Dynamic properties of nuclear pore complex proteins in gp210 deficient cells

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Abstract Gp210, an integral membrane protein of the nuclear pore complex (NPC), is believed to be involved in NPC biogenesis. To test this hypothesis, we have investigated dynamic properties of the NPC and distribution of NPC proteins in NIH/3T3 cells lacking gp210. POM121 (the other integral NPC protein) and NUP107 (of the NUP107/160 complex) were correctly distributed at the nuclear pores in the absence of gp210. Furthermore, fluorescence recovery after photobleaching experiments showed that POM121 and NUP107 remained stably associated at the NPCs. We conclude that gp210 cannot be required for incorporation of POM121 or NUP107 or be required for maintaining NPC stability.

Keywords: Nuclear pore complex; Nucleoporin; Integral membrane protein; Fluorescence recovery after photobleaching

1. Introduction

Molecular exchange between the nucleus and the cytoplasm takes place through the thousands of nuclear pores covering the nuclear surface (for review, see [1–3]). Each pore harbors a multiprotein structure called the nuclear pore complex, which conducts nucleocytoplasmic trafficking of proteins and RNA molecules in a highly regulated manner. Virtually all nuclear pore complex proteins, collectively termed nucleoporins (NUPs), of the yeast and vertebrate nuclear pore complex (NPC) are known [4,5]. In vertebrates, only two pore complex proteins, gp210 [6] and POM121 [7], have domains that span the nuclear membrane. These proteins are termed pore membrane proteins and are believed to play important roles in nuclear pore formation and anchoring of the peripheral NUPs to the nuclear membrane.

Earlier this year, Olsson et al. [8] reported cell type specific expression of the pore membrane protein gp210 during organogenesis in the mouse. In a number of tissues and cell lines, including NIH/3T3 cells, gp210 expression was absent or at least very low at transcriptional as well as translational level. The absence of gp210 cannot be explained by differential splicing since mRNA expression was investigated by Northern blotting using specific probes hybridizing to the 5′-, middle and 3′- regions of the transcript. The absence of gp210 from various cell types is rather surprising considering the proposed role of gp210 in nuclear pore formation [6,9,10].

To gain further insights in the functions and interactions of the individual NUPs, a number of research teams have analyzed defective nuclear pore complex assembly after depletion of specific NUPs (reviewed in [11]). However, so far changes in dynamic properties of NPCs in cells missing specific NUPs have not been studied. NUP107, a component of the NUP107/160 subcomplex [12,13], and the pore membrane protein POM121 have both been shown to be extremely tightly associated to the NPCs and are ideal markers for NPC distribution [14,15]. Thus, POM121 and NUP107 could be used to monitor NPC stability and alterations in their dynamic properties may be very informative for the understanding of how the NPC is functionally organized.

Here we have studied the distribution and dynamic properties of nuclear pores and individual nucleoporins, including POM121 and NUP107, in cells naturally deficient in gp210. However, the absence of gp210 did not have any effect on these parameters. The results are surprising since, being a pore membrane protein, gp210 has been suggested to play a central role in nuclear pore biogenesis.

2. Materials and methods

2.1. Cell culture and plasmids

NIH/3T3 cells (ATCC number: CRL-1658) and neuro-2a (N2a, ATCC number: CCL-131) cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 and transiently transfected with GFP_3-NUP107 in pEGFP-C1 [14], POM121-GFP_3 in pcDNA1neo [16] or GFP_3-NUP153 in pEGFP-C3 [15]. Before and during 12 h fluorescence recovery after photobleaching (FRAP) experiments, cells were treated with 100 μg/ml cyclohexamide.

2.2. Antibodies

Total proteins of extracts of N2a and NIH/3T3 cells were separated on 8% SDS–PAGE, electrotransferred to nitrocellulose and subjected to Western blotting as described [7].

Mouse monoclonal antibodies mAb414 (BabCO, Nordic BioSite AB, Täby, Sweden) recognizing a subfamily of NPC proteins containing phenylalanine glycine (FG)-repeats [17] but not POM121 [18]

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Abbreviations: N2a, neuro-2a; NPC, nuclear pore complex; NUP, nucleoporin; FG, phenylalanine glycine; FI, fluorescence intensity; FRAP, fluorescence recovery after photobleaching

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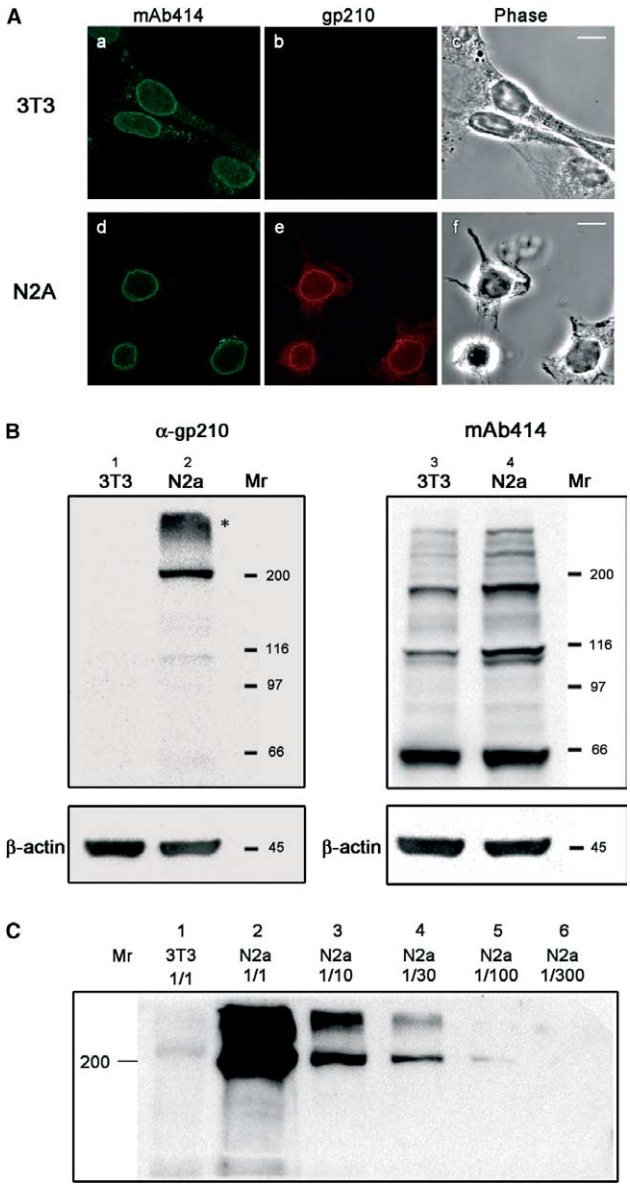

Fig. 1. NIH/3T3 cells, but not N2a cells, lack gp210. (A) Confocal laser scanning fluorescence micrographic images of monolayer cultures of fixed and permeabilized NIH/3T3 cells (a–c) and N2a cells (d–f) subjected to double immunostaining using antibodies against mAb414 (a and d) and anti-gp210 (b and e). Corresponding phase contrast images (c and f). Note the complete absence of anti-gp210 staining in NIH/3T3 cells (b) using the same dilution of anti-gp210 antibodies as for the N2a cells (e). Scale bar: 10 μm. (B) Western blot analysis of total proteins of NIH/3T3 cells (lanes 1 and 3) and N2a cells (lanes 2 and 4). Equivalent amounts of protein (20 μg) of whole cell lysates were separated by SDS–PAGE and analyzed by Western blotting, using anti-gp210 (lanes 1 and 2) and mAb414 (lanes 3 and 4) antibodies. Bars to the right indicate the migration of the molecular weight markers. The asterisk denotes high Mw oligomers of gp210. Note the complete absence of gp210 in NIH/3T3 cells (lane 1). (C) Detection limit of gp210 by western blot analysis. In the different lanes, equal amounts of total protein (40 μg) from NIH/3T3 (lane 1, 1/1) and N2a (lane 2, 1/1) cells and serial dilutions of the N2a cell lysate ranging from 4 to 0.13 μg (lanes 3–6, 1/10–1/300) were loaded. To be able to detect any signal at all in NIH/3T3 cells the exposure time during ECL detection was exaggerated. Note that the weak band at ~220 kDa in lane 1 (NIH/3T3 1/1 cell lysate) is equally weak as the band in lane 5 (N2a 1/100 cell lysate).

and a rabbit polyclonal anti-gp210 antibody [8] were used as primary antibodies. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit IgG and Cy3™-conjugated donkey anti-mouse IgG.

2.3. Confocal laser scanning microscopy (CLSM) and photobleaching

Imaging was performed on a Leica TCS-SP laser scanning confocal microscope (Leica, Heidelberg, Germany) with a 63.0×1.32 oil immersion objective using a 488 nm 20 mW argon laser line and a 568 nm 20 mW krypton laser line. Emission spectra were collected between 500–550 nm (GFP and FITC) and 580–650 nm (TRITC and Cy3), respectively. For colocalization, the laser lines scanned sequentially.

For FRAP experiments, defined regions of the nuclear envelope in cells expressing GFP_3-NUP107, POM121-GFP_3 or GFP_3-NUP153 were bleached for 0.5–1 s at full laser power of the 488 nm laser. The bleached area was then monitored and images were acquired at low laser power. To correct for unintentional bleaching during scanning, the fluorescence intensity (FI) was calculated as $FI = (I_{Bt} + I_{Ut})/I_{Bt}$, where I_{Bt} is the fluorescence intensity of the bleached area at each time point and I_{Ut} is the intensity of the unbleached area at the corresponding time points. $RFI = FI_{t0}/FI_{tn}$ was then plotted versus time. Images were processed using Adobe PhotoShop 5.5 software (Adobe Systems Inc., CA, USA).

3. Results and discussion

In order to investigate possible alterations of nuclear pore properties due to deficiency of gp210 in NIH/3T3 cells, we needed to find another mouse cell line with ''normal'' levels of gp210 as a reference. For this, we compared the levels of gp210 in NIH/3T3 cells with N2a cells by indirect immunofluorescence microscopy and Western blot analysis. Immunostaining using polyclonal anti-gp210 antibodies at concentrations resulting in a strong immunofluorescence at the nuclear rim in

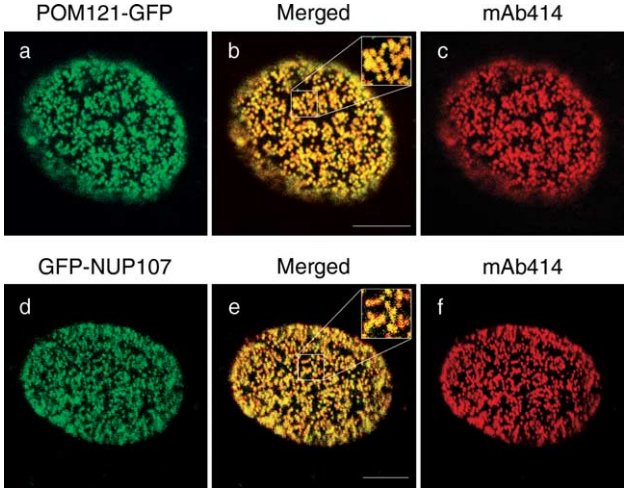

Fig. 2. Gp210 deficient cells display normal distribution of NPC proteins. High resolution confocal laser scanning fluorescence micrographic images of fixed and permeabilized NIH/3T3 cells (a–f) expressing POM121-GFP_3 or GFP_3-NUP107. Images show distribution of the fluorescence from POM121-GFP_3 (a), GFP_3-NUP107 (d) and immunostaining using antibodies against mAb414 (c and f). The fluorescence from POM121-GFP_3 and GFP_3-NUP107 shows perfect colocalization with the mAb 414 staining on the lower nuclear surface (b and e, respectively). Enlargement of a region of the nuclear surface (inset in overlay b and e). Scale bar: 5 μm.

N2a cells did not give rise to a detectable signal in NIH/3T3 cells (Fig. 1A). However, equally strong signals using mAb414 (recognizing a group of NUPs containing FG-repeats) were obtained in both cell types.

Western blot analysis of total proteins of N2a cells gave rise to a strong 200 kDa band, corresponding to gp210 (Fig. 1B). In contrast, gp210 was undetectable in total cell extracts of NIH/3T3 cells, although FG-repeat containing NUPs reacting with mAb414 were present in both cell types. Even after enhancing the blots (resulting in overloading of the gp210 signal in N2a cells), the NIH/3T3 cells only displayed a faint band with a slightly lower mobility (Fig. 1C), probably representing unspecific background. In order to define an upper limit of gp210 in NIH/3T3 cells, we measured its abundance relative to the gp210 signal in N2a cells after serial dilution (Fig. 1C). We conclude that NIH/3T3 cells contain at least 100-fold lower

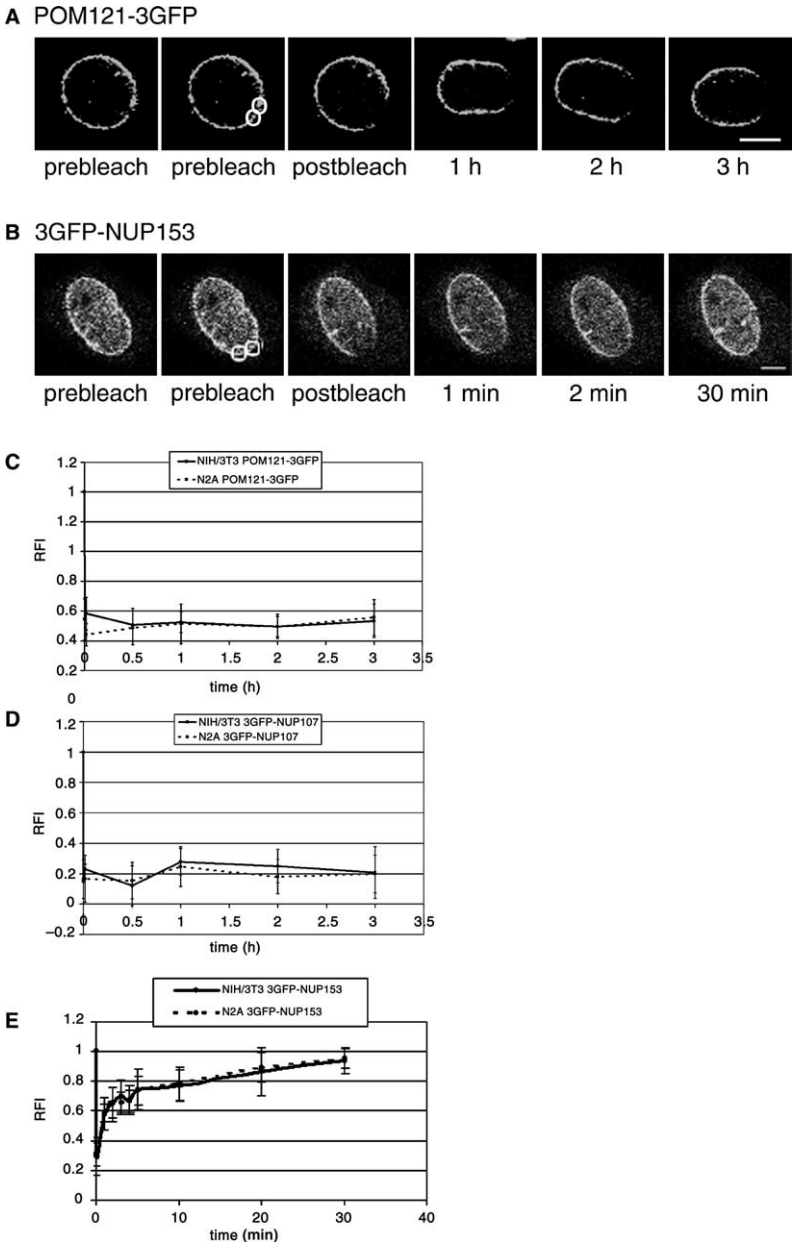

Fig. 3. The lack of gp210 does not alter the dynamic properties of nuclear pores of NIH/3T3 cells. (A and B) FRAP experiments on NIH/3T3 cell expressing POM121-GFP_3 and GFP_3-NUP153, respectively. Two adjacent regions at the nuclear rim (white circles) were bleached for 1 s each at full laser power. Images were acquired before bleaching (pre-bleach), immediately after bleaching (post-bleach) and at different time points after bleaching. Scale bar: 5 μm. (C) Quantitative FRAP analyses of POM121-GFP_3. RFI values ± S.D. for N2a cells ($n = 7$, dashed line) and NIH/3T3 cells ($n = 4$, solid line) were plotted versus time. (D) Quantitative FRAP analysis of GFP_3-NUP107. RFI values ± S.D. for N2a cells ($n = 6$, dashed line) and NIH/3T3 cells ($n = 4$, solid line) were plotted versus time. (E) Quantitative FRAP analysis of GFP_3-NUP153. RFI values ± S.D. for N2a cells ($n = 5$, dashed line) and NIH/3T3 cells ($n = 5$, solid line) were plotted versus time. There was no difference in dynamic properties of POM121-GFP_3 (C), GFP_3-NUP107 (D) or GFP_3-NUP153 (E) between N2a and NIH/3T3 cells.

concentration than N2a cells, corresponding to less than one molecule of gp210 per six NPCs [4]. This is consistent with the results from Ekblom and co-workers [8].

In order to investigate if the lack of gp210 in NIH/3T3 cells had any effects on the distribution of nuclear pores or nuclear pore proteins we analyzed the distribution of an integral membrane protein marker for the pore membrane (POM121-GFP_3) by CLSM. POM121-GFP_3 (Figs. 2a–c) distributed in a characteristic punctate pattern over the nuclear surface, perfectly colocalizing with immunostaining using mAb414 antibodies, showing that POM121 was correctly targeted to the nuclear pores in NIH/3T3 cells. A similar distribution of GFP fluorescence was obtained in cells expressing GFP_3-NUP107, a marker of the NUP107/160 subcomplex (Figs. 2d–f) and GFP_3-NUP153, located on the nucleoplasmic aspect of the NPC (Fig. 3B), showing that also NUP107 and NUP153 were correctly targeted to the NPCs in spite of the lack of gp210.

The density of NPCs in the NE was normal in both cell lines (NIH/3T3, 3.4 ± 0.1 NPCs/μm^2, $n = 5$; N2a, 3.0 ± 0.2 NPCs/μm^2, $n = 5$) consistent with NPC density in NRK cells [15]. We did not observe abnormal patterns of NPC distribution or formation of annulate lamellae. Furthermore, we did not observe any differences in cell cycle progression or generation time (NIH/3T3, 16.2 h; N2a, 16.4 h) between the two cell types. Our results indicate that gp210 cannot be required for targeting of POM121, NUP107 or the majority of FG-repeat containing peripheral NUPs including NUP153. Also, the lack of gp210 did not seem to limit the normal growth of NIH/3T3 cells, change NPC distribution or otherwise interfere with biogenesis of normal nuclear membranes.

Even if gp210 did not seem to be required for incorporation of nuclear pore proteins and formation of nuclear pores in NIH/3T3 cells, it is possible that gp210 may play a role in stabilizing the NPC. To compare dynamic properties of NPCs in NIH/3T3 and N2a cells, we performed FRAP experiments on cells expressing POM121-GFP_3 or GFP_3-NUP107, two of the most stable components of the NPC. The recovery of fluorescence in bleached regions of the nuclear membranes of NIH/3T3 or N2a cells expressing POM121-GFP_3 or GFP_3-NUP107 was extremely slow and did not exceed 20% 3 h after bleaching (Figs. 3C and D). Even after 12 h, recovery was not different between the two cell lines and did not exceed 40% (not shown). The data from N2a cells are consistent with previous investigations in NRK cells displaying a halftime of $\approx$20 and $\approx$16 h for POM121 [15] and NUP107 [14], respectively. There were no significant differences in dynamic properties of POM121-GFP_3 and GFP_3-NUP107 between NIH/3T3 and N2a cells, indicating that gp210 is not required for NPC stability. We also conclude that the NPCs remain immobile in the NE in the absence of gp210, since NPCs from the adjacent unbleached nuclear membrane did not migrate into the bleached region of NIH/3T3 cells after 3 h (Fig. 3A). As a reference to the dynamically stable NPC proteins we also performed FRAP analysis on the dynamically associated nucleoporin NUP153 (Fig. 3E), having a reported half time of recovery ($t_{1/2}$) of 15 s [15]. The $t_{1/2}$ for GFP_3-NUP153 was 60 s in NIH/3T3 cells and 70 s in N2a cells, suggesting that gp210 had no effect on the dynamic properties of NUP153.

Gp210 has been suggested to play a central role in nuclear pore biogenesis [6] and several different investigations have pointed in this direction. For instance, gp210 has been shown to be able to form multimers [19] and has a non-membrane spanning hydrophobic stretch of amino acids with potential fusiogenic properties [20]. Targeting of gp210 depends on its transmembrane segment and its C-terminal tail [21], suggesting that these domains mediate oligomerization of interactions with other NUPs. Furthermore, in *Xenopus* assembly reactions in vitro, antibodies directed against the C-terminal tail of gp210 inhibited NPC formation and led to formation of structures potentially representing fusion arrested pores [9]. In agreement, RNAi experiments suggested that gp210 in HeLa cells and *Caenorhabditis elegans* is essential for viability and that absence of gp210 led to similarly perturbed nuclear membranes as in *Xenopus* assembly extracts [10]. However, the latter study was contradicted by a later investigation [22], reporting 100% viable *C. elegans* embryos displaying wt phenotype. Recently, Olsson et al. [8] reported the absence of gp210 from several developing mouse tissues and actively dividing cell lines further challenging the proposed role of gp210 in nuclear pore biogenesis. Other circumstances speaking against a central role of gp210 in pore complex assembly are the fact that gp210 is recruited very late (in telophase or G1) during post-mitotic nuclear membrane formation [23], whereas many other nuclear membrane proteins including POM121 [15] and LAP2 [23] are recruited as early as in anaphase.

Our data presented in this study rule out any requirement for gp210 in assembly of the NPC core structure, NPC stability or immobilization in the nuclear envelope, since these function would be impossible to carry out at sub-stoichiometric concentrations. Although concentrations differing by a factor of at least 100-fold had no effect on generation time or cell cycle progression, our data cannot exclude the possibility that gp210 is involved in the membrane fusion event during pore formation. Such a function could in principle be carried out by a "hit-and-run" mechanism and does not require a continuous presence at the NPC. It is clear that extensive further studies are needed in order to elucidate the exact function of gp210.

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