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The maximal size of protein to diffuse through the nuclear pore is larger than 60 kDa

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Abstract **It has generally been believed that the diffusion limit set by the nuclear pore for protein is 60 kDa. We here studied the cellular localization of several artificial proteins and found that the diffusion limit set by the nuclear pore is not as small as previously thought. The results indicate that the maximal size of protein to diffuse through the nuclear pore complex could be quite larger than 60 kDa, thus greatly extending the diffusion limit that the nuclear pore can accommodate.**

Keywords: Nuclear pore; Protein nuclear translocation; Diffusion limit

1. Introduction

The nucleus is separated from the cytoplasm by a double membrane called the nuclear envelope (NE) in eukaryotes. The NE is penetrated by nuclear pore complexes (NPCs), through which the cytoplasm communicates with the nucleus and permits exchange of contents between the two organelles. With improvements in detection methods and instrumentation, the structure of the NPC has been refined over the years. It has been shown that the overall structure is similar in different species and is thus believed to be conserved in all eukaryotes [1]. The NPC is a huge structure of around 120 million Daltons in size and is constructed from ~30 different proteins that are often called nucleoporins [2–4]. The canonical feature of the NPC includes the central plug/transporter(CP/T), three rings with cytoplasmic filaments attached to the cytoplasmic ring and a basket in the nuclear sides of the NPC. The diameter of the cytoplasmic, lumenal spoke and distal ring determined by the latest studies is around 125 nm, 60 nm and 40 nm, respectively [5].

All nuclear proteins are made in the cytoplasm and must be translocated into the nucleus, while RNA products, including transfer RNA (tRNA), ribosomal RNA (rRNA) and messenger RNA (mRNA), are transcribed in the nucleus and subsequently exported to the cytoplasm for protein synthesis. Currently, the exchange of cytoplasmic and nuclear contents is believed to mainly involve two processes: (1) passive diffusion; and (2) an active process that is coupled to energy input. Both processes are mediated through NPCs. For more than two decades, it has generally been believed that for proteins to passively diffuse through the nuclear membrane, the limit set by the NPC is about 9–12 nm in diameter [6–8]. This was later interpreted to allow for the diffusion of proteins with a maximal size of 60 kDa [9–14]. Alternatively, proteins may shuttle between the cytoplasm and the nucleus in an active way that is mediated by nuclear localization signals (NLSs) or nuclear export signals (NESs). These signals are specifically recognized by corresponding adaptor proteins, dubbed as importins and exportins that chaperon the transported proteins into or out of the nucleus, respectively [12,15].

We have repeatedly observed the nuclear localization of a GFP3 oligomer protein, whose size is around 90 kDa. This phenomenon contradicts the long-term view that the maximal size for protein diffusion through the nuclear pore is around 60 kDa and it stimulates us to revisit the diffusion limit set by the NPC. For this study, the cellular localization of three artificial chimeric proteins was investigated. The results reveal that proteins with sizes from 90 to 110 kDa are allowed to diffuse through the nuclear pore, which may vary with different proteins studied. Thus, the capability of the nuclear pore for allowing macromolecules to diffuse through is greatly increased. In addition, this study does not exclude that the nuclear pore allows the diffusion of proteins that are even larger than those used in this study.

2. Materials and methods

2.1. Cell culture and transfection

Human cervical cancer cell line HeLa, prostate cancer cell line DU145 and lung cancer cell line A549 were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. Human melanoma cell line Colo38 was cultured in RPMI1640 supplemented with 10% FBS. Human colon cancer cell line HCT116 was cultured in McCoy's 5a medium supplemented with 10% FBS. All cells were cultured at 37 °C in a humidified incubator containing 6% CO_2. Experiments involving transient transfection were conducted with exponentially growing cells. Transfections of cells were conducted using Lipofectamine 2000 as described by the manufacturer (Invitrogen).

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Abbreviations: NPC, nuclear pore complex; ERK2, extracellular signal-regulated kinase 2; PDK1, 3′-phosphoinositide-dependent kinase; aa, amino acid(s); NLS, nuclear localization signal; NES, nuclear export signal

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2.2. Plasmid constructs

The vector pEGFP-C1 (from Clontech, Inc.) was used for GFP1 protein expression. The plasmid expressing GFP5 was as described previously [16]. The constructs expressing GFP2, GFP3 and GFP4 were generated by cutting the corresponding GFP portions from the parental vector for expression of GFP5 and ligating into pcDNA 3.1+ vector (Invitrogen). In addition, the constructs expressing chimeric proteins based on Myc-ERK2 or Myc-PDK1 were made in the pcDNA 3.1+ vector. Myc-ERK2 was created by polymerase chain reaction (PCR) using a 5′ oligonucleotide encoding the Myc-tag sequence (EQKLISEEDL) in addition to the extracellular signal-regulated kinase 2 (ERK2) sequence. The resulting product was subcloned into EcoRV and Xba1 sites of the vector. Myc-PDK1 was provided by Dr. Alessi (University of Dundee, UK). All the constructs expressing chimeric proteins based on Myc-ERK2 or Myc-PDK1 were generated based on PCR method.

2.3. SDS–PAGE and Western blot

At 16 h after transfection, cells were lysed in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 1 mM PMSF, 1 mM Na_3VO_4) with brief vortexing. After incubation on ice for 30 min, the supernatants were collected by centrifugation at maximal speed for 10 min. Lysates were dissolved in 2× sample buffer (100 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.03% bromophenol blue and 1.5% β-mercaptoethanol). Samples were subjected to SDS–PAGE followed by transferring to PVDF membranes. Membranes were blocked with TTBS (20 mM Tris–HCl, pH 7.0, 0.5 M NaCl, 0.05% Tween-20) containing 5% non-fat milk for 2 h. After blotting with specific primary antibodies and washing with TTBS, the membranes were then incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc) and the resulting signals were visualized by ECL detection (Amersham).

2.4. Immunofluorescence

Cells were grown on 6 well dishes containing coverslips, fixed in 2% formaldehyde/PBS at 4 °C for 1 h, and permeabilized in 0.2% Triton/PBS for 10 min. After washing once with PBS, the cells were blocked with 10% horse serum/PBS for 40 min and washed once again with PBS. The coverslips were then incubated with 4 μg/ml primary antibody/PBS at room temperature for 1 h followed by washing with PBS. The primary antibody against Myc tag was 9E10 from Santa Cruz Biotechnology, Inc. The samples were further blocked with rhodamine-conjugated goat anti-mouse immunoglobulin G antibody (Jackson ImmunoResearch Laboratories) for 1 h (1:100 dilution). The coverslips .were stained with 4,6-diamidino-2-phenylindole (DAPI) and were washed twice with PBS. The coverslips were mounted on glass slides with Aquamount (Polyscience, Inc., Warrington, PA). Images were captured under fluorescent microscopy using the indicated filter with a Hamamatsu 16-bit digital camera mounted on a Zeiss Axioplan microscope using a 63× objective and processed with Adobe Photoshop software.

3. Results and discussion

To determine the size limit for proteins that are able to diffuse through the nuclear pore, serial constructs for GFP oligomer fusion proteins were made, which include GFP1, GFP2, GFP3, GFP4 and GFP5. The GFPs are expressed in frame with each other and the number indicates the number of GFP proteins that are fused together (Fig. 1A). When these GFP fusion proteins were expressed, their molecular weights ranged from 28 kDa to 140 kDa (GFP1–GFP5, respectively) (Fig. 1B). When the localization of these fusion proteins was determined in HeLa cells after transient transfection for 8 h, it was found that GFP1, GFP2 and GFP3 were capable of translocating into the nucleus of essentially all the transfected cells, while GFP5 was predominantly localized in the cytoplasm (Fig. 1C). GFP4 protein was detected in the nucleus of most transfected cells (~90%), while it could be sparsely detected to be localized in the cytoplasm (~10%). This implied that the size of GFP4 protein is very near to or exceeds the diffusion limit set by the nuclear pore, though most of the protein is still able to "squeeze" through the nuclear pore. In addition, it was found that in the cells transfected with GFP1, GFP2 and GFP3, most of them demonstrated a higher GFP nuclear staining when compared to that of the cytoplasm (Fig. 1C). These results are consistent with previous observations that the nucleus generally demonstrates a higher fluorescent staining than the cytoplasm of the small diffusion proteins in fixed cells [17,18]. In contrast, the distribution of GFP signal in the cytoplasm was very similar to that in the nucleus of the GFP4 transfectants and the distinction of the nucleus from the cytoplasm became unclear in many cells (Fig. 1C), suggesting that protein nuclear translocation is a relative slow process and becomes inefficient as the protein size increases to that of GFP4 protein. In a separate experiment (data not shown), cells were fixed every one half hour after transfection with GFP3 or GFP4. Due to the time it took DNA to enter the cells and its subsequent transcription and translation, the GFP signal was barely detectable after 2 h transfection. Only after 2.5 h were the cells bearing the GFP signal clearly identified in both type of transfectants. GFP3 transfectants already demonstrated obvious nuclear localization by this time, indicating the nuclear translocation of GFP3 protein is a relatively efficient process. In contrast, the nuclear translocation of GFP4 protein could only be rarely identified after 3.5 h and most GFP4 transfectants still demonstrated a cytoplasmic localization pattern, demonstrating that the nuclear translocation of GFP4 protein is a relative slow process.

It is well established that GFP1 protein is able to diffuse into the nucleus due to its small size and GFP1 itself does not contain an NLS, otherwise GFP5 will be translocated into the nucleus too. The above results indicated that in order for protein to passively diffuse through the NPC, its size does not need to be smaller than 60 kDa as established before. In contrast, large proteins, such as GFP3 and GFP4, whose size are around 90 and 110 kDa respectively, are able to diffuse into the nucleus, though the process becomes less efficient as the protein size increases to that of GFP4.

To extend this study, additional cell lines were investigated (HCT116, colo38, DU145 and A541 cells). Interestingly, although GFP3 was still effectively translocated into the nucleus and GFP5 was predominantly localized in the cytoplasm of all the cell lines studied, the efficiency of the GFP4 nuclear translocation varied among the different cell lines. For example, it appears that the GFP4 is much poorer at diffusing through the nuclear pore in HCT116 cells than in HeLa cells and is mainly restricted to the cytoplasm. The small discrepancy of the cellular distribution pattern of GFP4 in different cell lines reflects that there is a subtle difference on the diffusion limit set by the NPCs in different cells studied, which is consistent with previous observations [7].

To further disprove that the nuclear pore only allows the diffusion of proteins with a maximal size of 60 kDa, the nuclear translocation of ERK2 protein was studied. ERK2 is a member of mitogen-activated protein (MAP) kinase family that regulates many cellular events, such as cell proliferation and differentiation [19,20]. ERK2 has frequently been reported to be localized in the nucleus of many cell lines. Due to its small size (42 kDa), it is generally believed that ERK2 can be transported into the nucleus by diffusion. Currently, it is not clear