Available online at www.sciencedirect.com
ScienceDirect
Methods 39 (2006) 309–315
METHODS
www.elsevier.com/locate/ymeth

Ultrastructural nuclear import assay

Hualin Zhong [a], Helen Shio [b], Nabeel R. Yaseen [c,*]

[a] Department of Biological Sciences, Hunter College, City University of New York, New York, NY 10021, USA
[b] Bio-Imaging Resource Center, The Rockefeller University, New York, NY 10021, USA
[c] Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

Accepted 20 June 2006

Abstract

Electron microscopy (EM) has been used for several decades to study the mechanisms of nuclear transport. In early studies of nuclear import, gold-conjugated nuclear proteins were microinjected into cells and followed by EM. As the components of the nuclear pore complex (NPC) and soluble mediators of nuclear import were cloned and characterized, gold-conjugated antibodies were utilized to sublocalize the components of the nuclear transport machinery by immuno-EM. Further, gold-conjugated recombinant proteins were used to probe permeabilized cells or isolated nuclear envelopes and characterize binding sites for these proteins at the NPC. More recently, recombinant gold-conjugated nuclear proteins were used in *in vitro* nuclear import assays to help dissect the mechanisms of nuclear import. We have used this ultrastructural nuclear import assay to study the nuclear import of the transcription factor PU.1. The results showed that this import requires energy but is carrier-independent. In the presence of energy, gold-conjugated PU.1 shifted to the nuclear side of the NPC and the inside of the nucleus. In conjunction with biochemical assays, these results indicated that this shift involved Ran-dependent binding of PU.1 to NUP153, a nucleoporin situated at the nuclear side of the NPC. Here we describe in detail the methods used in the ultrastructural nuclear import assay including preparation of recombinant protein, gold conjugation, *in vitro* nuclear import assay, electron microscopy, and data analysis.

Keywords: Nuclear import; *In vitro* assay; PU.1; Electron microscopy; Gold conjugation; Colloidal gold

1. Introduction

In early ultrastructural studies of nuclear transport gold-conjugated nuclear proteins were microinjected into the cytoplasm of amoebas [1,2] or amphibian oocytes [3]. These studies demonstrated that nuclear import of macromolecules occurs through the nuclear pores. Subsequent studies revealed that nuclear pores consist of large complexes of proteins (nucleoporins) that span the nuclear envelope. The mammalian nuclear pore complex (NPC) consists of multiple copies of each of 29 different nucleoporins [4].

The development of the digitonin-permeabilized cell assay for nuclear import [5] facilitated the elucidation of nuclear import pathways and the identification of a large group of nuclear import carriers. In this assay, cells are treated with digitonin to permeabilize the plasma membrane while leaving the nuclear membrane relatively intact. The cytosol is then washed away and a fluorescently labeled protein containing a nuclear localization signal (NLS) is added. By performing the assay in the presence of different cytosolic fractions, factors needed for the nuclear import of various substrates were identified [6,7]. In most cases, the NLS is recognized by a carrier of the karyopherin (importin) family of proteins. For example, the classic SV40 large T antigen NLS is recognized by a heterodimer of karyopherin α (importin α) and karyopherin β1 (importin β). Similarly, the M9 NLS of the hnRNP A1 protein binds to karyopherin β2 (transportin). Members of the karyopherin β family mediate the interaction between the import complex and the NPC by binding to a subset of nucleoporins

* Corresponding author. Fax: +1 312 503 8240.
E-mail address: n-yaseen@northwestern.edu (N.R. Yaseen).

1046-2023/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymeth.2006.07.012

that contain FG-repeats. Carrier-mediated nuclear import usually requires energy. This is provided by another soluble protein, the small GTPase Ran that cycles between GDP- and GTP-bound forms.

Although in most cases nuclear protein import is carrier-dependent, some proteins can enter the nucleus unassisted by soluble proteins. Examples include SMAD transcription factors [8,9], STAT transcription factors [10], hnRNP K [11], β-catenin [12–14], the HIV Vpr protein [15], ERK2 [16,17], and the transcription factor PU.1 [18]. The import of these proteins is thought to involve a direct interaction between the imported protein and nucleoporins. The role of energy in receptor-independent nuclear import is not clear in most cases, but energy has been shown to be required for the carrier-independent nuclear import of PU.1 [18].

While the use of fluorescently labeled protein cargo in the digitonin-permeabilized cell system has allowed the characterization of conditions and carriers required for nuclear import, the finer sublocalization of proteins within the NPC and subcellular compartments cannot be adequately performed by fluorescence microscopy. Nucleoporins and NPC-associated proteins have been sublocalized in cells and isolated nuclear envelopes by immuno-electron microscopy using gold-conjugated antibodies [19–25]. Electron microscopy using gold-conjugated proteins in digitonin-permeabilized cells and isolated nuclear envelopes has been used to identify where within the NPC certain proteins bind and under what conditions [21,26,27]. More recently, nuclear import assays in permeabilized cells were performed using gold-conjugated proteins and the results were visualized by electron microscopy to help elucidate the mechanisms of carrier-mediated [28] and carrier-independent [18] nuclear import.

In this article, we describe in detail the procedures that we used for ultrastructural nuclear import assays of the transcription factor PU.1 [18] including recombinant protein preparation, gold conjugation, import reactions in permeabilized cells, transmission electron microscopy, and quantitative analysis of the data.

2. Preparation of gold–protein conjugates

2.1. Preparation of recombinant PU.1

The human PU.1 open-reading frame was obtained by PCR from a pGEX-3X vector kindly provided by Dr. Elizabeth Eklund and subcloned into the *Bam*HI and *Xho*I sites of pGEX-6P1 (Amersham Biosciences). BL21(DE3) bacteria (Stratagene) transformed with the 6P1-PU.1 vector were used to produce recombinant PU.1. All cultures contained 50 μg/ml ampicillin. Seven milliliters of a saturated overnight culture of bacteria was diluted into 500 ml of LB media and incubated with shaking for 2.5 h at 37 °C. Protein expression was induced with 0.1 mM IPTG for a further 2 h. Bacteria were pelleted by centrifugation at 7000 rpm in a Sorvall SLA-1500 rotor for 10 min at 4 °C and resuspended in 200 ml transport buffer (20 mM Hepes·KOH, pH 7.3, 110 mM potassium acetate, and 2 mM magnesium acetate) with 0.1% Tween 20 (TB-T). The bacteria were sonicated until >90% were lysed as judged by microscopy and the lysate was clarified by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 20 min at 4 °C. The supernatant was incubated for 1 h at 4 °C with constant rotation with 4 ml glutathione–Sepharose 4B beads (Amersham Biosciences) that had been washed four times with TB-T. The beads were washed four times with TB-T and once with Pre-Scission protease cleavage buffer (Amersham Biosciences). One unit of Pre-Scission protease (Amersham Biosciences) in 1 ml cleavage buffer per ml of beads was added to cleave PU.1 from the GST tag and the digest mix was incubated overnight at 4 °C with rotation. The beads were sedimented in a microcentrifuge at 3000 rpm for 2 min at 4 °C and the supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at −80 °C. While these conditions yielded good quantities of PU.1, the conditions needed for the preparation of each individual protein need to be determined in small-scale preparations prior to scale-up. The information obtained from such preliminary studies would include how much protein is expressed by the bacteria, how much of it is soluble, whether detergent is needed for solubilization, the quantity of glutathione–Sepharose 4B beads needed, and the amount of protease and digest conditions required for optimal cleavage. In addition, some proteins may show better solubility if the IPTG induction is carried out at a lower temperature, for example 4 h at 30 °C.

2.2. Preparation of gold-PU.1 conjugates

The minimum amount of recombinant protein needed to stabilize colloidal gold particles (BB International, Cardiff, UK) was determined by titration, and the amount used at the preparative stage was 20% above the minimum to ensure complete conjugation. Addition of a large excess of the protein, however, would result in the excess remaining in solution and competing with the gold-conjugated fraction for binding to other molecules.

The addition of H_2O_2 and NaCl to 5 nm colloidal gold results in aggregation of the gold particles and a change in the color of the colloidal gold from red to grey. On the other hand, 10 nm gold particles will aggregate in the presence of NaCl alone. Aggregation is prevented by the addition of an amount of protein that is sufficient to coat the gold particles. It is important to note that the presence of DTT concentrations approaching 0.01 mM in the colloidal gold will result in aggregation and color change even in the absence of H_2O_2 and NaCl.

Colloidal gold (5 nm particles) was adjusted to pH 8 with 9 μl of 0.2 M K_2CO_3 and divided into 100 μl aliquots. Different amounts of PU.1 protein, ranging from 0 to 0.5 μg per aliquot, were added, followed by 10 μl of 30% H_2O_2 and 10 μl of 10% NaCl. The reactions were mixed by vortexing, incubated for 5 min at room temperature, and inspected for a change in color against a white background. The minimum amount of PU.1 required to prevent gold aggregation