Up-regulation of nuclear protein import by nuclear localization signal sequences in living cells

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Abstract Using an in vivo assay system, nuclear import ability in individual cells was determined by examining the nuclear import rate. It was found that when a small (not excess) amount of SV40 T-NLS peptides was co-injected, the nuclear import rate of SV40 T-NLS-containing substrates apparently increased. This up-regulation was reproduced by the co-injection of peptides containing bipartite type NLS of CBP80, but not mutated non-functional NLS peptides, which suggests that these phenomena are specific for functional NLSs. It was further shown that although, in growth-arrested cells, the nuclear import rate was down-regulated compared to growing cells, the elevation of the functional import rate by co-injected NLS peptides reached the same level as in proliferating cells. This up-regulation was abolished by the addition of a protein kinase inhibitor, staurosporine. These results suggest that although potential nuclear import ability does not vary in each cell, the rate of nuclear import may be controlled by the amount of karyophilic proteins, which need to be carried into the nucleus from the cytoplasm, possibly via an NLS-dependent phosphorylation reaction.

Key words: Nuclear protein import; Nuclear localization signal; Phosphorylation

1. Introduction

Nuclear protein import, which occurs through the nuclear pore complex, requires energy and soluble factors, and is mediated by the nuclear localization sequence (NLS) [1,2]. A characteristic feature of the NLS is a basic amino acid cluster in the primary amino acid sequence of the karyophilic proteins, although there might be no obvious consensus sequences. It has been proposed that the conventional NLSs can be divided into two groups: (1) single basic type (for example, the SV40 T-antigen) and (2) bipartite basic type (such as nucleoplasmin and Cap-binding protein, CBP80).

Recently, soluble factors required for nuclear protein import have been identified and characterized, mainly via the use of an in vitro import assay with digitonin-permeabilized semi-intact cells [3]. It is a well-known fact that NLS triggers the formation of a stable complex in the cytoplasm, which is termed the nuclear pore-targeting complex (PTAC) [4]. Two essential components which are involved in complex formation are referred to as PTAC58/PTAC97, importin-α/importin-β, karyopherin-α/karyopherin-β, and the NLS receptor/p97 [5–14]. This complex, including a karyophile, binds to the nuclear pore via importin-β, and the karyophile is then translocated into the nucleus, with the support of a small G protein, Ran [15,16], and Ran-interacting protein, p10/NTF2 [17,18]. In addition, several additional factors, which regulate the Ran GTPase cycle, also have been shown to be directly or indirectly involved in nuclear protein import [19].

It has also been shown that the intracellular behavior of some karyophilic proteins is regulated by the cell cycle or an extracellular signal. The nuclear accumulation of viral Jun (v-Jun) was found to be cell cycle-dependent, and this dependence is regulated by the phosphorylation of a serine residue which is located close to the NLS [20]. A transcription factor SWI5 is located in the cytoplasm when it is phosphorylated near its NLS in the G_2 and M phase, but is translocated into the nucleus after dephosphorylation in the G_1 phase [21]. In contrast, a transcription factor, NF-κB, does not enter the nucleus until its cytoplasmic anchoring protein, IκB, is phosphorylated and released from NF-κB after some extracellular stimuli [22]. In response to interferon-γ, a transcription factor, Stat1, is phosphorylated by the Jak family of tyrosine kinases, and then is translocated into the nucleus and directly activates target genes [23]. Recently, it was demonstrated that the interferon-γ-dependent nuclear import of Stat1 also requires Ran and the formation of PTAC with NPI-1, but not Rch1 of the importin-α family [24,25].

As described above, although our understanding of the mechanism of karyophilic protein-specific control is now increasing at the molecular level, little is known about whether or how the import machinery itself is regulated. It has previously been demonstrated that proliferating cells have a higher import efficiency than serum-starved growth-arrested cells [26]. In addition, in the course of an in vivo study of p10/NTF2, we recently reported that the injection of p10/NTF2 inhibits the nuclear import of co-injected NLS substrates in a dose-dependent manner. From these results, we speculated that p10/NTF2 not only plays an essential role in nuclear protein import, but also regulates nuclear transport efficiency in cells [27]. However, these regulatory mechanisms or components have not yet been studied at the molecular level.

The present study describes some experiments which are designed to better understand the molecular mechanisms which control nuclear protein import. Using an in vivo assay system, we found that when a small dose of SV40 T-NLS

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Abbreviations: NLS, nuclear localization signal; CBP, Cap binding protein; PTAC, nuclear pore-targeting complex; NTF2, nuclear transport factor 2; BSA, bovine serum albumin; hsc70, 70-kDa heat-shock cognate protein; RCC1, regulator of chromosome condensation; RanGAP1, Ran GTPase activating protein 1; RanBP, Ran binding protein

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peptides is co-injected with SV40 T-NLS-containing substrates into the cytoplasm of living cells, the kinetics of nuclear import were stimulated compared to the case when the substrates alone were injected. The results herein suggest that the ability of nuclear protein import can be up-regulated by the amount of nuclear proteins which are present in the cytoplasm. The present study is directed toward the identification of regulatory components which have not yet been identified and to develop a better understanding of the regulation of nuclear protein import.

2. Materials and methods

2.1. Cell culture and synchronization

Normal baby hamster kidney (BHK21) cells were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's minimum essential (DME) medium (Gibco BRL) supplemented with 5% fetal bovine serum (Dainippon). For growth arrest, cells were cultured for 72 h in minimum essential medium containing 0.5% serum.

2.2. Synthetic peptides

Synthetic peptides containing SV40 T-antigen wild type NLS (CYGGPKKKRKVEDP), its reverse type NLS (CYGGPDEVKRKKKP) and CBP80 NLS (CYMSRRRHSDENDGGQPHKRRKTSDANETED) were purchased from the Peptide Institute (Osaka, Japan).

2.3. Preparation of NLS-conjugated protein

As a substrate for nuclear import assay, fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) was chemically conjugated to a synthetic peptide (CYGGPKKKRKVEDP) containing NLS of SV40 T-antigen as described previously [28] (FITC-T-BSA). Two types of FITC-T-BSA were prepared. 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 2–4) of peptides per BSA molecule.

2.4. Microinjection and quantitation of nuclear import kinetics

Cells were seeded on indexed glass coverslips. Materials were microinjected through a glass capillary into the cytoplasm of cells. Injected cells were incubated for the indicated time at 37°C prior to fixation with formaldehyde. For quantitation of the nucleocytoplasmic ratio of cytoplasmically injected FITC-T-BSA, after fixation with a time sequence, the cells were analyzed on a confocal LSM310 Laser Scan Microscope system (Carl Zeiss). The fluorescence values of nuclear and cytoplasmic areas in each cell were measured using MacSCOPE software (Mitani Co.) and the nucleus/whole cell ratio was calculated.

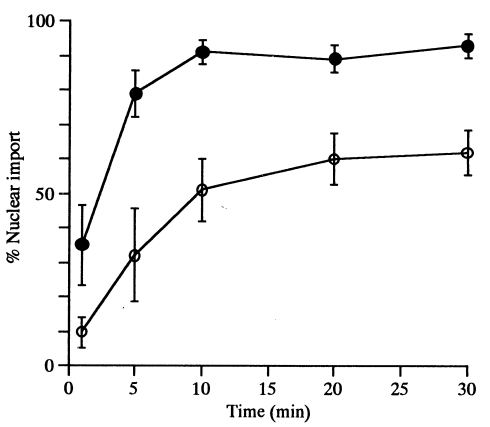

Fig. 1. Nuclear import kinetics of NLS-containing substrates. FITC-T-BSA (15 μM) containing a large (●) or small (○) number of SV40 T-NLS peptides was microinjected into the cytoplasm of BHK cells. These cells were then incubated at 37°C for the indicated times prior to fixation. The nuclear import of injected substrates was quantitated (see Section 2).

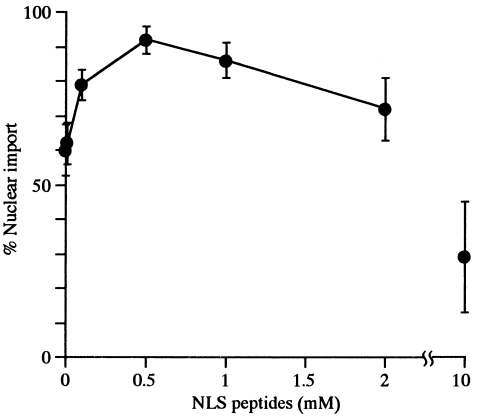

Fig. 2. The increase in nuclear import rate of NLS-containing substrates is dependent on the amount of co-injected NLS peptides. 15 μM FITC-T-BSA was microinjected with the indicated concentration of SV40 T-NLS peptides. These cells were then incubated at 37°C for 20 min prior to fixation and nuclear import of FITC-T-BSA was quantitated.

Data are expressed as mean ± S.E.M. of values obtained from 40 cells in two independent experiments (20 cells per experiment).

3. Results

3.1. An in vivo system for analyzing the kinetics of nuclear protein import

We assumed that each cell has a unique ability with respect to nuclear protein import and this ability would be expected to vary in response to the extracellular environment or intracellular conditions. In order to evaluate this ability, we attempted to examine the rate of nuclear protein import for each cell. In order to analyze the nuclear protein import kinetically, we initially prepared two types of transport substrates, FITC-labeled BSA conjugated with a large number (9–11 molecules per BSA) of SV40 T-NLS peptides or a small number (2–4 molecules per BSA). After the fluorescent-labeled karyophiles were microinjected into the cytoplasm of cultured mammalian (BHK) cells, the cells were incubated for various periods of time and the nuclear accumulation of injected substrates was quantitated as described in Section 2. As shown in Fig. 1, when FITC-T-BSA containing a large number of NLS peptides was injected into the cytoplasm, more than 90% of the injected substrates accumulated in the nucleus within 10 min and reached a plateau level. In contrast, we found that FITC-T-BSA which contained a small number of peptides was translocated relatively slowly into the nucleus reaching a plateau state 20–30 min after cytoplasmic injection (Fig. 1). Therefore, we concluded that the substrates which contained a large number of NLSs are not suitable for kinetic analysis because of the difficulty of following the detailed time course of the import of proteins precisely just after microinjection. It was further confirmed that when injected into a nucleus of a multinucleated homokaryon, the substrates which contained a small number of NLS peptides did not migrate from the injected nucleus to other nuclei within 1 h (data not shown), meaning that the substrates injected into the cytoplasm do not shuttle back from the nucleus to the cytoplasm within 1 h. Therefore, it was assumed that the import kinetics can be evaluated precisely only if the data are collected within 30 min after the cytoplasmic injection. On the basis of these

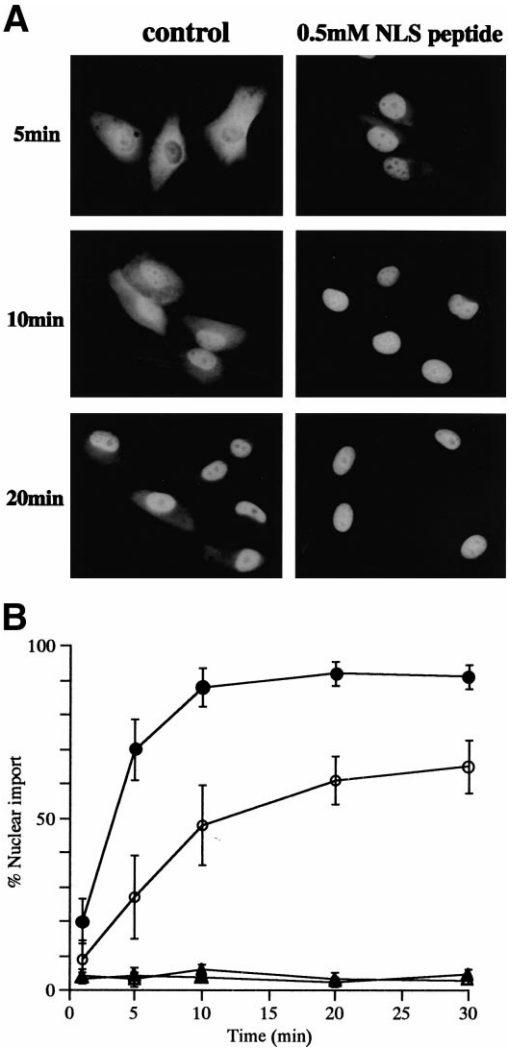

Fig. 3. Time course of nuclear import of NLS-containing substrates in the presence of 0.5 mM NLS peptide. A: 15 μM FITC-T-BSA was microinjected with buffer (control) or 0.5 mM SV40 T-NLS peptide. Injected cells were incubated for the indicated times at 37°C. The location of injected FITC-T-BSA is indicated. B: 15 μM FITC-T-BSA (○, ●) or 15 μM FITC-BSA (△, ▲) was microinjected with (●, ▲) or without (○, △) 0.5 mM SV40 T-NLS peptide. Injected cells were incubated for the indicated times at 37°C and nuclear import of injected substrates was quantitated.

results, for further in vivo analysis, we used FITC-T-BSA containing a small number of NLS peptides.

3.2. Kinetics of nuclear import of NLS-containing substrates are affected by co-injection of free NLS peptides

It is known that NLS-mediated protein import is competitively inhibited in the presence of an excess of NLS-containing protein or NLS peptides, which suggests that nuclear protein import is a saturable and receptor-mediated pathway. Using our in vivo assay system, we examined whether it is possible to titrate the saturability of a limiting number of nuclear import components in each mammalian cell. FITC-T-BSA (15 μM) was co-injected with various amounts of free SV40 T-NLS peptides into the cytoplasm of cells. As shown in Fig. 2, the co-injection of an excess (10 mM) of NLS peptides caused suppression of the nuclear import of the FITC-T-BSA, which is in agreement with a previous report [29]. However, unexpectedly, it was found that co-injection of low concentrations (0.1–1 mM) of NLS peptides conversely increased the rate of the nuclear import of the FITC-T-BSA. Because cycloheximide was found not to affect this phenomenon (data not shown), new protein synthesis is not a prerequisite for the increase of nuclear import rate.

When the concentration of NLS peptides was 0.5 mM, a maximal effect on the nuclear import was observed. Therefore, we next examined the time course of nuclear import of the FITC-T-BSA in the presence of 0.5 mM of NLS peptides. The subcellular distribution view of microinjected FITC-T-BSA is shown in Fig. 3A. In Fig. 3B, the nucleus/whole cell ratio of the fluorescence is plotted as a function of time after injection within 30 min. As shown in Fig. 3A,B, the nuclear accumulation of the FITC-T-BSA reached a plateau within 10 min after injection. As a control experiment, co-injection of NLS peptides with FITC-BSA had no effect on the distribution of FITC-BSA (Fig. 3B). These findings suggest that the co-injection of lower concentrations of NLS peptides than required for the complete competition conversely stimulates the kinetics of nuclear import.

3.3. Increase of nuclear import rate by other types of NLS peptides

As described in Section 1, the conventional basic type of NLS can be divided into two classes [30]. SV40 T-NLS (single type)- and CBP80-NLS (bipartite type)-containing substrates are both known to be transported into the nucleus via PTAC formation [14,31]. Therefore, in order to understand whether the up-regulation of the nuclear import rate of SV40 T-NLS-containing substrates is limited to the same NLS peptides, we examined the nuclear import of FITC-T-BSA when CBP80-NLS peptides were co-injected. For experimental convenience, we examined the nuclear import rate by analyzing the data at 20 min after injection. As shown in Fig. 4, the rate of nuclear import of FITC-T-BSA was increased by the presence of CBP80-NLS peptides to the same extent as T-NLS peptides. Furthermore, as shown in Fig. 4, the co-injection of the non-functional reverse type of NLS peptides of SV40 T-antigen had no effect on the nuclear import of FITC-T-BSA at all. These results indicate that the up-regulation of the nuclear

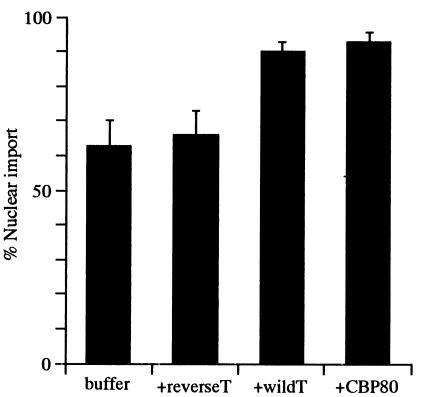

Fig. 4. Co-injection of not only single type but also bipartite type NLS peptides increases nuclear import efficiency of NLS-containing substrates. 15 μM FITC-T-BSA was microinjected with buffer, 0.5 mM SV40-T wild type NLS (wild T), its reverse type (reverse T), CBP80 NLS peptides into the cytoplasm of cells. These cells were then incubated at 37°C for 20 min and nuclear import of FITC-T-BSA was quantitated.

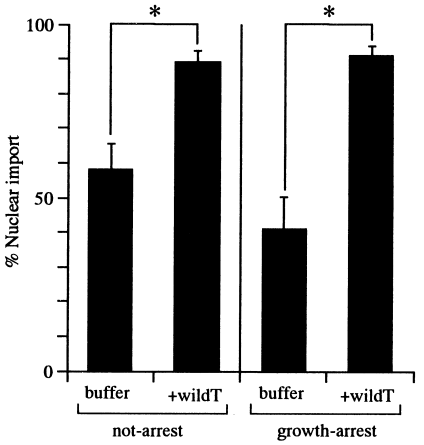

Fig. 5. The increase in nuclear import efficiency in the presence of NLS peptide in growth-arrested cells. FITC-T-BSA was microinjected with buffer or 0.5 mM SV40 T-NLS peptides (wild T) into the cytoplasm of non-arrested (left hand) or growth-arrested (right hand) cells (see Section 2). These cells were then incubated at 37°C for 20 min and nuclear import of FITC-T-BSA was quantitated. $*P < 0.05$ versus buffer control (Student's *t*-test for unpaired data).

import rate observed in this study is specific to functional NLS peptides of both the single and bipartite types.

3.4. *Effect of co-injected NLS peptides on the nuclear import in growth-arrested cells*

It has been shown, using microinjection of nuclear protein-coated colloidal gold, that the nuclear import efficiency in growth-arrested cells is lower than in proliferating cells [26]. To test whether the up-regulation of nuclear import as shown in this report is related to cell growth, we examined the effect of the SV40 T-NLS peptides on the nuclear import rate in growth-arrested cells. As shown in Fig. 5, in growth-arrested cells, the nuclear import rate was significantly reduced compared with that in non-arrested cells, which is consistent with the previous report [26]. On the other hand, when free NLS peptides (0.5 mM) were co-injected into the cytoplasm of growth-arrested cells, the rate of nuclear accumulation of FITC-T-BSA increased to the same level as that stimulated in non-arrested cells. These results indicate that growth-arrested cells have the same potential ability for nuclear import as growing cells, although the functional import rate is down-regulated in growth-arrested cells.

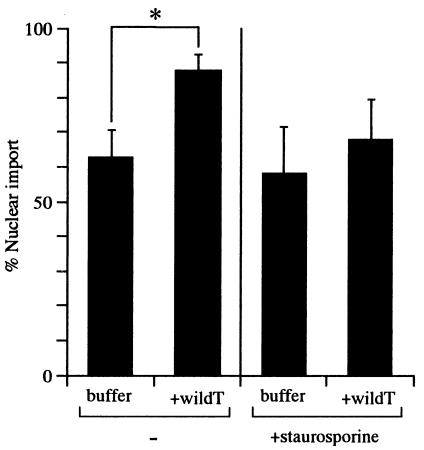

Fig. 6. Staurosporine, a protein kinase inhibitor, inhibits the increase in nuclear import efficiency by co-injected NLS peptides. BHK cells were treated with (right) or without (left) 100 nM staurosporine for 20 min. 15 μM FITC-T-BSA was then microinjected with buffer or 0.5 mM SV40 T-NLS peptides (wild T). The cells were then incubated at 37°C for 20 min in the presence (right hand) or absence (left hand) of staurosporine, and nuclear import of FITC-T-BSA was quantitated. $*P < 0.05$ versus buffer control (Student's *t*-test for unpaired data).

3.5. *Up-regulation of the nuclear import rate by co-injection of NLS peptides was suppressed by treatment of cells with a protein kinase inhibitor*

We speculated that the rapid response of the nuclear import rate to the co-injected NLS peptides may be due to intracellular protein modification, most likely protein phosphorylation. In order to demonstrate this hypothesis, we examined whether the phosphorylation reaction is involved in the up-regulation of nuclear import. FITC-T-BSA was microinjected with or without SV40 T-NLS peptides into the cytoplasm 20 min after the cells were treated with staurosporine, a general kinase inhibitor. As shown in Fig. 6, although the nuclear import rate of FITC-T-BSA in the absence of free NLS peptides was not affected, the up-regulation of nuclear import observed by co-injection of NLS peptides was suppressed by staurosporine treatment. This finding suggests that the up-regulation of the nuclear import rate observed in the presence of free NLS peptides may be mediated via a protein phosphorylation reaction.

4. Discussion

In order to understand the regulatory mechanism of nuclear import, we focused on the rate of import as the index. Using SV40 T-NLS substrates which contain a small number of peptides, we were able to monitor the kinetics of transport in living mammalian cells. Using this in vivo assay system, we observed that the nuclear import of the SV40 T-NLS-containing substrate is kinetically stimulated by co-injection of amounts of two types of NLS peptides at concentrations less than required for complete inhibition, even though we expected that the competition would be observed in a dose-dependent manner. The stimulation was found to be specific for functional NLSs. These results indicate that the co-injection of the NLS peptides induces a stimulation in the rate of nuclear protein import and that a regulatory system may well exist that monitors and responds to an intracellular alteration of the amount of karyophiles to be transported into the nucleus.

Several possible explanations exist for the stimulation of the nuclear import rate. First, regulatory factors, which have not yet been identified, may elevate the efficiency of NLS recognition by importin-α or the formation of the nuclear pore-targeting complex. One such candidate is a molecular chaperon such as hsc70. Although the exact role of hsc70 on nuclear protein transport is not yet clear [32], it has been found that elevated hsc70 levels suppress the nuclear import defects in a yeast srp1/NLS receptor mutant [33], suggesting the possibility that a molecular chaperon may promote the formation and stability of the nuclear pore-targeting complex. Second, the recycling rate of transport factors such as importin-α and -β may be stimulated, resulting in an apparent elevation of the import rate. Third, a portion of the transport factors may be routinely stored as inactive forms in a reservoir, for later

activation in response to cell activity. Fourth, other types of transport factors which are ordinarily involved in different transport pathways may be mobilized in response to some type of NLS. Alternatively, the elevated function of the nuclear pore complex, such as the binding of higher concentrations of SUMO-1-modified RanGAP1 to RanBP2, must be considered [34]. Lastly, the movement of the targeting complex from the cytoplasm to the nuclear pores may be facilitated in some manner, although how the complex moves in the cytoplasm is currently unknown. Further studies will be required to elucidate these or other possibilities.

In the cells cycling from the mitotic phase to interphase, after complete reformation of the functional nuclear envelope, an enormous number of nuclear proteins which are scattered in the cytoplasm during mitotic phase must accumulate into the nucleus. Therefore, it is suspected that the protein import machinery may be activated in order for nuclear proteins to be quickly and efficiently imported into the nucleus just after mitosis. In fact, it was previously reported that nuclear import efficiency is greatest at 1 h post anaphase during the cell cycle [35]. In addition, it has been shown that the arrest of cell growth leads to a decrease in nuclear import efficiency [36]. Consistent with this report, as shown in Fig. 5, the rate of nuclear protein import was found to be reduced in growth-arrested cells, compared with growing cells. In contrast, the stimulation of the rate of nuclear import induced by co-injection of NLS-peptides in the growth-arrested cells was the same as that in the growing cells (Fig. 5), suggesting that the potential ability of nuclear protein import in the growth-arrested cells is nearly equivalent to that in the growing cells. In proliferating cells, much more nuclear proteins may need to be constitutively transported into the nucleus compared with those in quiescent cells. Therefore, the nuclear import rate may be maintained at a higher level in proliferating cells compared with resting cells.

It has been shown that a co-injected protein kinase A inhibitor blocks the nuclear import of NLS-containing substrates [37], and that while injected SV40-transformed cell extracts stimulate nuclear import, the effect is abolished by a protein kinase inhibitor or protein phosphatase [36]. Moreover, the above workers showed that the injection of various protein kinases (PKA, PKC, MAPK, but not CK-I and II) activates nuclear import. Recently, we identified a protein kinase which is activated by NLS peptides [38]. In this study, it was shown that the up-regulation of nuclear protein import by co-injection of NLS peptides is suppressed by the treatment of cells with a protein kinase inhibitor (Fig. 6). Although the relationship between the phenomena investigated in this report and the above mentioned kinases is not clear, the possibility of the existence of NLS-activated kinase(s) which up-regulate nuclear protein import cannot be ignored. Such kinase(s) may phosphorylate nuclear transport factors including cytosolic factors such as the PTAC components or nuclear pore complex protein. Alternatively, we previously showed that the efficiency of nuclear protein import is regulated by the Ran-GTPase cycle via the use of tsBN2 cells which have a mutation in the Ran GDP/GTP exchange factor gene, RCC1 [39]. It is possible that target molecules of NLS-dependent kinase(s) may be Ran-regulating proteins such as RanGAP1 and RanBP1. The identification and characterization of such kinase(s) and their target molecules would be expected to contribute greatly to our understanding of the molecular mechanism of the regulation of nuclear protein import.

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