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Mammalian genome organization and its implications for the development of gene therapy vectors

Review Article

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Summary

The nuclear pore complex (NPC) is the site for macromolecular traffic between the nucleus and cytoplasm. NPCs are composed of 30-40 distinct proteins (termed nucleoporins) in yeast, and an estimated 50 distinct nucleoporins in vertebrates. Most nucleoporins are soluble proteins. In contrast, the number of integral membrane nucleoporins is small and includes only four proteins in yeast (Pom152, Ndc1, Snl1 and Pom34), and two proteins in metazoans (gp210 and Pom121). We discuss the known membrane nucleoporins, and present for the first time the sequences of putative gp210 orthologs from *Drosophila, C. elegans* and *Arabidopsis*. Our results suggest that Gp210 is conserved among all multicellular eukaryotes, including plants, consistent with a fundamental role in NPC structure or biogenesis.

I. Nuclear pore complexes (NPCs) mediate nucleo-cytoplasmic traffic

In all eukaryotic cells the nuclear envelope (NE) separates the nucleoplasm from the cytoplasm. The NE is composed of outer and inner membranes (OM and IM, respectively), a nuclear lamina and nuclear pore complexes (NPCs) (**Figure 1**). The OM is continuous with the rough endoplasmic reticulum (ER) (Gant and Wilson, 1997). At the end of mitosis, the two nuclear membranes fuse to form a channels or pores as a prerequisite for NPC assembly. This process forms a third domain, termed the pore membrane domain At the pore membrane there is ordered recruitment of nuclear pore complex proteins, known as nucleoporins, to form a mature NPC that mediates the selective transport of molecules between the nucleus and cytoplasm. The NPC is anchored to the pore membrane via interactions with integral pore membrane proteins, which are known as pore membrane proteins (POMs) in yeast (Pante and Aebi, 1996).

In yeast, the NPC has an estimated mass of 50 MDa and consists of multiple copies of about 30 distinct proteins (Rout et al, 2000). The vertebrate NPC is larger (estimated mass, 120 MDa) and is thought to contain at least fifty different proteins (Bagley et al, 2000; Miller and Forbes, 2000). Nucleoporins can be divided into two groups: soluble proteins and integral membrane proteins. The vast majority of nucleoporins is soluble and disperses into the cytoplasm during mitosis. In multicellular eukaryotes many soluble nucleoporins contain phenylalanine-glycine (FG) repeats which are important

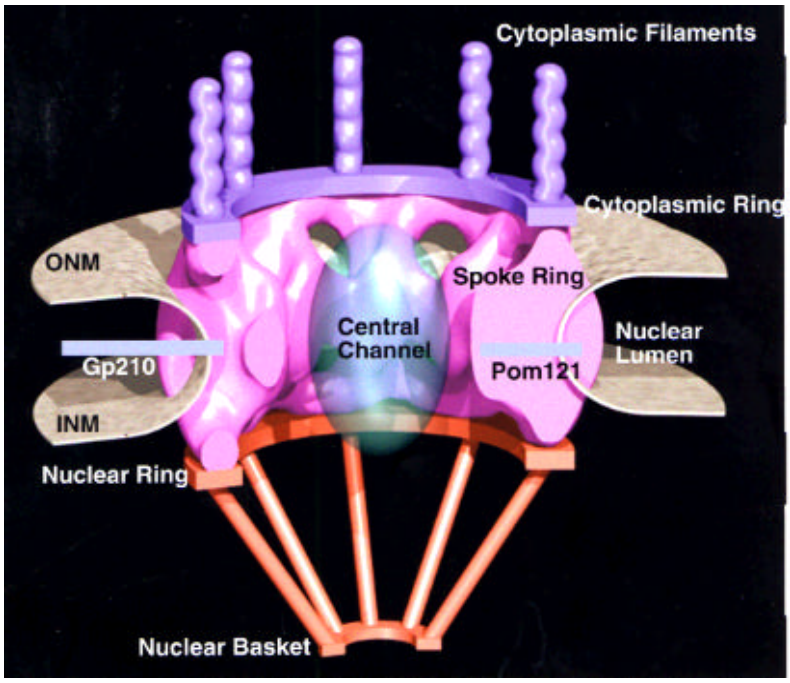

Figure 1. A model of vertebrate NPC structure (adapted from Pante and Aebi, 1996). The orientation of POM121 and Gp210 proteins is shown.

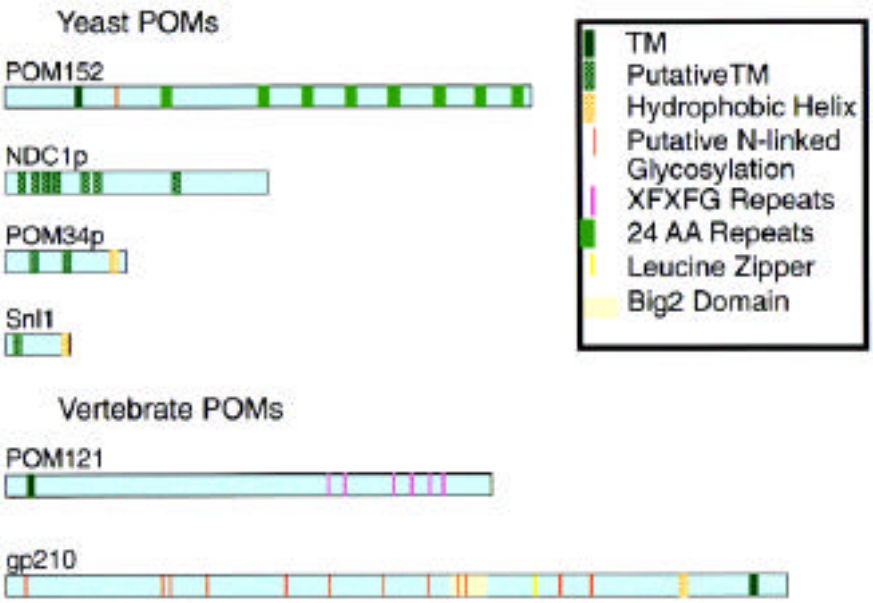

Figure 2. Motifs and domains found in Pore Membrane (POM) proteins from yeast and vertebrates. TM, transmembrane domain.

for transport through the NPC (Talcott and Moore, 1999). In contrast, very few membrane nucleoporins have been identified. Because NPCs assemble at pore membrane domains, membrane nucleoporins are proposed to play central roles in pore formation and NPC assembly (Greber et al, 1990; Soderqvist and Hallberg, 1994).

II. Yeast POMs

We will first discuss the four POM proteins identified in the yeast *Saccharomyces cerevisiae* (**Figure 2**), namely Pom152 (Wozniak et al, 1994), Ndc1 (Chial et al, 1998), Snl1 (Ho et al, 1998) and Pom34 (Rout et al, 2000). The yeast genome has been completely sequenced and no other candidate POMs have been identified. Thus, these four proteins might represent the full set of yeast membrane nucleoporins. Interestingly, another nucleoporin named nup53, which lacks a transmembrane domain, interacts with the nuclear inner membrane via an amphipathic α-helix (Marelli et al, 2001).

III. POM 152

Pom152 is posttranslationally modified by *O*-GlcNAc, and was isolated biochemically because it binds to a lectin, concanavalin A (conA; Wozniak et al, 1994). Pom152 was subsequently localized to the NPC (Yang et al, 1998). Pom152 is a type II integral membrane protein with a relatively short N-terminal head (175 residues) that faces the NPC and a long C-terminal domain (1141 residues) that resides in NE lumen. Sequence analysis predicted that Pom152 had two closely-spaced putative transmembrane domains. Nevertheless, Pom152 spans the pore membrane only once (Tcheperegine et al, 1999). In this regard, Pom152 is similar to gp210, a membrane nucleoporin in multicellular eukaryotes that is proposed to mediate membrane fusion (discussed below).

Deletion mutants of *POM152* are viable alone, but lethal in combination with mutations in either Nup188 or Nup170 (Aichison et al, 1995), both of which are proposed to help establish the functional diameter of the NPC central channel (Shulga et al, 2000). Over-expression of Pom152 reduces the growth rate of cells for reasons that are not understood (Wozniak et al, 1994). When expressed in mammalian cells, Pom152 localizes correctly to the pore membrane domain, suggesting that the microenvironment of the pore membrane domain is functionally conserved between yeast and mammals (Wozniak et al, 1994). We found a putative ortholog for Pom152 in *S. pombe* (GenBank accession number AL034463.2). Because *S. pombe* is evolutionarily distant from *S. cerevisiae*, the function of Pom152 appears to be highly conserved among single-cell eukaryotes.

IV. Ndc1

Ndc1 is an unusual nucleoporin first characterized due to its function at a late stage of spindle pole body (SPB; microtubule organizing center) duplication. In contrast to mammalian centrosomes, the *S. cerevisiae* SPB is embedded in the nuclear envelope, like pore complexes. *NDC1* is an essential gene that encodes a 74 kDa protein with six or seven putative transmembrane domains (Winey et al, 1993). By indirect immunofluorescence analysis, Ndc1 localizes to the membrane domains of both NPCs and SPBs (Chial et al, 1998).

In Ndc1-null cells, SPBs are not inserted into the nuclear envelope, but there is no detectable phenotype related to NPC structure or distribution. The lack of an NPC phenotype in cells that lack either Ndc1 or Pom152 suggests a redundancy among NPC components. The Ndc1 localization results further suggest a common assembly mechanism for NPCs and SPBs in yeast. Interestingly, the SPB phenotype of *ndc1-1* mutant cells is suppressed by deletion of Pom152. It was proposed that the loss of Pom152 releases 'defective' Ndc1 from NPCs, allowing all copies of 'defective' Ndc1 to function at the SPB (Chial et al, 1998). It is not known if Pom152 is also present in SPBs.

The *S. pombe* Ndc1 homologue, cut11+, encodes a protein with seven predicted membrane spanning regions. Cut11+ is also localized to both SPBs and NPCs and affects SPB function, consistent with being a functional homologue of Ndc1p (West et al, 1998).

V. Snl1

Snl1 was isolated in a baroque genetic screen for high-copy suppressors of the lethal over-expression of the carboxy-terminal 200 amino acids of the nucleoporin Nup116p (Nup116-C) in a nup116 null background (Ho et al, 1998). Nup116 localizes to the cytoplasmic face of the NPC (Ho et al, 2000) and is a docking site for nuclear import and export factors (Iovine et al, 1995, 1997). Snl1, when expressed at high copy, suppressed temperature sensitive mutations in two genes: *gle2*, which is essential for NPC assembly (Murphy et al, 1996) and *nic96*, which is involved in the transport of polyadenylated RNA and perhaps also protein transport (Gomez-Ospina et al, 2000). Snl1 has a predicted mass of 18.3 kDa, a putative transmembrane domain, and limited sequence homology to Pom152p. The C-terminal region of Snl1 faces the NPC. Results from both fluorescence localization and biochemical fractionation confirm that Snl1 is an integral pore membrane protein, but unexpectedly show that it is also present in the ER. Cells that lack both snl1 and Pom152 show no obvious growth defects and are viable, suggesting that both proteins are non-essential, and may be functionally redundant with at least one other pore membrane protein (Ho et al, 1998).

VI. POM34

POM34 was identified by mass spectrometry as a component of biochemically-purified yeast NPCs, and localizes to the pore membrane (Rout et al, 2000). Biochemical extraction experiments revealed that Pom34 is an integral membrane protein. By hydropathy analysis, Pom34 is predicted to have two putative transmembrane domains (Rout et al, 2000).

However, the topology of POM34 has not been determined.

VII. Vertebrate POMs

Only two integral pore membrane proteins have been identified in vertebrates; Pom121 and gp210, which are shown schematically in **Figure 2**.

VIII. Pom121

Pom121 was isolated from rat liver nuclei as a wheat germ agglutinin (WGA) binding glycoprotein, and subsequently localized to the NPC (Hallberg et al, 1993). Pom121 contains six XFXFG repeats, which are common to nucleoporins that are posttranslationally modified by *O*-linked N-GlcNAc. Antibody accessibility experiments showed that Pom121 spans the pore membrane only once; its short N-terminal head is in the lumen and its C-terminal domain, comprising 90% of its mass, is exposed to the NPC (Soderqvist and Hallberg, 1994). The signal that targets Pom121 to pores is found in a region of 310 residues near its transmembrane domain, on the cytosolic side, suggesting that Pom121 is localized by interacting with soluble nucleoporins (Soderqvist et al, 1997).

IX. Gp210

Gp210, the other membrane nucleoporin in vertebrates, was isolated almost twenty years ago from rat liver nuclei and identified as an integral glycoprotein associated with the NPC (Gerace et al, 1982). The cDNA sequences of gp210 have been determined for rat (Wozniak et al, 1989) and mouse (Olsson et al, 1999). We have now identified proposed orthologs for gp210 in *C. elegans*, *D. melanogaster*, and the plant *A. thaliana*, *and* compare these proteins below.

Rat gp210 has a predicted mass of 210 kDa but migrates with an apparent mass of ~185 kDa on SDS gels. Topological analysis showed that gp210 has a small cytoplasmic tail, one membrane spanning region and a long N-terminal domain, comprising 95% of its mass, positioned within the nuclear envelope lumen (Greber et al, 1990). Gp210 is localized by two independent sorting signals; the transmembrane domain, which is sufficient for localization at the pore membrane domain, and the carboxy-terminal tail, which encodes a weaker sorting signal (Wozniak and Blobel, 1992).

Similar to yeast Pom152, the lumenal domain of gp210 is core glycosylated by N-linked high mannose oligosaccharides and therefore binds to con A (Wozniak and Blobel, 1992). Rat gp210 is phosphorylated at Ser^{1880} during mitosis, by cyclin B-$p34^{cdc2}$ or a related kinase (Favreau et al, 1996). When expressed in rat cells, antibodies that recognize the lumenal tail of gp210 decreased both active and passive transport through the NPC, suggesting that gp210 is linked structurally to the NPC (Greber and Gerace, 1992).

For reasons that are not yet understood, patients with biliary cirrhosis and other hepatic auto-immune diseases produce autoimmune antibodies against lumenal epitopes of gp210 (Nickowitz and Worman, 1993; Nickowitz et al, 1994).

Gp210 is conserved in metazoans and plants. A proposed gp210 ortholog in *Xenopus* migrates in SDS gels as a 200-215 kDa conA-binding protein, and was localized to the NPC by fluorescence (Gajewski et al, 1996). A short cDNA sequence corresponding to Xe-gp210 is now available in the GenBank (GenBank accession number: AW642061), and has been shown to encode a bona fide nucleoporin (Drummond and Wilson, *manuscript in preparation*).

The first putative homolog of gp210 in invertebrates was described in *Drosophila* and localized to the NPC (Berrios et al, 1995), but was not cloned. A blast search in the Berkeley *D. melanogaster* Genome Project (http://www.fruitfly.org/) revealed an EST clone similar to rat gp210. We sequenced this EST clone (Dm-Gp210, GenBank accession number AF322889) and found that it encodes the complete Dm-Gp210. *Drosophila* gp210 is a protein of 1870 residue s and calculated mass of 209 kDa. We showed that Dm-gp210 is a unique gene that maps to the 41F11 band of the right arm of chromosome 2 by *in situ* hybridization to polytene chromosomes (**Figure 3**).

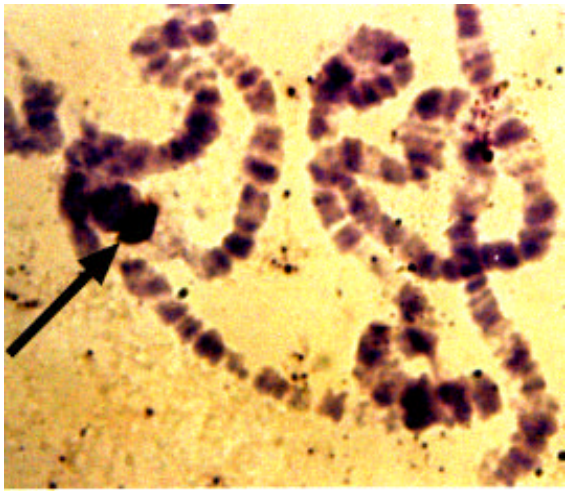

Figure 3. Dm-gp210 maps to the 41F11 band (marked by an arrow) on the right arm of *D. melanogaster* chromosome 2. Mapping was carried out by *in situ* hybridization on *D. melanogaster* polytene chromosomes, using full length *Drosophila* Gp210 cDNA as a probe.

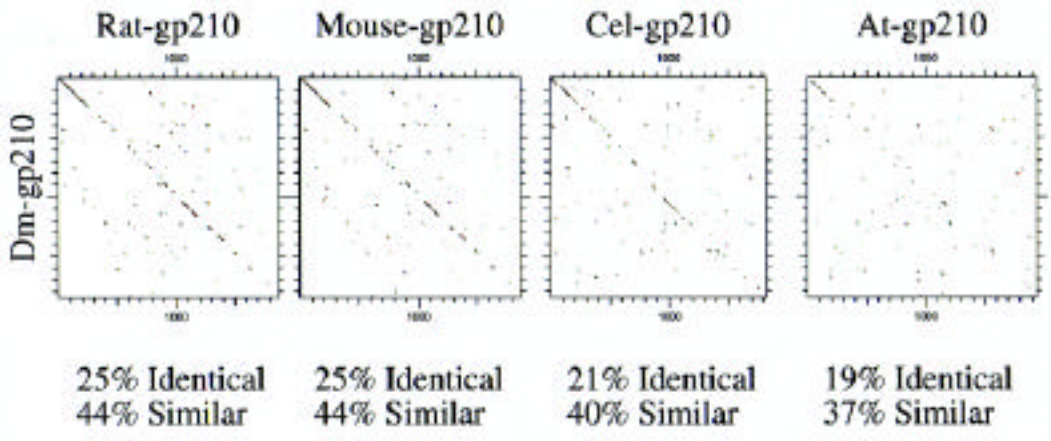

Figure 4. Dot matrix plot comparing the amino acid sequence of *D. melanogaster* (Dm) Gp210 to Gp210 in rat, mouse, *C. elegans* (Cel) and *A. thaliana* (At). Matrix was made with a window of 23 residues, using DNA Strider 1.3. The percent similarity and identity for Gp210 from each species relative to *Drosophila* Gp210 are, indicated below each plot.

A search in the *C. elegans* genome database (http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml) revealed a worm gp210 homologue (Ce-Gp210, GenBank accession number: U80033.1), which encodes a predicted 1847 residue protein with a calculated mass of ~200 kDa. Antibodies raised against Ce-Gp210 peptides produce a punctate staining pattern at the nuclear envelope rim in *C. elegans* embryos (our unpublished observation), which is characteristic of NPC staining. We also identified a gp210 homolog in *Arabidopsis thaliana* (GenBank accession number: AB017062). At-gp210 encodes a 1962 residue protein with a calculated mass of 215 kDa. We compared the amino acid sequences of the predicted gp210 proteins from rat, *Drosophila*, *C. elegans* and *Arabidopsis* using the UWGCG and BOXSHADE programs (http://www.ch.embnet.org/software/BOX_form.html). This analysis showed that Dm-Gp210 is 25%, 21% and 19% identical and 44%, 40% and 37% similar to the rat, nematode, and plant gp210 proteins, respectively (**Figure 4**). Hydrophobicity plots of these four proteins all predict a conserved transmembrane domain at the C-terminus (**Figure 5**).

Rat gp210 was originally predicted to have two membrane-spanning hydrophobic domains (Wozniak et al, 1989). However, topological and biochemical analyses showed definitively that only one of these domains actually crosses the pore membrane, while the other remains in the lumen. Based on this topology, Wozniak and Gerace, (1992) hypothesized that the rat gp210 may promote membrane fusion between the IM and OM to generate pores, through mechanisms analogous to the influenza hemagglutinin protein (Skehel, and Wiley, 2000). Gp210 is present in16-24 copies per NPC (Gerace et al, 1982), which theoretically allows it to assemble in a similar way to the trimers of HA, which co-operate to mediate fusion (Danieli et al, 1996). This assembly is probably not mediated by the gp210 second hydrophobic domain, since the hydropathy plots shown in **Figure 5** did not reveal significant evolutionarily conservation of this putative second hydrophobic domain between species.

X. Cell cycle dynamics of NPCs

Single-cell organisms such as yeast undergo a 'closed' mitosis, in which the NE and NPCs do not break down. In contrast, higher eukaryotes exhibit an open mitosis in which the nuclear lamina and NPCs disassemble and nuclear membranes merge into the ER (Ellenberg et al, 1997). Vertebrate and *Drosophila* NPCs start to disassemble at prophase (Georgatos et al, 1997; Harel et al, 1989), whereas *C. elegans* NPCs begin to disassemble later, after prometaphase (Lee et al, 2000). NE re-assembly occurs during late anaphase/telophase.

The assembly of NPCs does not require *de-novo* protein synthesis, suggesting that NPC components are recycled (Maul, 1977). NPC breakdown and reassembly are regulated by cell cycle-dependant phosphorylation of several nucleoporins including gp210, Nup153, Nup214 and Nup358 (Macaulay et al, 1995; Favreau et al, 1996). *In vitro*, NPC assembly is initiated as soon as membranes bind to chromatin and form flattened membrane patches (Lohka and Masui, 1984; Macaulay and Forbes, 1996; Wiese et al, 1997). A pathway for NPC assembly has been proposed based on the discovery of structures termed dimples, holes and star-rings on nuclei assembling in *Xenopus* cell-free extracts (Goldberg et al, 1997). NPCs can assemble prior to the full enclosure of chromatin within the NE; assembly is rapid (6-7 minutes in *Xenopus* extracts) and asynchronous (Wiese et al, 1997).

The recruitment of nucleoporins into assembling nuclei has been studied in a number of experimental systems, although many questions remain as to the order

of nucleoporin recruitment during NPC assembly. NPC assembly begins at late anaphase, possibly with early recruitment of Nup153 (a constituent of the nuclear basket; Pante et al, 2000). Assembly continues throughout telophase, with sequential accumulation of Pom121, p62 (a constituent of the central channel; Davis and Blobel, 1986), CAN/Nup214 (Fornerod et al, 1997), and finally Gp210 and Tpr (which forms fibers extending the nucleus, Cordes et al, 1997). Haraguchi et al, (2000) studied the

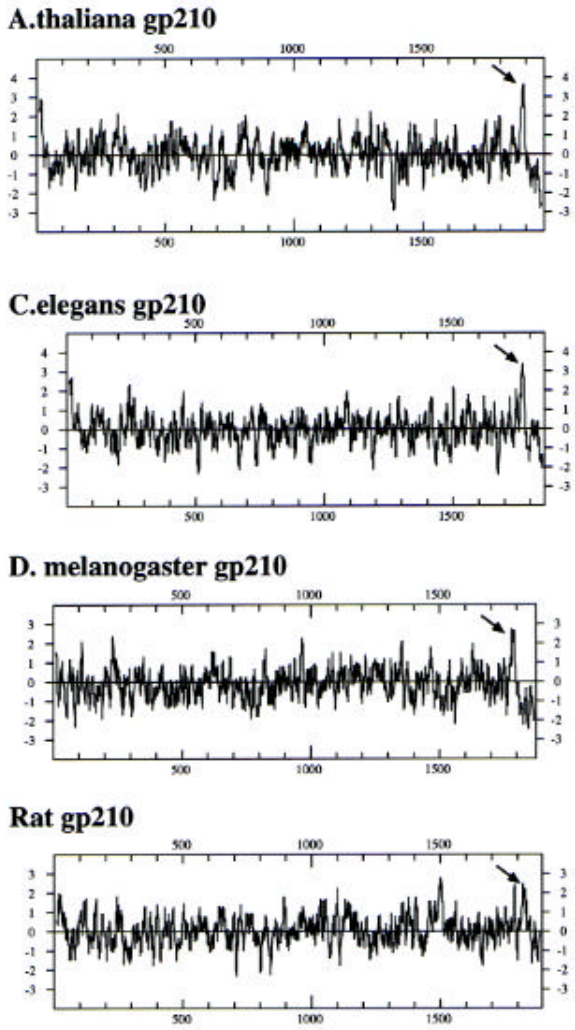

Figure 5: Hydropathy plots for Gp210 from *A. thaliana* (At), *D. melanogaster* (Dm), *C. elegans* (Cel) and rat, were made over a window of 7 amino acids using DNA Strider 1.3. Hydrophobicity was calculated according to Kyte and Doolittle (Kyte and Doolittle, 1982). An arrow indicates the transmembrane domain.

timing of NPC assembly by immunofluorescence staining of human cells fixed at precise times after the onset of anaphase. Their results showed that several NPC components (RanBP2, Nup153, p62), but not Tpr, reconstitute around chromosomes very early in telophase prior to the recovery of nuclear import activity. However in *Xenopus* extracts, NUP153 is recruited very late in the assembly process and binds to lamins, which mostly also assemble late (Smythe et al, 2000).

XI. Membrane fusion and NPC formation

Membranes do not tend to fuse spontaneously due to strong electrostatic repulsion between phospholipid head groups in an aqueous environment is high. To fuse, membranes must first circumvent this repulsion. The stalk hypothesis of membrane fusion (Siegel, 1993, 1999), predicts several stages in fusion, beginning with the formation of a "stalk" between facing phospholipid monolayers. The stalk then forms a dimple. Further pulling produces a hemifusion diaphragm followed by an opening of a fusion pore by the opening of a fusion pore by an unknown mechanism (Jahn and Sudhof, 1999).

Membrane fusions are key events in the secretory pathway, synaptic release, endocytosis, ER dynamics and certain viral infections, The formation of the nuclear pore is mediated within the lumen of the NE, and is therefore likely to resemble viral fusion rather than cytosol-mediated events observed throughout the secretory pathway.

Viral fusion proteins are glycoproteins, with a single membrane spanning region, and a relatively large tail, exposed on surface of the virus (Jahn and Sudhof, 1999). Viral fusogenic proteins differ in sequence between viruses, but all have a similar key feature; a short helical amphiphilic domain with alternating hydrophobic and charged amino acid residues. In the case of hemagglutinin (HA), fusion is triggered by the low pH (pH 5-6) within endosomes, from whence the virus fuses to enter the cytoplasm. Low pH induces a conformational change in HA exposing the N-terminal fusion peptide. The fusion peptide is then inserted into the target membrane (Li et al, 1993; Durrer et al, 1995) and possibly also into the viral membrane (Weber et al, 1994). This insertion is thought to produce a stalk structure, which connects the two membranes. At this stage of fusion, the stalk structure expands in such a way that the inner leaflets form a hemifusion diaphragm, where a fusion pore is opened. The expansion of the fusion pore completes the fusion event (Gaudin, 2000).

The fusion mechanism that produces nuclear pores is an open question. However, Gp210 remains an excellent candidate for the fusogen as originally proposed by Wozniak and Blobel, (1992). Our work suggests that Gp210 is conserved in all multicellular eukaryotes including plants, as expected for a protein with a fundamental function. We anticipate that the identification of gp210 homologs from insects, worms and plants, here reported for the first time, will contribute to a better

understanding of gp210 function and nuclear pore formation.

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