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Inner Nuclear Membrane Protein Transport Is Mediated By Multiple Mechanisms

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Abbreviations used: Transmembrane (TM), nuclear envelope (NE), inner nuclear membrane (INM), outer nuclear membrane (ONM), pore membrane (PoM), endoplasmic reticulum (ER), nuclear pore complex (NPC), nuclear localization signal (NLS), lamin B-receptor (LBR), lamina-associated polypeptide 2 (LAP2), isoelectric point (pI), fluorescence recovery after photobleaching (FRAP), phenylalanine-glycine (FG), FK506 binding protein (FKBP), FKBP-rapamycin binding domain (FRB).

Abstract

Work in the nuclear transport field has led to an incredibly detailed description of protein translocation through the central channel of the nuclear pore complex, yet the mechanism by which nuclear envelope transmembrane proteins reach the inner nuclear membrane after synthesis in the endoplasmic reticulum is still hotly debated. Three different translocation models have gained experimental support: (1) simple lateral diffusion through the nuclear envelope membrane system, (2) translocation by vesicle fusion events and (3) a variation on classical transport mediated by the nuclear pore complex. Although these models appear to be mutually exclusive, here we argue that they probably all function for different inner nuclear membrane proteins according to their unique characteristics.

Introduction

The defining characteristic of eukaryotes is the presence of the nuclear envelope (NE), a double membane system that separates nuclear and cytoplasmic activities. The two NE membranes are respectively called the outer (ONM) and inner (INM) nuclear membranes and they connect where they curve around the nuclear pore complexes (NPCs) at what is sometimes called the pore membrane (PoM)(reviewed in [1,2]). Thus the NE provides an impenetrable diffusion barrier except where the NPCs, ~60 MDa protein complexes, regulate bi-directional transport of molecules in and out of the nucleus. Cryo-electron microscopy of NPCs indicates that the central channel can accommodate proteins up to 39 nm in diameter, but also indicates that there are peripheral channels between the NPC core and the membrane that could accommodate proteins of up to 10 nm in diameter [3,4]. A 10 nm diameter unobstructed channel is consistent with the measured diffusion limits for soluble dextrans [5]. The focus of study on nuclear-cytoplasmic transport has been on soluble proteins (reviewed in [6,7]), but transmembrane (TM) proteins must also access the INM as several have been shown to bind lamins that form an intermediate filament polymer under the INM and chromatin (reviewed in [8,9]). Many of these proteins, moreover, have been linked to human disease (reviewed in [10-12]).

While several lower eukaryotes divide by NE fission, higher eukaryotes mostly disassemble and reassemble the NE during mitosis at each cell division. Though INM proteins in these organisms could access the nuclear compartment during NE assembly, new INM proteins must also be able to gain access during interphase because the nuclear surface area roughly doubles as chromatin is replicated but the density of proteins in the NE does not diminish during this growth [13-15]. In particular, the spacing between NPCs does not change throughout interphase because new NPCs are inserted into the membrane at a rate corresponding to NE growth [14,16]. Thus, TM proteins must be continuously transported to the INM after their synthesis in the endoplasmic reticulum (ER) throughout interphase. As the ONM is continuous with the ER [15,17], TM proteins can diffuse freely in the membrane

between these two compartments. However, the only possible pathways for a newly synthesized TM protein to reach the INM during interphase are by vesicle fusion through both membranes or to enter at the NPCs either going around the outer face or through the central channel.

The Lateral Diffusion-Retention Hypothesis

The observation of a ~10 nm channel on the outer face of the NPC [3] together with the ability of an INM protein to move between nuclei in fused cells [18] led to the development of the lateral diffusion-retention hypothesis. This proposed that both ER and INM proteins normally rapidly diffuse in the membrane between the ER and the INM at equilibrium, but INM proteins can bind to peripheral chromatin or lamins leading to their retention and accumulation in the nucleus. This mechanism was supported by the observation that an ER resident protein could accumulate in the INM when its TM segment was fused to lamin binding sequences from an INM protein [19] (Figure 1). Subsequent experiments reaffirmed these observations using lamin-binding sequences from different INM proteins [20,21] (Figure 1). The retention part of the model was further supported by observations using fluorescence recovery after photobleaching (FRAP) that over 60% of pre-bleach fluorescence was not recovered for the INM protein lamin B-receptor (LBR), consistent with its being mostly immobile in the INM [22]. More compellingly, it was recently shown that the mobility of INM protein emerin was much faster in cells disrupted for its intermediate filament binding partner lamin A [23].

The 10 nm channel observed between the outer face of the NPC and the PoM should be able to accommodate a protein of up to between 40 and 60 kDa based on average stokes radius calculations for globular proteins. This is consistent with the measured diffusion limit [5] and should thus set an upper limit for the nucleoplasmic mass of a TM protein that can be transported to the INM. Increasing the mass of the reporter fusion used in the original lateral diffusion study from 22.5 kDa to 70 kDa, above the diffusion limit, blocked its accumulation in the INM [24]. Two later studies found that a reporter with a 55 kDa nucleoplasmic mass

could freely access the INM while a reporter with a 58 kDa nucleoplasmic mass was slowed but could still accumulate in the INM [21,25] (Figure 1). This is consistent with earlier observations that soluble protein diffusion across the NPC slows greatly as the diameter/mass of the protein approaches the measured diffusion limit [5].

Vesicle Fusion in the NE

For many years the lateral diffusion-retention hypothesis went unchallenged. However, with the exception of the correlation between the size of the peripheral channels and the measured diffusion limit, the results supporting lateral diffusion are equally consistent with a translocation mechanism involving vesicle fusion. Vesicle fusion has been extensively studied in the ER, the Golgi aparatus and the plasma membrane. Fusion events are energy and temperature dependent and require calcium (reviewed in [26-28]). Within the cell most membranes are supported by protein meshworks (e.g. spectrins, clathrin, lamins) and also use specific proteins (e.g. SNAREs, NSF) to mediate fusion events. Principal among the proteins regulating vesicle fusion are the p97 and p47 proteins [29,30]. To test if these proteins are required for NE reassembly at the end of mitosis, they were depleted from vesiculated *Xenopus* oocyte extracts that were then mixed with demembraned sperm chromatin. Undepleted extracts reformed NEs, while those depleted for p97 did not [31]. The nuclei formed in this assay system can recapitulate many characteristics of interphase including DNA replication and NE growth [32]. Depletion of p47 was further found to block the growth phase [31].

Although a mechanism clearly exists for vesicle fusion, the dependency on this mechanism observed in these studies may be an artifact of the in vitro experimental system. The ER is not vesiculated in intact interphase cells, but has a tubular structure; therefore interphase nuclear membrane growth is more likely to derive from membrane channeled from the ER where it connects to the NE. Indeed, RNA interference-mediated reduction of p97 and p47 orthologs in *C. elegans* yielded no NE deficits [33]. Furthermore, a vesicle fusion mechanism would be costly to the cell because it would require continuous remodeling of

INM protein connections to the lamin polymer and chromatin. Thus it is likely that vesicle fusion functions only during the NE reassembly step in intact cells. Recent work indicates that in addition to p97 this step requires certain NPC proteins including the integral NPC protein gp210 and the GTPase Ran [34-37].

Gated Lateral Diffusion

This challenge to the lateral diffusion-retention hypothesis did not go unnoticed and a new inducible live reporter assay system was quickly developed that allowed for testing of some of the requirements for translocation to the INM. Here a TM segment lacking any nuclear retention sequences was fused to the FKBP-rapamycin binding domain (FRB) and also to GFP for live visualization. This reporter diffused at equilibrium between the ER and the INM. Cells were co-transfected with a second soluble fusion protein that contained both lamin binding sequences of the INM protein LAP2ß and the FK506 binding protein (FKBP). Upon treatment of the cells with the drug rapamycin the FRB bound to FKBP and so the TM reporter construct gained a lamin-binding domain and rapidly accumulated in the INM [25]. Vesicle fusion requires energy, calcium, p97 and is sensitive to temperature whereas lateral diffusion within the membranes of the ER and Golgi compartments has no such requirements [27]. Addition of calcium chelators or inhibitors of p97 to the system had no effect on accumulation of the reporter in the INM [25]. Thus the process here does not require vesicle fusion.

Nonetheless, the process was shown to be more complicated than simple free diffusion as temperature reduction and ATP-depletion significantly inhibited accumulation of the reporter in the INM while having no effect on its mobility within the ER [25]. Strikingly, accumulation in the INM was also inhibited by injection of cells with antibodies to the integral NPC protein gp210 [25]. Together these results suggested a modification of the lateral-diffusion hypothesis wherein gp210 acts as a gatekeeper and requires a toll of energy for a conformational change that would allow TM proteins to pass.

Classical NPC-Mediated Transport

More recent work argues that ER to INM translocation of TM proteins is mediated by components of the classical nuclear import pathway. Transport receptors such as importin alpha bind to nuclear localization signals (NLSs) on transport cargos. The receptors then interact with phenylalanine-glycine (FG)-repeats on core NPC proteins in the central channel of the NPC to negotiate translocation of their cargos across the NPC. The Ran-GTPase forms a gradient with Ran-GDP in the cytoplasm and Ran-GTP in the nucleus, so Ran-GTP binds to the receptor-cargo complex when it reaches the nucleus and facilitates release of the cargo from the receptor. Depletion of importin alpha or blocking cycling of the Ran-GTPase strongly inhibited correct targeting of the yeast INM proteins Heh1 and Heh2, both of which have NLSs [38]. In an independent study, a translocation signal for an insect TM protein targeted to the INM was found to bind to an isoform of importin alpha [39]. Further analysis of the small set of characterized INM proteins revealed that roughly 2/3 also had predicted NLSs [40].

The requirement for mediators of classical NPC transport pathways for TM proteins would appear to indicate that the INM-destined cargos utilize the central channel of the NPC; however, there is no reason that importin alpha and Ran could not similarly negotiate the peripheral channels with TM protein cargos. Though the bulk of the mass of FG-repeat NPC proteins resides in the central channel, recent improvements in the resolution of NPC structural organization indicate that some of these FG-repeat proteins are positioned on the outer ring facing the membrane [41,42]. Thus these data would appear to further refine the model such that TM proteins are synthesized in the ER and then diffuse freely between the ER and the ONM where they are recognized by transport receptors and Ran due to encoded NLSs and these facilitate their translocation through the peripheral channels of the NPC while still in the membrane in an energy and temperature-dependent process.

It did not take long for this new model to be challenged as the same year another study found that a third yeast INM protein, Doa10, was unaffected by the same yeast NPC disruption strain that blocked translocation of Heh2 [43]. Each of the earlier studies used

different reporters and assay systems that could have explained in part their different results. However, in this case the yeast strains and assay systems were identical indicating that there are differing requirements for INM transport of Doa10 and Heh2.

Which model is correct?

The contradictions in the data published so far indicates that the targeting of INM proteins is much more complex than first assumed. Either a heretofore unclear mechanism exists that can somehow account for all these data or multiple translocation mechanisms exist and each individual INM protein has a unique set of characteristics that direct it to a preferred mechanism. The existence of multiple translocation mechanisms should further enable essential proteins to access the nucleus when the favored mechanism is overburdened or inhibited. Along the same lines, unique combinations of translocation signals on individual INM proteins could contribute to differential regulation of their transport at distinct stages of the cell cycle or under distinct physiological conditions of the cell (Figure 2). Another factor that may contribute to how a particular INM protein is to be translocated is its nucleoplasmic mass. As translocation was slowed as nucleoplasmic mass approached the diffusion limit, the requirement for energy in transport might only be for larger proteins (Figure 2).

To learn how varied the modes of transport actually are it will be necessary to systematically sample a large number of native INM proteins instead of the varied artificial constructs that account for most experiments to date. Recent work in our laboratory has directly compared sixteen different INM proteins for translocation from the ER to the INM finding that they have a wide range of translocation rates and different subgroups are sensitive to energy depletion or Ran depletion (N. Zuleger, D. A. Kelly, and E. C. Schirmer, in preparation). Thus it appears that there is considerable variation in the details of translocation mechanisms.

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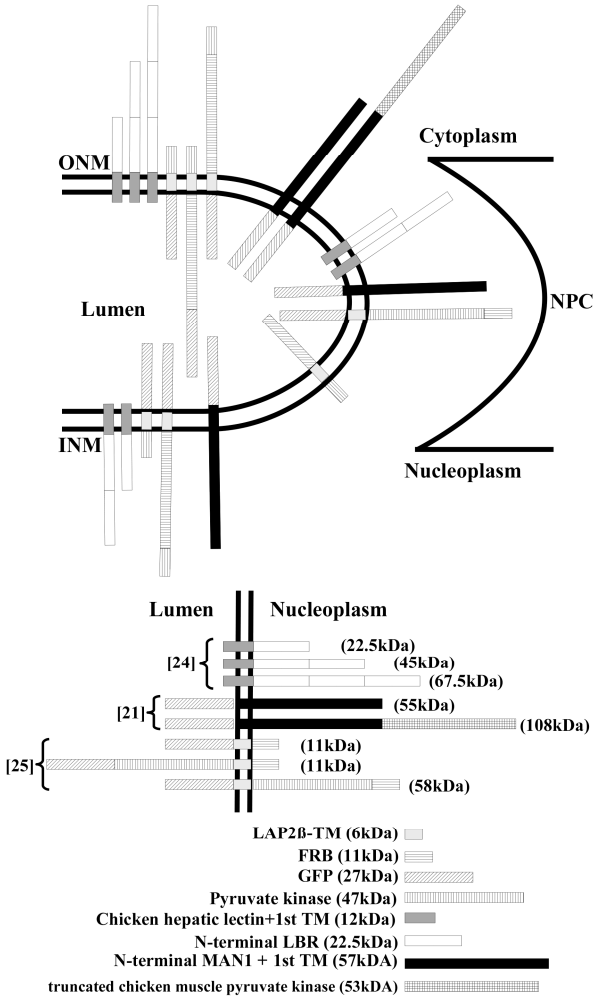
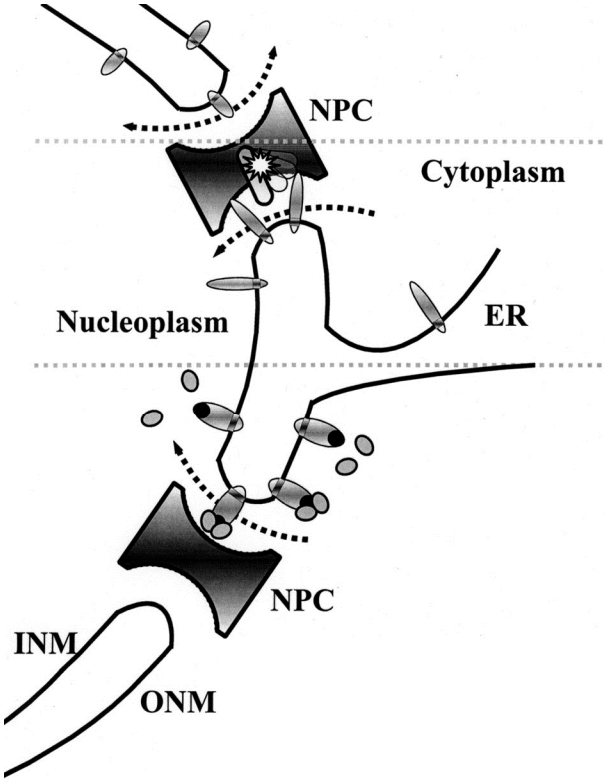
Figure Legends

Figure 1.



Lateral diffusion and the diffusion limit. After synthesis in the ER proteins can freely diffuse to the outer nuclear membrane (ONM), but to access the inner nuclear membrane (INM) they must pass through the peripheral channels of the nuclear pore complex (NPC). To study translocation from the ONM to the INM, several studies used different reporter fusion proteins. The component segments are listed and their assembled structure shown. Each had the different cyto/nucleoplasmic masses listed. Those that had nucleoplasmic masses above 70 kDa did not accumulate in the INM and the reporter protein with a nucleoplasmic mass of 58 kDa translocated very slowly. Presumably this is because the lateral channels are too small to accommodate proteins above a certain size limit.

Figure 2.



Multiple translocation mechanisms may operate for INM proteins depending on their individual characteristics. (A) A protein with a small nucleoplasmic mass may freely diffuse between the ONM and INM. (B) A protein with a large nucleoplasmic mass may require energy (ATP hydrolysis) for an undefined gating step to translocate through the peripheral channel. (C) A protein with a nuclear localization signal (NLS) may require assistance from transport receptors (Importin) to pass through the peripheral channel.

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