Journal of Cellular Biochemistry 80:388–396 (2001)

Investigation of Nucleo-Cytoplasmic Transport Using UV-Guided Microinjection

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Abstract Active nucleo-cytoplasmic transport is mediated by dynamic signal-mediated pathways. We investigated the effects of transcription inhibitors or fluorescent lectins on nuclear import mediated by nuclear localization signals (NLSs). Therefore, a novel experimental approach that allows the controlled sequential introduction of fluorescent substances into living cells was established. A microinjection system equipped with an UV-source enabled us to identify fluorescent-labeled cells for the subsequent introduction of additional fluorescent compounds, in order to study their interactions in vivo. Cells were initially labeled either by expression of autofluorescent proteins or by microinjection of fluorescent substances. Transcription inhibitors did not affect nuclear transport mediated by classical NLSs but inhibited import mediated by the M9-domain of hnRNPA1. Comparison of a mono- and bipartite NLS revealed that the bipartite signal was more active in import. Sequential injection of differentially labeled nuclear import and export substrates allowed monitoring of import and export simultaneously in the same living cell. The introduced experimental approach will also be useful to analyze a variety of biological processes in living mammalian cells. J. Cell. Biochem. 80:388–396, 2001. © 2001 Wiley-Liss, Inc.

Key words: nucleo-cytoplasmic trafficking; transcription inhibitors; lectins; M9; microinjection; GFP/BFP/RFP

A hallmark of eukaryotic cells is their spatial and functional separation into the nucleus and the cytoplasm by the nuclear envelope. Although this separation introduces a potent and sophisticated level of regulation not existing in prokaryotes, it also requires a highly effective and selective transport machinery. Therefore, transport of proteins and nucleic acids through the nuclear pore is one of the most active research areas. Biochemical, genetic or in vitro assays led to the basic insights into the structure of the nuclear pore complex (NPC), the composition of nuclear import and export signals and the identification of receptors and cofactors required for transport (for detailed reviews see Corbett and Silver, 1997; Feldherr, 1998; Izzaurralde and Adam, 1998; Mattaj and Englmeier, 1998; Nakielny and Dreyfuss, 1997). However, it becomes more and more obvious that the orchestration of nuclear import/export requires a controlled interplay of kinetically fine-tuned processes. Thus, the living cell with its multi-dimensional information will be the ultimate test tube to investigate nucleo-cytoplasmic trafficking. Consequently, the combination of autofluorescent proteins (AFP) (e.g., GFP/BFP or newly discovered *Anthozoa* proteins [Matz et al., 1999]) together with chemically labeled fluorescent compounds represent an ideal in vivo experimental set-up. In order to study the controlled interactions of multiple fluorescent markers we introduce UV-guided microinjection. A microinjection

Abbreviations used: ActD, actinomycin D; AFP, autofluorescent proteins; BFP, blue fluorescent protein; ConA, concavalin A; DRB, 5,6-dichloro-ribofuranosylbenzimidazole; GFP, green fluorescent protein; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; RFP, red fluorescent protein; WGA, wheat germ agglutinin.

Grant sponsor: Johannes and Frieda Marohn-Stiftung, Wilhelm-Sander Stiftung; Grant number: 98.090.1/96.042.2; Grant sponsor: Kalkhof-Rose Stiftung, Deutsche Forschungsgemeinschaft; Grant number: SFB466.

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Received 7 March 2000; Accepted 26 June 2000

This article published online in Wiley InterScience, November XX, 2000.

stage equipped with an UV source allowed us to label cells by microinjection of fluorescent compounds (recombinant autofluorescent fusion proteins or chemically labeled substances) and to re-identify the labeled cells for a subsequent round of microinjection, in order to study the effect(s) of different molecules in the same living cell. Alternatively, cells initially marked by the transient expression of AFP-tagged fusion proteins were identified and specifically targeted with additional fluorescent molecules.

Our report not only describes a novel approach to study nucleo-cytoplasmic trafficking but also demonstrates, for the first time, the use of recombinant purified blue fluorescent protein fusions for in vivo applications, which increases the potential of AFP-tagged proteins in general.

MATERIALS AND METHODS

Plasmids

The bacterial expression vector pGEX-GFP encoding a fusion of glutathione S-transferase (GST) linked to GFP has been described before [Rosorius et al., 1999]. To generate the plasmid pGEX-BFP, GFP was replaced by BFP. For this purpose the BFP-coding region was amplified by PCR using specific oligonucleotides containing *BamHI*- or *EcoRI*-restriction sites and the plasmid pCMV-GFPsg50 [Stauber et al., 1998b] as a template. Plasmid pGEX-RFP expresses a fusion of GST linked to RFP. The RFP-coding region was PCR amplified using specific oligonucleotides containing *BamHI*- or *EcoRI*-restriction sites and the plasmid pDsRed-N1 (Clonetech, Germany) as a template and used to replace GFP in the pGEX-GFP vector. Plasmid pGEX-SV40NLS-BFP encodes a GST–BFP fusion protein containing the classical SV40 large T-antigen nuclear import signal [Kalderon et al., 1984] (amino acids TPPKKKRKVEDP) and was constructed using synthetic double-strand oligonucleotides followed by ligation into the *BamHI*/*NheI*-cut vector pGEX-BFP. The construct pGEX-NLS-GFP [Rosorius et al., 1999] codes for a GST–NLS–GFP fusion protein containing a typical bipartite NLS of the Herpes Simplex Virus ICP22 protein (amino acids 14–35; KARRPALRSPP LGTRKRKRPSR). The construct pGEX–M9–GFP encodes a GST–M9–GFP fusion protein containing the M9 domain of the hnRNPA1 protein (amino acids 264–308; GNYNNQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGYGGS). The plasmid was constructed by PCR amplification of the M9 sequence using specific primers containing *BamHI*- or *NheI*-restriction sites and cloning into the vectors pGEX-GFP. Plasmid pGEX–E1BNES–GFP encodes a GST–GFP fusion with the adenovirus type 5 E1B-55K amino acids 83–93 (LYPELRRILTI) and was described before [Krätzer et al., 2000]. pBrev–GFP expressing a GFP-tagged HIV-1 Rev protein was already described [Stauber et al., 1998a]. The coding regions of all constructs were confirmed by sequence analysis.

Purification of GST-GFP/BFP Fusion Proteins

Recombinant GST–BFP/GFP hybrid proteins were expressed in *E. coli* BL21 and affinity purified from crude lysates with glutathione-sepharose 4B according to the specifications of the manufacturer under non-denaturing conditions (Pharmacia Biotech., Freiburg, Germany). Briefly, 500 ml of exponentially growing cultures were induced with 0.5 mM IPTG for 4 h at 33°C. Bacterial lysates were incubated with 500 µl glutathione-sepharose 4B for 2 h at 4°C in an overhead rotator. The bound GST–BFP/GFP fusion proteins were eluted using 15 mM glutathione, dialyzed extensively against cold PBS and stored at −70°C. SDS-polyacrylamide gel electrophoresis revealed a major single band of approximately 54 kDa.

Chemicals

Texas-Red-labeled Concavalin A (ConA), Texas-Red-labeled wheat germ agglutinin (WGA), FITC conjugated IgG, the transcription inhibitors Actinomycin D and 5,6-dichlororibofuranosylbenzimidazole (DRB) were purchased from Molecular Probes Europe BV (Leiden, the Netherlands) or Sigma (Deisenhofen, Germany), respectively.

Microscopy and Microinjection

Vero or HlTat cells were prepared for microinjection and transfected as described [Rosorius et al., 1999]. To identify and microinject specifically fluorescent-labeled cells a CompiC INJECT computer-assisted injection system equipped with an UV source and detection system was used (Cellbiology Trading, Hamburg, Germany). Typically, 10 fl of the fluorescent transport substrates (adjusted to

approximately 2–3 mg/ml) were injected into the cytoplasm or nucleus of somatic cells, respectively. Following injection, cells were immediately analyzed using the appropriate fluorescence filters as described [Heger et al., 1999; Rosorius et al., 1999].

Treatment with Transcription Inhibitors

Cells were incubated for 2 h with a medium containing either ActD (concentration 2 μg/ml) or DRB (concentration 50 μg/ml) prior to microinjection and the drugs were present throughout the experiment.

RESULTS AND DISCUSSION

Nuclear Import Mediated by Classical NLSs or the M9-Domain Does not Depend on Active Transcription

Previously, it was described that transcription inhibitors appear to block nuclear import of hnRNPA1 [Pinol-Roma and Dreyfuss, 1992] or the HIV-1 Rev protein [Meyer and Malim, 1994; Richard et al., 1994] and affect nuclear export of the von Hippel–Lindau tumor suppressor protein (VHL) [Lee et al., 1999] or the poly-A binding protein 1 (PABP1) [Afonina et al., 1998]. However, it is still controversial if nucleo-cytoplasmic transport is directly inhibited or the effects are indirect by affecting cytoplasmic/nuclear retention. This demands study of import/export in the absence of other functional domains of the proteins in the context of a defined system. Consequently, we investigated the influence of transcription inhibitors (ActD/DRB) on nuclear import mediated by classical NLSs using UV-guided microinjection in living cells. To control the biological effect of drug treatment on a single cell basis we used the HIV-1 Rev protein as an in vivo indicator. Rev responds highly sensitively to treatment with transcription inhibitors since ActD concentrations reported to affect preferentially RNA polymerase I (0.04 μg/ml) or RNA polymerase II (0.5 μg/ml) resulted in the cytoplasmic localization of Rev [D'Agostino et al., 1995; Dundr et al., 1995; Richard et al., 1994; Stauber et al., 1995]. To ensure a robust inhibition of all classes of RNA polymerases cells were treated with ActD (2 μg/ml) or DRB (50 μg/ml) for 2 h which caused cytoplasmic accumulation of Rev-GFP in less than 1 h. Subsequently, a GST–BFP fusion protein harboring the well-characterized monopartite SV40 large T-antigen NLS was microinjected into the cytoplasm of Rev-GFP expressing cells (Fig. 1). Nuclear import was completed after 40 min (Fig. 1A and B, time = 0 min postinjection not shown), even if the cells had been pretreated with ActD or DRB (Fig. 1C and D; data not shown). Likewise, nuclear import of a fluorescent substrate containing a bipartite NLS of the Herpes Simplex Virus ICP22 protein [Rosorius et al., 1999] was not affected by these inhibitors of RNA-polymerase I and II (data not shown). In our GST–GFP system the bipartite ICP22 NLS was more active compared to the SV40 NLS as judged by the kinetics of nuclear import (see Fig. 2). Similar results were obtained in at least three independent microinjection experiments (data not shown). Interestingly, Feldherr and colleagues reported that a monoparite NLS was more effective in mediating nuclear import than a bipartite NLS in *Amoebae* [Feldherr and Akin, 1999], implicating changes in signal-mediated nuclear transport during evolution. The existence of qualitatively different nuclear import signals might also contribute to regulate the biological activity of the respective NLS-containing proteins.

Nuclear import and export of hnRNPA1 is mediated by the so called M9 domain and involves the import receptor transportin instead of the classical importin α/β axis [Bogerd et al., 1999; Izaurralde et al., 1997a; Nakielny et al., 1996]. ActD treatment was reported to cause slow cytoplasmic accumulation of the complete hnRNPA1 [Pinol-Roma and Dreyfuss, 1992]. However, it was not clear if nuclear import mediated by the M9 domain itself depends on active transcription. To probe the influence of transcription on the M9-mediated import independent of nuclear export, a GST–M9–GFP fusion protein was injected into the cytoplasm of cells treated with ActD or untreated control cells. While nuclear import was completed after 2.5 h in the control cells (Fig. 3 A–C), import was significantly impaired in the absence of active transcription (Fig. 3D–F). Similar results were observed in three independent injection experiments (data not shown). Using heterokaryon analysis, Piñol-Roma and Dreyfuss [1992] reported that hnRNPA1 shuttled in about 4 h. Thus, the observed import time for the GST–M9–GFP fusion was still in the biological relevant time frame. Siomi and colleagues [1997] described the cytoplasmic accumulation

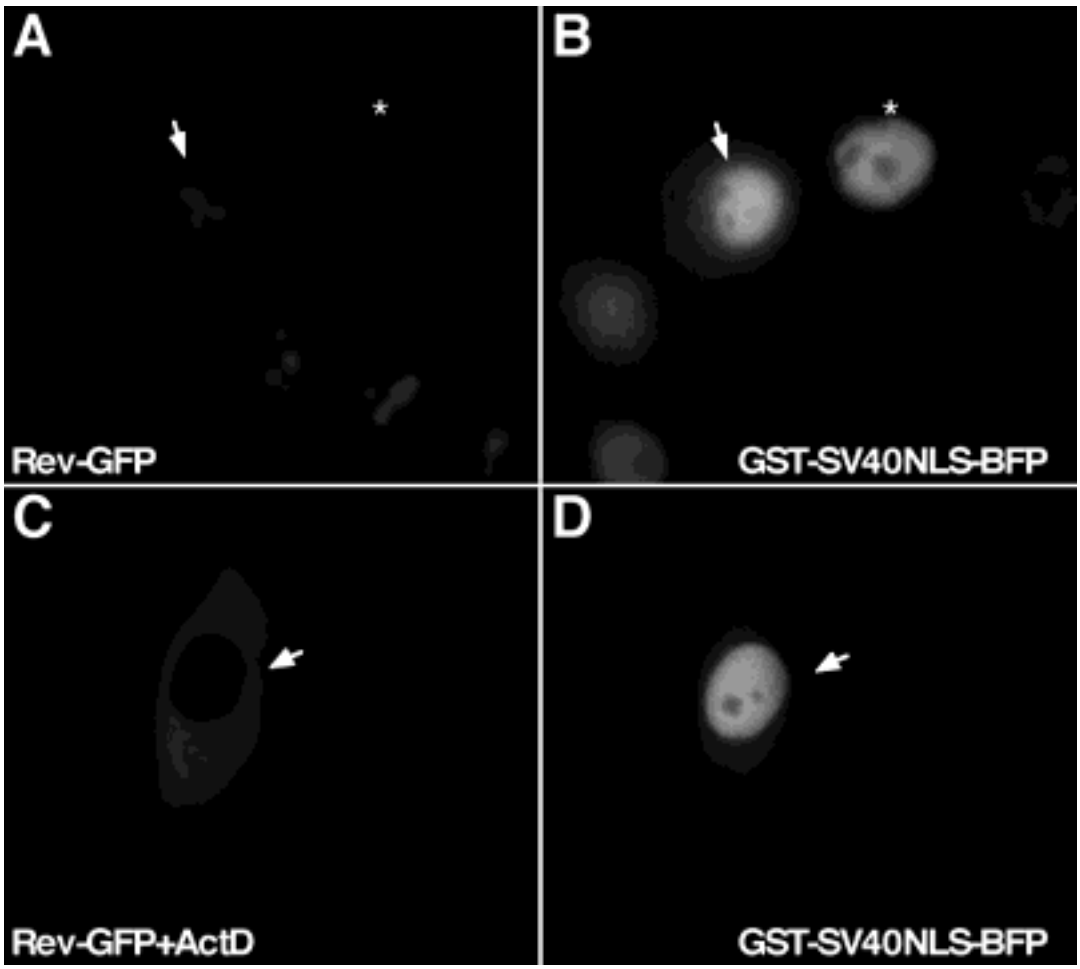

Fig. 1. Nuclear import of a GST–SV40NLS–BFP hybrid is not affected by ActD in living cells. Purified GST–SV40NLS–BFP was microinjected into the cytoplasm of HlTat cells previously transfected with pBrev–GFP and nuclear import observed directly by fluorescence microscopy. **A**, **B**: cells 40 min post-injection. Nuclear import was completed in cells expressing Rev-GFP (marked by the arrow) or in control cells (marked with asterisks). **C**, **D**: cells pretreated prior to injection with ActD (2 μg/ml for 2 h) at 40 min post-injection. The cytoplasmic accumulation of Rev-GFP assures efficient ActD treatment (C) which did not inhibit nuclear import (D). Arrows mark the same cells. GFP or BFP, respectively, were detected independently using the appropriate fluorescence filters.

of a nuclear pyruvate kinase–M9 fusion when RNA polymerase II was inhibited supporting our observation. The M9 domain itself appears therefore to be a specific sensor for transcription-dependent nuclear transport.

We concluded that nuclear import mediated by classical NLSs does not depend on active transcription in contrast to M9 domain-dependent transport. Since import of HIV-1 Rev appears to be directed by a NLS resembling the SV40 T-antigen NLS and also uses the importin β-pathway [Henderson and Percipalle, 1997] the effects of transcription inhibitors on trafficking could also be indirect. Preventing active transcription might affect secondary binding sites in the nucleus (e.g., by the degradation of 5S RNA at the nucleolus which binds the HIV-1 Rev protein [Lam et al., 1998] and therefore changes the steady-state localization of the protein, rather than interfering directly with transport. Additional studies in defined import/export systems are necessary to completely understand the effect of transcription inhibitors on the trafficking of HIV-1 Rev, VHL or PABP1.

Specific Lectins to Probe Nuclear Import

Several groups reported that lectins are able to bind to the NPC and effectively block nuclear import in mammalian cells and *Xenopus* oocytes. However, careful inspection of the literature reveals some discrepancies regarding

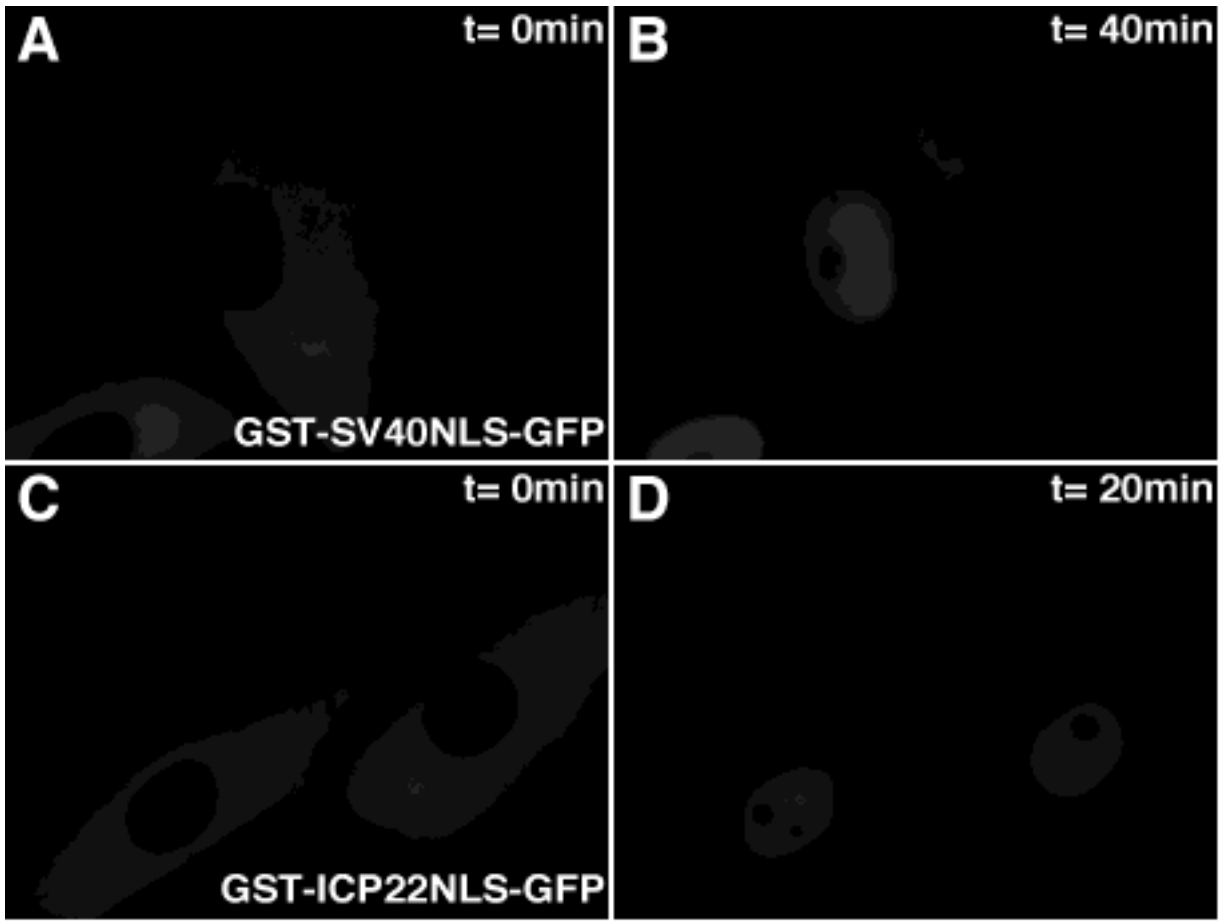

Fig. 2. Nuclear import mediated by the ICP22 bipartite NLS is more efficient than the monopartite SV40 NLS. Equal amounts of purified GST–NLS–GFP (2 mg/ml) was microinjected into the cytoplasm of Vero cells and nuclear import monitored directly by fluorescence microscopy. **A**: 0 min post-injection. **B**: 20 min. **C**: 0 min. **D**: 40 min. While nuclear import of the GST–ICP22NLS–GFP (A/B) was completed after 20 min the GST–SV40NLS–GFP (C/D) took about 40 min at 37°C.

the intracellular localization of the injected lectins and their effects on nuclear transport. To avoid a potential masking of the NLS by coinjection of the import substrate together with lectins we first injected cells with the fluorescent-labeled lectins, re-identified the injected cells and applied the autofluorescent import substrates by another round of injection. Microinjected fluorescent WGA (concentration 2 mg/ml) accumulated in the nucleus and at the nuclear envelope in living cells and completely blocked nuclear import mediated by the SV40 NLS and the bipartite NLS (Fig. 4A and B and data not shown). Coinjection of FITC-labeled IgG served as a microinjection control (Fig. 4C). Although the extensive study by Rutherford et al. and others [Dabauvalle et al., 1988; Rutherford et al., 1997; Yoneda et al., 1987] reported that WGA localized primarily in the cytoplasm and to the nuclear envelope, Wolff and colleagues [1988] also observed WGA in the nucleus of living cells. How WGA gets access to the nucleus and if the reported differences in localization are due to the preparation of WGA, the cell type used (e.g., mammalian cells vs. *Xenopus* oocytes) or influenced by the fixation procedure awaits further investigation.

Injection of fluorescent ConA (concentration 2 mg/ml), at concentrations higher than reported to partially affect nuclear import in in vitro assays (concentration 0.1 mg/ml) [Finlay et al., 1987], did not block nuclear import in living cells as assayed by the subsequent injection of the fluorescent import substrates (data not shown). ConA localized to the cytoplasm but did not accumulate at the nuclear envelope (data not shown). Similar results were reported

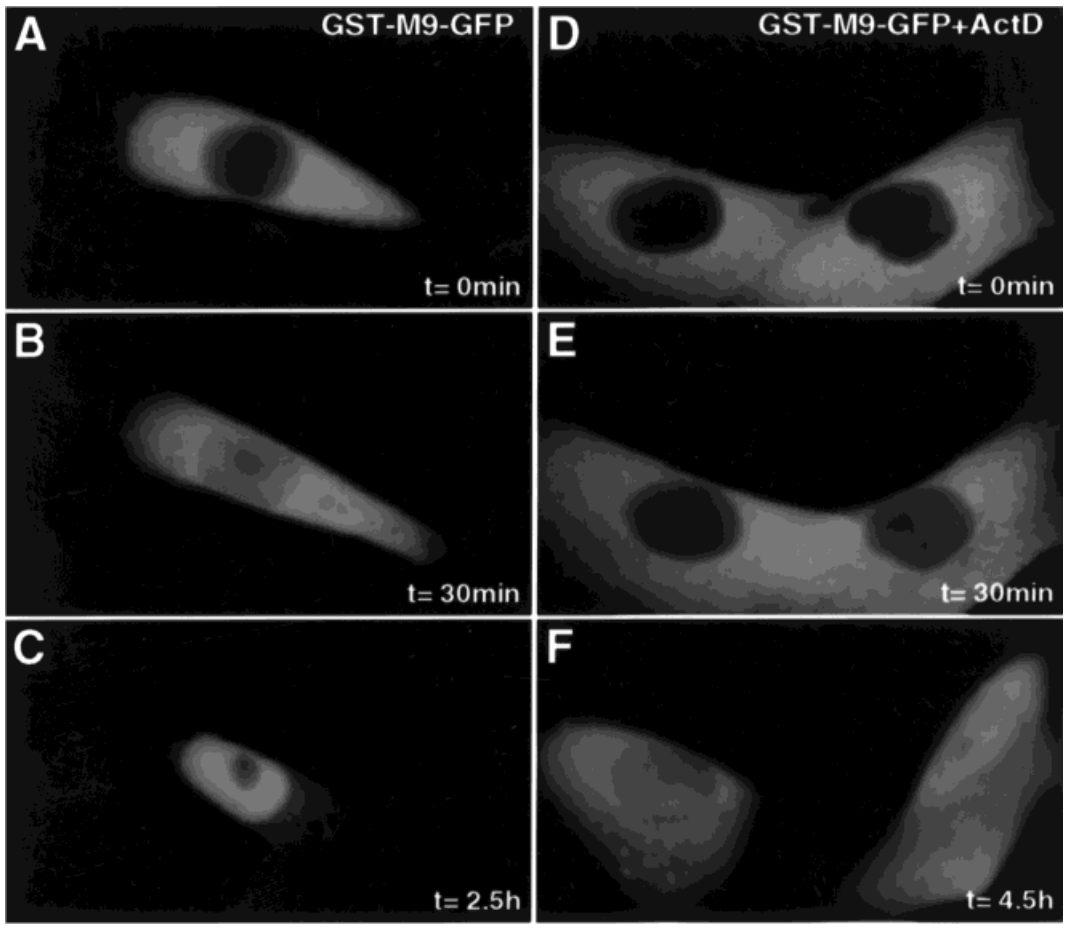

Fig. 3. ActD treatment impairs nuclear import mediated by the M9 domain. Purified GST–M9–GFP was microinjected into the cytoplasm of Vero cells and nuclear import observed directly by fluorescence microscopy. **A–C**: Untreated cells at different time points after injection. M9-mediated import was completed after 2.5 h. **D–F**: Cells were treated prior to injection with ActD (2 μg/ml for 2 h). In contrast to untreated cells import was significantly impaired even after prolonged incubation (F).

by Wolff et al. [1988] and Yoneda et al. [1987] by coinjecting ConA together with an import substrate. One could speculate that the higher molecular weight of ConA (104 kDa) compared to WGA (36 kDa) prevents the access to lectin-binding sites inside intact NPCs, since ConA is generally used as a marker to probe for intact nuclear envelopes [Newmeyer et al., 1986]. Thus, treatment with detergents (e.g., cell permeabilization) might be required to unmask ConA binding site. Results obtained in in vitro systems should therefore be interpreted with caution and verified in living cells. It is striking that out of ten different lectins [Finlay et al., 1987; Yoneda et al., 1987] only WGA which recognizes *N*-acetyl-glucosaminyl residues was able to block efficiently the passage through the nuclear pore. Although WGA does not unspecifically damage the NPC [Rutherford et al., 1997 and references therein] it is still not clear if WGA binds specifically to nucleoporins directly involved in import or sterically prevents the interaction of essential factors with the transport cargoes. Our system sets the stage to examine the effects of various lectins on nucleo-cytoplasmic transport in living cells. However, the functional relevance of identified lectin-binding proteins have to be reevaluated by biochemical [Pante et al., 1994] and genetic approaches.

Of note, the attempt to include the newly discovered red fluorescent protein (RFP) from *Anthozoa* [Matz et al., 1999] in our system failed since a GST-RFP displayed extensive aggregation and could not be used in microinjection (data not shown). Thus, although BFP is more affected by bleaching compared to GFP, the BFP mutant used in our study [Stauber

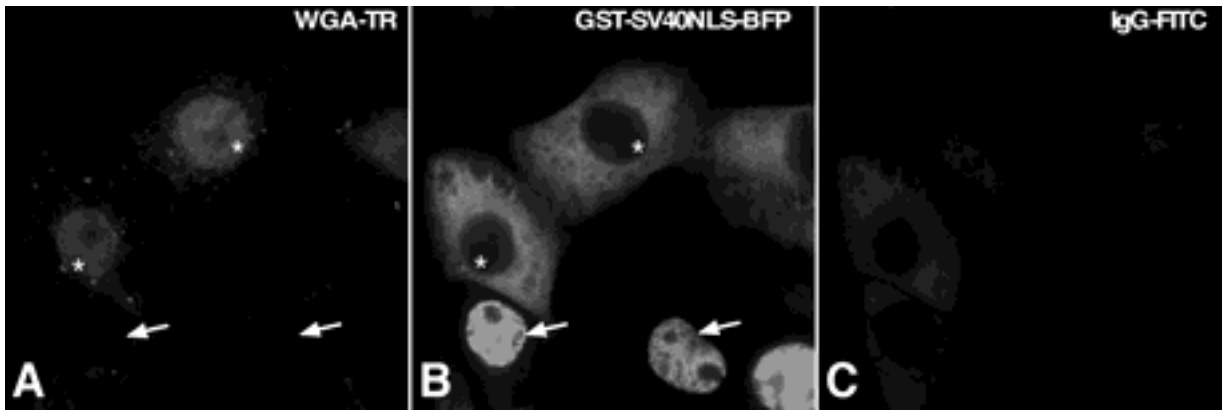

Fig. 4. WGA blocks nuclear import in living cells. Texas Red conjugated WGA (TR-WGA) was microinjected into the cytoplasm of Vero cells (**A**). Subsequently, purified GST–SV40NLS–BFP was injected into the cytoplasm (**B**). The presence of WGA prevented the nuclear migration of the substrate (cells marked by asterisks) whereas import was completed in control cells (indicated by arrows) in 40 min. Coinjected FITC conjugated IgG served to control for cytoplasmic injection. The different fluorescent signals were recorded using the appropriate filter sets.

et al., 1998b] allows the application of recombinant purified BFP-hybrids in vivo.

Nuclear Import and Export Appear not to Interfere With Each Other

The majority of transport events requires the interaction with at least one member of the importin β superfamily and the small GTPase Ran which determines the directionality of transport [Izaurralde et al., 1997b]. Recently, it was reported that the direction of transport through the NPC can be inverted [Nachury and Weis, 1999] and that *Xenopus* pores allow transport in both directions [Dworetzky and Feldherr, 1988; Keminer et al., 1999]. In spite of this, the nucleus is a highly structured organelle and transport of proteins and RNAs has been observed to occur on specific tracks [Kimura et al., 2000; Lawrence et al., 1989; Meier and Blobel, 1992; Rosbash and Singer, 1993]. Thus, in mammalian cells certain pores might preferentially mediate export or import which would represent another level of regulation for transport processes. We therefore asked, if the simultaneous application of relatively large import and export substrates (approx. 60 kDa) could significantly affect the kinetics of the respective transport processes. A recombinant nuclear export substrate (GST-E1BNES-GFP) was microinjected into the nucleus (concentration 30 μM) and subsequently an import cargo (GST-SV40NLS-BFP) was microinjected into the cytoplasm (concentration 30 μM) of the same cells (Fig. 5A and B). Of note, the localization of the GST-AFP substrates was not flawed by passive diffusion [Rosorius et al., 1999]. To prevent the onset of export in between microinjections the cells were kept in ice cold medium. We observed that export and import occurred simultaneously and were not significantly delayed compared to control cells which had been injected with the respective transport substrate only (Fig. 5C and D; data not shown). Under our experimental conditions, the cells were capable of handling even a sudden burst of import and export cargoes. Taking the mean value of a Vero cell (approximately 450 fl) and the mean number of NPCs per nucleus (approximately 3300) [Kubitscheck et al., 1996], the estimated import rates of about 2 molecules/s per NPC and 4–6 molecules/s per NPC for export [Keminer et al., 1999], underlines the efficiency of the nuclear transport machinery. Although these measurements were performed in the absence of cargo destined for transportation in the reverse direction [Keminer et al., 1999], they explain why transport was not saturated by the amounts of substrate applied in our assay. We did not attempt to microinject extremely concentrated protein solutions which might result in unspecific side effects on nuclear transport. Clearly, additional studies are necessary to investigate if certain combinations of import and export signals are able to affect each other. In particular, it will be interesting to analyze the effect of the so-called M9 sequence which can function as a NLS and NES [Bogerd et al., 1999] on import and export. In addition, electron micro-

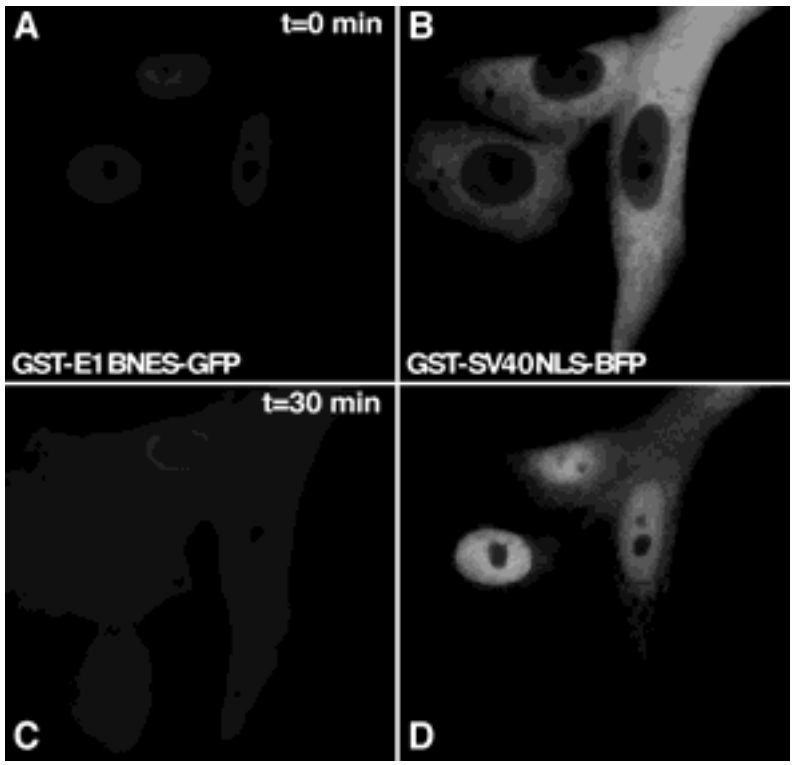

Fig. 5. Nuclear import and export do not affect each other. Vero cells were microinjected into the nucleus with an export substrate (GST–E1BNES–GFP) and subsequently an import substrate (GST–SV40NLS–BFP) was injected into the cytoplasm of the same cells (**A**, **B**). Nuclear export and import occurred simultaneously and did not significantly inhibit each other (**C**, **D**). Similar results were obtained in independent experiments.

scopy studies will help to visualize if NPC are capable of handling import and export substrates simultaneously.

Taken together, the presented work demonstrates how UV-guided microinjection can be applied to study nucleo-cytoplasmic transport in living cells. Transcription inhibitors appear not to affect the importin α/β axis in contrast to M9-mediated import. Lectins, in combination with specific antibodies, will be important tools in identifying additional factors involved in nuclear transport. The finding that there are qualitatively different nuclear import signals suggests also a potential regulatory role for the NLS. Our approach to study the interactions of multiple fluorescent compounds can be generally applied to investigate a variety of biological processes inside the living cell (e.g., protein interactions, signal transduction, apoptosis, etc.). Since the living cell also becomes more and more attractive in high content drug screening assays, the combination of live fluorescent markers will increase the power of these approaches in the future.

ACKNOWLEDGMENTS

R. Stauber and F. Krätzer contributed equally to this work. We thank Bernhard Fleckenstein for continuos support. R. S. is supported by the DKFZ AIDS-Stipendium Program.

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