# Exogenously injected nuclear import factor p10/NTF2 inhibits signal-mediated nuclear import and export of proteins in living cells

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**Abstract p10/NTF2 is a cytosolic factor which is required for the translocation step in nuclear protein import in an in vitro assay with digitonin-permeabilized cells. To study the functional roles of p10/NTF2 on protein transport between the nucleus and cytoplasm in living cells, recombinant p10/NTF2 was microinjected into cultured mammalian cells. Cytoplasmically injected p10/NTF2 strongly inhibited the nuclear import of co-injected NLS-containing substrates in a dose-dependent manner but had no effect on the diffusive import of small non-nuclear proteins. Moreover, when injected into the cell nucleus, p10/NTF2 inhibited the nuclear export of NES-containing substrates. The results suggest that the nuclear import factor p10/NTF2 may also be involved in the nuclear export of proteins and that the protein transport efficiency between the nucleus and cytoplasm may be regulated by the intracellular level of p10/NTF2.**

*Key words:* Nuclear protein import; Nuclear protein export; p10/NTF2

## 1. Introduction

Nuclear localization signal (NLS)-mediated protein import into the nucleus can be divided into two distinct steps, (a) binding to the nuclear pore complex (NPC) and (b) translocation to the interior of the nucleus [1–3]. Studies involving both in vivo and in vitro nuclear import assay systems have identified several factors which are required for these steps [4]. Hsc70 (a 70 kDa heat shock cognate protein) constitutes one of these factors [5–7]. During the initial stage of nuclear import, an NLS-containing substrate forms a stable complex (referred to as the nuclear pore-targeting complex, PTAC) with 58 kDa and 97 kDa components to target the nuclear pore [8,9]. The 58 kDa component of PTAC (PTAC58) has been shown to bind directly to the NLS and has been designated the NLS receptor/importin-α/karyopherin-α [10–14]. The 97 kDa component of PTAC (PTAC97) is required for interaction of the substrate-58 kDa component complex with the NPC and is referred to as p97/importin-β/karyopherin-β [9,15–18]. Using digitonin-permeabilized cells, a small GTPase Ran/TC4 [19,20] and its interacting protein, p10 [21], have also been identified as cytosolic factors which are required for the translocation step in nuclear import. The Ran GTPase cycle has been shown to regulate nuclear import in living cells [22]. p10 has been shown to interact with nuclear pore protein p62 and is referred to as NTF2 [23]. The mechanism by which p10/NTF2 acts on nuclear protein transport in living cells, however, remains unknown.

Recently, the signal required for the nuclear export (nuclear export signal, NES) of certain proteins has been identified [24–28]. The NES-mediated nuclear export has been shown to be temperature- and ATP-dependent. Little, however, is known about the machinery involved in this export.

In this paper, we wish to report on a study involving the microinjection of recombinant p10/NTF2 protein into cultured mammalian cells in an attempt to better understand the functional roles of p10/NTF2 on nuclear transport in living cells. The results suggest that p10/NTF2 may regulate not only nuclear import, but also nuclear export.

## 2. Materials and methods

### 2.1. Cell cultures

Human embryonic lung (HEL) and Madin-Darby bovine kidney (MDBK) cells were cultured in 5% $CO_2$ at 37°C in Dulbecco's modified Eagle's minimum essential (DME) medium (Gibco BRL) supplemented with 10% bovine calf serum (Hyclone).

### 2.2. Cloning and purification of recombinant human p10/NTF2

Human p10/NTF2/pp15 gene [29] was amplified from HeLa cell cDNA by the polymerase chain reaction (PCR) using the primers 5′-AGCTAGCATATGGGAGACAAGCCAATTTGGGAG-3′ and 5′-AGCTAGAGATCTTCAGCCAAAGTTGTGCAGGGC-3′. The PCR product was digested with *Nde*I and *Bgl*II and inserted into *Nde*I-*Bam*HI-digested pET3a vector (Novagen). The plasmid was introduced into *E. coli* strain BL21(DE3). The expression of p10/NTF2 proteins was induced by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside to exponentially growing cultures ($OD_{600} = 0.3$) in 50 ml of LB medium containing ampicillin. The cultures were incubated for 6 h at 37°C, after which the bacteria were harvested by centrifugation and frozen at −20°C. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1.0 mM ethylenediamine tetraacetic acid, 0.5 mM dithiothreitol (DTT) and 0.1 mM (*p*-amidinophenyl)methanesulfonyl fluoride). After two freeze-thaw cycles, the cells were disrupted by sonication. After centrifugation for 30 min at 15000×*g*, the supernatant was subjected to chromatography on a Hitrap Q column (1 ml) in FPLC system (Pharmacia) at a flow rate of 0.2 ml/min using a linear gradient of 0–500 mM NaCl in 20 mM HEPES-NaOH buffer (pH 7.3). p10/NTF2 eluted between 150 and 200 mM NaCl. Peak fractions containing p10/NTF2 were pooled, the pooled fractions sieved on a Superose 12 FPLC column (HR30/10, Pharmacia) and equilibrated with 20 mM HEPES-NaOH, pH 7.3 and 100 mM KOAc. Peak fractions containing p10/NTF2 were pooled and concentrated in a Millipore centrifugal concentrator (Ultra Free, C3), and 10 μl aliquots were frozen in liquid nitrogen and stored at −80°C.

### 2.3. Preparation of NLS conjugated protein and GST-NES-GFP protein

As a substrate for nuclear import assays, fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) or allophycocyanin (APC) (Calbiochem) was chemically conjugated to the synthetic peptide (CYGGPKKKRKVEDP) containing the nuclear localization signal of SV40 T-antigen as described previously [22,30] (FITC-T-BSA, T-APC). The number of conjugated peptides was estimated to be 3–5 per BSA molecule and 10–12 per APC molecule by SDS-PAGE.

As a substrate for nuclear export assays, GST-NES-GFP (green fluorescent protein) fusion protein was used. A full-length cDNA of GFP (containing the S65T mutation) (Clontech) was inserted into the

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*Sma*I/*Xho*I site of pGEX-5X-2 (Pharmacia) to generate pGST-GFP. The synthesized oligonucleotide, corresponding to the NES of PKI (heat-stable protein kinase inhibitor), LALKLAGLDI [24], was inserted into the *Bam*HI/*Sma*I site of pGST-GFP. The GST-NES-GFP fusion protein was expressed in *E. coli* strain JM109 and purified to homogeneity using glutathione Sepharose (Pharmacia).

*2.4. Preparation of recombinant proteins for the cell-free import assay*

Recombinant PTAC58 [12], PTAC97 [16] and Ran/TC4 [31] proteins were prepared from *E. coli* as previously described.

*2.5. Microinjection*

Cells were seeded on indexed glass coverslips. Proteins were microinjected through a glass capillary into the cytoplasm or nucleus of cells, along with RITC- or FITC-conjugated BSA to monitor the actual site of injection. Injected cells were incubated for the indicated time at 37°C before fixation. Localization of injected RITC- or FITC-conjugated protein or GFP fusion protein was examined directly after fixation. In order to determine the nuclear/cytoplasmic ratio of the amount of cytoplasmically injected FITC-p10/NTF2, FITC-p10/NTF2-injected cells were analyzed on a confocal LSM410 Laser Scan Microscope system (Carl Zeiss, Inc.). RNA synthesis was visualized as described below.

*2.6. Indirect immunofluorescence*

The BrUTP-injected cells were fixed with 3.7% formaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and rinsed in 3% skim milk in PBS. The cells were incubated with mouse anti-BrdU mAbs and then with FITC-conjugated anti-mouse IgG (Cappel Co.) After extensive washing, the cells were then examined with an Axiophot microscope (Carl Zeiss, Inc.).

*2.7. Cell-free import assay*

Digitonin-permeabilized MDBK cells were prepared using methodology reported by Adam et al. [32]. Testing solutions contained 100 μg/ml T-APC and transport buffer (20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 5 mM sodium acetate, 0.5 mM ethylene glycol bis-β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 2 mM DTT and 1 μg/ml each of aprotinin, leupeptin and pepstatin). For the nuclear-binding assay, incubation was performed on ice for 30 min in the presence of 40 μg/ml PTAC58 and 40 μg/ml PTAC97, with or without 100 μg/ml of p10/NTF2. For the nuclear import assay, the incubation was performed at 30°C for 20 min in the presence of 40 μg/ml PTAC58, 40 μg/ml PTAC97, 100 μg/ml Ran/TC4, 10 or 100 μg/ml p10/NTF2, 1 mM ATP, 5 mM creatine phosphate and 20 U/ml creatine phosphokinase.

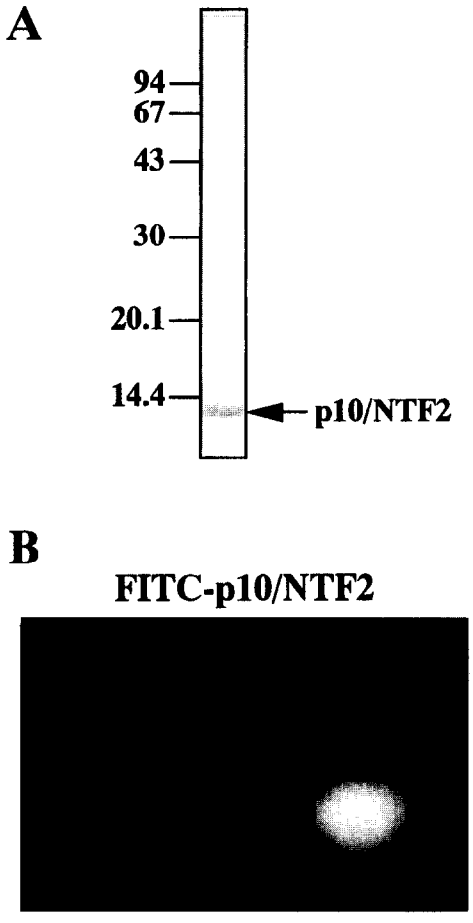


Fig. 1. A: Purified human p10/NTF2. p10/NTF2 was purified from *E. coli* as described in Section 2. The purified p10/NTF2 is >90% pure as evidenced by SDS-PAGE. Molecular mass standards are indicated on the left of the panel. B: Subcellular location of injected p10/NTF2 cross-linked with FITC. FITC-p10/NTF2 was injected into the cytoplasm of HEL cells. The cells were then incubated at 37°C for 20 min before fixation. This FITC-p10/NTF2 supports nuclear protein import in the in vitro import assay (data not shown).

## 3. Results and discussion

Human p10/NTF2 was expressed as recombinant protein in *E. coli*. The purified recombinant protein yielded a single band having the expected electrophoretic mobility by SDS-PAGE analysis (Fig. 1A). This recombinant p10/NTF2 protein was confirmed to support the nuclear import of NLS-containing substrate (T-APC) in an in vitro assay with digitonin-permeabilized cells when added at a concentration of 5–20 μg/ml in the presence of recombinant Ran, the 58 kDa and 97 kDa components of PTAC (Fig. 5A) as described previously [18].

To elucidate the functional roles of p10/NTF2 in nuclear protein import in living cells, various concentrations of the recombinant p10/NTF2 were injected, along with the transport substrate (FITC-T-BSA), into the cytoplasm of cultured mammalian cells. After incubation at 37°C for 30 min, the cells were fixed and the localization of injected FITC-T-BSA was examined by fluorescence microscopy. As shown in Fig. 2A, p10/NTF2 at a concentration of 0.2 mg/ml had no effect on nuclear protein import. In contrast, injection of more than 1 mg/ml of p10/NTF2 inhibited the nuclear import of FITC-T-BSA in a dose-dependent manner. Similar results were obtained by substituting nucleoplasmin (*Xenopus* karyophilic protein) for FITC-T-BSA as a transport substrate (data not shown).

In these experiments, as shown in Fig. 1B, injected p10/NTF2 was found to localize in the cytoplasm, nucleus and on the nuclear rim. Ran has been found to be involved, not only in nuclear protein import, but also in the nuclear export of NLS-containing protein [33] and mRNA [34,35]. In order to determine whether p10/NTF2 has any effect on the nuclear export of proteins, we injected GST-NES-GFP with and without recombinant p10/NTF2 into the nucleus of cultured HEL cells. After incubation at 37°C for 30 min, the localization of injected GST-NES-GFP was examined. As shown in Fig. 2B, nuclear injection of p10/NTF2 at a concentration of 0.2 mg/ml had no effect on the nuclear protein export. In contrast, as in the case of the nuclear protein import, nuclear export of GST-NES-GFP was strongly inhibited by the injection of more than 1 mg/ml of p10/NTF2. This suggests that the nuclear import factor, p10/NTF2, may well participate in the nuclear export of NES-containing proteins as well. Similar results were also obtained using HeLa, BHK, and MDBK cells (data not shown).

It is well known that small non-nuclear molecules rapidly

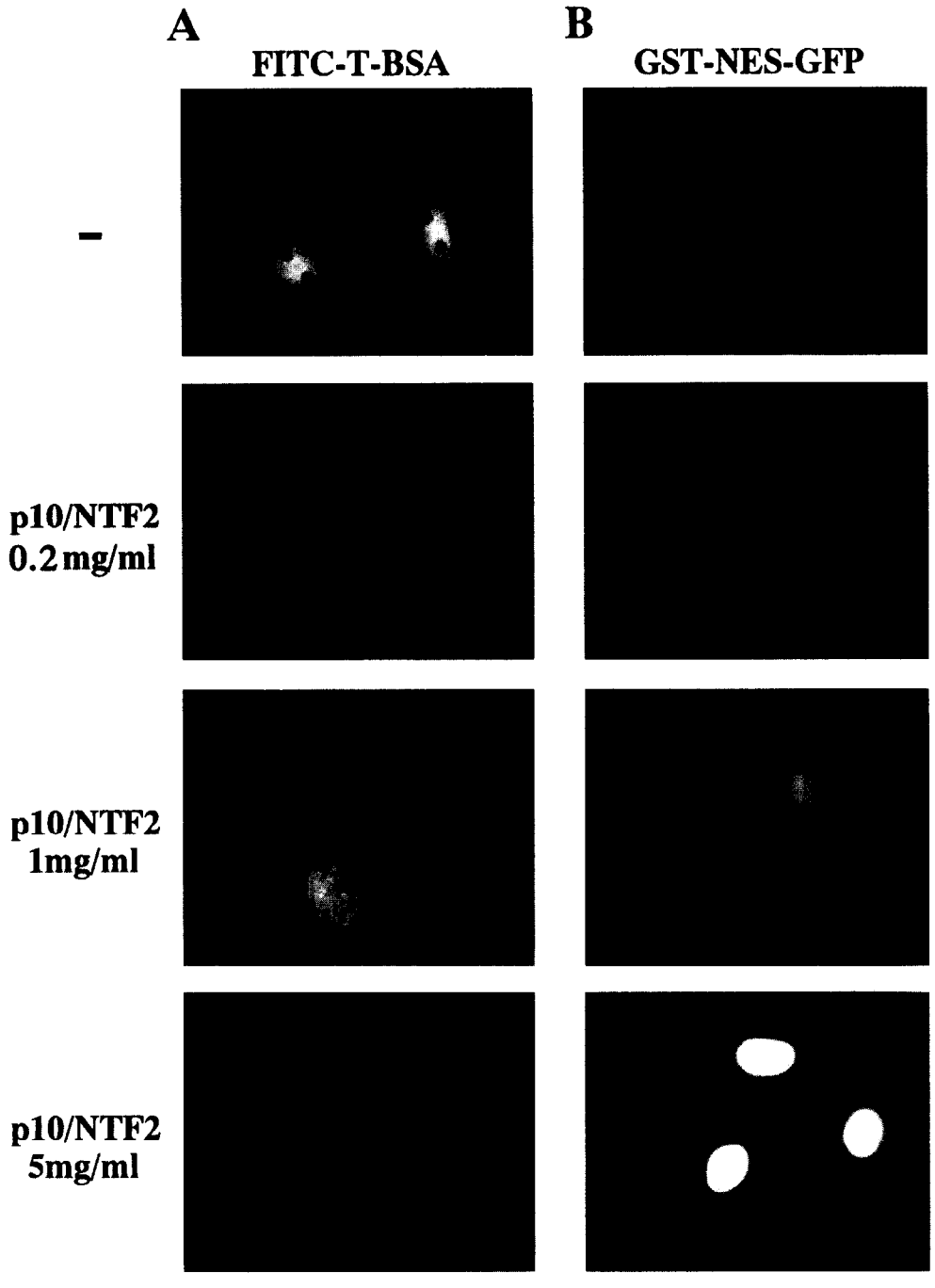


Fig. 2. Effects of injected p10/NTF2 on nuclear protein import and export. FITC-T-BSA (1 mg/ml, A) or GST-NES-GFP (1 mg/ml, B) was injected with 0.2 mg/ml, 1 mg/ml, 5 mg/ml or without (−) recombinant p10/NTF2 protein into the cytoplasm (A) or nucleus (B) of HEL cells. These cells were then incubated at 37°C for 30 min before fixation.

diffuse into the nucleus [36]. In another series of experiments, we attempted to determine if the injected recombinant p10/NTF2 specifically inhibits signal-mediated protein transport. RITC-myoglobin was injected with and without recombinant p10/NTF2 into the cytoplasm or the nucleus of HEL cells. After incubation at 37°C for 30 min, the localization of injected RITC-myoglobin was examined (Fig. 3). The injected p10/NTF2 did not inhibit the diffusive import and export of myoglobin, a small non-nuclear protein. These results indicate that the nuclear pores were not physically obstructed and the inhibitory effect of injected p10/NTF2 is specific to signal-mediated nuclear import and export of proteins.

To determine if RNA was synthesized in the cells after injection of p10/NTF2, BrUTP was injected with p10/NTF2 into the nucleus of HEL cells. As shown in Fig. 4, p10/NTF2 did not block RNA synthesis. After injection of p10/NTF2, therefore, the cells were viable and the inhibition of nuclear import and export of proteins was not caused by cell death.

The mechanism by which injected p10/NTF2 inhibits nuclear import and export of proteins in living cells remains an open question. As shown in Fig. 1B, the injected FITC-p10/NTF2, which had the same inhibitory activity on nuclear import and export of proteins as the non-labeled one, was located in the cytoplasm (30–40%) and nucleus (60–70%). More-

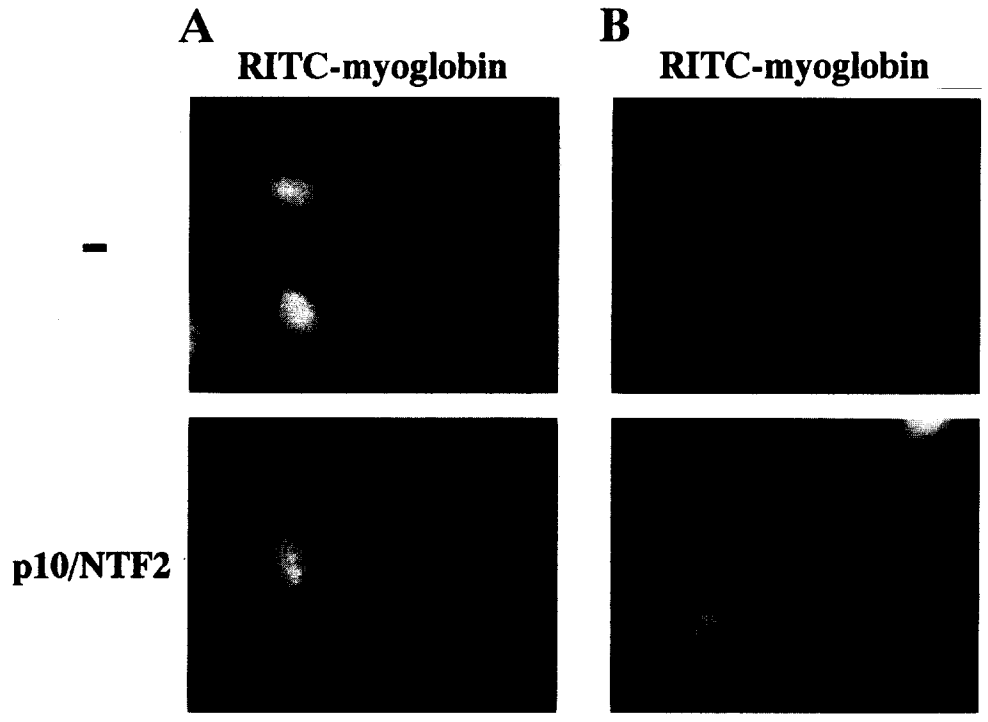


Fig. 3. Effects of injected p10/NTF2 on the passive diffusion of small molecules to/from the nucleus. RITC-myoglobin (1 mg/ml) was injected with (lower) and without (upper) recombinant p10/NTF2 protein into cytoplasm (A) or nucleus (B) of HEL cells. These cells were then incubated at 37°C for 30 min before fixation.

over, nuclear rim accumulation was often observed. Similar results were obtained when the FITC-p10/NTF2 was injected into the nucleus (data not shown). It has already been shown that p10/NTF2 interacts with nuclear pore protein p62. It is likely that excess p10/NTF2 may interact immoderately with nuclear pore proteins which are required for nuclear transport such as p62 and thus inhibit nuclear protein import and export. While this work was in progress, Nehrbass and Blobel reported that p10/NTF2 binds directly to several peptide repeat-containing nucleoporins, and supports GDP-GTP exchange for Ran on nucleoporins and the dissociation of the karyopherin-α (58 kDa component of PTAC) from the karyopherin αβ heterodimer, after targeting to nucleoporins [37]. Exogenously injected excess p10/NTF2 may disrupt this series of reactions.

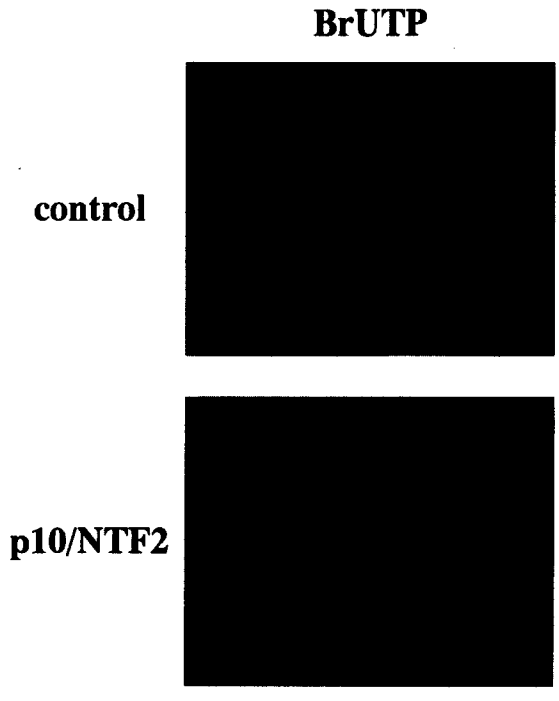


Fig. 4. Effects of injected p10/NTF2 on RNA synthesis. p10/NTF2 (5 mg/ml, lower) or BSA (5 mg/ml, upper) was injected with BrUTP (50 mM) into the nucleus of HEL cells. The cells were then incubated at 37°C for 30 min before fixation. RNA synthesis was detected by indirect immunofluorescence staining with mouse anti-BrdU mAbs, and then with FITC-conjugated antibodies to mouse IgG.

We also found that the concentration of p10/NTF2 utilized in the in vitro assay using recombinant factors (p10/NTF2, Ran, PTAC58 and PTAC97) required for nuclear import is critical. That is, when Ran (100 μg/ml), PTAC58 (40 μg/ml) and PTAC97 (40 μg/ml) were used, 5–20 μg/ml of p10/NTF2 permitted the efficient nuclear import of the substrate, but a larger amount (~100 μg/ml) of p10/NTF2 inhibited import (Fig. 5A). In contrast, when 100 μg/ml of p10/NTF2 was added to the in vitro assay on ice in the presence of PTAC58 (40 μg/ml) and PTAC97 (40 μg/ml), the binding of T-APC to the nuclear rim was not inhibited (Fig. 5B). These results suggest that the in vivo inhibition of nuclear transport by exogenously injected p10/NTF2 may be due to an imbalance in the concentration of intracellular p10/NTF2 relative to other import factors and that the inhibition by p10/NTF2 may occur at the translocation step of nuclear import. From these findings, it is plausible to assume that the nuclear transport efficiency between the cytoplasm and the nucleus may be regulated by controlling the expression of p10/NTF2 in cells. In contrast, the expression of p10/NTF2 in cells may be

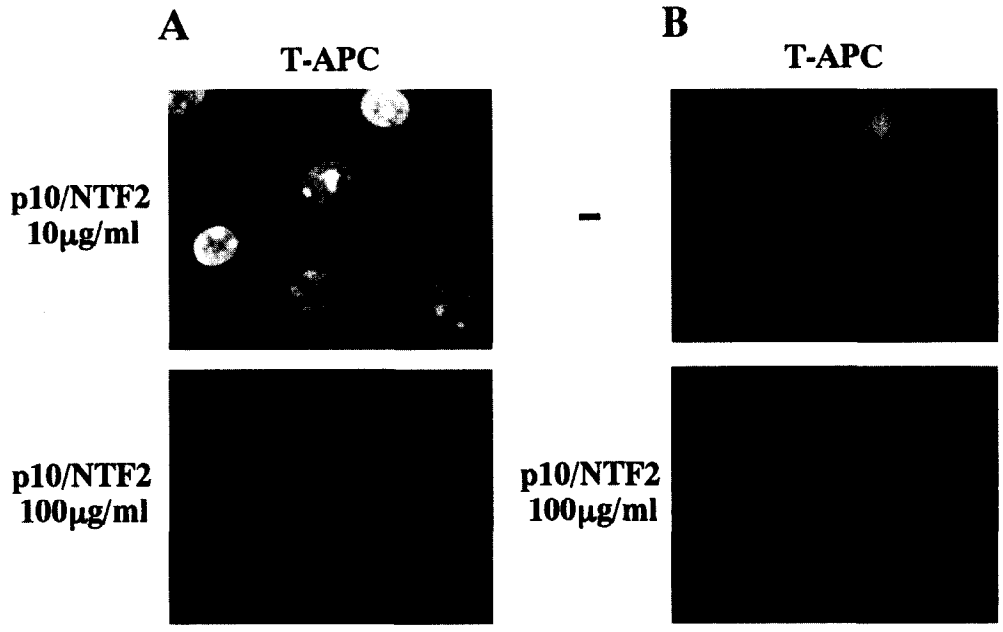


Fig. 5. Effects of p10/NTF2 on nuclear protein import in the in vitro assay. MDBK cells permeabilized with digitonin were incubated at 30°C for 20 min with transport buffer in the presence of 100 μg/ml T-APC, 40 μg/ml PTAC58, 40 μg/ml PTAC97, 100 μg/ml Ran/TC4, 10 or 100 μg/ml p10/NTF2, 1 mM ATP and its regeneration system (A) or on ice for 30 min in the presence of 100 μg/ml T-APC, 40 μg/ml PTAC58 and 40 μg/ml PTAC97, with or without 100 μg/ml p10/NTF2 (B).

strictly controlled so as to support constant and efficient nuclear transport.

Clearly, additional in vivo and in vitro experiments are needed in order to understand the exact roles of p10/NTF2 on the regulation of nuclear protein transport. The data collected here, however, suggest that p10/NTF2 may participate, not only in nuclear import, but also in nuclear export, and that nuclear import and export may be actually regulated by changes in the levels of p10/NTF2 in living cells.

Moreover, the present study showed that injected p10/NTF2 inhibits signal-mediated nuclear import and export but not passive diffusion in living cells, and that the p10/NTF2 protein is easy to handle because it is small and soluble. As a result, recombinant p10/NTF2 protein will be useful as a tool to examine physiological phenomena which involve active protein transport between the nucleus and cytoplasm in living cells.

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