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FUNCTIONALIZATION OF A NANOPORE: THE NUCLEAR PORE COMPLEX PARADIGM

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Summary

Biological cells maintain a myriad of nanopores which, although relying on the same basic small-hole principle, serve a large variety of functions. Here we consider how the nuclear pore complex (NPC), a large nanopore mediating the traffic between genetic material and protein synthesizing apparatus, is functionalized to carry out a set of transport functions. A major parameter of NPC functionalization is a lining of it external and internal surfaces with so-called phenylalanine glycine (FG) proteins. FG proteins integrate a multitude of transport factor binding sites into intrinsically disordered domains. This surprising finding has given rise to a number of transport models assigning direct gating functions to FG proteins. However, recent data suggest that the properties of FG proteins cannot be properly assessed by considering only the purified, transport-factor-stripped NPC. At physiological conditions transport factors may shape FG proteins in a way allotting an essential role to surface diffusion, reconciling tight binding with efficient transport. Thus, NPC studies are revealing both general traits and novel aspects of nanopore functionalization. In addition, they inspire artificial molecule sorters for proteomic and pharmaceutical applications.

Keywords

intrinsically disordered proteins; membrane transport; nanopores; nuclear pore complex; reduction of dimensionality

1. Introduction

The biological cell maintains a myriad of nanopores [4]. Most nanopores serve as “transporters”, i.e. transmembrane proteins mediating the transport of matter through membranes [48,111]. But there are also soluble nanopores such as the proteasomes which have protein-degrading functions [44]. Even microtubules may be classified as nanopores although little is known about the functional significance of their internal space and surface.

All cellular nanopores rely on a simple principle which is essentially the formation of a small shielded compartment with an entrance and an exit. In spite of the simple principle, nanopores acquire a large degree of versification by variation of geometry, electrostatics, adsorption/

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desorption kinetics, surface roughness and other parameters. The functionalization of nanopores is only partly understood and its further elucidation is crucial for cell biology. The study of nanopore functionalization is also inspiring the creation of artificial devices for novel technical applications [18,66].

Here, we consider the nuclear pore complex (NPC), a nanopore which mediates the traffic between genetic material and protein synthesizing apparatus. NPCs are huge and, in spite of modularity [94] and stratification [33], refined protein complexes of eukaryotic cells with a peculiar architecture and several distinct functions (for review, see [63,79,104]). Occupying large holes in the nuclear envelope, NPCs are the only gateway between nuclear contents and cytoplasm (Figure 1). NPCs have crucial functions in chromatin organization and gene expression [60], cell cycle progression [21] and possibly cellular senescence [96]. In the present context, however, only the transporter functions of the NPCs will be dealt with.

As a transporter (for review, see [79,80,89,100]), the NPC supports two processes, hindered diffusion (passive permeability) and facilitated diffusion (mediated transport). For molecules which do not bind to structural elements of the NPC referred to as phenylalanine glycine (FG) repeats the NPC has properties of a nanopore ~10 nm in diameter [55,72,76] and 45 nm in length [55]. FG-binding molecules, however, are transported through the NPC at high speed even if their molecular diameter greatly exceeds [73] the exclusion limit for non-binding molecules [86,98]. Thus, counter-intuitively, transport through the NPC is enhanced and sped up by binding. Facilitated diffusion through the NPC involves nuclear transport receptors (NTR) such as karyopherins (kap). Kaps are rather peculiar, snake-like molecules which can bind both cargos containing nuclear localization signals and FG repeats and thus ferry cargos through the NPC [24,25,42,70,100]. Remarkably, kap-mediated transport of proteins through the NPC is a passive process, not requiring metabolic energy [34,95] nor involving motor proteins [26,90] but driven exclusively by stochastic thermal motion. The transport properties of the NPC have given rise to a substantial number of transport models [2,5,11,15,35–37,56, 61,64,65,78,84,86,91,97,108,116].

In the following, we summarize basic features of the NPC, paying particular attention to the FG domains. We then focus on recent data on the relations between NTRs and FG domains and consider the implications of these data for the transport mechanism. Finally, we discuss why the NPC can serve as paradigm for cellular nanopores and provide inspiration for novel artificial molecule sorters.

2. The nuclear pore complex is a nanopore

In electron micrographs of cells or native preparations of the nuclear envelope the NPC is seen to consist of a large cylindrical structure, which occupies a hole in the nuclear envelope, and of filaments which radiate from the cylindrical structure into cytoplasm and nucleus (Figure 1A). A closer inspection shows that the cylindrical structure, also referred to as the central scaffold, in vertebrates measures ~125 nm in diameter and 50–70 nm in height and that there are eight cytoplasmic and eight nuclear filaments. The nuclear filaments are jointed at their ends forming a basket-like structure. It is not immediately obvious, however, that the NPC scaffold forms a large pore. Instead, the centre of the scaffold is usually occupied by a polymorphic mass, the central granule. The nature of the central granule has been debated for a long time [67]. But recent cryo-electron microscopic studies of the NPC in living cells [9] have provided further evidence that the central granule is not a genuine component of the NPC but predominantly represents cargo in transit.

Recent three-dimensional reconstructions of the NPC by cryo-electron microscopy [9,101] suggest that the scaffold forms a large central pore (Figure 1B). The central pore has an hour-glass-like shape which in vertebrates has a smallest diameter of ~40 nm and a length of ~50–

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70 nm. According to reconstructions of the scaffold by hybrid computational methods [3,33] the wall of the pore is 25–30 nm thick and formed by three concentric layers: A peripheral "membrane layer", an intermediate "scaffold layer" and an innermost "FG layer". Recent studies, in which the structures of individual nups or nup pairs were analyzed by X-ray diffraction, have provided a more detailed picture of these layers. The so-called Nup84 complex consisting of seven nups provides the NPC with a flexible coat [16,29,49] while other nups such as Nup96 provide stability and rigidity through β-propellers and non-canonical α-helical structures [53,93]. Overall, the scaffold has an eight-fold symmetry with respect to the long axis of the nuclear pore and a two-fold symmetry with respect to the plane of the nuclear envelope. Most of the nups are "symmetric" in that they occur in both the cytoplasmic and the nuclear half of the NPC. A small fraction of the nups is "asymmetric", occurring only in the cytoplasmic or nuclear half. Altogether, the vertebrate NPC is made up of 30–32 different nups yielding according to the proteomic analysis of the isolated NPC [26] a total of ~600 peptide chains and a mass of 60 MDa. However, previous mass estimated of the vertebrate NPC by scanning transmission electron microscopy [83] yielded a much larger mass, 125 MDa, which supports the view [80] that transport factors are genuine components of the NPC.

In addition to the central pore, eight peripheral channels or holes of ~5 nm diameter have been observed in both in detergent extracted samples [108] and cryo preparations [9,101] of the NPC. In certain [101] but not all [9] cryo electron microscopic reconstructions the peripheral channels or holes seem to terminate at the nuclear membrane. In reconstructions of the NPC by hybrid computational methods peripheral channels are surrounded by a cloud of FG repeats [3]. Thus, the nature and physiological significance of peripheral channels or holes are still unresolved.

That the NPC is, in essence, a nanopore is also strongly supported by recent work with artificial nanopores [54]. Extending the methods used in optical single transporter recording [77,107] track-etched membrane filters containing cylindrical pores of nanometer dimensions were coated with a monolayer of Nsp1 or Nup100, both major FG nups of *Saccharomyces cervisiae*. Transport through Nsp1-/Nup100-coated nanopores was measured in a two-compartment system. When the pore diameter was chosen to be ~30 nm and the transport solution contained a NTR the nanopores displayed a NPC-like transport specificity, transporting NTRs and NTR-cargo complexes substantially faster than cargos of the same size which did not bind to FG domains.

3. The nuclear pore is functionalized by FG repeats

Conspicuously, about one third of the nups contain large FG domains, each comprising 150–700 amino acid residues. FG domains consist of up to 40 FG repeats in which the amino acid motifs FG, FXFG, GLFG, SAFG or PSFG are separated by linkers of 5–50 predominantly hydrophilic residues. Each NPC contains ~180 FG domains with a total of ~2,700 FG repeats which accounts for ~12% of the NPC mass [58]. Most FG domains are constituents of the nuclear pore wall. But FG repeats are also abundant on cytoplasmic filaments and at the basis of the nuclear basket.

4. FG motifs are binding sites of nuclear transport receptors

It has been shown by several independent methods ranging from biochemical binding assays, X-ray analysis, NMR spectroscopy and molecular dynamics simulation [7,8,10,23,27,51,52, 57,62,69] to the molecular genetic modification of living yeast cells [102,114] that FG motifs are NTR binding sites. According to X-ray analysis [6] the binding of FG repeats to a NTR molecule involves the insertion of one or sometimes two phenylalanine residues of an FG motif into hydrophobic pockets of the NTR. All studied NTRs have at least two FG binding sites per functional unit (which is usually a monomer, sometimes a dimer). Molecular dynamics

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simulations have indicated, however, that there may be more binding sites. Thus, karyopherin β1 seems to have ten FG binding sites which cover the whole convex surface of the molecule in form of a continuous stripe [51].

The equilibrium dissociation constants of NTR-FG repeat complexes have been measured employing purified proteins [8,11,20,81,85] and permeabilized cells [106]. The association-dissociation reaction involves multiple binding sites on both NTRs and FG domains and therefore yields apparent dissociation constants and not the dissociation constant pertaining to single binding sites and single FG motifs. With these preliminaries the apparent dissociation constant ranged predominantly in the lower nM range. The kinetics of the reaction has been determined so far at low time resolution only [82,85] but seems to be amazingly slow with off-times in the second-to-minute range.

A systematic deletion of FG motifs in living yeast cells revealed a considerable redundancy [102]. Only when 50% or more of the native FG motifs were deleted viability was completely compromised. At a smaller extend of FG removal the viability was affected but depended on the mutated nups and their combinations. The deletion or swapping of FG motifs in asymmetric nups had little effect on viability and transport [114].

5. FG proteins belong to the class of intrinsically disordered proteins

Intrinsically disordered proteins (IDP) and intrinsically disordered protein regions (IDR) contain little well-defined secondary structure in the isolated state but assume a folded state upon binding of ligands such as nucleic acids, proteins or small ligands (for review, see [38, 39,50,109,112]). IDPs and IDRs are malleable elements which can undergo large conformational changes and adapt to a large number of functions and binding partners. On the basis of their amino acid composition, which is characterized by a low content of aromatic residues and a high net charge, IDPs and IDRs can be predicted from primary sequence data with a success rate of 50–70% [110]. Such predictions suggest that IDPs and IDRs are very abundant in complex organisms, accounting possibly for up to 30% of proteins and protein domains [38]. They occur predominantly in the nucleus being involved in the regulation of transcription but are also associated with cell cycle control and translation. They have not been found in transporters other than the NPC so far.

Purified FG domains were found to fulfil all the criteria of IDPs with regard to small hydrophobicity, large net charge, large hydrodynamic radius, little structure according to circular dichroism and protease hypersensitivity [31,32]. A protease hypersensitivity of FG nups was also observed when isolated yeast nuclei instead of isolated FG nups were employed. Notably [31], in addition to IDRs FG nups have structured domains, which serve to anchor these nups to other components of the NPC scaffold. The structured domains of the vertebrate nups Nup214 [71], Nup58/45 [68] and Nup98 [103] have been crystallized and their structures elucidated at the atomic level. In the case of Nup58/45, which is a component of the so-called Nup62 complex and lines the wall of the central channel, the structured domains contain α-helical elements which may confer flexibility to the channel diameter by intermolecular sliding [68].

6. Conformation, disposition and function of FG repeats are debated

By incubation of ultrathin sections of embedded cells and tissues with polyspecific primary antibodies against FG nups and subsequent incubation with gold-labeled secondary antibodies (so-called post-embedding immune gold electron microscopy) we found [45] that irrespective of the cell type antibodies predominantly bound within a radius of 25 nm around the center of the nuclear pore complex (NPC), thus suggesting that FG nups coat the whole length of the nuclear pore. Similar conclusions were drawn from our single-molecule studies of

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permeabilized cells which showed that a number of different NTRs such as NTF2, Kapβ1 and Kapβ2 are bound to the NPC with a maximum close to the NPC center [28,59].

However, Rout et al. [90] assumed that FG domains, although anchored in the pore wall by the folded domains of the FG nups, form a phalanx of docking filaments bristling out from the NPC towards karyoplasm and cytoplasm. It was suggested that the filamentous FG nups, by vigorous thermal motion, prevent non-FG-binding molecules from entering the channel but that molecules which bind to FG motifs have a larger probability for entering and permeating the transport channel, provided binding is not too strong and rapidly reversible. This mechanism was termed Brownian affinity gating or virtual gating. By theoretical studies it was shown that binding to FG filaments can enhance transport [115,117]. The transport efficiency is in principle positively correlated with the avidity of transported molecules for their binding sites in the channel. Only if the number of ligands per binding site is restricted, the transport efficiency reaches a maximum with increasing binding affinity and then decreases again because the channel becomes clogged. The value of the optimal binding strength is a function of the flux through the channel. The transport of molecules which bind non-specifically to sites in the channel (i.e. have a small affinity) is also positively correlated with affinity. However, when cargos with low and high affinity are simultaneously present, the transport efficiency of the low-affinity cargos is reduced because the high affinity cargos occupy the binding sites. Thus, the competition between specific and non-specific transport substrates can, in principle, increase selectivity.

In contrast, Ribbeck and Gorlich [86] suggested that FG domains form a hydrogel filling the lumen of the nuclear pore. The FG phase was assumed to have a high solubility for NTRs so that, in analogy to the permeation of lipophilic molecules through lipid bilayers, NTRs and transport complexes would permeate the selective phase by a solubility-diffusion process. In support of the selective phase model the authors showed [87] that the transport rate of NTR cargo complexes depends, on a qualitative basis, inversely on the size of the complex. Also, a cargo with two NLSs was transported faster when in a complex with two rather than one NTR. Furthermore, cyclohexane-1,2-diole, a compound interfering with hydrophobic interactions, disrupted the permeability barrier of the NPC in a reversible manner. Recently, the authors found [41] that concentrated solutions of recombinant FG domains in deed can form hydrogels. Such FG hydrogels are temperature stable but dissolved by chaotropic agents. Mutation of FG motifs (from FSFG to SSSG) abolishes the capability of recombinant FG domains to form hydrogels. Strikingly, FG hydrogels display sorption properties which are analogous to the transport characteristics of the NPC [40]. NTRs and NTR-cargo complexes were rapidly taken up by FG gels, whereas molecules which do not bind to FG motifs were excluded to a very high degree.

To better discriminate between the virtual gating model and the selective phase model Patel et al. [75] recently studied interactions between FG nups by a qualitative bead assay and found that all FG domains of yeast nups which contain GLFG motifs would bind to each other. In contrast, nups containing FxFG motifs would not bind to each other or FG domains with GLFG motifs. In binding pairs the affinity was very small with apparent dissociation constant of 5 – 70 μM. The binding depended on the F-residues of the FGFL motifs and was abolished by mutation of F to A. FG domains which interacted *in vitro* seem also to interact *in vivo* because such domains, when over expressed, formed visible precipitates in live yeast cells. By depleting yeast cells of FG nups and measuring nucleocytoplasmic transport in living cells it was confirmed that FG nups are involved in maintaining the characteristic passive permeability of the NPC. Patel et al. [75] concluded, combining the virtual gating with the selective phase model, that the center of the nuclear pore is occupied by a FG hydrogel while FG domains at the entrances of the nuclear pore form filaments bristling out from the NPC towards karyoplasm and cytoplasm.

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7. At physiological conditions FG domains are saturated with NTRs

Using purified proteins the apparent dissociation constant of the NTR-FG association-dissociation reactions were determined *in vitro*, as mentioned above, to be in the lower nM range [11,81]. However, the concentration of NTRs in the living cells is 10–20 μM [74,81]. This suggests that FG domains are saturated with NTRs at physiological conditions. Recent studies support this notion: In living cells [74] and in permeabilized cells at physiological NTR concentration [106] the NPC is associated with large numbers of NTRs, e.g. 104 Kapβ molecules, 48 Kapα molecules and 6 NTF2 molecules per NPC [74]. Furthermore, in permeabilized cells the apparent k_d-value of the FG-Kapβ complex was found to be in the lower nM range [106], very similar to the values found with isolated compounds [11,81].

As to be expected for IDPs, the conformation of FG domains is affected in a very sensitive way by association with other molecules. When gold dots of 100 nm diameter were covered with a monolayer of the recombinant FG domain of Nup153, the gold-attached FG proteins were found by atomic force microscopy to form a polymer brush of ~30 nm height [64]. Upon addition of minute concentrations of Kapβ1 (full effect at 11.3 nM) the FG brush collapsed to form a more compact layer of ~10 nm height. Upon removal of Kapβ1 the polymer brush was fully restored.

8. A role for surface diffusion in transport through the nuclear pore

The data summarized in preceding sections led to the following hypothesis for the mechanism of transport through the NPC [78,80]: At physiological NTR concentrations the FG domains of the NPC, which seem to be distributed all along the nuclear pore and cytoplasmic filaments, are saturated with NTRs. Being a kind of IDPs the FG domains response to ligand binding in a sensitive way and form a compact layer coherently lining pore wall and cytoplasmic filaments. NTRs are firmly bound to the condensed FG layer but, because of the extended FG binding surfaces, nevertheless able to move laterally on the FG layer at a substantial speed. Transport through the NPC is brought about by the attachment of NTRs and NTR-cargo complexes to the FG layer at the pore entrance, a two-dimensional random walk of NTRs and their cargo complexes on the FG layer, and the Ran-induced or RanGAP-triggered release of cargo or NTR-cargo complexes at the pore exit.

Surface diffusion [1] is a common phenomenon in both artificial and biological systems (for review, see [14,30,43,105]). In the case of several DNA-binding proteins [14,43,46,88] and signalling molecules [13] surface diffusion leads to a dramatic [88] decrease of capture times, i.e. the mean time ligands need to find its target. The theory underlying the reduction of capture times by surface instead of bulk diffusion, a phenomenon also known as reduction of dimensionality (ROD), has been discussed in an extraordinarily lucid and inspiring way in [12].

However, in the outlined ROD model of transport through the NPC surface diffusion does not yield a decrease of capture time. The distances involved in transport through the NPC (50–200 nm) neither support nor require that effect, as estimated below. Instead, surface diffusion assumes a novel, unrelated function, the reconciliation of tight binding with effective transport.

By binding of a ligand to a surface, e.g. the binding of a NTR or transport complex to the postulated FG layer coating internal and external surfaces of the NPC, the dwell time of the ligand at the surface is increased. The dwell time depends on the binding affinity or avidity. At the apparent dissociation constants reported for kap-FG association reactions [8,11,20,81, 85,106] the off-rates are expected to be large (second-to-minute range), in accordance with experimental determinations so far available [82,85].

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Tight binding to a surface can lead to an immobilization of the bound molecule. However, if binding involves delocalized binding patches, stripes or surfaces on the ligand, as described for the kap-FG system [52,69], and binding forces such as electrostatic attraction or hydrophobic interactions, the bound molecule may retain a substantial degree of lateral mobility in the surface plane. Importantly, for surface diffusion to be effective the energy landscape of sliding has to be flat with a roughness smaller that about one k_BT [99].

Experimental studies of surface diffusion in a variety of artificial and biological systems have shown that the surface diffusion coefficients of bound molecules is frequently reduced by 2–3 orders of magnitude as compared to bulk diffusion coefficients ([43,113]). Assuming that this holds also for NTRs sliding on the FG layer of the nuclear pore, a diffusion coefficient of $D = 0.05 – 0.5\ \mu m^2/s$ is predicted. With a pore length of L = 50 nm the time a NTR molecule placed initially at the pore entrance needs on average to capture the pore exit by two-dimensional diffusion on the pore wall is $\tau = L^2/2D$ [13], i.e. 0.25 – 2.5 ms. This rough estimate agrees well with the residence times of several NTRs and NTR-cargo complexes at the NPC which we have measured previously [28,59].

Frequently, surface and bulk diffusion coexist and are convoluted [22]. Whether surface or bulk diffusion prevails depends essentially on the strength of surface attachment. On this basis, we envision three basic mechanisms for transport through the NPC: Pure bulk diffusion, bulk-mediated surface diffusion, and pure surface diffusion (Figure 2). The available data favour an almost pure surface diffusion. However, a part of the data this hypothesis is based on has been obtained so far only *in vitro* with purified components and more experimental evidence and direct *in vivo* measurements are required.

Thus, in comparison to other transport models [5,11,15,35,36,61,64,65,78,86,91,97,116] , the ROD model assumes that the physical phenomenon which reconciles the puzzling observation that in the NPC effective transport seems to be linked to tight binding is surface diffusion. The ROD model is also distinguished by the assumption that the FG domains are not directly involved in “gating”, i.e. in preventing small macromolecules from passing through the nuclear pore. In the ROD model FG domains provide a coherent and energetically smooth layer on pore wall and filaments, making the sliding of NTRs and NTR-cargo complexes possible and efficient. The exclusion of small macromolecules is simply a consequence of the pore diameter (~40 nm diameter) and the height of the FG/NTR bilayer (~15 nm) [80]. The geometric parameters provide for a patent diffusion tube of ~10 nm diameter in the pore centre. However, this tube has no rigid boundaries and is even temporarily obstructed when bulky hydrophilic cargos such as ribonucleoprotein particles are transported. The organization of FG domains in form of a compact layer appears to be also compatible with the observation [102] that in yeast up to half of the FG motifs can be deleted without seriously compromising viability. In the context of the ROD model the deletion of FG motifs would lead, in principle, to a reduction of the surface density of FG motifs on the FG layer. However, this may be compensated to a certain extend by slight rearrangements of the FG layer exposing more of the previously buried FG motifs on the surface. A coherent semi-fluid FG layer seems also to be compatible with recent suggestions [29,68] that the NPC has a flexible coat so that the transport channel can accommodate large transport substrates.

9. Conclusions and prospects

The NPC is an excellent system for studying how cellular nanopores are functionalized to fulfil a vast range of tasks: The NPC is relatively well characterized on a proteomic basis [26,91]; its structural characterization is making fast progress [3,17,29,93]; at least in yeast the NPC is accessible to genetic manipulations [102]; versatile techniques for transport measurements [76], also on a single-NPC [55] and single-molecule level [59], are available. Yet the NPC is

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still associated with a great deal of mystery. Recent studies of the NPC have yielded surprises, e.g. the unprecedented occurrence of IDPs in a transporter [31] and the implicated role of surface diffusion [78]. Thus, more work is required to better understand the mechanism underlying transport through the NPC and to better characterize other functions of the NPC. Only with an improved knowledge it will be possible to fully appreciate the role of the NPC in cell function. Insight gained by studying the NPC should also help to better understand how other cellular nanopores are tuned for their specific functions.

Insight into the functionalization of nanopores gained by studying the NPC has also biotechnological implications. The NPC is able to sort proteins, protein complexes and ribonucleoprotein complexes in native form at high precision and speed. It would be of great value if such capabilities could be reproduced in technical systems. Filtration techniques have been a keystone of biomolecular separation for a long time. With the advent of nanotechnology is has become possible to create filters with nanoscopic pores and to integrate such nanopore filters into microfluidic devices, yielding novel analytical and preparative devices [47]. Thus, nanopores have been isolated from biological sources and integrated into technical set-ups. An example is the incorporation of α-haemolysin pores into artificial lipid bilayers for the (still projected) high-speed low-cost sequencing of nucleic acids [18]. Alternatively, fully artificial nanopores have been built from bottom up taking biological nanopores as model [19,66]. We have followed a “hybrid” approach by attaching recombinant FG proteins to artificial nanopores [54]. Such nanopores have NPC-like transport specificity but do not yet match the NPC. We assume, however, that by the further analysis of the NPC and a corresponding refinement and tuning of hybrid nanopores the gap between the cellular and the artificial system can be reduced.

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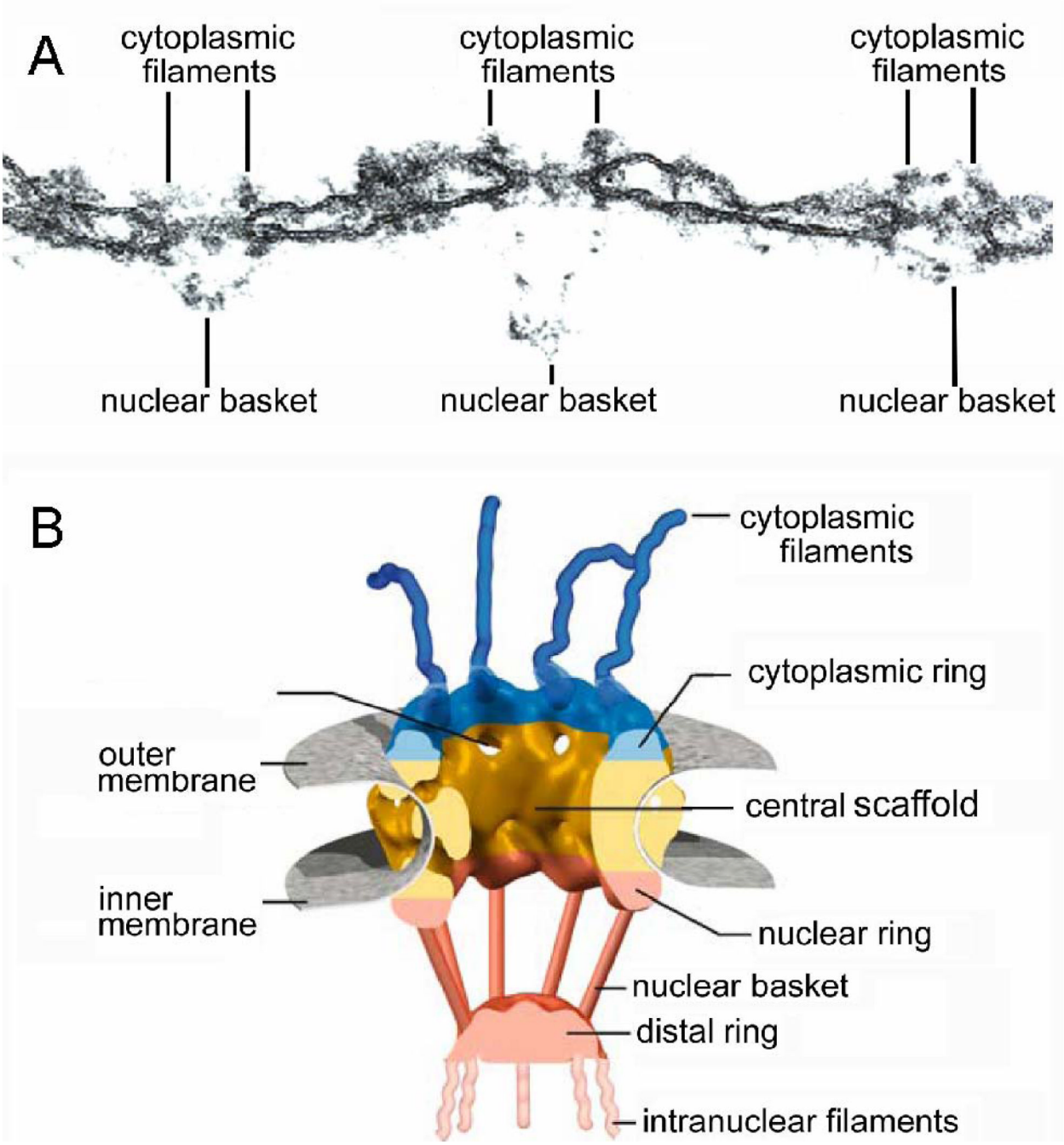

Figure 1. Architecture of the nuclear pore complex

A) In nuclear envelopes isolated from *Xenopus* oocytes the NPC can be seen to occupy a large pore in the nuclear envelope and to consists of a central scaffold, cytoplasmic filaments and nuclear filaments forming a basket-like structure. B) The three-dimensional reconstruction of the *Xenopus* NPC based on cryo-electron microscopy reveals that the NPC is, in essence, a nanopore. Figure 1A from [92] with permission. Figure 1B from [35] with permission.

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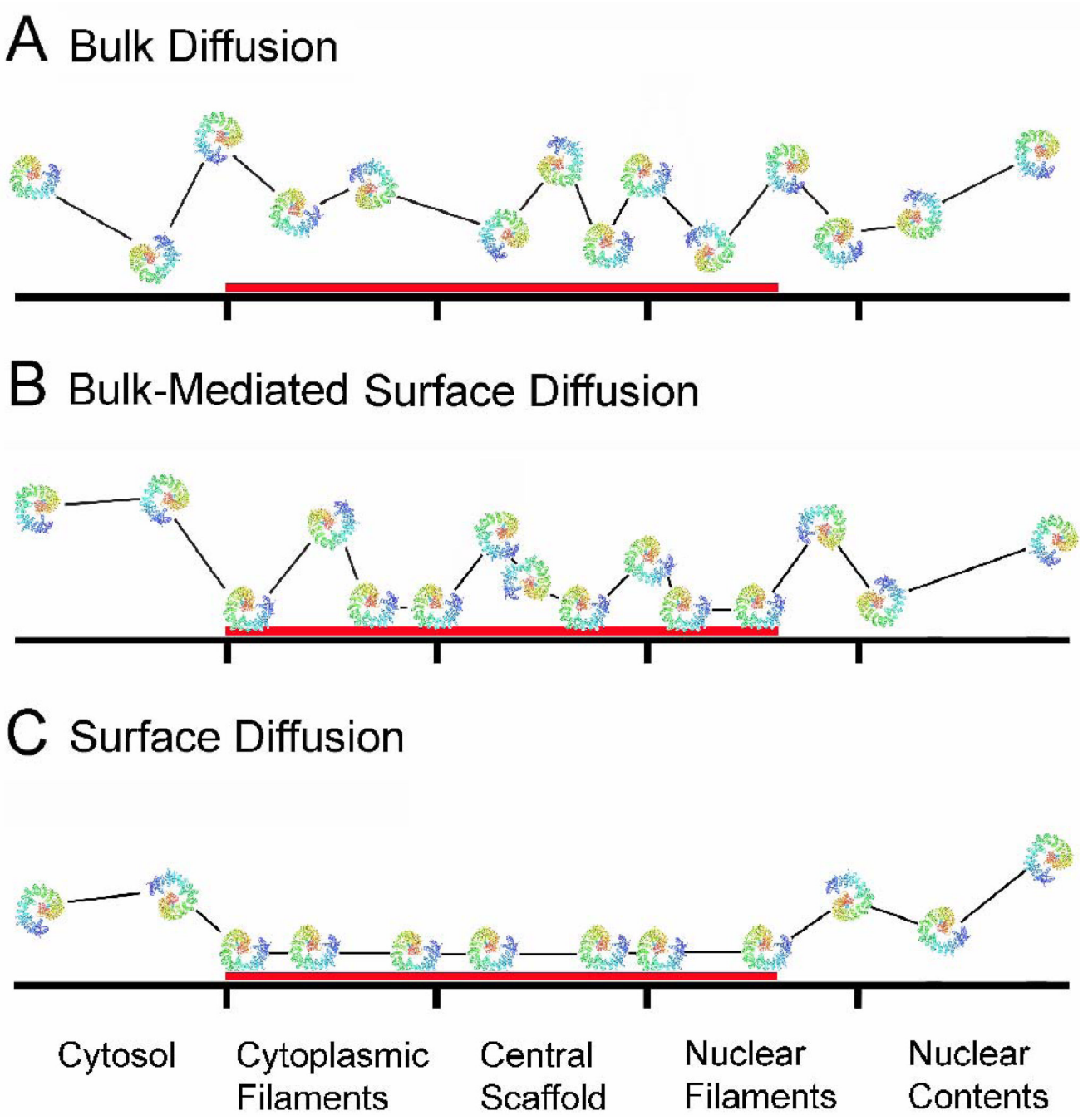

Figure 2. Three general mechanism of diffusion through the nuclear pore

On the way from cytosol to nuclear contents or vice versa a transport factor passes through various regions: cytoplasmic filaments, central scaffold and nuclear filaments. In some regions surfaces are lined by FG domains, as indicated in red. In the regions lined with FG domains diffusion can be constrained A) to the aqueous phase, or C) to the pore wall. In addition (B), both sites may be involved. The relative prevalence of the three indicated mechanism depends in essence on the avidity of NTRs for FG domains.