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AN NLS IS SUFFICIENT TO ENGAGE FACILITATED TRANSLOCATION BY THE NUCLEAR PORE COMPLEX AND SUBSEQUENT INTRANUCLEAR BINDING

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Summary: We investigated the nuclear transport of a fusion protein consisting of a nuclear localization signal linked to β-galactosidase, normally a cytoplasmic protein. We microinjected the radiolabeled fusion protein into the cytoplasm of living Xenopus oocytes or supplied it directly to the surface of the oil-isolated oocyte nucleus and measured its transport into the nucleus. Our data confirm that a nuclear localization signal is sufficient to entrain a protein's facilitated transport through the nuclear pore complex and its subsequent nuclear accumulation. Moreover, nuclear envelope micropuncture experiments determine that the fusion protein's accumulation results from its intranuclear binding, demonstrating that no specific region of a transported protein – other than the nuclear localization signal itself – is required for facilitated transport and intranuclear binding. Finally, we present evidence that the intranuclear binding of a transported protein requires not only its nuclear localization signal, but also its prior facilitated transport through the nuclear pore complex.

Proteins containing one or more nuclear localization signals (NLS-proteins) are selectively transported through the nuclear pore complex (NPC) of the interphase nuclear envelope more rapidly than is possible by diffusion, and many accumulate to much higher (e.g., > 100:1) nuclear vis-a-vis cytoplasmic concentrations (reviewed in 1). Because NLS-proteins include nuclear enzymes and structural proteins, hormone receptors and other transcription factors, as well as known oncogene proteins, it is crucial to understand the mechanisms responsible for their transport and accumulation.

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Abbreviations: NLS, nuclear localization signal; NPC, nuclear pore complex; Np, nucleoplasmin; OIN, oil-isolated nucleus; B, bead; C, cytoplasm; WGA, wheat germ agglutinin; β-gal, beta galactosidase; SV40 lgT, Simian virus 40 large T antigen; ATPrs, ATP regenerating system.

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Studies of a prototypical NLS-protein, nucleoplasmin (Np), in an oil-isolated nucleus (OIN) transport system identified two previously unresolved mechanisms: (i) facilitated translocation through the NPC by an energy-dependent mechanism, and (ii) subsequent accumulation in the nucleus by intranuclear binding (2). Intranuclear binding of Np has also been identified in intact, living cells (3). The sequential actions of these two mechanisms fully account for the translocation and accumulation of Np, and eliminate the necessity for 'active transport' – a hypothetical, single-step mechanism by which the NPC would establish and maintain gradients in the concentration of diffusive species of NLS-proteins across the envelope (4,5). However, despite considerable indirect evidence, intranuclear binding has not been proven for NLS-proteins other than Np, and a role for active transport of NLS-proteins remains controversial. Therefore, we sought to determine whether the facilitated transport/intranuclear binding model applies to NLS-proteins other than Np, and, if so, whether the NLS is sufficient to entrain intranuclear binding or whether some additional region(s) of the transported proteins is/are required.

Materials and Methods

Fusion Protein Variants (Transportants): We expressed in E. coli vectors (kindly provided by R. Peters) encoding the fusion proteins p4K and p4T. We labeled the proteins in vitro with ^{35}S (SLR, Amersham) or in vivo by supplying the bacteria with [^{35}S]methionine (TRAN^{35}S-LABEL, ICN). In vivo labeling provided a higher specific activity, important in tracer studies with individual cells and nuclei, and obviated the risk of altering the native properties of the proteins. In practice, in vitro labeling was adequate, but we consistently measured higher levels of nuclear accumulation (by a factor of 2-3) with in vivo labeled p4K (e.g., Table 1). [^{14}C]ovalbumin (A 6418) was purchased from Sigma.

Intact Cell Procedures and Isolation of Nuclei and Cytoplasm: Xenopus laevis frogs were maintained at room temperature in dechlorinated water and fed twice weekly. Ovaries were surgically removed into an extracellular medium containing (in mM) 82.5 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 1.0 $MgCl_2$, 1.0 Na_2HP0_4, 3.3 NaOH, 5.0 HEPES, and 2.0 sodium pyruvate, pH 7.6. Full-grown (stages V-VI) (6), folliculated oocytes were manually isolated with microscissors and microinjected with radiolabeled transportants using standard methods (7). Microinjected cells were incubated in extracellular medium nd then transferred to mineral oil for incubation and subsequent enucleation. (Oocytes mineral oil remain viable and display normal metabolic characteristics [8], as well as ective nuclear transport of NLS-proteins [3], for over 12 h.) At different times after oinjection nuclei and cytoplasms were manually isolated from individual oocytes under l, a method which prevents the loss of nuclear contents entailed in aqueous nuclear on procedures, and the radioactivity in each compartment was measured. iately after microinjection and transfer to oil, some oocytes were impaled with a edle (15-20 µm shaft diameter) 4-6 times at the animal pole to a depth > 600 µm to pass entirely through the nucleus). This micropuncture procedure irreversibly zes the nuclear envelope and permits diffusion of unbound proteins between and cytoplasm (3,9,10). Such punctured oocytes were then incubated and under oil, and the nucleocytoplasmic distributions of the microinjected were measured.

OIN-Transport Pairs: The oil-isolated nucleus (OIN) retains normal structure and functions for several hours, including selective transport and accumulation of NLS proteins (11), thus allowing its utilization in OIN:bead (OIN:B) or OIN:cytoplasm (OIN:C) 'transport pairs'(2). Briefly, while observed through a dissecting scope, an OIN is conjoined with either an aqueous, cross-linked agarose bead or a bolus of cytoplasm also isolated under oil (either approximately the size of the nucleus). Prior to forming pairs, the aqueous bead or cytoplasm is preloaded with a radiolabeled transportant. Prior to formation of some pairs, we micropuncture the OIN's envelope 4-6 times with a 22-guage hypodermic needle, permitting diffusion of proteins between the two compartments. At known times after pair formation, nuclei are separated from their paired bead or cytoplasm and the amount of radioactivity in each compartment is measured.

Results and Discussion

We expressed in E. coli the fusion protein p4K which includes the NLS region (amino acids 111-135) of the SV40 large T antigen (lg T) linked to the amino terminus of β-galactosidase (β-gal) (Fig. 1). We also expressed p4T (Fig. 1), identical to p4K except for a single substitution $K^{128} \rightarrow T^{128}$ which abolishes selective nuclear transport. We term lg T residues 111-135 the 'extended NLS', because this region includes a 'minimal NLS' (126-132) – reported to be sufficient to engage selective nuclear transport of hybrid protein constructs (12-14) – plus the amino flanking sequence (111-125) which greatly enhances transport. In earlier studies, p4K conjugated in vitro with 5-iodoacetamidofluorescein and microinjected into the cytoplasm of cultured cells was transported rapidly into the nucleus and accumulated 10-20 fold vis-a-vis the cytoplasm within 30-40 m, while a variant including the minimal NLS but not the flanking sequence showed no measurable nuclear uptake over this time (15). We therefore used p4K with the extended NLS in the present studies.

Intact Cell Transport Experiments: We microinjected radiolabeled p4K and p4T into the cytoplasm of intact, living oocytes, incubated the cells for 18-24 h under aqueous media or oil (the results did not differ), and measured the nucleocytoplasmic distributions of the transportant proteins in individual cells. We found that p4K was selectively transported into the nucleus and accumulated therein, while p4T and the control

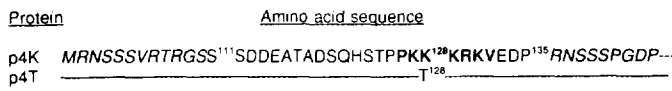

Fig. 1. **Primary structures of NLS-β-gal variants, p4K and p4T.** Beginning with the amino terminus on the left, there is a linker region *(italics)*, the extended lgT NLS residues 111-135 including the minimal NLS *(bold)*, another linker *(italics)*, and E. coli β-galactosidase residues 9-1023 *(horizontal dashed line)*.

Table 1. **Nuclear transport and accumulation in living oocytes.** The [N]/[C] values are means ± s.e.m. for individual cells and include correction for the known fraction of water unavailable as solvent in the oocyte cytoplasm (2).

Transportant	[N] / [C] In Vitro-Labeled Transportant		In Vivo-Labeled Transportant
	Intact Envelope	Punctured	Intact Envelope
p4K	5.3 ± 0.3	4.6 ± 1.3	13.3 ± 2.2
p4T	0.44 ± 0.07*	0.72 ± 0.06	----------
[^{14}C]ovalbumin	0.35 ± 0.03*	0.78 ± 0.14	----------

*These non-zero values potentially include *(i)* slight diffusional entry into the intact nucleus, particularly for ovalbumin which has a molecular weight ~40,000 (p4T is a tetramer, molecular weight 480,000), *(ii)* trace amounts in residual cytoplasm on the surface of the oil-isolated nucleus and, *(iii)* amounts of p4T which may be specifically bound to the cytoplasmic side of the NPCs (but not translocated).

[^{14}C]ovalbumin (which has no NLS) were not transported (Table 1). These data confirm in the oocyte the selective and rapid nuclear transport and accumulation of p4K previously observed in other cells. To resolve active transport and intranuclear binding mechanisms, we punctured nuclear envelopes within oocytes under oil with a microneedle (prior to incubating the living oocytes for 18-24 h) – a procedure which eliminates the envelope's sieving barrier to protein diffusion (9,10) and permitted microinjected p4T (as well as control [^{14}C]ovalbumin) to enter the nucleus by diffusion (Table 1). In contrast, p4K's nuclear accumulation was not significantly altered by puncturing – demonstrating that p4K accumulates in the oocyte nucleus by binding. We confirmed and extended this conclusion with experiments (below) utilizing the oil-isolated nucleus (OIN).

OIN Transport Experiments: Since in vivo-labeled p4K accumulated significantly more in the nuclei of intact oocytes than did in vitro-labeled p4K (Table 1), we used in vivo-labeled transportants in experiments with the OIN. The OIN maintains its in vivo structure and functions – including the selective transport of NLS-proteins – for many hours (2,16), and can be conjoined under oil to an aqueously-equilibrated agarose bead (B) or a bolus of oocyte cytoplasm (C). Preloading the B or C with a radiolabeled transportant permits formation of OIN:B and OIN:C 'transport pairs'. The experimental advantages of transport pairs include (i) immediate presentation of transportants to the nuclear surface (eliminating the time delay encountered in intact cells by microinjected proteins in transport from their injection site to the nucleus) and (ii) the ability to supply transportants to the nucleus in the absence of bulk cytoplasm (i.e., in OIN:B pairs). By separating transport pairs at known times after their formation and measuring the amount

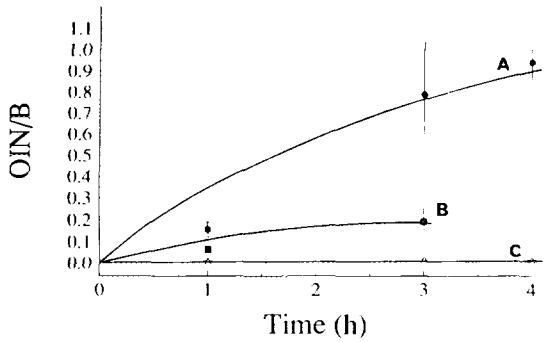

Fig. 2. **Transport of fusion proteins into the intact nucleus in OIN:B pairs.** *Curve A:* ●, p4K and an ATP-regenerating system (ATPrs) initially loaded into the bead; *Curve B:* ■, p4K, with no ATPrs; *Curve C:* Δ, p4T, with ATPrs.

of radiolabeled transportant in each compartment, accurate quantitation of the transport kinetics of NLS-proteins into individual, in vivo-like nuclei is achieved.

Previous studies with nucleoplasmin (Np) in OIN:B transport pairs demonstrated that this prototypical NLS-protein moves into the nucleus by a facilitated transport mechanism: Np asymptotically approaches equal concentrations in the two compartments within 3-4 h, while similar size transportants which do not contain an NLS remain excluded by the intact nuclear envelope. We now report this same behavior for p4K (Fig. 2, curve A). The facilitated transport of p4K in OIN:B pairs is dependent upon ATP (Fig. 2, curve B) and is abolished by the $K^{128} \rightarrow T^{128}$ substitution in p4T (Fig. 2, curve C). Thus, the extended NLS of Ig T is necessary and sufficient to engage β-gal, a normally cytoplasmic protein, with the energy-dependent, NPC facilitated transport mechanism.

Np was found to not accumulate within the nucleus in OIN:B pairs, but it did accumulate in the nucleus in OIN:C pairs, implying a requirement for cytoplasmic factors in intranuclear accumulation (2). Furthermore, Np accumulated in OIN:C pairs even if the nuclear envelope was permeabilized by puncturing prior to pair formation, demonstrating that its accumulation is due to intranuclear binding (2). We now report similar behavior for p4K. When supplied to the OIN via cytoplasm, p4K enters the nucleus and accumulates therein, with kinetics significantly accelerated in comparison with p4K transport in intact cells (Table 2). When we permeabilized the nuclear envelope by micropuncture, p4K still accumulated > 25-fold in the nucleus within 3 h (Table 2). Micropuncture reduces p4K's binding relative to that in the unpunctured OIN; we attribute this effect — not detected in intact cells — to puncture-induced disturbance to the OIN's internal structure, since the degree of reduction is proportional to the number of times the

Table 2. **Accumulation of p4K in OIN from Cytoplasm.** Means ± s.e.m. of individual oocytes from four frogs. Oocytes vary among frogs and within each frog.

Frog	Time (h)	Intact OIN	Punctured OIN
I	1	4.3 ± 1.3	2.0 ± 0.1
II	1	5.7 ± 2.4	1.7 ± 0.5
III	1	5.6 ± 3.2	----------
IV	1	3.5 ± 1.3	----------
I	2	53.9 ± 16.1	12.0 ± 2.1
II	2	51.4 ± 20.5	36.4 ± 14.5
III	2	25.2 ± 5.7	----------
IV	2	23.7 ± 8.8	----------
I	3	45.4 ± 11.5	27.5 ± 13.5
II	3	64.2 ± 24.5	27.0 ± 13.5
III	3	55.4 ± 20.8	----------
IV	3	34.7 ± 10.0	----------

OIN is punctured (data not shown). We conclude – as we did above for the intact cell studies – that p4K's accumulation in the OIN is due to its intranuclear binding.

Intranuclear Binding Requires Prior Facilitated Transport: Wheat germ agglutinin (WGA) is a lectin which binds NPC glycoproteins and inhibits the translocation of many NLS-proteins through the NPC (17-19). In the presence of WGA, Np enters the punctured OIN from cytoplasm by diffusion through the puncture holes, but instead of accumulating in the nucleus (as it does when WGA is not present) it only equilibrates between the two compartments (2). We now report the same results for p4K (Fig. 3). We conclude that while an NLS is sufficient to engage the intranuclear binding of Np or p4K, such binding requires that the protein first be translocated by the NPC facilitated transport mechanism. If either protein diffuses into the nucleus through puncture holes – the only means of entry available in the presence of WGA – it does not bind. We previously hypothesized, based upon the Np results, that proteins containing an NLS are somehow altered during the facilitated transport step to become 'activated' or enabled to bind within the nucleus. The fact that we have obtained the same result for p4K indicates that the activation/binding process, like the engagement of NPC facilitated transport, requires no specific region(s) of the transported protein, other than a competent NLS.

The data reported herein extend the facilitated transport/intranuclear binding model to NLS-β-gal, demonstrating that the extended NLS of the SV40 lg T antigen enables β-gal to be selectively translocated through the NPC via facilitated transport and

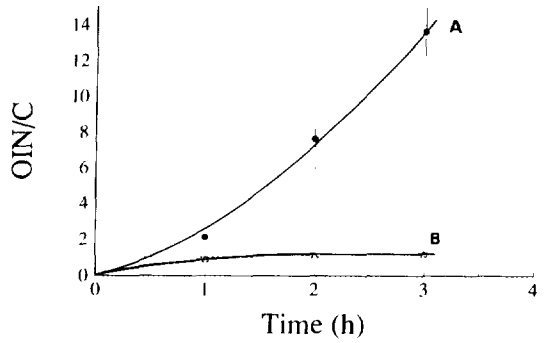

Fig. 3. **Transport of fusion proteins into the punctured nucleus in OIN:C pairs.** *Curve A:* ●, p4K, with no WGA; *Curve B:* Δ, p4K, with WGA.

subsequently accumulated by binding within the nucleus. Because β-gal is ordinarily a nonnuclear protein, NLS-β-gal's facilitated transport by the NPC and its intranuclear binding must result from the NLS per se. Unlike the case of the nuclear protein Np, there is no reason to believe that any moiety of β-gal itself is responsible for the intranuclear binding of p4K, and we conclude that the extended NLS is responsible. Details of how an NLS engages first facilitated transport and then intranuclear binding remain unclear. It is known that cytoplasmic factors are involved (20-22). Our data herein for p4K (and earlier for Np [2]) show that cytoplasmic factors are required for the intranuclear binding step. In addition, translocation through the NPC is a prerequisite for the intranuclear binding of both p4K and Np. Because the phosphorylation states of specific amino acids (S^{112},T^{124}) within the Ig T extended NLS dramatically influence transport behavior (23,24), we are currently utilizing additional NLS-β-gal variants to dissect the roles of these specific sites in NPC facilitated transport and intranuclear binding.

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