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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 (NLS). The results showed that loss of RCC1 function leads to the decline of nuclear import competence of the cytoplasm in 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)-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 G1 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.

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.
Regulation of Nuclear Protein Import in RCC1 Mutant Cell

A

B

C

D

E

F

G

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 G1 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. 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. 1A), 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. 1B) and into BHK21 nuclei at 39.5 °C (Fig. 1F), it did not accumulate efficiently into tsBN2 nuclei at 39.5 °C (Fig. 1C), and the efficiency varied from cell to cell. When T-BSA containing a small number of NLS peptides was injected into G1-arrested tsBN2 cells at 39.5 °C, nuclear accumulation was not detected in any cells within 30 min after injection (Fig. 1E). These results indicate that nuclear protein import was not inhibited completely, but its efficiency greatly decreased in G1-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 G1-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. 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

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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. 3A, 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. 3A, 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 heterokaryons containing only one tsBN2 nucleus and seven BHK21 nuclei (Fig. 3A, 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. 1G). Just after fusion at 39.5 °C, T-BSA did not accumulate into the tsBN2 nuclei within 30 min (Fig. 3B, first column and Fig. 3C, first column), similar to the tsBN2-BHK21 heterokaryon. But in the tsBN2-Δ8–29ts" heterokaryon.
ons cultured for 3 h after fusion at 39.5 °C, microinjected T-BSA accumulated in the nuclei of permeabilized tsBN2 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 can easily enter 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. 3B, 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 coimmunoprecipitated from the cell extract and used for in vitro import.

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 can 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. 3C, 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. 3B, 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.

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