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Diffusion of Proteins Across the Nuclear Envelope of HeLa Cells

BioTechniques 24:668-674 (April 1998)

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ABSTRACT

We describe an experimental system to study nucleocytoplasmic diffusion of proteins in living HeLa cells. To localize proteins to the nucleus, substrates were created that contain a nuclear localization sequence fused to Aequorea victoria green fluorescent protein (GFP). Transiently and stably transfected HeLa cells were used for these assays. A protein of 29-kDa molecular mass that harbors GFP and the bipartite Xenopus nucleoplasmin nuclear localization sequence (NLS) accumulates efficiently in nuclei of HeLa cells. However, in the absence of active facilitated nuclear import, the reporter protein exits the nucleus and equilibrates between nucleus and cytoplasm. We define different conditions that promote the diffusion of small nuclear proteins across the nuclear envelope of mammalian culture cells. Our results set the stage to analyze the competence of nuclear pore complexes for nucleocytoplasmic diffusion of macromolecules in living cells.

INTRODUCTION

The distribution of proteins between nucleus and cytoplasm depends on their size as well as the presence of specific targeting signals (7,8). Diffusion and facilitated transport between nucleus and cytoplasm are mediated by nuclear pore complexes. Proteins with a molecular mass of 40 kDa or less rapidly equilibrate between both compartments (12,13). Although such small proteins can traverse the nuclear envelope by diffusion, accumulation within the nucleus requires the presence of specific signals such as nuclear localization sequences (NLSs) or nuclear retention sequences.

The presence of an NLS promotes nuclear accumulation by facilitated import of the protein, a process that requires energy and a specialized transport apparatus (5,6,8,11). In the absence of an NLS, small nuclear proteins that are not retained in the nucleus will diffuse into the cytoplasm unless the nuclear pore channel for passive diffusion is occluded. The aqueous diffusion channel of the nuclear pore complex has a functional diameter of approximately 9 nm, which can be altered under certain conditions such as depletion of calcium stores in the perinuclear space (9). This indicates that cells might regulate the passive translocation of molecules across these channels. Additional studies with isolated nuclei and nuclear membranes verified that diffusion of intermediate-sized molecules is dependent on nuclear calcium stores. By contrast, diffusion of molecules of less than 0.5 kDa is not affected by depletion of calcium in the perinuclear space (16). At present, mechanisms that control the diameter of nuclear diffusion channels are not understood. Moreover, the components that respond to the concentration of nuclear calcium stores are not defined, and an assay system is required to study diffusion across the nuclear envelope. To this end, we have developed experimental strategies to follow nucleocytoplasmic diffusion of proteins in living mammalian cells. Fusion proteins containing green fluorescent protein (GFP) and the bipartite NLS of Xenopus laevis nucleoplasmin or the C-terminal half of nucleoplasmin were generated. These reporter proteins served as substrates to define conditions that promote the diffusion of proteins from the nucleus into the cytoplasm in higher eukaryotes. Our results describe a test system for future studies to analyze the effect of chemical compounds and mutant components of the nuclear envelope on the physiological state of diffusion channels of the nuclear pore complex.
MATERIALS AND METHODS

Cell Culture and Transfection

HeLa cells (HTA-1) were grown in Dulbecco’s modified Eagle medium (DMEM) containing penicillin G (50 U/mL), streptomycin (50 µg/mL) and 8% fetal bovine serum (complete medium). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For transient transfections, cells were subcultured in 6-well tissue culture plates. DNA was introduced with the calcium phosphate/DNA co-precipitation method (14) and CalPhos Maximizer™ (Bio/Can, Mississauga, ON, Canada) as described by the manufacturer. To generate stable cell lines, HTA-1 cells were transfected following the modified transfection protocol described (14).

Identification of HeLa Cells Synthesizing GFP Fusion Proteins

Cells were incubated overnight with DNA followed by addition of fresh medium. Approximately 7 h after the change of medium, cells were trypsinized and transferred to Lab-Tek® Tissue Culture Chamber Slides (Nalge Nunc International, Rochester, NY, USA). Cells were grown for another 16–44 h in complete medium supplemented with 50 nM dexamethasone. To study diffusion, cells were washed and incubated in dexamethasone-free medium for 1 h at 37°C before analysis. To test different conditions for their effect on nucleocytoplasmic traffic, cells were washed with phosphate-buffered saline (PBS) and incubated in PBS in the cold for times indicated in Figure 2a. Alternatively, cells were treated with 10 mM sodium azide and 10 mM 2-deoxy-D-glucose in PBS as described for Figures 2b and 3. All subsequent steps were carried out at room temperature. Slides were washed in PBS, incubated for 25 min in PBS/3.7% formaldehyde and rinsed with PBS. Samples were incubated with PBS containing 4',6-diamidino-2-phenylindole (DAPI) and 2 mg/mL bovine serum albumin (BSA), washed in PBS and mounted in 30% glycerol in PBS. Slides were sealed with rubber cement.

Generation of Stable Cell Lines and Detection of Nuclear Reporter Proteins

HTA-1 cells were grown and transfected as described above. Cells were kept under standard conditions for at least two weeks without selection. One day after addition of 50 nM dexamethasone, cells were sorted by fluorescence-activated cell sorting into 24-well plates under sterile conditions. A maximum of one cell per well was obtained after sorting. Cells were further grown for two to three weeks and tested for the synthesis of nuclear reporter proteins. Towards this end, stable cell lines were incubated in the presence of 50 nM dexamethasone (2 days) and analyzed by fluorescence microscopy as detailed above.

Plasmid Constructions

Plasmid pGRE5-2 (4,10) was used as a vector for genes encoding reporter proteins. Gene expression is controlled by glucocorticoid response elements and can be induced by dexamethasone (10). The generation of a mutant form of GFP carrying a Ser65→Thr65 mutation was described earlier (4). The mutant GFP allele was cloned into pBlue-script® KS(+) (Stratagene, La Jolla, CA, USA) digested with KpnI and EcoRI (4).

The generation of a gene encoding the C-terminal half of Xenopus nucleoplasmin fused to a single copy of GFP has been described (4). This reporter protein is referred to as nucleoplasmin-GFP. A gene fusion encoding nucleoplasmin NLS and GFP, termed NP-GFP, was created by fusion of oligonucleotides encoding the bipartite NLS (1) to GFP. Gene fusions containing two copies of GFP were created by
removal of the UAG stop codon of GFP followed by insertion of a NotI linker (12-mer; New England Biolabs, Beverly, MA, USA). In addition, a NotI linker was inserted into the KpnI site of plasmid p580 (4). Both copies of the GFP coding sequence were fused in-frame by their NotI sites.

RESULTS

Nuclear Reporter Proteins Containing GFP

We have generated genes encoding substrates for nuclear transport and nucleocytoplasmic diffusion. Polypeptide nucleoplasmin-GFP has a predicted molecular mass of 38 kDa, and it contains the C-terminal half of Xenopus nucleoplasmin including the bipartite NLS and the DNA-binding region (4). The 29-kDa protein NP-GFP carries only the bipartite NLS of nucleoplasmin and lacks the DNA-binding region of nucleoplasmin.

Figure 2. Nucleocytoplasmic diffusion of NP-GFP. (a) Chilling of cells. HeLa cells transiently expressing the NP-GFP gene were incubated for 2 h at 4°C, followed by 2 h on ice. The distribution of fluorescence was determined before (A and B) and after (C and D) chilling. After incubation on ice, cells were returned to 37°C and incubated overnight in the absence of the inducer dexamethasone to test for reimport of NP-GFP into the nucleus (E and F). Panels A, C and E show phase contrast, Panels B, D and F show the distribution of fluorescence. (b) Treatment with sodium azide/deoxyglucose at different temperatures. Transiently transfected HeLa cells containing NP-GFP were incubated with azide/deoxyglucose as described in Materials and Methods. The localization of NP-GFP was determined before (B and H) and after treatment with sodium azide/deoxyglucose for 45 min (D and J). Slides were washed in PBS and incubated overnight with medium lacking dexamethasone to analyze accumulation of NP-GFP in the nucleus (F and L). Identical experiments were carried out on ice (A–F) and at room temperature (G–L). Phase contrast is presented in Panels A, C, E, G, I and K, and the location of fluorescent proteins is shown in Panels B, D, F, H, J and L.
domain. GFP2 contains an in-frame fusion of two copies of GFP with a molecular mass of 54 kDa.

**Transient Expression of GFP-Containing Substrates in HeLa Cells**

To determine the cellular localization of transport substrates under normal growth conditions, HeLa cells were transiently transfected with plasmids that express fusion proteins under the control of a regulatable promoter. Gene expression is controlled by glucocorticoid response elements and is induced in the presence of dexamethasone. When synthesized in HeLa cells, NP-GFP is efficiently targeted to the nucleus because of the presence of the bipartite NLS derived from *Xenopus* nucleoplasmin (Figure 1B). Nontransfected cells display only a low level of autofluorescence (Figure 1, B, D and F); low autofluorescence signals were also obtained for noninduced cells (Figure 4 and below).

By contrast, GFP lacking an NLS was detected in both cytoplasm and nucleus (Reference 4 and Figure 1D). This distribution is expected because the small size of the protein permits its translocation across the nuclear envelope by passive diffusion. In addition, we expressed a gene encoding two copies of GFP (GFP2) in HeLa cells. GFP2, a protein with a molecular mass of approximately 54 kDa, also traverses the nuclear envelope. However, because GFP2 is missing an NLS, it does not accumulate in nuclei (Figure 1F).

**Diffusion from the Nucleus into the Cytoplasm**

Analysis of protein diffusion across the nuclear envelope requires substrates that are small enough to translocate across the nuclear pore in the absence of facilitated transport. NP-GFP, a 29-kDa protein that contains the *Xenopus* nucleoplasmin NLS fused to GFP, accumulated in nuclei under normal growth conditions (Figure 1B). However, its small size should permit NP-GFP to also traverse the nuclear pore complex by passive diffusion. A similar approach has been reported for *Saccharomyces cerevisiae* using a 43-kDa protein harboring simian virus 40 (SV40)-NLS (15). The smaller-sized NP-GFP described by us, however, is likely to improve its equilibration between nucleus and cytoplasm under conditions that abolish facilitated nuclear transport. Chilling of cells and depletion of cellular ATP pools differently affect facilitated transport and diffusion across the nuclear envelope. Whereas facilitated transport is temperature- and energy-dependent, passive diffusion across nuclear pore complexes still occurs upon chilling or energy depletion (5,6,8,11). These treatments can therefore be used to study diffusion across nuclear pore complexes in the absence of facilitated nuclear transport.

To test whether NP-GFP diffuses across the nuclear envelope, the inducer dexamethasone was removed, and cells were transferred to 4°C. Although this led to diffusion of NP-GFP from the nucleus into the cytoplasm, even after 4 h of incubation at 4°C, the nuclear fluorescence was more intense than the fluorescence signal seen for the cytoplasm (data not shown).

If cells were incubated for 4 h at 4°C and returned to 37°C in growth medium, cytoplasmic NP-GFP was reimported into nuclei, demonstrating that facilitated transport was regained under normal growth conditions. Prolonged incubation at 4°C resulted in rounding of HeLa cells, which was also reversible. Round cells flattened again and spread on the slide after overnight incubation at 37°C.

A more pronounced exit of NP-GFP from the nucleus into the cytoplasm was obtained when cells were incubated 2 h at 4°C and subsequently 2 h on ice. Under these conditions, fluorescence signals observed for nuclei and for the cytoplasm were of similar intensity, indicating the efficient equilibration of NP-GFP between nucleus and cytoplasm (Figure 2a, Panel D). These results support the idea that upon chill-

![Figure 3. Kinetic of the diffusion of NP-GFP across the nuclear envelope in HeLa cells treated with sodium azide/deoxyglucose. HeLa cells were incubated at room temperature with sodium azide/deoxyglucose, and the nucleocytoplasmic distribution of the fluorescence signal was determined for 0, 10, 30, 45, 60 and 90 min of the incubation with sodium azide/deoxyglucose as indicated in the figure.](image)
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ing, NP-GFP left the nucleus by diffusion, and nuclear accumulation of NP-GFP is prevented because of the absence of facilitated nuclear import.

To determine whether nuclear exit of NP-GFP in chilled cells is reversible, cells were first treated 2 h at 4°C and 2 h on ice and subsequently incubated in normal growth medium at 37°C (Figure 2a, Panel F). Under these conditions, cytoplasmic NP-GFP was reimported into nuclei, demonstrating that cells recovered from previous chilling.

In addition to cold treatment, we also tested the effect of sodium azide/2-deoxy D-glucose (azide/deoxyglucose) on the localization of NP-GFP. After incubation of transiently transfected cells with azide/deoxyglucose, NP-GFP equilibrated efficiently between nucleus and cytoplasm (Figure 2b, Panel D). We further determined whether incubation with azide/deoxyglucose led to nuclear exit of NP-GFP at other temperatures. Treatment at room temperature (Figure 2b, Panel J) or at 37°C (data not shown) results in diffusion of NP-GFP across the nuclear envelope. For all the different temperatures tested, reimport of NP-GFP upon return of cells to normal growth medium and 37°C is observed (Figure 2b, Panels F and L). Note, however, that after incubation with azide/deoxyglucose at 37°C, fewer cells seem to recover when compared with those pretreated at room temperature or incubated on ice.

To study diffusion of NP-GFP across the nuclear envelope in more detail, we followed the distribution of fluorescence in cells that were incubated with azide/deoxyglucose at room temperature. Upon treatment with azide/deoxyglucose, a gradual increase of cytoplasmic fluorescence was observed (Figure 3, B–F). The equilibration of NP-GFP between nucleus and cytoplasm is complete at approximately 45 min, and prolonged incubations with inhibitors do not lead to further changes. Some fading of the fluorescence signal is detected after one hour of incubation. This could be explained by bleaching of the fluorescence signal possibly accelerated by azide, which seems to increase photobleaching of GFP (2).

When incubated with azide/deoxyglucose, few of the transiently transfected cells are resistant to these inhibitors, and their fluorescence signal remains associated with nuclei (data not shown). At present, it is not known why some cells do not equilibrate NP-GFP between nucleus and cytosol when facilitated nuclear import of proteins is inhibited. However, it is possible that for a certain population of cells, the physiological state of nuclear diffusion channels does not allow NP-GFP to exit the nucleus.

![Figure 4. Stably transfected HeLa cells expressing nucleoplasmin-GFP.](image)

(a) HeLa cells were transfected with DNA encoding nucleoplasmin-GFP, and stable transfectants were selected as described in Materials and Methods. Green fluorescence obtained after induction of gene expression with dexamethasone (B) or in the absence of dexamethasone (D) was determined. Nuclei were detected by staining with DAPI (A and C). (b) Stably transfected cells expressing nucleoplasmin-GFP were treated with sodium azide/deoxyglucose at room temperature for the times indicated. Cells were located by phase contrast (A, C and E); green fluorescence for nucleoplasmin-GFP is shown in Panels B, D and F.

Generation of a Stable Cell Line Expressing Nucleoplasmin-GFP

When induced with dexamethasone, transiently transfected cells show variability in fluorescence intensities, which reflects different copy numbers of plasmids introduced into each cell. In addition, transiently transfected cells lose the plasmid after prolonged culturing. For many studies, it is therefore advantageous to have a more uniform population of cells with comparable fluorescence. To this end, we created a stable cell line that carries the gene for nucleoplasmin-GFP. Cells were transfected and grown for two weeks without selection. Dexamethasone was added to the growth medium, and cells displaying fluorescence were sorted by fluorescence-activated cell sorting. Under these conditions, approximately one in 10,000 cells gave a fluorescence signal. Single cells were isolated and further cultured. Characterization of stable transfectants demonstrated that the fluorescence signal varied among different clones. This most likely reflects variable levels of gene expression due to differences in the integration sites of the plasmid. However, fluorescence signals for single cells derived from the same clone were comparable (Figure 4a, Panel B). In the absence of the inducer dexamethasone, no fluorescence signal was obtained, demonstrating the tight control of gene expression in this experimental system (Figure 4a, Panel D).

We further analyzed the diffusion of nucleoplasmin-GFP across the nuclear envelope under conditions that promote nuclear exit of NP-GFP. However, neither cold treatment nor incubation with NaN₃/deoxyglucose for up to 3 h results in nucleocytoplasmic diffusion of nucleoplasmin-GFP (Figure 4b, Panels D and F), indicating that the protein is retained in the nucleus although facilitated nuclear import of proteins is inhibited. The failure of nucleoplasmin-GFP to exit the nucleus is not a specific characteristic of the stable cell line because transiently transfected cells synthesizing nucleoplasmin-GFP gave the same result (data not shown).

DISCUSSION

We have generated an experimental system for mammalian culture cells to analyze passive diffusion of proteins from the nucleus into the cytoplasm. Components that control channels for nucleocytoplasmic diffusion of macromolecules are currently unknown. Studies to understand diffusion channels of the nuclear pore complex therefore require methods that permit the rapid analysis of traffic across the nuclear pore in the absence of facilitated transport. We have now established such procedures for growing HeLa cells.

As described here and previously (4), GFP enters nuclei by passive diffusion and therefore localizes to both the nucleus and the cytoplasm. Moreover, for the experimental conditions and incubation times used by us, nuclear pore complexes of HeLa cells can accommodate the diffusion of GFP2, a protein of about 54-kDa molecular mass. This translocation of GFP2 into nuclei is not mediated by a cryptic NLS because a protein containing four copies of GFP is excluded from nuclei (3).

Fusion of the bipartite nucleoplasmin NLS to GFP efficiently mediates nuclear accumulation of this reporter protein. The small molecular mass of NP-GFP (i.e., 29 kDa) makes it an ideal tool to study diffusion from the nucleus into the cytoplasm. The experiments reported here demonstrate that NP-GFP exits the nucleus at low temperatures even in the absence of metabolic inhibitors. Migration of NP-GFP into the cytoplasm of HeLa cells during chilling is not caused by irreversible damage of the cells or the nuclear envelope. HeLa cells survived this treatment, and NP-GFP was reimported into nuclei after shifting to normal growth conditions.

Our results for NP-GFP differ from data recently reported for diffusion of another fluorescent substrate across the nuclear envelope of the yeast S. cerevisiae. Shulga et al. (15) found that cytoplasmic fluorescence signals for a 43-kDa, GFP-containing protein did not increase if yeast cells were kept for several hours on ice. There are several conceivable explanations for these discrepancies. The smaller size of the transport substrate used by us (i.e., about 29 kDa) is likely to result in a higher diffusion rate when compared to a 43-kDa protein. Alternatively,
sequences provided by the yeast transcription factor Gal4p present in the 43-kDa protein might retain this transport substrate in the nucleus under certain experimental conditions. At present, we cannot rule out, however, that diffusion across yeast and mammalian nuclear pore complexes is differently affected by chilling of cells or by energy depletion.

Whatever the mechanisms that allow NP-GFP to rapidly exit the nucleus of HeLa cells during chilling, this substrate enables us to study diffusion in the absence of metabolic inhibitors that are likely to interfere with a variety of cellular processes. Cells synthesizing NP-GFP are therefore an ideal tool for future studies of nucleocytoplasmic diffusion.

We also analyzed diffusion of the 38-kDa fluorescent reporter protein nucleoplasmin-GFP in stably transfected cells. We do not detect an equilibration of the fluorescence signal between nucleus and cytoplasm if cells are chilled or treated with sodium azide/deoxyglucose. Different interpretations that are not mutually exclusive could explain this result: (i) the larger size of nucleoplasmin-GFP as compared to NP-GFP prevents its rapid diffusion across the nuclear envelope; and (ii) nucleoplasmin-GFP still contains the DNA-binding region of Xenopus nucleoplasmin, and binding to DNA could retain the reporter protein in the nucleus even in the absence of facilitated transport across the nuclear pore complex. Because GFP2, a protein of 54-kDa molecular mass, can traverse the nuclear envelope of HeLa cells by diffusion, we favor the interpretation that nucleoplasmin-GFP is retained within nuclei because of its binding to DNA. This nuclear retention of nucleoplasmin-GFP in cells that do not support facilitated transport makes it a perfect substrate to probe for the integrity of the nucleus under various experimental conditions.

Taken together, the procedures described by us provide a novel system to study nucleocytoplasmic diffusion in higher eukaryotic cells. This will enable us to determine the effect of different drugs or of mutant components of the nuclear envelope on the physiological state of diffusion channels provided by nuclear pore complexes.

ACKNOWLEDGMENTS

We are grateful to Dr. Hans-Martin Jäck (Loyola University, Chicago) for providing us with HtTA-1 cells used in this study. This work was supported by grants from MRC Canada and NSERC to U.S. (Nos. MT-12558, MT-13659 and OGP0155509). U.S. is a scholar of the Deutsche Forschungsgemeinschaft (DFG), Germany.

REFERENCES