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Loss of RCC1 Leads to Suppression of Nuclear Protein Import in Living Cells*

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The role of RCC1-Ran/TC4 in nuclear protein import was examined in living cells using a temperature-sensitive RCC1 mutant cell line, tsBN2, and tsBN2 transformed with a RCC1 cDNA lacking the nuclear localization sequence domain, Δ8–29. Substrate, containing a small number of SV40 T antigen nuclear localization sequence peptides, injected into the cytoplasm of tsBN2 cells cultured at the non-permissive temperature of 39.5 °C did not accumulate efficiently in the nucleus. When the same substrate was injected into the cytoplasm of heterokaryons of tsBN2 and wild type BHK21 cells, import efficiency into the tsBN2 nuclei was not restored. Import into the BHK21 nuclei gradually decreased after fusion. In contrast, import efficiency into tsBN2 nuclei gradually recovered after fusion with tsBN2 cells transformed with Δ8–29 in which functional RCC1 was diffusely distributed in both the nuclei and cytoplasm. Substrate did not accumulate in the nuclei of digitonin-permeabilized tsBN2 cells cultured at 39.5 °C even in the presence of normal cytosol. These results suggest that loss of RCC1 function leads to the decline of import competence of the nucleus and accumulation of a factor in the cytoplasm that suppresses nuclear import. These results indicate that the RCC1-Ran/TC4 system may regulate nuclear import.

Nuclear protein import is a receptor-mediated active cellular process that requires ATP (1–4). Selective import can be divided into at least two steps (5–7), nuclear localization sequence (NLS)[1]-dependent binding to nuclear pores and translocation through the pores, with the aid of factors such as the 54/56-kDa NLS-binding protein (8), p97 (9), and 70-kDa heat shock cognate protein (Hsc70) (10–12). Recently, Ran/TC4 was found to be required for cell-free nuclear protein import (13, 14). Ran/TC4 is a member of the Ras superfamily of small GTP-binding proteins (15, 16). It has been suggested to play a key role in the regulation of diverse cellular processes (17–21). The chromatin-associated protein RCC1 (17–20, 22–26) acts as a guanine nucleotide release protein for Ran/TC4 (27). tsBN2 is a temperature-sensitive mutant derived from the BHK21 cell line (28). It has a point mutation in RCC1 (23) and arrests in G_1 or induces premature chromosome condensation at the non-permissive temperature. An examination of the transport of NLS-containing substrates in tsBN2 cells reveals a role for RCC1-Ran/TC4 in nuclear protein import in living cells.

EXPERIMENTAL PROCEDURES

Preparation of NLS-containing Substrates—NLS-containing substrates (T-BSA and T-APC (fluorescein isothiocyanate-labeled bovine serum albumin and allophycocyanin, coupled to peptides containing the SV40 T antigen NLS)) were prepared as described previously (29). For the preparation of two populations of T-BSA, 2 or 0.5 mg of synthetic peptides (CYGGPKKKRKVEDP) were mixed with 4 mg of fluorescein isothiocyanate-labeled BSA activated with sulfosuccinimidyl 4-(*p*-maleimidophenyl) butyrate (Sulfo-SMPB; Pierce). The number of peptides conjugated was estimated by mobility shift assays on SDS-polyacrylamide gels. The preparations had a large number (average 9–11) or a small number (average 3–5) of peptides per BSA molecule.

Cell Lines and Culture Conditions—The tsBN2 cell line is temperature-sensitive and was derived from the BHK21 cell line. Δ8–29 ts$^+$ is the tsBN2 cell line transfected with a deleted RCC1 cDNA clone containing the Δ8–29 allele (30). These cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 33.5 °C (tsBN2), 37 °C (BHK21), and 39.5 °C (Δ8–29 ts$^+$ transformants). For synchronization at G_1, cells were cultured for 48 h in isoleucine-free minimum essential medium-5% dialyzed serum (28).

Microinjection Experiment—T-BSA was microinjected through a glass capillary into the cytoplasm of cells grown on a coverglass. After incubation for 30 min, the cells were fixed with 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1 mM KH_2PO_4).

Cell Fusion by the Hemagglutinating Virus of Japan (HVJ, Sendai Virus)—Cells grown on coverglass were washed in cold BSS-Ca (10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 5.4 mM KCl, and 2 mM $CaCl_2$) and incubated with UV-inactivated HVJ (500 hemagglutinating units/ml) in BSS-Ca on ice for 5 min (31). After washing with BSS-Ca, they were transferred to 39.5 °C in cultured medium. Cells started to fuse within 10 min after transfer.

Indirect Immunofluorescence—The cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and rinsed in 3% skim milk in PBS. They were incubated with rabbit anti-RCC1 or anti-Ran/TC4 antiserum and then with rhodamine isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Co.). After washing with PBS, the DNA was stained by Hoechst 33342. The cells were then examined using an Axiophot microscope (Carl Zeiss, Inc.).

Preparation of Ehrlich Ascites Tumor Cell Cytosolic Extract—Ehrlich ascites tumor cells were freshly harvested from the abdominal cavity of mice, and a cytosolic extract was prepared as described by Adam *et al.* (32).

In Vitro Import Assays—*In vitro* import assays were performed as described previously (12, 32). Digitonin-permeabilized cells were incubated with incubation buffer (20 mM Hepes pH 7.3, 110 mM CH_3COOK, 5 mM CH_3COONa, 2 mM $(CH_3COO)_2Mg$, 1 mM glycoletherdiaminetetraacetic acid, 2 mM dithiothreitol, and 1 μg/ml each of aprotinin, leupeptin, pepstatin, 1 mM ATP, 5 mM creatine phosphate, and 20 units/ml creatine phosphokinase) containing T-APC and cytosolic extract (10 mg/ml) prepared from Ehrlich ascites tumor cells. After incubation at 30 °C for 20 min, the cells were fixed in 4% paraformaldehyde in PBS for 10 min and examined using an Axiophot microscope.

RESULTS AND DISCUSSION

We prepared two populations of SV40 T-antigen NLS peptide-BSA conjugates (T-BSA) containing a large number (average 9–11) or a small number (average 3–5) of peptides per BSA

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[1] The abbreviations used are: NLS, nuclear localization sequence; BSA, bovine serum albumin; APC, allophycocyanin; HVJ, hemagglutinating virus of Japan (Sendai virus); PBS, phosphate-buffered saline; BSS, balanced salt solution.

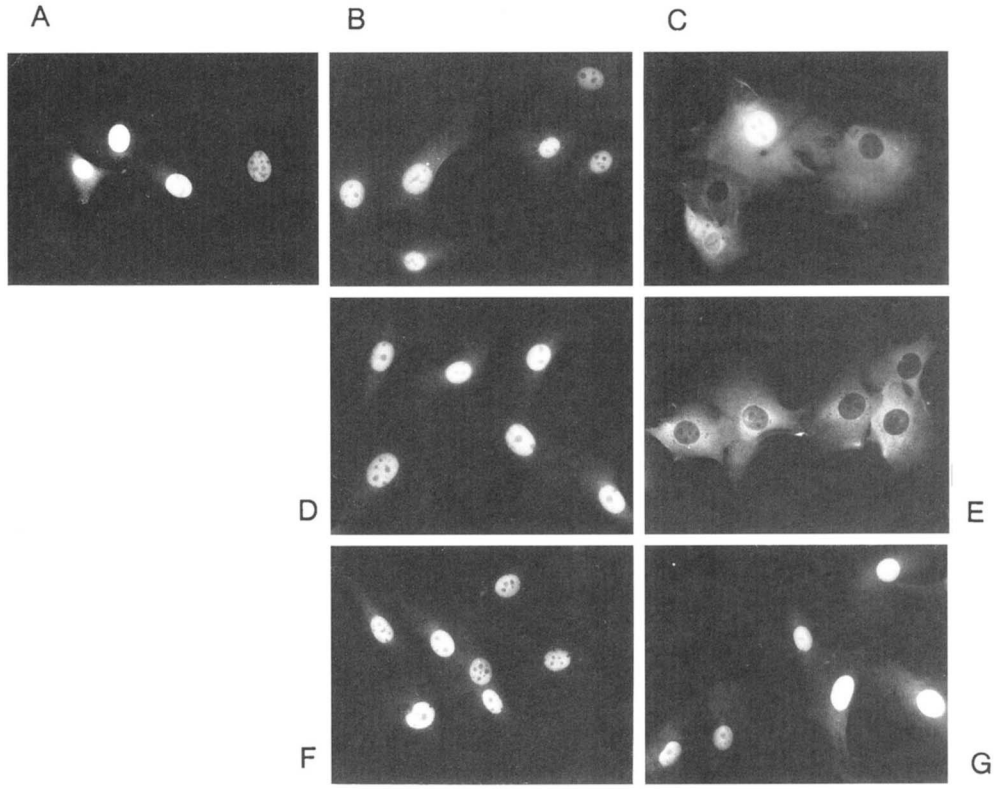

FIG. 1. **Nuclear import of T-BSA microinjected into tsBN2, BHK21, and Δ8–29ts⁺ cells.** tsBN2 (*A–E*), BHK21 (*F*), and Δ8–29ts⁺ (*G*) cells were grown in Dulbecco's modified Eagle's medium-10% fetal calf serum (*A–C*) or isoleucine-free minimum essential medium-5% dialyzed serum at the permissive temperature of 33.5 °C for 48 h for synchronization at G_1 phase (*D–G*) and incubated for 6 h at 33.5 °C (*B* and *D*) or at the non-permissive temperature of 39.5 °C (*A*, *C*, *E–G*). Then T-BSA (1 mg/ml) containing a large number (*A*) or a small number (*B–G*) of NLS peptides was microinjected into the cytoplasm of each cell. After further incubation at 33.5 °C (*B* and *D*) or 39.5 °C (*A*, *C*, *E–G*) for 30 min, the cells were fixed.

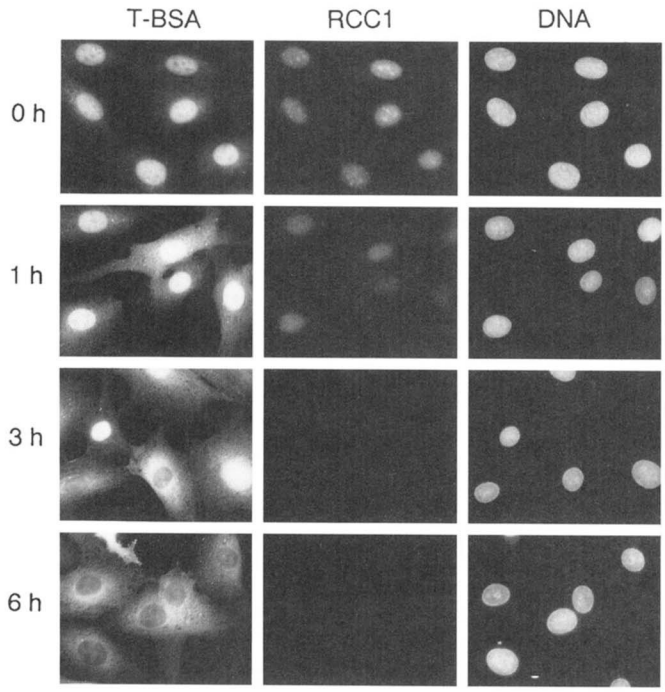

FIG. 2. **Kinetic experiment on nuclear import efficiency in tsBN2 cells after temperature shift to 39.5 °C.** tsBN2 cells were grown at 33.5 °C in isoleucine-free medium for 48 h for synchronization at G_1 phase, transferred to the non-permissive temperature of 39.5 °C, and incubated for 0, 1, 3, or 6 h. Then T-BSA (1 mg/ml) containing a small number of NLS peptides was microinjected into the cytoplasm. After further incubation at 39.5 °C for 30 min, the cells were fixed. The localization of RCC1 was determined by indirect immunofluorescence using rhodamine isothiocyanate-labeled anti-rabbit IgG as second antibody.

molecule for use as substrates. T-BSA containing a large number of peptides efficiently accumulated in tsBN2 nuclei at the non-permissive temperature of 39.5 °C after injection (Fig. 1*A*), which is consistent with a previous report (18). On the other hand, although T-BSA containing a small number of peptides accumulated efficiently within 30 min into tsBN2 nuclei at the permissive temperature of 33.5 °C (Fig. 1*B*) and into BHK21 nuclei at 39.5 °C (Fig. 1*F*), it did not accumulate efficiently into tsBN2 nuclei at 39.5 °C (Fig. 1*C*), and the efficiency varied from cell to cell. When T-BSA containing a small number of NLS peptides was injected into G_1-arrested tsBN2 cells at 39.5 °C, nuclear accumulation was not detected in any cells within 30 min after injection (Fig. 1*E*). These results indicate that nuclear protein import was not inhibited completely, but its efficiency greatly decreased in G_1-arrested tsBN2 cells cultured at 39.5 °C. Therefore, for further *in vivo* analysis, we used T-BSA containing a small number of NLS peptides and G_1-arrested cells.

To determine whether RCC1 affects nuclear import directly, we examined the relationship between loss of RCC1 and nuclear import efficiency by following the time course after a temperature shift. As shown in Fig. 2, after incubation at 39.5 °C for 3 h, no RCC1 could be detected immunocytochemically. When the tsBN2 cells normally grown at 33.5 °C are transferred to 39.5 °C, the guanine nucleotide release protein activity of RCC1 is lost within 20 min.[2] However, microinjected T-BSA still accumulated in some nuclei of tsBN2 cells cultured at 39.5 °C for 3 h. These results show that loss of RCC1 triggers

[2] F. R. Bischoff, personal communication.

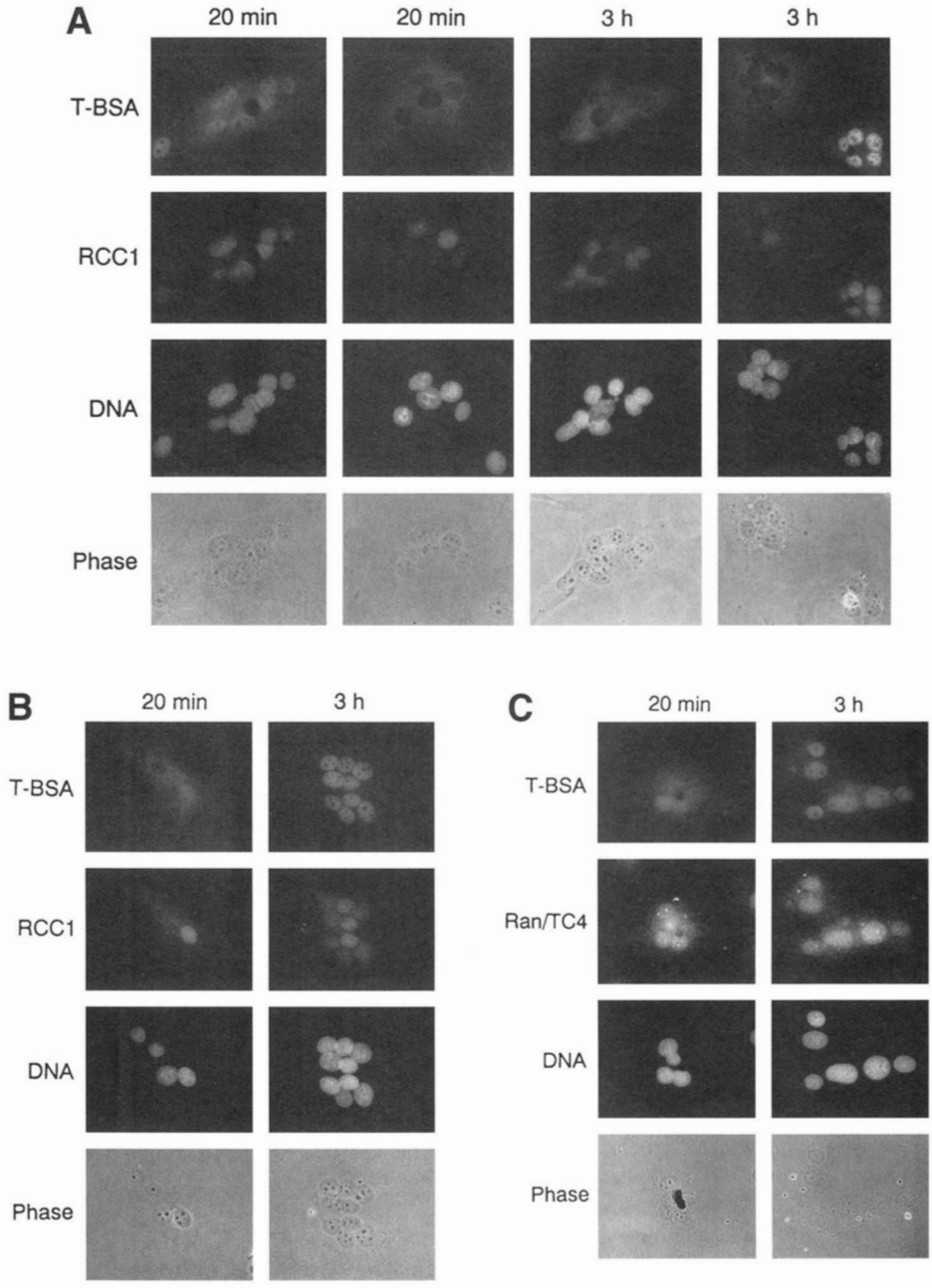

FIG. 3. **Nuclear protein import in tsBN2-BHK21 and tsBN2-Δ8–29ts⁺ heterokaryons.** tsBN2 cells were co-cultured with wild type BHK21 cells (*A*) or Δ8–29ts⁺ cells (*B* and *C*) in isoleucine-free medium at 33.5 °C for 48 h, transferred to 39.5 °C, and incubated for 6 h. They were then fused using HVJ (Sendai virus) (31), and T-BSA containing a small number of NLS was microinjected into the cytoplasm of the heterokaryons at 20 min or 3 h after fusion. After microinjection, the cells were incubated at 39.5 °C for 30 min and fixed. The subcellular localization of RCC1 (*A*, *B*) and Ran/TC4 (*C*) was determined by indirect immunofluorescence using rhodamine isothiocyanate-labeled anti-rabbit IgG as second antibody.

the decrease of nuclear import efficiency, but RCC1 itself does not act directly on nuclear import.

To examine whether the supply of RCC1 leads to recovery of nuclear import efficiency *in vivo*, we used the HVJ (Sendai virus)-mediated cell fusion method (31). After co-cultivation at 39.5 °C for 6 h, we constructed heterokaryons between wild type BHK21 and tsBN2 cells and then microinjected T-BSA into the cytoplasm. T-BSA microinjected at 20 min after fusion did not accumulate into the tsBN2 nuclei within 30 min after injection, even in the heterokaryon constructed of six BHK21 cells and only one tsBN2 cell (Fig. 3*A*, *first column*). On the other hand, as the ratio of tsBN2 nuclei to BHK21 nuclei in the heterokaryon increased, the nuclear accumulation of T-BSA into the BHK21 nuclei was worse (Fig. 3*A*, compare *first* and *second column*). Moreover, T-BSA did not accumulate within 30 min after injection into either the BHK21 nuclei or the tsBN2 nuclei in heterokaryons cultured for 3 h at 39.5 °C after fusion. This is true even in heterokaryon containing only one tsBN2 nucleus and seven BHK21 nuclei (Fig. 3*A*, *third column*), although the BHK21 nuclei still contain RCC1 protein. Similar results (not shown) were obtained using Swiss 3T3 cells. These results indicate that neither wild type cell cytoplasm nor nuclei restore nuclear import efficiency into tsBN2 nuclei. They also show that tsBN2 cell cytoplasm suppressed import into BHK21 nuclei *in trans*. Therefore, loss of RCC1 activity may make nuclei incompetent for nuclear protein import, and a factor that suppresses nuclear import may accumulate in the cytoplasm.

If an inhibitory factor accumulates in the cytoplasm and it can be converted there into an ineffective form, nuclear import should be restored. Based on this hypothesis, we fused tsBN2 to tsBN2 cells transformed with an RCC1 cDNA lacking the NLS domain, Δ8–29. The latter have functional RCC1 lacking its NLS in both nuclei and the cytoplasm (30). T-BSA accumulated efficiently into the nuclei of Δ8–29ts⁺ cells cultured at both 33.5 and 39.5 °C (Fig. 1*G*). Just after fusion at 39.5 °C, T-BSA did not accumulate into the tsBN2 nuclei within 30 min (Fig. 3*B*, *first column* and Fig. 3*C*, *first column*), similar to the tsBN2-BHK21 heterokaryon. But in the tsBN2-Δ8–29ts⁺ heterokary-

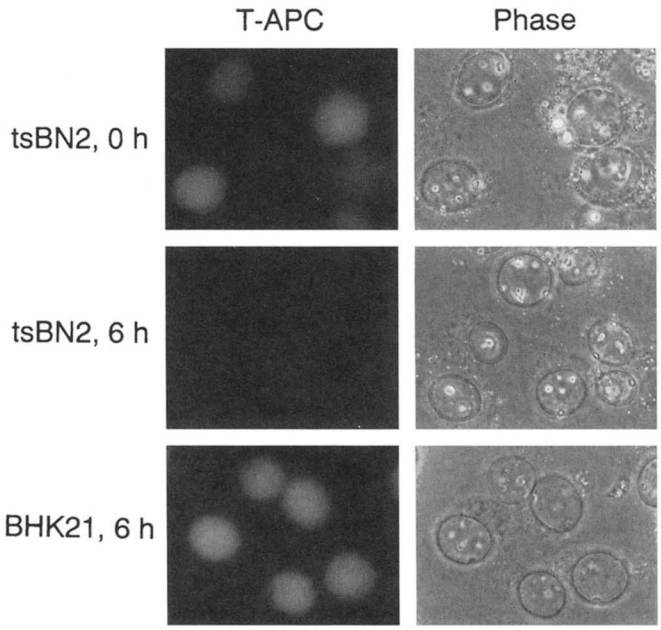

FIG. 4. **Nuclear protein import in digitonin-permeabilized tsBN2 and BHK21 cells.** tsBN2 and BHK21 cells were synchronized in isoleucine-free medium at 33.5 °C for 48 h and then incubated at 39.5 °C for 0 or 6 h. Cells permeabilized with digitonin were incubated with incubation buffer containing T-APC and normal cytosolic extract at 30 °C for 20 min.

ons cultured for 3 h after fusion at 39.5 °C, microinjected T-BSA accumulated efficiently into both tsBN2 and Δ8–29ts$^+$ nuclei (Fig. 3*B*, *second column* and Fig. 3*C*, *second column*).

Next, to confirm that the nuclei of tsBN2 cells cultured at 39.5 °C become incompetent for nuclear protein import, we used an *in vitro* semi-intact cell system (32). Digitonin-permeabilized tsBN2 cells were incubated at 30 °C for 20 min with NLS-containing substrate (T-APC) and normal cytosol prepared from Ehrlich ascites tumor cells. As shown in Fig. 4, T-APC accumulated in the nuclei of permeabilized tsBN2 cells cultured at 33.5 °C but did not accumulate, even in the presence of normal cytosol, when the cells were cultured at 39.5 °C for 6 h before the *in vitro* assay. These results indicate that loss of RCC1 in the nucleus diminishes import competence of the nucleus in living cells.

The fusion experiments and the *in vitro* assay using tsBN2 cells show that loss of RCC1 activity affects the import competence of nuclei. They also suggest that a factor that suppresses nuclear import may abnormally accumulate in the cytoplasm after the loss of RCC1 activity. The only obvious difference between BHK21 and tsBN2 transformed with an RCC1 cDNA lacking the NLS is the location of RCC1 protein. Therefore, we supposed that the abnormally accumulated cytoplasmic suppressor could be converted into an ineffective form by cytoplasmic RCC1.

Because RCC1 was found not to directly affect nuclear import efficiency, we assumed that the phenomena observed due to the loss of RCC1 may be mediated by Ran/TC4. So, we examined the subcellular localization of Ran/TC4 in tsBN2. Ran/TC4 primarily localized in the nucleus at 33.5 °C, but after a temperature shift to 39.5 °C, substantial Ran/TC4 staining of the cytoplasm was detected in tsBN2 cells (not shown), consistent with a previous report (19). When the nuclear import efficiency into tsBN2 nuclei was gradually restored after fusion with Δ8–29ts$^+$ cells, Ran/TC4 re-accumulated in the tsBN2 nuclei (Fig. 3*C*, *second column*).

We speculate that the inhibitory factor accumulating in the cytoplasm may be a GDP-bound form of Ran/TC4 and that decrease of a GTP-bound form in the nucleus may cause import incompetence. From this perspective, our results can be explained if a GDP Ran/TC4 is a cytoplasmic suppressor that may be converted to a GTP-bound form by cytoplasmic RCC1 of Δ8–29ts$^+$ cells. The converted GTP-bound form may then accumulate in tsBN2 nuclei to make the nucleus competent for nuclear protein import. This predicts that the GDP-bound form of Ran/TC4 hardly enters the nucleus even though it is small enough to diffuse passively into the nucleus, whereas the GTP-bound form of Ran/TC4 can efficiently pass through the nuclear envelope. In addition, because RCC1 was also detected in tsBN2 nuclei when their import competence recovered (Fig. 3*B*, *second column*), passive diffusion into the nucleus of RCC1 lacking its NLS and conversion of the GDP-bound form to the GTP-bound form by nuclear RCC1 may also be required for the nucleus to recover its competence.

It is also possible that inhibitory factors other than a GDP-bound form of Ran/TC4 accumulate. Further tests include analysis of nuclear protein import where an NLS-containing substrate and GDP or GTP form of Ran/TC4 are coinjected into the heterokaryon and use of cytosol prepared from tsBN2 cells cultured at a non-permissive temperature in digitonin-permeabilized cell nuclear import assay.

Although the precise function of the RCC1-Ran/TC4 system in nuclear protein import remains unclear, our study indicates that the loss of RCC1 function leads to a decrease of nuclear import efficiency *in vivo*. The results raise the possibility that nuclear import in living cells may actually be regulated by a mechanism mediated by the RCC1-Ran/TC4 system.

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