Short Technical Reports

Monitoring Nuclear Import with GFP-Variant Fusion Proteins in Digitonin-Permeabilized Cells

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ABSTRACT

A convenient assay for monitoring nuclear localization signal-mediated nuclear import of green fluorescent protein (GFP)-variant fusion proteins has been developed. This modified assay relies upon indirect immunofluorescence microscopy for visualization of transported substrates. The use of GFP-variant fusion proteins allows for the rapid assessment of optimal digitonin concentration and permits nuclear import to be monitored with minimal sample preparation in real time.

INTRODUCTION

Monitoring nuclear localization signal (NLS)-mediated nuclear protein import in digitonin permeabilized cells was first described by Adam et al. (1). Concentrated cell cytosol containing an NLS-bearing import substrate and an ATP-regenerating system is incubated with digitonin-permeabilized cells, and nuclear uptake is visualized by indirect immunofluorescence microscopy. Digitonin is used to selectively permeabilize plasma membranes of cells [because of their relatively high cholesterol content (2)], leaving the nuclei intact. All that is required to reconstitute a functional nuclear import system are cytosolic extract (or reticulocyte lysate) and an energy source. This assay has proven to be a key tool in deducing the mechanisms of NLS-mediated nuclear import processes. Complementing immunocytochemistry, nuclear uptake in digitonin-permeabilized cells allows for a variety of parameters to be easily altered to determine their effects on import. The effects of point mutations, exogenous proteins, peptides, antibodies, and inhibitors can be readily examined. This method for studying nuclear import processes has been successfully applied to a variety of

cell types for applications such as the examination of steroid receptor (4) and aryl hydrocarbon receptor nuclear translocator (3) nuclear import.

In adapting this assay for our own applications, we have simplified it considerably, resulting in a rapid, novel assay. By utilizing NLS-bearing substrates that are fused to variants of green fluorescent protein (GFP) expressed by transient transfection of cells, we forego the need for extensive processing of samples for visualization by indirect immunofluorescence microscopy or the need to label substrates before import with fluorescent molecules such as fluorescein-5-isothiocyanate (FITC). In addition, the protocol described here allows visualization of the import process in real time, if so desired.

MATERIALS AND METHODS

Cell Culture

Cos-1 cells were grown in α -minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in 94% air with 6% CO₂.

Plasmids and Transient Transfection

The plasmids pEYFP, pEYFP-Nuc, and pECFP-Nuc were obtained from Clontech Laboratories (Palo Alto, CA, USA). Transient transfections were performed using LIPOFECTAMINE™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, using 9 μ g DNA, 30 μ L LIPOFECTAMINE, and 20 μ L PLUS reagent (Invitrogen) for each approximately 80% confluent 100-mm dish.

Cytosol Preparation

Cells (one 100-mm dish/import reaction) were harvested about 24 h after transfection by treatment with trypsin/EDTA and centrifuged at 120 $\times$ g at 4°C for 4 min. Cells were typically near 100% confluent, and protein concentrations of lysates were 7–10 mg/mL. Cell pellets were briefly washed twice with ice-cold PBS and once with

Wash Buffer [10 mM HEPES, pH 7.3, 110 mM Kac, 2 mM MgAc, 2 mM dithiothreitol (DTT)] (1). Washed cell pellets were supplemented with protease inhibitor cocktail (Sigma) and dithiothreitol (final concentration 2 mM) before homogenization by 10 passes through a 30-gauge syringe. Protease inhibitors and DTT were made up at 10 $\times$ concentrations in 10 $\times$ transport buffer (TB) (20 mM HEPES, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM MgAc, 1 mM EGTA), allowing dilution of cell pellets by only 10% before homogenization. Crude lysates were centrifuged at 100 000 $\times$ g for 30 min at 4°C to give the cytosolic supernatant.

Determination of Optimal Digitonin Concentration

Cos-1 cells grown in 12-well microplates were transiently transfected with pEYFP (0.5 μ g) and pECFP-Nuc (0.5 μ g) using LIPOFECTAMINE according to the manufacturer's protocol (total DNA 1.5 μ g made up with the empty vector pCI). Twenty-four hours after transfection, cells (near 100% confluence) were rinsed once with PBS and once with TB. Cells were then incubated for 5 min on ice with 1 mL of complete TB (CTB: TB + 2 mM DTT and 1 $\times$ protease inhibitor cocktail) containing 10, 20, 40, or 60 μ g/mL digitonin. Cells were rinsed again with TB and fixed in 4% formaldehyde in PBS for 30 min at room temperature. Cover slips were mounted and observed by fluorescence microscopy. Cells were compared for (i) depletion of YFP from cytoplasm and (ii) retention of CFP-Nuc in nuclei. Optimal digitonin concentration was determined to be 40 μ g/mL digitonin.

Real-Time Nuclear Import Assay in Digitonin-Permeabilized Cells

Cos-1 cells grown in 24-well culture dishes were rinsed with 500 μ L PBS, followed by 500 μ L ice-cold complete TB. Cells were then incubated in 500 μ L ice-cold CTB containing 40 μ g/mL digitonin (from 40 mg/mL stock in DMSO) for 5 min on ice. Cells were rinsed once with ice-cold CTB, and 75 μ L cytosol (see above) from COS-1 cells transfected with either pEYFP or

pEYFP-Nuc were added. Cells were immediately visualized with a Nikon® Eclipse™ TE300 inverted fluorescence microscope with Nikon Pan Fluor ELWD 60×/0.70 objective and SPOT RT color cooled CCD camera model 2.2.0 (Diagnostic Instruments, Sterling Heights, MI, USA).

RESULTS AND DISCUSSION

The starting point for this type of assay is to determine the optimal concentration of digitonin required to permeabilize the plasma membrane while leaving the nuclear membrane intact. GFP-variant proteins proved valuable

Figure 1. Use of co-expressed YFP and CFP-Nuc to optimize digitonin concentration for selective permeabilization of COS-1 cells. Panels A–D show depletion of cytoplasmic YFP fluorescence upon treatment of cells with 0 (A), 20 (B), 40 (C), and 60 µg/mL (D) digitonin. Panels E and F show retention of CFP-Nuc fluorescence upon treatment of cells with 0 (E), 20 (F), 40 (G), and 60 µg/mL (H) digitonin. Note that nucleolar staining in panel D is carryover into the green channel from the intense cyan fluorescence of CFP-Nuc (see panel G).

Short Technical Reports

for determining the optimal digitonin concentration for selective permeabilization of COS-1 cells. Cells transiently expressing yellow fluorescent protein (YFP) and CFP-Nuc [cyan fluorescent protein (CFP) with three tandem repeats of the NLS of simian virus 40 (SV40) large T antigen] were treated with a range of concentrations of digitonin (10, 20, 30, 40, and 60 $\mu\text{g/mL}$) for 5 min on ice and directly examined by fluorescence microscopy (Figure 1). Cells transfected with YFP show diffuse localization throughout the cytoplasm and nuclei, while those expressing CFP-Nuc show mostly nuclear localization with concentration in nucleoli, as is typically seen with this construct. Loss of cytoplasmic YFP and retention of nuclear CFP-Nuc were used to assess the treatments. The optimal treatment of 40 $\mu\text{g/mL}$ resulted in the nearly complete loss of cytoplasmic YFP from all cells and retention of CFP-Nuc in approximately 90% of treated cells compared with non-treated controls. This method for optimization of digitonin concentration could prove useful regardless of the type of import assay that will be performed.

Cytosol prepared from COS-1 cells transiently expressing either YFP or YFP-Nuc was chosen as a convenient source of substrate for the assay because of its high transfection efficiency. Cytosol was overlaid directly onto adherent, digitonin-permeabilized COS-1 cells, both grown and permeabilized in the wells of a 24-well microplate. Larger wells may be used but require increased amounts of cytosol to sufficiently cover the cells. Cells were immediately visualized by direct fluorescence microscopy on an inverted microscope with the appropriate filters. The cytosol containing the fluorescent substrate causes a visible background signal. However, the cells rapidly accumulate the NLS-bearing substrate and are easily visualized (Figure 2), while the NLS-lacking YFP did not show any signs of uptake into the cells and actually showed exclusion from the semi-permeabilized cell nuclei (Figure 3). In developing this assay, we found that the successful visualization of import requires that cytosol be prepared as concentrated as possible and that the GFP-fusion protein expresses well in COS-1

cells. For this reason, we found that lysing cells with minimal to no dilution provides the best results.

As a control, to demonstrate that the observed uptake was an energy-requiring process, the assay was performed with cytosol containing YFP-Nuc that was pre-incubated with 10 U apyrase (Sigma) for 15 min at 30°C. Apyrase treatment did not completely inhibit nuclear uptake; however, it was markedly diminished, even after 60 min, and considerable cytoplasmic/perinuclear localization is seen that is not observed in the absence of apyrase

(Figure 4). Examination of the nuclear uptake of YFP versus YFP-Nuc in the presence and absence of apyrase thus demonstrates that it is NLS mediated and energy dependent.

Monitoring NLS-mediated nuclear import in digitonin-permeabilized cells by this technique has numerous advantages. First, the assay can be quickly set up to analyze import of already created fusion proteins. Second, although this system may not accurately reflect import kinetics in intact cells, it can easily be utilized to qualitatively examine alterations in import induced by temper-

Figure 2. Time course demonstrating nuclear import of YFP-Nuc in digitonin-permeabilized COS-1 cells visualized in real time by fluorescence microscopy.

Figure 3. Time course demonstrating the lack of nuclear import of YFP in digitonin-permeabilized COS-1 cells. Arrows depict some of the nuclei that show exclusion of YFP.

Figure 4. Time course showing nuclear import of YFP-Nuc (upper) and inhibition of YFP-Nuc import by apyrase treatment of cytosol (lower).

ature, exogenous peptides and proteins, or inhibitors of import. In addition, import can be monitored in real time, and the extent of import at different time points can be recorded in single samples, with no need for a separate import reaction for each time to be examined.

In summary, the use of GFP-variant fusion proteins to analyze nuclear import processes in digitonin-permeabilized cells has distinct advantages over the traditional assay and may allow for more detailed study of complex nuclear import mechanisms.

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This work was supported in part by National Institutes of Environmental Health Sciences grant no. ES04869. Address correspondence to Dr. Gary H. Perdew, Center for Molecular Toxicology and Carcinogenesis, Department of Veterinary Science, 226 Fenske Lab, The Pennsylvania State University, University Park, PA 16802, USA. e-mail: ghp2@psu.edu

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John R. Petrulis and Gary H. Perdew
The Pennsylvania State University
University Park, PA, USA

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