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Signal-mediated nuclear transport in simian virus 40-transformed cells is regulated by large tumor antigen

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ABSTRACT **Transformation of cultured cells with simian virus 40 (SV40), or transfection with the early region of the SV40 genome, causes a significant increase in both the rate of signal-mediated nuclear transport and the functional size of the transport channels (located in the pore complexes). By microinjecting purified large tumor (T) antigen into the cytoplasm of murine BALB/c 3T3 cells, we have demonstrated that this protein alone can account for the increase in transport capacity. The T antigen-dependent changes can be partially inhibited by cycloheximide and require a functional nuclear localization sequence. Although necessary, the nuclear localization sequence by itself cannot produce the observed variations in nuclear permeability and presumably functions in a "helper" capacity, in association with another, as yet unidentified domain.**

A variety of biological processes, including hormonal stimulation (1), cell division (2), and differentiation (3–5), are dependent on changes in the nucleocytoplasmic distribution of specific polypeptides. Regulation of the intracellular protein distribution can be achieved by (*i*) reversible binding to cytoplasmic anchors, (*ii*) masking the nuclear localization sequence (NLS), (*iii*) posttranslational changes that alter NLS activity, or (*iv*) modification of the transport machinery. Consistent with the last mechanism, i.e., that the nuclear pores or associated cytoplasmic factors are directly involved in controlling cellular activity, we recently reported significant differences in signal-mediated nuclear transport between proliferating and quiescent cells (6, 7). These changes involved a reduction in the functional diameter of the transport channels from ≈230 Å in proliferating populations, to 190 Å in growth-arrested 3T3-L1 cells, and <140 Å in quiescent BALB/c 3T3 cells. In addition, the relative rate of nuclear import decreased as cells entered the resting state. These differences in transport capacity could significantly affect intracellular exchanges of ribonucleoprotein particles and protein aggregates and, at least in part, account for the overall decrease in metabolism that occurs during quiescence.

In this study we have used simian virus 40 (SV40)-transformed cells to further examine the relationship between nucleocytoplasmic exchange and cell function. Transformation is dependent on the expression of the early region of the SV40 genome that encodes the large (T) and small (t) tumor antigens (8, 9). In numerous cell types, T antigen alone can induce the transformed state (10); however, in some cells the "helper effect" of t antigen is also necessary (11, 12). T antigen is a multifunctional protein that contains a NLS (13, 14), has specific binding sites for viral DNA (15) and tumor-suppressor proteins (16–19), and, among other activities, can regulate transcription of the SV40 genome (20, 21), initiate viral DNA synthesis (22, 23), and stimulate host cell DNA and RNA synthesis (24, 25). The objective of this study was to determine whether the increased level of nuclear activity that is required for transformation is accompanied by changes in the functional properties of the nuclear pores.

METHODS

Cell Cultures. BALB/c 3T3 A31 cells (mouse embryo fibroblast line) and the transformed cell lines MOP-8 and SV-T2 were obtained from the American Type Culture Collection. The MOP-8 line resulted from transformation of NIH 3T3 cells with a transcription unit consisting of the SV40 promoter fused to the early region of polyomavirus. SV-T2 was derived from BALB/c 3T3 by transformation with SV40. 3T3-L1 cells (preadipocyte line derived from NIH 3T3) were a gift from Susan Frost (University of Florida). All cells were grown in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium containing 4 mM glutamine and 4.5 mg of glucose per ml and supplemented with 10% calf serum, penicillin G (10,000 units/ml), streptomycin (10 μg/ml), and amphotericin B (250 μg/ml). Subcultures were prepared every 3 days. Culture reagents were obtained from GIBCO/BRL. To facilitate subsequent electron microscopy analysis, experimental cells were plated on gridded ACLAR coverslips 24 hr prior to injection, as described by Feldherr and Akin (6).

Gold Preparations and Microinjection. Gold particles 80–290 Å in diameter were prepared by reducing gold chloride with sodium citrate (26). Smaller particles, 20–120 Å in diameter, were obtained by using a saturated solution of phosphorus and ether as a reducing agent (27). The gold particles were coated with nucleoplasmin, which was isolated from *Xenopus* oocytes (27), or bovine serum albumin (BSA, Sigma) according to Dworetzky *et al.* (27). The protein coat increased the diameter of the particles by ≈30 Å. The coated gold particles were microinjected into the cytoplasm of the experimental cells by using a continuous flow system (6). The injection medium was 117 mM KCl/10 mM NaCl/6 mM K_2HPO_4/4 mM KH_2PO_4, pH 7.0.

Transformation Procedures. Transformation of BALB/c cells with the plasmid pSV3neo (American Type Culture Collection, no. 37150), which contains the early region of the SV40 genome plus a gene that confers resistance to Geneticin (G418), was performed as follows. BALB/c cells were treated with calcium phosphate precipitates of linearized plasmids as outlined by Lanford *et al.* (28), and transformants were selected on the basis of colony formation in medium containing methylcellulose (29) and G418 (200 μg/ml) (GIBCO/BRL).

Preparation of T Antigen, the (cT)-3 Mutant, and the BSA-NLS Conjugates. T antigen and the (cT)-3 mutant were synthesized in insect cells (*Spodoptera frugiperda* Sf9 cell line) using baculovirus expression vectors (30). The baculo-

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Abbreviations: BSA, bovine serum albumin; N/C, nuclear/cytoplasmic; NLS, nuclear localization sequence; SV40, simian virus 40.

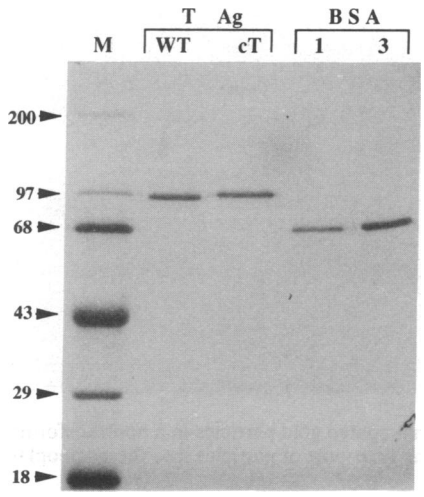

FIG. 1. Analysis of purified wild-type T-antigen (Ag) and (cT)-3 mutant preparations by SDS/PAGE. Lane M, molecular weight markers ($M_r \times 10^{-3}$). The wild-type (WT) and mutant (cT) lanes each contained 3 μg of antigen. For comparison, lanes containing 1 and 3 μg of BSA are included. The gel was stained with Coomassie blue.

virus expressing (cT)-3 was a generous gift from Kari van Zee and Ellen Fanning (Institute for Biochemistry, University of Munich). The proteins were isolated by antibody affinity chromatography. Densitometric analysis of SDS/12% polyacrylamide gels (Fig. 1) revealed that the T-antigen and (cT)-3 preparations were 90% and 93% pure, respectively. Nuclear import of the purified proteins was tested by microinjection into cultured cells and subsequent intracellular localization by immunofluorescence microscopy (31). As expected, the wild-type T antigen accumulated in the nucleoplasm, whereas the mutant remained cytoplasmic. Synthetic tridecapeptides containing either the wild-type T-antigen NLS or the (cT)-3 NLS were prepared and conjugated with BSA as reported by Lanford *et al.* (31, 32).

Electron Microscopy. Nuclear import of the injected gold particles was analyzed directly from electron micrographs. The nuclear/cytoplasmic (N/C) gold ratios were obtained by counting particles in equal and adjacent areas of nucleoplasm and cytoplasm. The size distribution of the particles that entered the nuclei was determined by measuring all particles in randomly selected areas of nucleoplasm. Separate controls were performed for each experimental group to compensate for variations in the size of the gold fractions. The size of the injected particles (particles available for transport) was also determined. Details of the electron microscopy procedures are provided in ref. 6.

RESULTS AND DISCUSSION

Initially, we investigated signal-mediated transport in two established transformed cell lines, MOP-8 (SV40/polyoma transformed) and SV-T2 (SV40-transformed). In separate experiments, these cells were compared, respectively, with proliferating 3T3-L1 and BALB/c 3T3 populations. Both MOP-8 and 3T3-L1 cells are derived from the NIH 3T3 line, and the SV-T2 cells are transformed BALB/c 3T3 A31 cells. The experimental cells were microinjected with nucleoplasmin-coated gold particles that ranged in diameter from 110 to 320 Å, including the 30-Å protein coat. Nucleoplasmin is a karyophilic *Xenopus* oocyte protein that contains a well-characterized NLS (33) and can effectively target gold particles to the nucleus (34). Thirty minutes after injection, the cells were fixed for electron microscopy and both the number and the size distribution of the particles that entered the nucleus were determined (Table 1, experiments A and B; Fig. 2). Relative nuclear uptake, expressed as the N/C gold ratio, was significantly higher in the transformed cells; in addition, measurements of particle size revealed a significantly increased nuclear accumulation of gold particles in the 200- to 240-Å size class. Particles larger than 280 Å (or 310 Å including the protein coat) were unable to enter the nuclei. These results demonstrate that the exclusion limit for particles entering the nucleus was ≈40 Å greater in transformed

Table 1. The N/C gold ratios and the size distribution of particles transported into the nucleus

Experiment*	No. of particles measured	Distribution, % particles† 80–120 Å	120–160 Å	160–200 Å	200–240 Å	240–280 Å	N/C ratio, mean ± SE (counts)	Significance‡ Dist.	N/C
A. 3T3-L1 controls (34)	1060	1.2	16.4	77.5	4.9	0.0	0.56 ± 0.07 (2161)	—	—
MOP-8(41)	1148	0.3	7.8	54.4	29.9	7.5	1.41 ± 0.12 (3354)	s	s
Size of injected gold	3020	0.6	6.2	46.3	31.6	15.3			
B. BALB/c controls (10)	675	1.3	29.3	65.0	4.3	0.0	1.00 ± 0.07 (829)	—	—
SV-T2 (12)	432	0.5	13.7	51.6	34.3	0.0	1.80 ± 0.15 (814)	s	s
Size of injected gold	584	1.2	17.5	44.5	32.9	3.8			
C. BALB/c controls (22)	428	5.8	28.7	60.0	5.4	0.0	0.99 ± 0.16 (1173)	—	—
pSV3neo-transformed (25)	707	2.4	20.4	60.0	16.4	0.8	2.11 ± 0.15 (1228)	s	s
Size of injected gold	766	2.1	17.6	57.2	18.0	5.1			
D. BALB/c controls (16)	519	6.2	37.0	52.6	4.2	0.0	0.71 ± 0.12 (1061)	—	—
BSA injection, 6 hr (16)	364	6.9	35.2	54.4	3.6	0.0	0.63 ± 0.07 (915)	ns	ns
T-Ag injection, 6 hr (19)	545	3.5	21.1	51.0	22.6	1.8	2.03 ± 0.11 (1492)	s	s
T-Ag injection, 1 hr (16)	402	5.2	32.1	53.7	9.0	0.0	0.72 ± 0.07 (1369)	ns	ns
(cT)-3 injection, 6 hr (16)	471	6.2	36.5	51.8	5.5	0.0	1.10 ± 0.11 (1188)	ns	ns
T Ag + cycloheximide (17)	447	2.5	23.7	64.4	9.5	0.0	1.36 ± 0.07 (1196)	s	s
Size of injected gold	793	1.4	18.0	50.3	22.6	7.6			
E. BALB/c controls (16)	434	4.6	26.0	63.4	6.0	0.0	0.90 ± 0.06 (676)	—	—
BSA-WT, 6 hr (15)	553	2.9	24.4	64.2	8.5	0.0	1.05 ± 0.08 (886)	ns	ns
BSA-(cT), 6 hr (17)	482	1.7	24.1	66.8	7.5	0.0	0.88 ± 0.08 (909)	ns	ns
Size of injected gold	554	0.7	14.8	55.6	21.5	7.4			

*No. of cells examined is given in parentheses. Size distribution of injected particles is included because different gold fractions were used in each experiment. T Ag, T antigen; BSA-WT and BSA-(cT), BSA conjugated to wild-type NLS and (cT)-3 NLS, respectively.
†Particle measurements do not include the 30-Å protein coat.
‡Statistical analyses indicate whether the results are (s) or are not (ns) significantly different from the controls. Probability values of 0.01 or below were considered statistically significant.

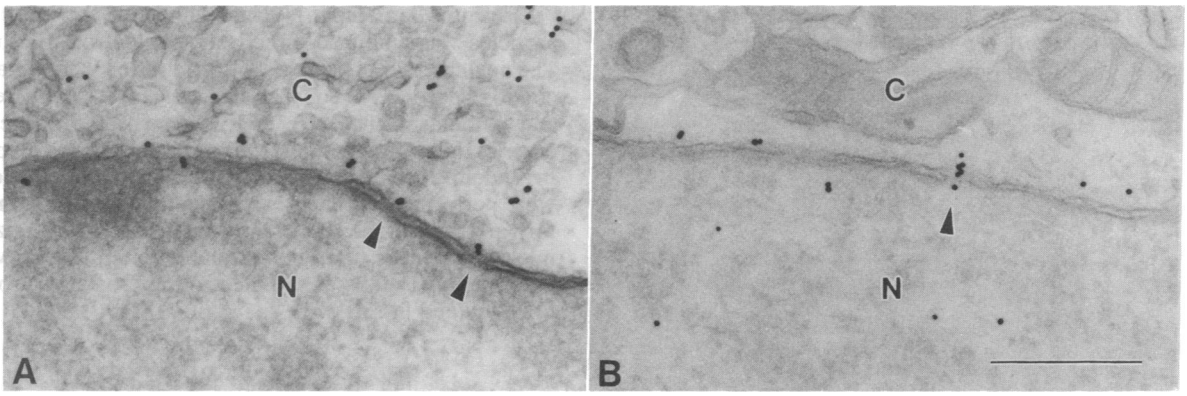

FIG. 2. Electron micrographs showing the intracellular distribution of nucleoplasmin-coated gold particles in a nontransformed 3T3-L1 cell (*A*) and a transformed MOP-8 cell (*B*). The cells were fixed 30 min after gold injection. Transport of particles into the nucleoplasm (N) occurs through the nuclear pores (arrowheads). C, cytoplasm. (Bar = 0.5 μm.)

cells compared with proliferating populations of nontransformed cells.

It is possible that the differences in nuclear import capacity in transformed versus nontransformed cells were due to a difference in the intracellular migration of the gold particles, perhaps reflecting variations in the organization of the cytoskeleton. To determine if this was the case, we compared the size distribution of particles located within 1000 Å of the nuclear pores in SV-T2 and BALB/c cells 30 min after injecting 110- to 320-Å nucleoplasmin–gold. The results, based on the analysis of 494 and 364 particles in transformed and nontransformed populations, respectively, were not significantly different ($P = 0.67$); furthermore, in both cell lines ≈30% of the pore-associated particles were >200 Å in diameter, demonstrating that even particles in this size range, which were largely excluded from the nuclei of BALB/c 3T3 cells, had equal access to the transport sites. In addition, we analyzed the overall cytoplasmic distribution of the gold particles 30 min after injection. SV-T2 and BALB/c 3T3 cells were randomly divided into quadrants, and the percentage of the total cytoplasmic particle count in each quadrant was determined. The percentages, progressing clockwise from the highest value (assumed to be the site of injection), were 38.3, 24.1, 13.5, and 23.9% for the SV-T2 cells and 39.3, 23.4, 17.3, and 19.9% for the BALB/c 3T3 line; 9 and 7 cells were examined, respectively. The difference in the gold distributions was not significant ($P = 0.17$). Taken together, the above results indicate that the intracellular migration of the particles was not a limiting factor in nuclear import.

Further confirmation that transformation increases nuclear transport capacity was obtained by studying BALB/c 3T3 cells that were transfected with the plasmid pSV3neo, which contains the early region of the SV40 genome and encodes T antigen and t antigen. Both the N/C gold ratios and the size of the particles present in the nucleoplasm were significantly larger in the transformed cells than in the nontransformed BALB/c cells (Table 1, experiment C). Nuclear permeability in cells transfected with pSV2neo, which lacks the SV40 genome but otherwise is identical to pSV3neo, was not significantly different from that in BALB/c controls (data not shown).

The effect of T antigen alone on nuclear transport was investigated by direct injection of the purified protein. T antigen was microinjected into proliferating BALB/c 3T3 cells at a concentration of 0.65 mg/ml (≈5% of the cell volume was injected). One and 6 hr after T-antigen injection, nuclear transport was analyzed by injection of nucleoplasmin–gold. In control experiments, the nuclear import of gold was examined in untreated cells or 6 hr after the injection of BSA at 0.65 mg/ml. T antigen caused a significant increase both in the N/C gold ratio and in the functional size of the pores (Table 1, experiment D). The nuclear transport capacity after 6 hr was comparable to that observed in transformed cells. The increase after 1 hr was not significant, demonstrating that the antigen had a gradual effect.

Although T antigen significantly increased signal-mediated transport, it had no effect on passive diffusion through the pores. Diffusion was assayed by using gold coated with BSA, which lacks a NLS. The particles ranged in diameter from approximately 50 Å to 150 Å, including the protein coat. The N/C gold ratios obtained 30 min after particle injection were 0.009, 0.008, and 0.006 for untreated BALB/c controls, 6-hr BSA control injections, and 6-hr T-antigen injections, respectively. Six cells were examined in each group, and the differences were not significant. These results indicate that T antigen does not alter the diameter of the aqueous diffusion channels but, instead, increases their ability to dilate in the presence of a NLS.

To determine whether the T antigen-induced permeability changes required protein synthesis, T antigen was injected into BALB/c cells that were incubated in medium with cycloheximide (50 μg/ml, Sigma) throughout the course of the experiment. This treatment reduced protein synthesis by over 94%. Six hours after T-antigen injection, nuclear transport capacity in the cycloheximide-treated cells was significantly greater than in uninjected BALB/c controls, but significantly less than in T antigen-injected cells maintained in cycloheximide-free medium for 6 hr (Table 1, experiment D; $P < 0.01$ for both the N/C ratios and the size distributions). Interpretation of these data is complicated by the fact that in control experiments a 6-hr treatment with cycloheximide reduced the N/C gold ratio by ≈20% in BALB/c 3T3 cells. This decrease in the basal transport level would offset the increase produced by T antigen. Although it is not possible to precisely determine the requirement for protein synthesis, the results do show that at least a partial increase in transport capacity can occur in the presence of cycloheximide.

Considering the possibility that the T-antigen NLS (Pro-Lys_3-Arg-Lys-Val, residues 126–132) might be involved, either directly or indirectly, in regulating the functional capacity of the pores, we prepared a mutant T antigen, designated (cT)-3, that is deficient in nuclear transport and remains localized in the cytoplasm (14). The deficiency is the result of a single amino acid substitution, in which Lys^{128} is replaced by Asn. The mutant protein was injected into BALB/c 3T3 cells at a concentration of 0.67 mg/ml, and nuclear import of nucleoplasmin-coated gold particles was analyzed 6 hr later. There were no significant permeability differences between these cells and the uninjected or BSA-

injected BALB/c controls (Table 1, experiment D), indicating that a functional NLS was necessary for the enhancement of nuclear transport capacity. In addition, these results demonstrate that the effect of T antigen on nuclear import was not due to impurities introduced during the preparative procedures. If this were the case, the (cT)-3 mutant, which was prepared by the same methods, should also have increased the uptake of nucleoplasmin–gold.

Next, we wished to establish whether the NLS alone is sufficient to regulate pore function, or whether it serves to facilitate the action of a second domain. For this purpose, BSA was conjugated with synthetic tridecapeptides that contained either the wild-type or the (cT)-3 NLS. The NLS is functional in this context, as demonstrated by the fact that the signal peptides can mediate protein transport across the nuclear envelope (e.g., refs. 31, 32, and 35). The conjugates, which contained ≈13 NLSs per BSA molecule, were microinjected into BALB/c cells at a concentration of 0.5 mg/ml. After 6 hr, neither of the conjugates increased nuclear transport beyond the control level (Table 1, experiment E), supporting the view that the NLS plays a "helper" role and possibly functions by directing the antigen to a specific region of the cell where it can exert its effect on transport. This could be either within the nucleus or, perhaps, at the pore complex.

In this investigation we have obtained further evidence that signal-mediated nuclear import is a dynamic process that can vary in response to specific cellular activities. We have demonstrated, in SV40-transformed cells, that transport capacity can be modulated by T antigen. In relation to the viral life cycle, a T antigen-induced increase in nuclear transport would occur following expression of the early genome and would accompany a number of metabolic events required for viral replication (36). These include synthesis of host cell enzymes necessary for nucleic acid production, viral DNA synthesis, the synthesis and translation of late stage mRNA, and the nuclear accumulation of capsid proteins. Since all of these processes depend on macromolecular exchanges across the nuclear envelope, even a nonspecific increase in transport would be expected to promote virion formation. It will be important to identify the T-antigen domain that is directly involved in regulating permeability and localize its site of action within the cell. It will also be important to determine whether T antigen acts directly on the transport machinery or whether it functions by mediating the activity of other factors.

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