, osteonectin) inhibits the mitogenic effect of vascular endothelial growth factor on microvascular endothelial cells. J. Biol. Chem. 273, 29635–29640

39. Hasselaar, P., and Sage, E. H. (1992) SPARC antagonizes the effect of basic fibroblast growth factor on the migration of bovine aortic endothelial cells. J. Cell. Biochem. 49, 272–283

40. Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A. 95, 14863–14868

41. Saldanha, A. J. (2004) Java Treeview: Extensible visualization of microarray data. Bioinformatics 20, 3246–3248

31.

32.

33.

34.

35.

36.

37.

38.

39.

40.

41.