Evidence Using a Green Fluorescent Protein-Glucocorticoid Receptor Chimera that the RAN/TC4 GTPase Mediates an Essential Function Independent of Nuclear Protein Import

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Abstract. The Ran/TC4 GTPase is required for the nuclear accumulation of artificial karyophiles in permeabilized cell assays. To investigate Ran function in a physiologically intact setting using mammalian cells, we examined the effects of several Ran mutants on cell growth and on the nuclear translocation of a glucocorticoid receptor-green fluorescent protein fusion (GR-GFP). Glucocorticoid receptor is cytosolic in the absence of ligand, but translocates to the nucleus on binding the agonist dexamethasone. After transfection into baby hamster kidney cells (BHK21), GR-GFP was detectable in living cells by direct fluorescence microscopy. Addition of dexamethasone caused a rapid translocation of the chimeric protein from the cytosol into the nucleus ($t_{1/2} \sim 5$ min). Cotransfection with epitope-tagged, wild-type Ran led to expression of HA1-Ran that was $\sim$1.6-fold higher than the level of the endogenous protein, but it had no deleterious effect on nuclear import of the GR-GFP. However, expression of the Ran mutants G19V, T24N, or a COOH-terminal deletion (ΔC) mutant dramatically reduced the accumulation of GR-GFP in the nuclei. An L43E mutant of Ran was without significant effect on nuclear GR-GFP import. Identical results were obtained following microinjection of recombinant Ran mutants into cells expressing GR-GFP. Significantly, all of the Ran mutants, including L43E, strongly inhibited cell growth. These results demonstrate the use of GR-GFP in real-time imaging of nuclear transport. They also show that multiple types of Ran mutant exert dominant effects on this process, and that normal Ran function requires cycling between the GTP- and GDP-bound states of the protein. Most importantly, the results with the L43E Ran mutant provide strong evidence that Ran mediates a function essential to cell viability that is independent of nuclear protein import.

APID progress has been made in recent years in elucidating the mechanism of transport through nuclear pore complexes. Proteins that contain nuclear localization signals (NLSs)[1] associate with a 56-kD receptor called α-importin, α-karyopherin, or hSRP1 (Gorlich et al., 1994; Moroianu and Blobel, 1995). The α-importin can associate with a second protein, p97, β-importin or β-karyopherin (Adam and Adam, 1994; Radu et al., 1995). Together, these proteins appear sufficient to dock cargo at the nuclear pore (Gorlich et al., 1995). Ran in the GTP-bound state can associate with β-importin (Rexach and Blobel, 1995) and addition of Ran to an in vitro assay allows the accumulation of cargo within the nuclear compartment (Moore and Blobel, 1993; Melchior et al., 1993; Melchior and Gerace, 1995). A 360-kD Ran-binding protein, NUP358/RanBP2 (Wu et al., 1995; Yokoyama et al., 1995), which contains four Ran-binding domains (Beddow et al., 1995), is located at the nuclear pore and may act as a docking site for Ran in the GTP-bound state. We have recently found that Ran-binding domains promote the interaction of Ran with β-importin/β-karyopherin, and they may therefore provide a link between the docking and translocation steps across the nuclear pore (Lounsbury et al., 1996).

A constitutively activated (GTP-bound) mutant of Ran can block nuclear protein import in budding yeast (Schlenstedt et al., 1995) and RNA1p, a GTPase-activating protein (GAP) for Ran (Becker et al., 1995; Bischoff et al., 1995), is needed for import, suggesting that GTP hydrolysis on Ran is an essential step in the nuclear transport mechanism (Corbett et al., 1995). The interaction of Ran: GTP with Ran-binding domains increases by severalfold its sensitivity towards Ran-GAP (Beddow et al., 1995; Richards et al., 1995; Bischoff et al., 1995).

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1. *Abbreviations used in this paper:* BSA, bovine serum albumin; GFP, green fluorescent protein; GR, glucocorticoid receptor; NLS, nuclear localization signal; RanBP, Ran-binding protein; RanBD, Ran-binding domain.

It remains unclear whether Ran possesses cellular functions other than the transport of nuclear protein cargo through the nuclear pore complex. The perturbation of the Ran GTP/GDP cycle, for instance by depletion of RCC1, can interrupt cell cycle progression, nuclear growth, RNA export, and RNA processing (Sazar and Nurse, 1994; Dasso et al., 1994; Kadowaki et al., 1993; Cheng et al., 1995) but many of these effects could be secondary to a defect in nuclear protein import. Ideally, Ran mutants are required that can, if possible, separately disrupt these processes.

Much of the work that has identified the factors necessary for nuclear transport has relied on the addition to permeabilized cells of transport factors such as Ran, plus artificial cargo bearing an SV40 nuclear localization signal (NLS). There is some evidence to suggest that the density of such signals on the nuclear cargo can affect the transport process (Cserpan and Udvardy, 1995). Moreover, cell permeabilization leads to a rapid depletion of Ran from the nucleus (Moroianu and Blobel, 1995). The addition of exogenous Ran to the permeabilized cells or isolated nuclei therefore produces a gradient of Ran across the nuclear envelope which is the *inverse* of that present in the intact cell. This inversion may significantly perturb the normal transport machinery. It is important therefore to relate data obtained using in vitro assays to the nuclear transport of endogenous cargo in living cells.

To address this issue we have developed a method for following the agonist-dependent nuclear accumulation of the glucocorticoid receptor in living cells. The glucocorticoid receptor (GR) possesses two distinct NLSs that can function independently when attached to heterologous proteins (Picard and Yamamoto, 1987). Unliganded GR is cytosolic and nuclear translocation occurs with a $t_{1/2}$ of ~5–10 min following addition of the artificial agonist, dexamethasone (Qi et al., 1989). Export after dexamethasone withdrawal is very slow. We reasoned that a GR-GFP chimera would provide an ideal system for studying nuclear translocation in intact living cells. We have created a fusion protein of human glucocorticoid receptor with an S65T mutant of the *Aquoria* green fluorescent protein (GFP) (Chalfie et al., 1994). GFP possesses an intrinsic fluorescence that does not require the presence of cofactors. The S65T mutation enhances the emission efficiency by ~10-fold and improves the excitation and emission spectral characteristics such that the protein can very easily be detected within cells using a standard epifluorescence microscope (Heim et al., 1995).

We show that the GR-GFP fusion protein translocates into the nucleus upon exposure to agonist with kinetics similar to those published for the glucocorticoid receptor, confirming that the system is useful for studying nuclear transport in vivo. We demonstrate both by cotransfection and by micro-injection of recombinant proteins that a constitutively activated mutant, G19V Ran, a constitutively inactive mutant, T24N, and a COOH-terminal deletion mutant of Ran, ΔC, all dominantly inhibit agonist-induced nuclear translocation of the GR-GFP chimera. An effector domain mutant, L43E, does not block nuclear translocation. Remarkably, however, all mutants including L43E, are toxic to cell growth. These results provide strong evidence that Ran mediates an essential function additional

to its role in nuclear protein import, and suggests that L43E will be a useful tool to elucidate that function.

Materials and Methods

Plasmid Constructions

A new vector, pK7-GFP, was constructed based on the eukaryotic expression vector pKH3 (Mattingly et al., 1994), in which the triple HA1 epitope-tag was removed and replaced by the S65T mutant of GFP (Heim et al., 1995). Cloning sites were included both at the 3' and 5' ends of the GFP open reading frame. Expression is driven from a cytomegalovirus promoter. GR was amplified by PCR using primers containing XbaI sites, and the DNA product was cloned into the XbaI site in pK7-GFP such that the stop codon of GR was deleted and the coding sequence continued in-frame with that of the GFP. The 5' GR XbaI site obeys Kozak's rule for efficient initiation of translation. This construct was called pK7-GR-GFP. The various Ran mutants were constructed by megaprimer PCR and cloned into pKH3, which encodes a triple HA1 epitope tag at the NH₂ terminus. They have been described and characterized previously (Richards et al., 1995; Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication). The ΔC mutant lacks the COOH-terminal six amino acid residues (Lounsbury et al., 1994).

Transfections

Plasmid DNAs were transfected into baby hamster kidney cells (BHK21) by the calcium phosphate method, without carrier DNA (Sambrook et al., 1989). When the concentrations of specific plasmid DNAs were to be varied, empty vector (pKH3) was added to maintain equal total molar quantities of DNA transfected. Cells were grown at 37°C in DMEM lacking phenol red, plus 10% FBS and penicillin/streptomycin. The serum was stripped of endogenous glucocorticoids by incubation with activated charcoal, before use (Eckert et al., 1982). After 24 h, the cells were rinsed with fresh medium and incubated an additional 16–20 h. Cells were then examined by fluorescence microscopy to detect expression of GR-GFP.

Microscopy

Transfected cells were grown in 2-well LabTek slides, and the medium was replaced with Hepes-buffered saline (20 mM Hepes, 125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 1 mg/ml bovine serum albumin) before microscopy because the DMEM exhibited a high background fluorescence. Living cells were examined using a Nikon Diaphot inverted microscope with a heated stage and epifluorescence attachment, using a fluorescein filter set. A 50% neutral density filter was placed in the incident light path to reduce cell damage due to local heating and photolysis. Photographs were taken using a 35-mm Nikon camera with 400 ASA color slide film (pushed to 800 ASA).

Cells were fixed for immunostaining using a freshly prepared solution of 4% paraformaldehyde in PBS, for 15 min at room temperature. Cells were then washed three times with PBS and permeabilized on ice for 2 min with −20°C methanol. After three additional washes with PBS, cells were blocked with 5% bovine serum albumin (BSA) in PBS and incubated with anti-HA1 monoclonal antibody, 12CA5 in ascites fluid at a dilution of 1:400 for 45 min. The cells were then washed with PBS/5% BSA and incubated in the dark for 45 min with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:800. After an additional three washes, the cells were mounted (Vectashield, Vector Laboratories, Burlingame, CA) and examined by epifluorescence and confocal microscopy.

For quantitation of nuclear/cytoplasmic ratios, an MC1000 confocal system was used (Bio-Rad Labs, Hercules, CA), attached to an Olympus BX50 microscope with a 40× objective lens. Data were collected and quantitated using Comos software (Bio-Rad). For each set of conditions, the intensities of pixels were summed within the individual nuclei and cytosols of at least 10 cells, using a Z-step value of 5 μm, and corrected for background fluorescence (<5% of pixel intensity within the cells). N/C fluorescence ratios were calculated and pooled for each time point to give the means ± 1 SD. Cells were randomly selected from those that were fluorescent in both the fluorescein (GFP) and rhodamine (Cy3) channels. Using the Kalman filter, each image was accumulated from seven scans, but no image enhancement was performed before quantitation.

In addition to these quantitative measurements, at least 100 cells were scored qualitatively (by eye) for each condition to assess the nuclear vs cytosolic distribution of the GR-GFP.

Micro-injections

Ran mutants were produced as GST fusion proteins in *Escherichia coli* and purified by glutathione-Sepharose affinity chromatography as described previously (Richards et al., 1995). The Ran proteins were cleaved from the GST by addition of thrombin. Thrombin was removed by addition of p-aminobenzamidine-Sepharose (Sigma Chem. Co., St. Louis, MO). The recombinant proteins were concentrated in a Centricon10 to ~4 mg/ml, in 10 mM sodium phosphate, pH 7.7, 70 mM KCl, 1 mM $MgCl_2$. The proteins were mixed with an equal volume of TRITC-dextran at 2 mg/ml (as an injection marker) in the same buffer and micro-injected into BHK21 cells that expressed GR-GFP. An Eppendorf 5242 system attached to the Nikon Diaphot inverted microscope was used for injection. Fluorescent cells were visualized as described above. Injected cells were then incubated at 37°C for 30 min to allow recovery, before addition of 1 µM dexamethasone. Cells were photographed after an additional 15 min at 37°C, without fixation.

Immunoblotting

BHK21 cells in 100-mm plates were transfected with 15 µg of pKH3-Ran or empty pKH3 vector, as described above. After 48 h, cell extracts were prepared by direct addition of Laemmli sample buffer and analyzed by SDS gel electrophoresis. Proteins were transferred to nitrocellulose (0.6 amps, 2 h) and immunoblotted using either the 12CA5 anti-HA1 antibody or an anti-Ran monoclonal antibody (Transduction Laboratories, Lexington, KY). An antiserum prepared against a unique peptide near the COOH terminus of Ran (Richards et al., 1995) gave similar results to the monoclonal antibody (not shown). After incubation of the nitrocellulose with a horseradish peroxidase–coupled secondary antibody (1:10,000 dilution), bands were detected by chemiluminescence using LumiGLO (Kirkegaard and Perry, MD). Bands detected by the anti-Ran antiserum that corresponded to endogenous Ran and to the larger HA1-tagged Ran were quantitated using a DPI densitometer. Recombinant Ran protein (1–100 ng), loaded in lanes adjacent to the cell extracts, was used as a standard to calibrate the densitometer.

Cell Growth Assay

To measure the growth of cells expressing mutant or wild-type HA1-Ran proteins, BHK21 cells were cotransfected with 10 µg of pKH3 alone or the pKH3-Ran vector of interest plus 10 µg of pK7-GFP, which expresses an unfused green fluorescent protein. We have determined (see Results section) that a highly reproducible cotransfection efficiency of ~74% is obtained under these conditions. Thus, most of the cells that express the green fluorescent protein also express the HA1-Ran protein encoded by the vector with which it was cotransfected. Transfected cells were seeded sparsely into 100-mm dishes and were then counted daily for 5 d to determine the number of green fluorescent cells per dish, and the fraction of green fluorescent cells in the population.

Results

Real-time Imaging of Nuclear Translocation of GR-GFP

To test the efficacy of using a GR-GFP fusion protein to study nuclear transport, we first characterized the ability of GR-GFP to respond to the agonist dexamethasone in a manner similar to that known for the glucocortocoid receptor. 40 h after transfection of BHK21 cells with plasmid that expresses the GR-GFP fusion protein, fluorescence was detected in ~20–30% of the cells, and was predominantly cytosolic. The percentage of transfected cells exhibiting exclusively cytosolic fluorescence was increased to >90% by growth of the cells in charcoal-stripped serum. Picard and Yamamoto (1987) have noted previously that phenol red and serum can affect the subcellular distribu-

tion of the glucocorticoid receptor resulting, in their hands, in a predominantly nuclear localization.

Heterogeneity in subcellular distribution was occasionally observable among transfected cells even in the absence of phenol red and with charcoal-stripped serum, which did not correlate with the level of expression of the chimeric fluorescent protein. The cause of this heterogeneity is not known. Addition of the agonist dexamethasone led to the rapid nuclear accumulation of the GR-GFP, was observable within individual living cells, and occurred with a half-time of ~5 min at 37°C (Fig. 1 *A*). This time course is similar to that which has been reported previously for the GR (Picard and Yamamoto, 1987), confirming that the addition of the GFP epitope to the COOH terminus of the receptor does not interfere with agonist-induced nuclear translocation. Most cells exhibited a similar time course for complete translocation. In ~20% of cells the fluorescent protein could be seen to concentrate at the nuclear envelope before entry.

Transfection of the parent vector, which expresses GFP alone, resulted in a diffuse fluorescence throughout the cytosol and nuclear compartments, the distribution of which is not altered by addition of dexamethasone (Fig. 1 *B*). Note that GFP is small enough (27 kD) to pass through the nuclear pores by passive diffusion (Melchior and Gerace, 1995).

Removal of dexamethasone allowed the slow return of GR-GFP to the cytosol with a half-time of ~4 h, which is similar to that reported previously for the wild-type GR (Qi et al., 1989).

To further determine if the chimeric receptor functions normally, we examined the effect of different concentrations of dexamethasone on nuclear translocation of GR-GFP. A half-maximal effect was observed at a concentration of ~50 nM (Fig. 2). This value is approximately fivefold higher than the $K_{1/2}$ for binding of dexamethasone to endogenous receptors and for induction of gene expression (Bloom et al., 1980). However, in the studies by Bloom et al. (1980), the dexamethasone was allowed to equilibrate with the receptor for at least 30 min, while in our studies nuclear import was assayed after incubation with dexamethasone for only 15 min. This difference may account for the slightly higher apparent $K_{1/2}$.

Expression Level of Transfected Ran

The Ran GTPase is an abundant nuclear protein, constituting ~0.3% of total cell proteins (Bischoff and Ponstingl, 1991). We wished to determine the effects of various Ran mutants on the nuclear transport of GR-GFP, and were concerned that the level of expression of transfected Ran might be too low in comparison to that of the endogenous protein to produce significant effects. It was therefore important to estimate the amount of HA1-Ran expressed in transiently transfected BHK21 cells relative to that of the endogenous Ran protein. This information can be obtained if the fraction of cells expressing HA1-Ran is known, and if the relative amounts of the HA1-Ran and endogenous Ran present in the same cell population can be measured. The fraction of cells that express HA1-Ran can be determined by cotransfecting with a marker such as the green fluorescent protein, GFP, if the propor-

tion of cells that take up DNA and express both proteins is reproducible. The relative amounts of HA1-Ran and endogenous Ran can both be determined by immunoblotting with an anti-Ran antibody, because the epitope tag causes the HA1-Ran protein to move with a reduced mobility in SDS-PAGE, so that it can be easily distinguished from the endogenous Ran protein.

To determine the cotransfection efficiency of the cells, equal amounts of the plasmids pK7-GFP and pKH3-Ran were mixed and transfected into BHK21 cells. After 48 h the cells were fixed and stained with the 12CA5 anti-HA1 antibody and Cy3-labeled secondary antibody to detect the HA1-Ran. Red and green fluorescent cells were then counted as a proportion of the total cell number in several randomly selected fields of 100-mm plates of cells, to determine the number of cells expressing either GFP alone, HA1-Ran alone, or both proteins. We found in three independent transfection experiments that 74% ± 8% of the

transfected cells expressed both the GR-GFP and the HA1-Ran. Another 6–7.5% expressed HA1-Ran alone; and 6–7.5% expressed GR-GFP alone. Therefore both plasmids are reproducibly transfected into the BHK21 cells with equal efficiency.

To determine the relative amounts of the tagged and endogenous Ran proteins in the same transfected cell population, cells were cotransfected with pKH3 and pK7-GFP as above. The plate was then examined by fluorescence microscopy, without fixation, to determine the proportion of cells expressing GFP. The fraction of the cell population transfected was 0.3 ± 0.03 (n = 150 cells). The same cells were then harvested and extracts were analyzed by SDS-PAGE followed by immunoblotting with an anti-Ran monoclonal antibody. As shown in Fig. 3 A, an intense band at ∼25 kD is detected in both samples, which corresponds to the endogenous Ran protein. A second band of ∼33 kD is present only in the sample from cells trans-
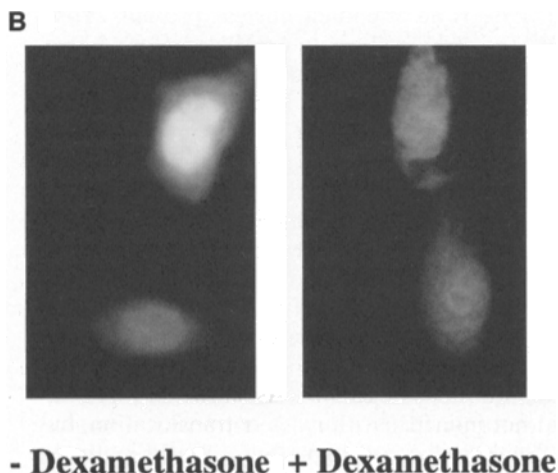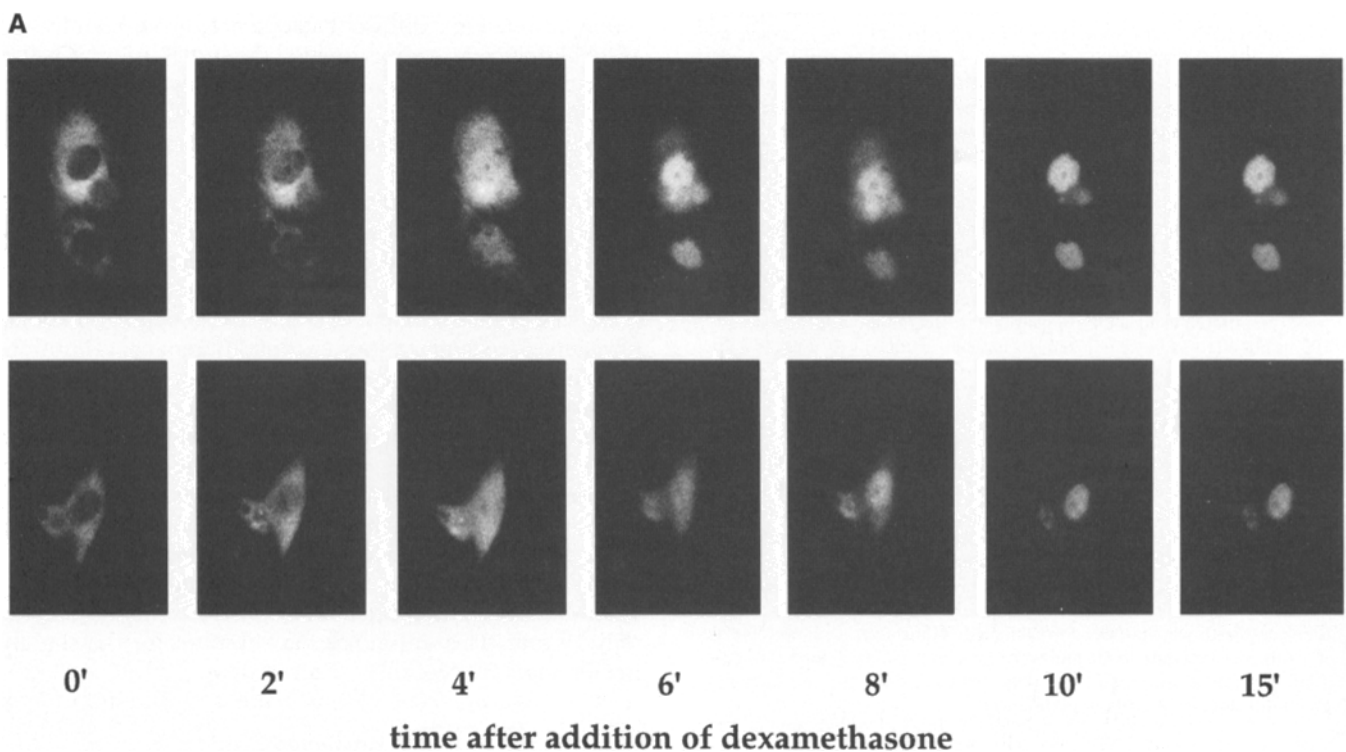
A

time after addition of dexamethasone

B

- Dexamethasone + Dexamethasone

Figure 1. Nuclear transport of GR-GFP in single living cells. (*A*) Agonist-dependent nuclear accumulation of GR-GFP. BHK21 cells were transfected with 2 μg of the vector pK7-GR-GFP + 2 μg of pKH3, as described in Materials and Methods. The pK7-GR-GFP vector expresses a fusion of the glucocorticoid receptor and a mutant green fluorescent protein, S65T. Living transfected cells were detected by epifluorescence. Single fluorescent cells were photographed at intervals after the addition to the medium of 1 μM dexamethasone. Two typical examples of nuclear transport in response to dexamethasone are shown. Each frame shows two cells, and in both sets one cell appears to be expressing significantly higher amounts of GR-GFP than the other. However, the kinetics of transport appear to be similar. (*B*) Effect of dexamethasone on GFP distribution. As a negative control, BHK21 cells were transfected pK7-GFP, which expresses unfused green fluorescent protein. The 27-kD protein is diffusely distributed within the cell, and no relocalization is detectable after treatment for 15 min with 1 μM dexamethasone. Bar, (*A*) 30 μm.
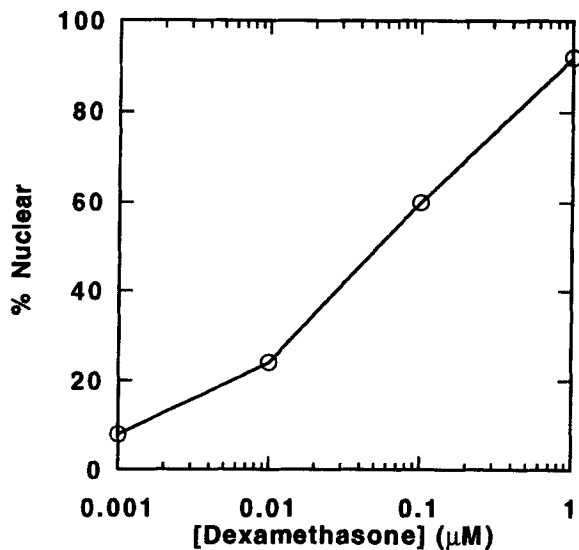
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Figure 2. Dose response for dexamethasone-dependent nuclear transport of GR-GFP. Cells were transfected with pK7-GR-GFP as described in Fig. 1. 2 d after addition of DNA, separate cultures were treated with various doses of dexamethasone for 15 min at 37°C. The living cells were washed with Hepes-buffered saline and examined by epifluorescence to count the proportion of cells in which the GR-GFP was predominantly nuclear. At least 100 transfected cells were counted for each concentration of agonist.

fected with HA1-Ran. This upper band corresponds in size to that of HA1-Ran detected using the anti-HA1 monoclonal antibody, 12CA5 (Fig. 3 A). When the bands were quantitated by densitometry, the density of the upper band was found to be ~47% of that corresponding to endogenous Ran. Given that the proportion of cells transfected with GFP was 0.3, and that the transfection efficiencies for pK7-GFP and pKH3-Ran were equal, we estimate the level of expression of HA1-Ran to be ~1.6-fold that of the endogenous Ran GTPase.

Western blots of cells transfected with the pKH3-Ran mutants indicate that all of these constructs are expressed at approximately equal levels (Fig. 3 B).

These data demonstrate that we can express epitope-tagged Ran at levels similar to those of endogenous Ran. This level is expected to be sufficient to interfere with Ran function, and is not so large that interference could be ascribed to nonspecific effects.

Effects of Cotransfection of Ran Mutants on GR-GFP Nuclear Translocation

To determine the effects of the heterologous expression of Ran on agonist-dependent GR-GFP nuclear accumulation, we cotransfected vectors expressing either wild-type or mutant HA1-epitope tagged Ran/TC4 GTPase together with pK7-GR-GFP.

Ran vectors were cotransfected with GR-GFP at ratios of 0:1, 0.25:1, 0.5:1, and 1:1. Higher concentrations of Ran mutants were rapidly toxic. The qualitative effects of Ran expression were determined by direct examination of living cells. However, to quantitate the import, transfected cells were fixed at intervals after addition of dexamethasone and examined by confocal microscopy. Images were

recorded and quantitated by summing pixel values separately within the nucleus and within the cytoplasmic compartment. Wild-type HA1-tagged Ran did not affect the rate of GR-GFP nuclear translocation at any concentration tested (Fig. 4, A and B).

A mutant Ran (G19V) that does not respond to Ran-GAP and is predominantly GTP-bound when isolated from transfected BHK21 cells (Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication), significantly slowed the rate of import of GR-GFP into the nucleus in response to dexamethasone. The effect was dose-dependent (Fig. 4, A and B). Therefore, G19V Ran appears able to dominantly interfere with nuclear protein import in intact cells.

ΔC-Ran, a mutant lacking the COOH-terminal acidic residues DEDDDL, inhibited GR-GFP import more potently than did Ran G19V (Fig. 4, A and B). While the nuclear/cytoplasmic ratio for 0.5 μg of G19V Ran was ~0.5, no nuclear GR-GFP was observed on transfection of 0.5 μg of ΔC-Ran over this time period.

A third mutant, T24N, is analogous to a dominant-loss-of-function mutation in Ras, and binds with high affinity to the exchange factor, RCC1 (Dasso et al., 1994; Klebe et al., 1995; Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication). Others have observed that T24N Ran interrupts cell cycle progression in Xenopus oocyte extracts, but has no effect on nuclear transport (Kornbluth et al., 1994). However, we found that T24N inhibited GR-GFP nuclear accumulation as efficiently as did the ΔC-Ran mutant (Fig. 4, A and B). This result is consistent with a mechanism for Ran function in which the protein must cycle between the GDP- and GTP-bound states.

A fourth mutant, L43E, has been found to interact only weakly with Ran-binding proteins, and to be insensitive to Ran GAP. In intact cells a significant fraction of L43E is GTP-bound, and it associates almost exclusively with the nuclear envelope (Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication). We were surprised, therefore, to observe no inhibition of GR-GFP nuclear transport by L43E (Fig. 4, A and B). The mutant Ran was expressed at a level similar to that of the other forms of Ran that were examined, but rates of nuclear accumulation were almost identical to those of mock-transfected cells.

Effects of Micro-injected Ran Mutants on GR-GFP Nuclear Translocation

The results described above were obtained about 40 h after cotransfection. During this period many cellular processes may have been perturbed by the Ran mutants. Therefore, the data do not prove that Ran is directly involved in the nuclear translocation of GR-GFP. To circumvent this problem, we prepared recombinant Ran mutant proteins and micro-injected them into BHK21 cells that expressed GR-GFP (as determined by fluorescent microscopy of the living cells). After permitting a brief recovery period (30 min), the injected cells were challenged with dexamethasone for 15 min. As shown in Fig. 5, wild-type Ran did not interfere with nuclear translocation, but the G19V, T24N, and ΔC mutants all potently inhibited
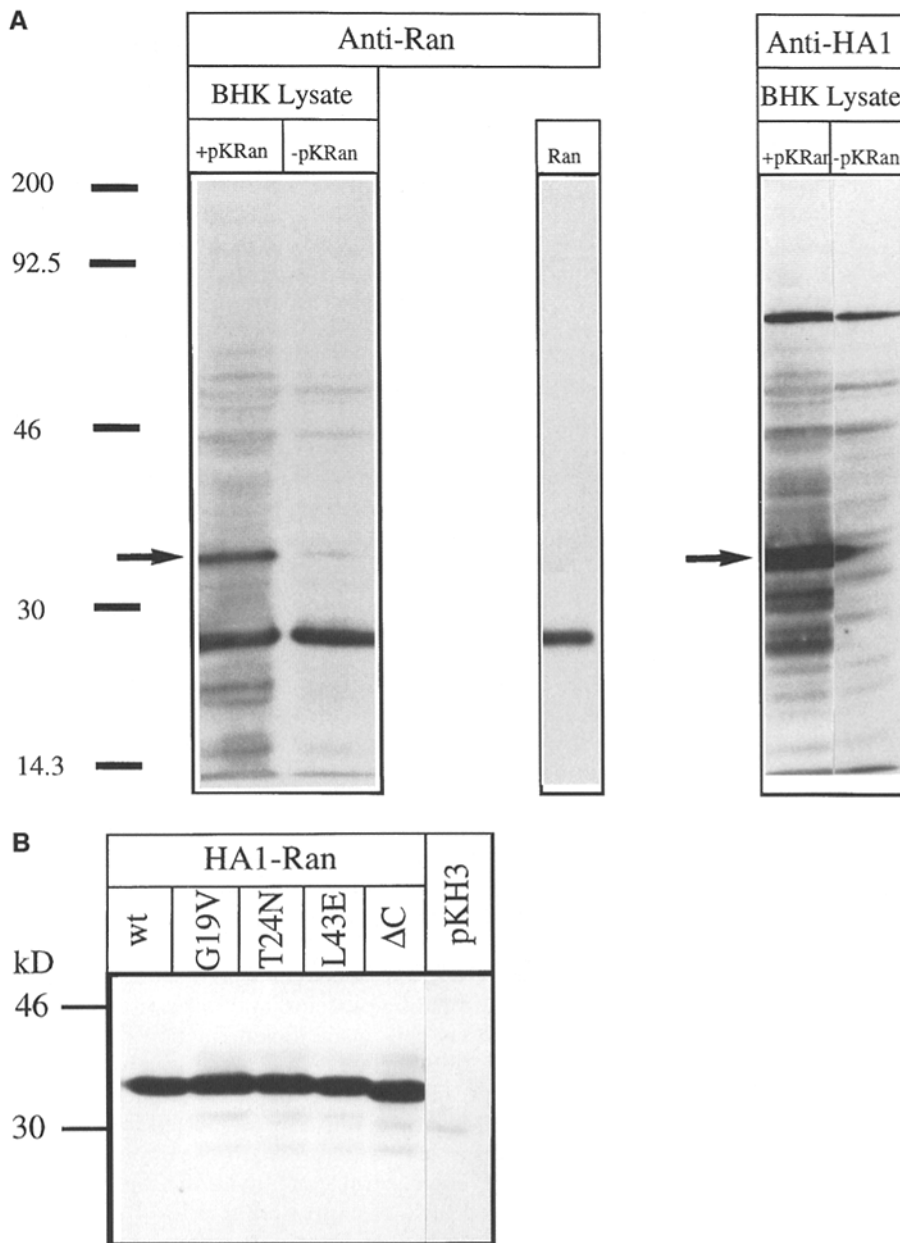
A

Anti-Ran

BHK Lysate

+pKRan -pKRan

Ran

Anti-HA1

BHK Lysate

+pKRan -pKRan

200

92.5

46

30

14.3

B

HA1-Ran

kD

wt G19V T24N L43E ΔC pKH3

46

30

Figure 3. Relative expression levels of endogenous Ran and heterologously expressed HA1-Ran. (*A*) Expression of wild-type HA1-Ran. BHK21 cells (100-mm plates) were transfected with 15 μg of pKH3-Ran or pKH3, as described in Materials and Methods. After 48 h, the cells were rinsed with phosphate-buffered saline, and then lysed directly into Laemmli sample buffer and analyzed by SDS-PAGE. Proteins were transferred to nitrocellulose from the gel and parallel lanes were probed with either anti-Ran antiserum (*left panel*) or anti-HA1 tag antibody 12CA5 (*right panel*). Detection was by chemiluminescence. 10 ng of recombinant Ran protein (*middle panel*) served as a control for the anti-Ran antiserum. The dark bands at ∼25 kD in the left and center panels correspond to the expected location of endogenous Ran. The arrow refers to a band corresponding to HA1-tagged Ran. (Note that in the right panel the intensity of the anti–HA1-stained band was so great that it spilled over slightly into the adjacent lane. Some degradation is apparent in the HA1-Ran transfected lane.) (*B*) Relative expression levels of HA1-Ran mutants. Transfections were performed as in *A*, and cell extracts containing equal amounts of total protein were analyzed by SDS gel electrophoresis and immunoblotting with 12CA5 antibody.

GR-GFP accumulation in the nucleus. Notice in Fig. 5 that an uninjected cell, neighboring two that were injected with G19V Ran, underwent normal nuclear translocation, proving that the manipulation of the plate during micro-injection did not adversely affect the ability of the cells to respond to dexamethasone.

Significantly, the micro-injected L43E mutant of Ran did not inhibit nuclear transport (Fig. 5).

These results support the data obtained by cotransfection, and indicate that the observed inhibition of GR-GFP translocation by Ran mutants is a direct effect on nuclear transport, rather than a long-term, indirect response.

Figure 4. Effect of Ran mutants on nuclear transport of GR-GFP. (*A*) Time courses for nuclear transport of GR-GFP in BHK cells cotransfected with 2 μg pK7-GR-GFP plus 0 μg (*open circles*), 0.5 μg (*closed triangles*), 1.0 μg (*open diamonds*), or 2.0 μg (*open squares*) of the indicated Ran mutant in pKH3. pKH3 vector containing no insert was added to equalize (to 4 μg) the total amounts of DNA transfected in each experiment. After 48 h the cells were treated with 1 μM dexamethasone for the indicated times, and then fixed and stained for HA1-Ran expression as described in Materials and Methods. Nuclear/cytoplasmic ratios of GR-GFP were quantitated for at least 10 cotransfected cells for each data point, using the Bio-Rad MRC-1000 confocal microscope as described in Materials and Methods. Error bars where large enough to be visible are ±1 SD. (*B*) Representative images of GR-GFP subcellular distribution in cotransfected BHK21 cells 15 min after addition of 1 μM dexamethasone. Bar, 20 μm.
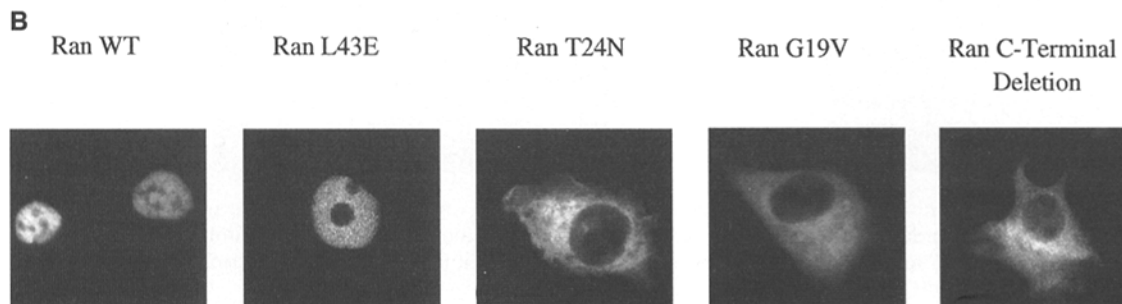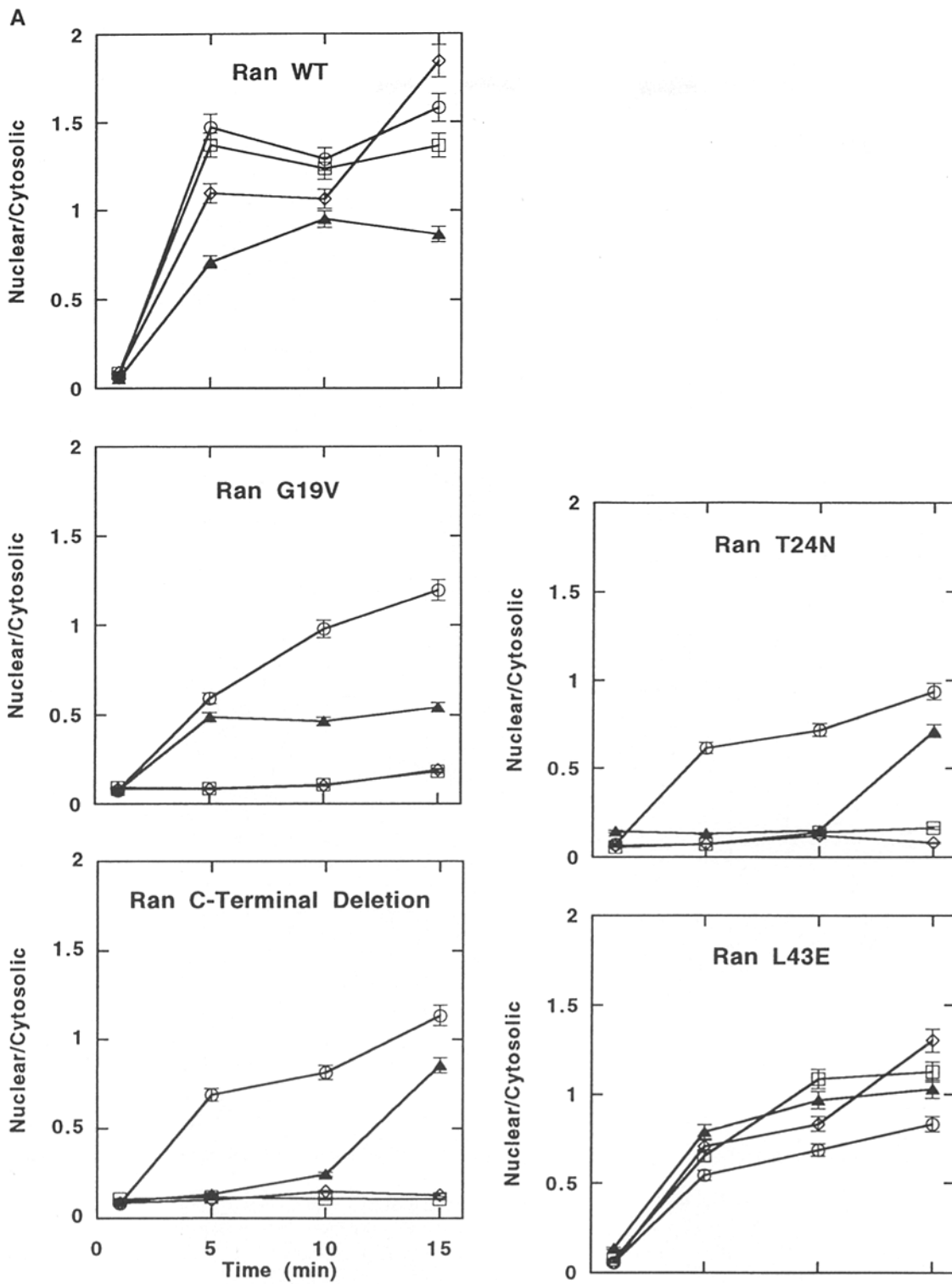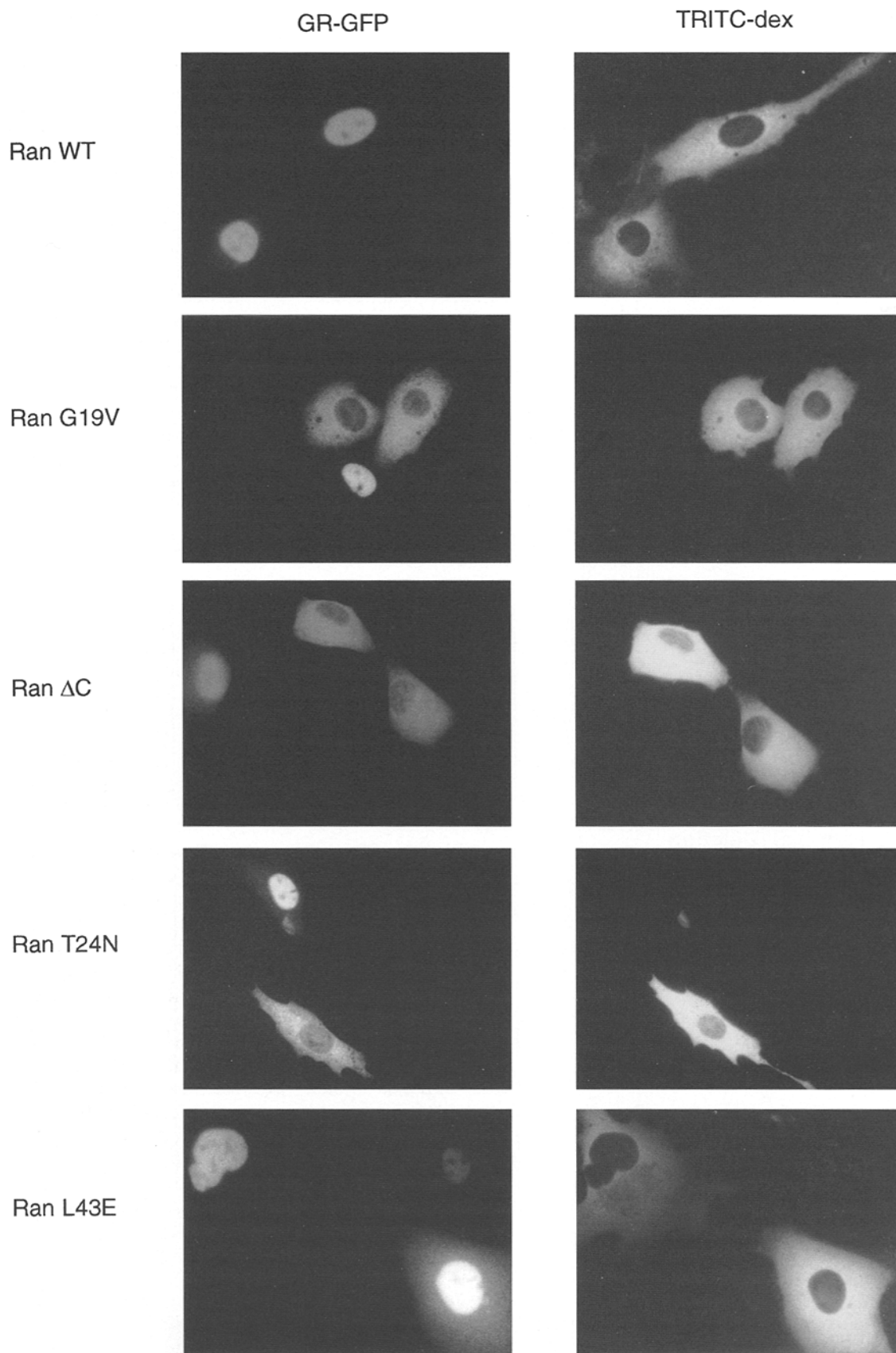
Figure 5. Effects of micro-injected recombinant Ran proteins on nuclear accumulation of GR-GFP. Cells were transfected with pK7-GR-GFP as in Fig. 4. After 48 h cells expressing GR-GFP were selected by fluorescence microscopy and micro-injected with recombinant Ran proteins (2 mg/ml) plus TRITC-dextran (1 mg/ml) in 10 mM sodium phosphate, pH 7.1, 70 mM KCl, 1 mM MgCl₂. Cells were
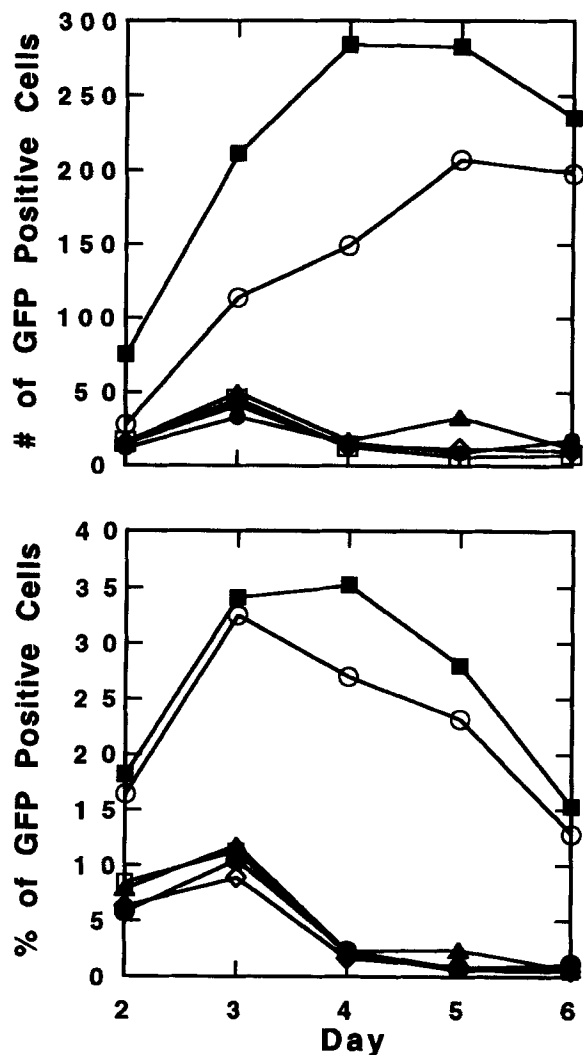
Figure 6. Effects of Ran mutants on cell growth. Cells were plated at 10% confluence and were transfected as in Fig. 1 with 10 μg of pK7-GFP plus pKH3-Ran (wild-type or mutants), or pKH3 as a vector control. The GFP was used as a transfection marker. On days 2–6, 10 random fields of cells were examined in each plate by bright-field and fluorescence microscopy, to score a total number of cells in the field, and a number of green fluorescent cells. Data are represented as a total number of GFP-positive cells (*A*) and as the number of GFP-positive cells as a proportion of the total number of cells in the field (*B*). ■---■, vector control; ○---○, wild-type Ran; □---□, G19V; ◇---◇, T24N; ●---●, ΔC; ▲---▲, L43E.

Effect of Ran Mutants on Cell Growth

We had observed that high doses of plasmid DNA encoding Ran mutants were rapidly toxic to cells. This effect was to be expected for those mutants that inhibit cyto-nucleoplasmic transport, but a similar response was apparent for the L43E mutant. We therefore examined the effect of

HA1-Ran expression on cell growth. BHK21 cells were cotransfected with pK7-GFP plus either pKH3 as a vector control, or with pKH3-Ran mutants, and cells were counted over 5 d. The pK7-GFP vector expresses unfused GFP, which we used as a marker for the transfected cells. Results are presented in Fig. 6. Expression of wild-type HA1-Ran has little effect on cell growth, as compared to the vector control. Note that the green cells increase both in absolute number and as a percentage of the total number of cells counted between days 2 and 3 (Fig. 6 *B*). This effect is most likely a result of the continuing accumulation of GFP, which causes more transfected cells to become visibly fluorescent. Then between days 4–6, the percent GFP-positive cells falls as the plasmid is diluted out of the cells as they divide. This effect is normal for transiently transfected cells.

Importantly, all of the Ran mutants, including L43E potently inhibit cell growth particularly from day 3 onwards. This result strongly suggests that Ran participates in some essential function other than the import of nuclear protein cargo, and that the L43E mutant dominantly interferes with this function.

Discussion

We have shown that a fusion protein of glucocorticoid receptor and green fluorescent protein (GR-GFP) translocates into the nucleus in response to the receptor agonist, dexamethasone. This translocation can be visualized over time in single living cells and obeys kinetics similar to those reported for the endogenous glucocorticoid receptor. The system provides a unique opportunity to observe the nuclear transport of physiologically relevant nuclear cargo in real time, and to test the effects of various components of the nuclear transport machinery in an in vivo situation. Cotransfections with unfused GFP proved useful for determining transfection efficiency without destruction of the cells, and for measuring cell growth rates of cotransfected cells.

It is important that results obtained using in vitro or permeabilized cell assays be corroborated by comparison with more physiologically relevant assays. Ran is rapidly depleted from the nucleus after permeabilization of the plasma membrane (Moroianu and Blobel, 1995), resulting in an inverted Ran gradient across the nuclear envelope in the in vitro assay system. This inversion very likely perturbs the normal transport process. Additionally, RNA molecules may become trapped within the nuclear pores as cytosolic components are washed out of the cells. Conceivably, therefore, in vitro assays of nuclear protein import may identify cytosolic components that are not directly involved in this process, but are instead essential either to re-establish a nucleo-cytoplasmic gradient or to release molecules from the pores that are inactivating the protein import machinery. Moreover, slightly different

allowed to recover for 30 min at 37°C, and were then challenged with 1 μM dexamethasone for 15 min. Cells were rinsed in isotonic buffer then examined by fluorescence microscopy to determine the subcellular location of the GR-GFP. Panels on the left show TRITC fluorescence, which was used as an injection marker. Panels on the right show GR-GFP fluorescence. At least 30 cells were injected for each Ran protein, and results identical to those shown in the photographs were obtained in two independent experiments.

protocols for the in vitro assays can lead to different conclusions about what factors are essential for nuclear protein import, depending on the level of depletion from the permeabilized cells (Melchior and Gerace, 1995).

One concern with using cotransfection as an in vivo approach to study nuclear transport is that the protein of interest is expressed for many hours before the assay, during which time it might interfere with multiple cellular processes. However, the advantage of GR-GFP is that the living cells that express this construct are fluorescent and can be micro-injected with recombinant proteins. The effects of these proteins can then be studied within minutes after introduction into the cells.

We have used the GR-GFP system to examine the effects of the expression of various mutants of the Ran GTPase on nuclear transport. Mutants were introduced into the cells either by cotransfection or by micro-injection of recombinant proteins. Importantly, results obtained by both methods were identical, and support the conclusion that the phenotypes observed are mediated directly by the Ran mutants on nuclear transport rather than through an indirect effect on other processes.

We found that while wild-type Ran has no effect on GR-GFP translocation into the nucleus, the constitutively activated Ran mutant G19V blocks the nuclear accumulation of GR-GFP. This result agrees with predictions from earlier in vitro work using nonhydrolyzable analogues of GTP (Melchior et al., 1993), or recombinant G19V Ran and Xenopus oocyte nuclei (Kornbluth et al., 1994), and from studies in yeast using a constitutively nuclear substrate (Schlenstedt et al., 1995). Therefore, it is likely that the G19V Ran is interfering directly with protein import. However, Schlennstedt et al. (1995) demonstrated that in yeast this mutant also blocks mRNA export, and it remains possible that the inhibition of GR-GFP import is a secondary consequence of a disruption of the nuclear export machinery by G19V Ran.

The effects of other Ran mutants in our GR-GFP assay diverge significantly from those obtained in alternate systems. Using an in vitro nuclear transport assay, Ren et al. (1995) observed no inhibition of nuclear import by a ΔC Ran mutant, whereas we find that this mutant potently inhibits the dexamethasone-induced nuclear translocation of GR-GFP. The ΔC Ran does not bind efficiently to RanBP1 and RanBP2, but it does interact in a Ran overlay assay with three other proteins, of ~90, 115, and 120 kD (Lounsbury et al., 1994, 1996). Wild-type Ran does not interact efficiently with these proteins in overlay assays except in the presence of RanBP1 or an isolated Ran-binding domain. We have identified the 90-kD protein as β-karyopherin (also called β-importin and p97) (Lounsbury et al., 1996). This protein is an essential component of the nuclear docking complex, and is required for transport of protein cargo through the nuclear pore complex (Adam and Adam, 1994; Radu et al., 1995; Gorlich et al., 1995). The inhibition of nuclear transport of GR-GFP by ΔC Ran may therefore be a result of its inappropriate interaction with β-karyopherin/importin. The absence of any effect on in vitro import may be a consequence of the abundance of β-karyopherin/importin in the cell extract used for the assay.

Ren et al. (1994) were also unable to detect any effect of the ΔC mutant of Ran on cell cycle progression in 293/Tag

cells, although the G19V mutant was inhibitory, arresting cells predominantly in the G2 phase of the cell cycle. We found that all of the Ran mutants tested were strongly inhibitory to cell growth when expressed in BHK21 cells. This difference may reflect the more extended period of time over which we were able to observe the BHK21 cells. The 293/Tag cells express the SV40 large T antigen and can be studied only over a period of about 48 h before the replicating plasmid begins to cause nonspecific toxic effects. There may be other differences related to the very high expression level of the Ran in 293/Tag cells because we find that ΔC Ran is primarily cytosolic (Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication), whereas in the 293/Tag cells it appears to be nuclear (Ren et al., 1994).

Kornbluth et al. (1994) observed no inhibition of nuclear import of an artificial karyophile into Xenopus oocyte nuclei by the T24N Ran mutant, whereas we find that this mutant potently inhibits GR-GFP nuclear translocation. One possible mechanism for inhibition by T24N is that the mutant binds to and sequesters the nuclear Ran exchange factor, RCC1, and thereby prevents regeneration of the GTP-bound state of the endogenous Ran GTPase (Dasso et al., 1994). A second possibility is that the T24N mutant of Ran binds to and sequesters another essential component of the transport machinery, such as NTF2, which can associate with Ran in the GDP-bound state (Melchior and Gerace, 1995; Lounsbury, K., unpublished observations).

The difference between our results in BHK21 cells and those with the Xenopus system may be reconciled if a significant run-down time is required before the disruption of RCC1 function can have an impact on nuclear protein import. For example, a significant fraction of the total endogenous Ran protein in the cell (0.3% of cell protein) may normally be GTP-bound and be competent to maintain transport, and it may take a long time to completely deplete the pool of GTP:Ran, especially within the large nucleus of the oocyte. In our BHK21 cell micro-injection assay the T24N Ran will have been present within the cell cytosol for only 30 min before addition of dexamethasone, but in these small cells 30 min may be sufficient time to reduce the abundance of GTP:Ran below a level required to sustain transport.

The most surprising outcome of these studies of Ran mutants in the GR-GFP translocation assay was the lack of any inhibitory effect by L43E. This mutation is in the putative effector domain of Ran (Sheffzek et al., 1995), and reduces the affinity of the GTPase for RanBP1 and -2 (Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication). L43E also is not sensitive to RanGAP activity, but undergoes normal RCC1-stimulated nucleotide exchange. When expressed in BHK21 cells it accumulates almost exclusively at the nuclear envelope, and shows a patchy distribution presumably because of association with the nuclear pore complexes (Lounsbury, K.M., S.A. Richards, K. Carey, and I.G. Macara, manuscript submitted for publication). One explanation of these data is that the association of the L43E mutant with the nuclear pores is sufficiently weak that it can be displaced by endogenous Ran, which is able to mediate nuclear transport. L43E Ran remains at the

pore because it cannot be converted to the GDP-bound state by RanGAP to complete the GTP:GDP cycle.

Despite the lack of any effect of L43E on nuclear transport of GR-GFP, this Ran mutant was inhibitory to cell growth. Wild-type HA1-Ran had no such inhibitory effect. These observations provide the first evidence that Ran may provide an essential cellular function additional to its known role in nuclear protein import. The L43E mutant will likely provide an important tool in further investigations of this function.

Taken together, the Ran mutant data support a cyclical mechanism for Ran function in nuclear transport, similar to that proposed by Bourne (1988) for regulating vesicle traffic, rather than the switch mechanism of the Ras GTPases (e.g., Satoh et al., 1992). In a switch mechanism, dominant gain-of-function and loss-of-function mutants of the switch have opposite phenotypes when expressed in cells. Gain-of-function Ras mutants, that are constitutively GTP-bound, are oncogenic in NIH 3T3 cells, while the T17N loss-of-function mutant is predominantly GDP bound and can inhibit cell growth and transformation. In a cyclical mechanism, on the other hand, arrest of the GTPase in either the GTP- or GDP-bound states results in the same phenotype. This mechanism is exemplified by ARF, a GTPase which is involved in vesicle movement. Both gain- and loss-of-function ARF mutants inhibit vesicle traffic from the endoplasmic reticulum. The underlying mechanism of this inhibition differs for the gain- or loss-of-function mutants (e.g., Dascher and Balch, 1994), but the end result is the same.

If the Ran GTPase functioned as a switch, we would predict that the G19V or T24N mutants would have opposite effects: one would activate nuclear transport, and the other would inhibit. However, we observe inhibition by both the activated and inactivated mutants, which supports the cyclical model. One caveat to this conclusion is that the G19V Ran may inhibit nuclear protein import indirectly, as a consequence of a block to mRNA export. We expect assays that can identify individual steps in nuclear translocation will distinguish the mechanisms by which these mutants exert their inhibitory effects.

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