

Early localization of NPA58, a rat nuclear pore-associated protein, to the reforming nuclear envelope during mitosis

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We have studied the mitotic reassembly of the nuclear envelope, using antibodies to nuclear marker proteins and NPA58 in F-111 rat fibroblast cells. In earlier studies we have proposed that NPA58, a 58 kDa rat nuclear protein, is involved in nuclear protein import. In this report, NPA58 is shown to be localized on the cytoplasmic face of the envelope in interphase cells, in close association with nuclear pores. In mitotic cells NPA58 is dispersed in the cytoplasm till anaphase. The targeting of NPA58 to the reforming nuclear envelope in early telophase coincides with the recruitment of a well-characterized class of nuclear pore proteins recognized by the antibody mAb 414, and occurs prior to the incorporation of lamin B1 into the envelope. Significant protein import activity is detectable only after localization of NPA58 in the newly-formed envelope. The early targeting of NPA58 is consistent with its proposed role in nuclear transport.

1. Introduction

The nuclear envelope forms an interactive boundary between the nuclear and cytoplasmic compartments in an eukaryotic cell. The envelope consists of a double membrane: the outer nuclear membrane is contiguous with the endoplasmic reticulum whereas the inner nuclear membrane is associated with the underlying nuclear lamina formed by a filamentous meshwork of proteins called lamins. Molecular trafficking across the nuclear envelope is regulated by large supramolecular aqueous channels termed the nuclear pore complexes (NPCs) that are embedded in the double membrane at intervals (reviewed by Gerace and Burke 1988; Görlich and Mattaj 1996; Nigg 1997; Nakielny and Dreyfuss 1999; Görlich and Kutay 1999).

In higher eukaryotes that undergo open mitosis, the nuclear envelope and its components including the NPCs are disassembled at the onset of mitosis at the end of prophase and subsequently reassembled in telophase in a stepwise manner to form an intact nuclear envelope competent for transport (Gant and Wilson 1997). These processes are believed to be regulated by mitotic phosphorylation and dephosphorylation cycles, the best-studied

example of which is the depolymerization and reformation of the nuclear lamina. The nuclear lamina is disassembled into soluble A-type and vesicle-associated B-type lamins in response to mitotically activated kinases, chiefly the cyclin B-dependent p34^{cdc2} kinase (Heald and McKeon 1990; Peter *et al* 1990). In addition the integral membrane proteins of the inner nuclear membrane such as the lamin associated polypeptides (LAPs) and lamin B receptor (LBR) also undergo mitotic phosphorylation (Foisner and Gerace 1993). During mitosis in mammalian somatic cells the integral membrane proteins are observed to be dispersed within the peripheral network of the endoplasmic reticulum (ER) which remains connected to the outer nuclear membrane (Yang *et al* 1997). The NPCs have been proposed to exist in soluble subcomplexes within the cytoplasm of the mitotic cells (Macaulay *et al* 1995). Towards the close of mitosis, the individual components are targeted sequentially to the chromatin and reassemble in steps that are not fully elucidated yet. Reassembly of the nuclear envelope is proposed to be initiated by binding and fusion of nuclear vesicles containing integral membrane proteins, followed by sequential recruitment of nucleoporins that form functional nuclear pores and finally by the assembly of an intact lamina (Gant and

Keywords. Mitotic reassembly; nuclear envelope assembly; nuclear pore complex

Abbreviations used: NPC, Nuclear pore complex; LAP, lamin associated polypeptide; LBR, lamin B receptor; ER, endoplasmic reticulum; DMEM, Dulbecco's minimum essential medium; DAPI, 4,6-diamidino-2-phenylindole.

Wilson 1997) and appears to require the activity of protein phosphatase 1 (Steen *et al* 2000).

A consensus model for the vertebrate NPC based on extensive electron microscopic (EM) analysis suggests that it is a tripartite assembly consisting of multisubunit rings on its cytoplasmic and nucleoplasmic faces connected to a central framework of eight radial multi-domain spokes, which together embrace a central transporter (Akey and Radermacher 1993). In addition to this core structure which has dimensions of $\sim 120 \times 80$ nm, NPCs possess peripheral extensions that project 50–100 nm into the cytoplasm and the nuclear interior, which are proposed to function as docking sites for cargo in transit through the pores. The mass of the vertebrate NPC is estimated to be about 125×10^6 Da and it is likely to be composed of multiple copies of at least 50 different polypeptides, collectively known as nucleoporins (Nups), of which less than 20 have been identified and localized within the NPC structure so far (Panté and Aebi 1995; Urt 1997).

We have recently identified a 58 kDa protein termed NPA58 that is phosphorylated at the onset of mitosis and dephosphorylated at the close of mitosis in F-111 rat fibroblast cells, using a monoclonal antibody mAb E2 (Ganeshan and Parnaik 2000). We have earlier shown that mAb E2 could specifically inhibit the signal-mediated import of nuclear proteins in transport assays performed with digitonin-permeabilized F-111 cells, and the antibody labelled the nuclear periphery in a punctate pattern characteristic of NPCs (Pandey *et al* 1994). Hence NPA58 may be involved in nuclear protein import. In the present study we have initially localized NPA58 to the cytoplasmic face of the envelope in close association with the nuclear pores. Subsequently, we have examined the mitotic distribution of NPA58 and compared its relocalization within the newly-formed nuclear envelope with respect to lamin B1 and NPC proteins recognized by mAb 414. MAb 414 recognises a family of related nucleoporins of sizes 62 kDa, 153 kDa, 214 kDa and 358 kDa (Davis and Blobel 1986; Favreau *et al* 1996) that have been well-characterized. Our results show that the localization of NPA58 to the nascent nuclear envelope overlaps with that of the NPC proteins recognized by mAb 414 and it is targeted earlier than lamin B1, and before significant import activity is detected.

2. Materials and methods

2.1 Cell culture and antibodies

F-111 rat lung fibroblast cells were routinely maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum in an atmosphere of 5% CO₂. Mouse mAb E2 has been described previously (Pandey *et al* 1994). Mouse mAb LA-2B3 to recombinant

rat lamin A and LB-P, a rabbit polyclonal antibody to recombinant rat lamin B1, have been described earlier (Jagatheesan *et al* 1999). A rabbit polyclonal antibody to U5-116 kDa (Fabrizio *et al* 1997) was a gift from Dr R Lüthmann (University of Marburg, Germany). A mouse monoclonal antibody to PCNA (PC10) a protein associated with DNA replication centres during S phase (Kill *et al* 1991) was obtained from Santa Cruz Biotech (USA). A rat monoclonal antibody to tubulin (YOL1) was obtained from Serotec (USA). MAb 414, a mouse monoclonal antibody, which recognizes a class of NPC proteins (Davis and Blobel 1986), was obtained from Berkeley Antibody Co. (USA). All antibodies were used at the recommended dilutions for immunofluorescence assays. MAb 414 was biotinylated by coupling with N-hydroxy-succinimidobiotin (Sigma Chemical Co., USA) in 0.1 M sodium borate buffer, pH 8.8, using standard procedures.

2.2 Immunofluorescence microscopy

F-111 cells were grown on coverslips to about 70% confluency prior to fixing. The cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.5% Triton X-100 for 5 min at room temperature for labelling with mAb E2, mAb 414, and antibodies to U5-116 kDa and tubulin. Alternatively cells were also fixed with methanol at -20°C for 10 min for staining with LA-2B3 and PC10, or with methanol:acetone (2 : 1 v/v) at 4°C for 15 min for labelling with LB-P. Further processing was carried out at room temperature. Cells were blocked with 3% BSA for 1–2 h (or with 0.5% gelatin for 1–2 h for LA-2B3), incubated with the primary antibody for 1–2 h, and then secondary antibody conjugated to FITC or alexa-488 for 1 h. For double labelling experiments, cells were fixed, blocked and incubated serially with mAb E2 and alexa-488-conjugated anti-mouse antibody and then with the primary antibody to tubulin or U5-116 kDa, followed by biotinylated secondary antibody and avidin-Cy3. Double labelling studies with mAb E2 and mAb 414 were done by incubating cells with mAb E2, followed by alexa 488-conjugated anti-mouse antibody, and then with biotinylated mAb 414 followed by avidin-Cy3. In a control experiment, when fixed F-111 cells were incubated with biotinylated mAb 414 followed by alexa-488-conjugated anti-mouse antibody no signal was obtained, indicating that the biotinylated antibody was unable to bind to the anti-mouse antibody, and excluding the possibility that free antigen-binding sites in the anti-mouse antibody could bind to biotinylated mAb 414 to give false colocalization. Secondary antibody conjugates were from Molecular Probes (USA), Jackson Immunoresearch Laboratories (USA) and Vector Laboratories (USA) and used at the recommended dilutions. Coverslips were mounted in Vectashield (Vector Laboratories, USA) containing

1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for DNA staining. All samples were routinely viewed under phase contrast also. Cells were permeabilized with digitonin by standard procedures (Adam *et al* 1990). Briefly coverslips containing cells were rinsed thrice in ice-cold buffer (50 mM HEPES pH 7.3, 110 mM K-acetate pH 7.5, 5 mM Na-acetate pH 7.0, 2 mM Mg-acetate, 1 mM EGTA, 2 mM DTT) and permeabilized with 40 µg/ml digitonin (Molecular Probes, USA) on ice for 10 min. Cells were rinsed gently and then incubated with antibodies as described above (but at 4°C). Confocal laser-scanning immunofluorescence microscopy (CLSM) was carried out on a Meridian Ultima scan head attached to an Olympus IMT-2 inverted microscope fitted with a 60X, 1.4 NA objective lens, with excitation at 515, 488, and 351–364 nm (Argon-ion laser). The 351–364 nm laser was used for excitation of DAPI, with a 485/40 band pass filter for detection. The 488 nm laser was used for excitation of dye conjugates, with a 560 nm short pass dichroic, a 530/30 band pass filter for detection of FITC and alexa-488, and a 580/30 band pass filter for the detection of Cy3. In double labelling experiments, the percentage crossover for each dye was calculated automatically after scanning singly labelled specimens under identical settings using Ultima master program V 4.15, and corrected for during image analysis. Corrected images of 0.5 µ or 0.2 µ optical sections are displayed. For quantitative analysis of colocalization, the data from individual sections (0.2 µ thickness) was queried by test lines through the nuclear periphery and fluorescence intensities for both dyes were viewed graphically. The number of overlapping and non-overlapping peaks were estimated for seven hundred peaks for each sample and per cent colocalized peaks were calculated. Images were analysed using DASY master program V 4.19 (Meridian Instruments Inc., USA.) and assembled using Adobe Photoshop 5.0.

3. Results

3.1 Association of NPA58 with the nuclear pore complex

The vertebrate nucleoporins that have been identified so far exhibit characteristic punctate staining of the periphery of the nucleus due to the perforation of the nuclear membranes at intervals by the NPCs. This pattern is typically observed in cells immunostained with mAb 414 (Davis and Blobel 1986; Kubitscheck *et al* 1996). We initially confirmed that mAb E2 labelled formaldehyde-fixed F-111 cells in a punctate pattern exclusively at the nuclear periphery by confocal microscopy (figure 1A–C). Further, we carried out confocal imaging of dual-labelled cells to study the localization of NPA58 with respect to

known pore proteins. In order to preserve the nuclear architecture and avoid fixation artifacts, unfixed F-111 cells were permeabilized with 0.004% digitonin. This treatment permeabilizes the plasma membrane, leaving the nuclear membrane intact (Adam *et al* 1990). Hence nuclear antigens facing the cytoplasm would be accessible to exogenously added antibodies, whereas proteins localized along the inner surface of the nucleus or within the nucleus would be inaccessible. Thus in the control experiments presented in figure 1H–K, when digitonin-permeabilized cells were stained with antibodies to lamin B1, which is associated with the inner nuclear membrane, and U5-116 kDa, a soluble nucleoplasmic protein, there was no labelling by either antibody. This confirmed that the digitonin treatment had not damaged the nuclear membrane and it was inaccessible to antibodies recognizing proteins on the nucleoplasmic face. Only further permeabilization of the nuclei with methanol produced the typical peripheral staining of lamin B1 and intranuclear staining of U5-116 kDa. Studies were then performed with digitonin-permeabilized cells that were immunostained with both mAb E2 and mAb 414. Since both the antibodies were of mouse origin, mAb 414 was biotinylated and detected by using an avidin-Cy3 conjugate in order to distinguish it from mAb E2, which was detected using anti-mouse alexa-488 conjugate. As seen in figure 1D and E, both mAb E2 and mAb 414 stained the nuclear membrane on the cytoplasmic face in a punctate pattern, with a high degree of coincident fluorescence as evident from the merged image (figure 1F) where the overlap of the fluorophores produced a yellow colour. The appreciable extent of overlap between the two signals which was calculated to be ~75% suggests that NPA58 is localized close to the nuclear pores. The nucleoporins recognized by mAb 414 have been shown to be situated near the centre of the NPC (p62) and in the cytoplasmic filaments (Nup214 and Nup358) or in the nuclear basket (Nup153) (Panté and Aebi 1995). In earlier biochemical studies, NPA58 was observed to fractionate with pore proteins and was not extracted with detergent alone, thus excluding the possibility that NPA58 might be associated with the outer nuclear membrane (Pandey *et al* 1994). These observations taken together provide a strong basis for proposing that NPA58 is closely associated with the NPCs on the cytoplasmic face of the nuclear envelope.

3.2 Localization of NPA58 during mitosis

In order to clearly demarcate the different mitotic stages in F-111 cells the localization of NPA58 was examined by immunolabelling F-111 cells with mAb E2 and antibodies to tubulin and staining for DNA with DAPI. The fragmentation of the nuclear envelope is initiated towards the end of prophase of mitosis after the chromatin begins to

condense and the spindle asters are clearly visible (figure 2B, D). At this stage the nuclear rim staining of NPA58 (observed in interphase cells) was disrupted, with the appearance of cytoplasmic labelling (figure 2A). The NPA58 labelling was also observed in regions of the nuclear envelope that were partially disrupted. By metaphase the distribution of NPA58 in these cells was entirely cytoplasmic and completely excluded from the mitotic apparatus (figure 2E–H) unlike the localization of PBC68, a pore protein which associates reversibly with the mitotic spindle (Theodoropoulos *et al* 1999). As cells entered anaphase, the paired chromosomes began separating and the spindle poles also moved apart. The localization of NPA58 remained cytoplasmic during anaphase and

there was little or no association with the spindle fibres or the chromosomes (figure 2I–L). By telophase, the daughter chromosomes were well separated, while the spindle fibres stretched to the poles. At this stage there was a substantial concentration of NPA58 at the periphery of the reformed nuclear envelope (figure 2M–P). The topology of the nascent nuclear envelope was generally uneven in comparison with that observed in interphase nuclei. The nuclear envelope formation had progressed by cytokinesis, where the chromatin had decondensed in the daughter nuclei and the spindle was reduced to a midbody that persisted between the cells with the nuclei appearing more rounded, and NPA58 was completely localized at the nuclear periphery (figure 2Q–T).

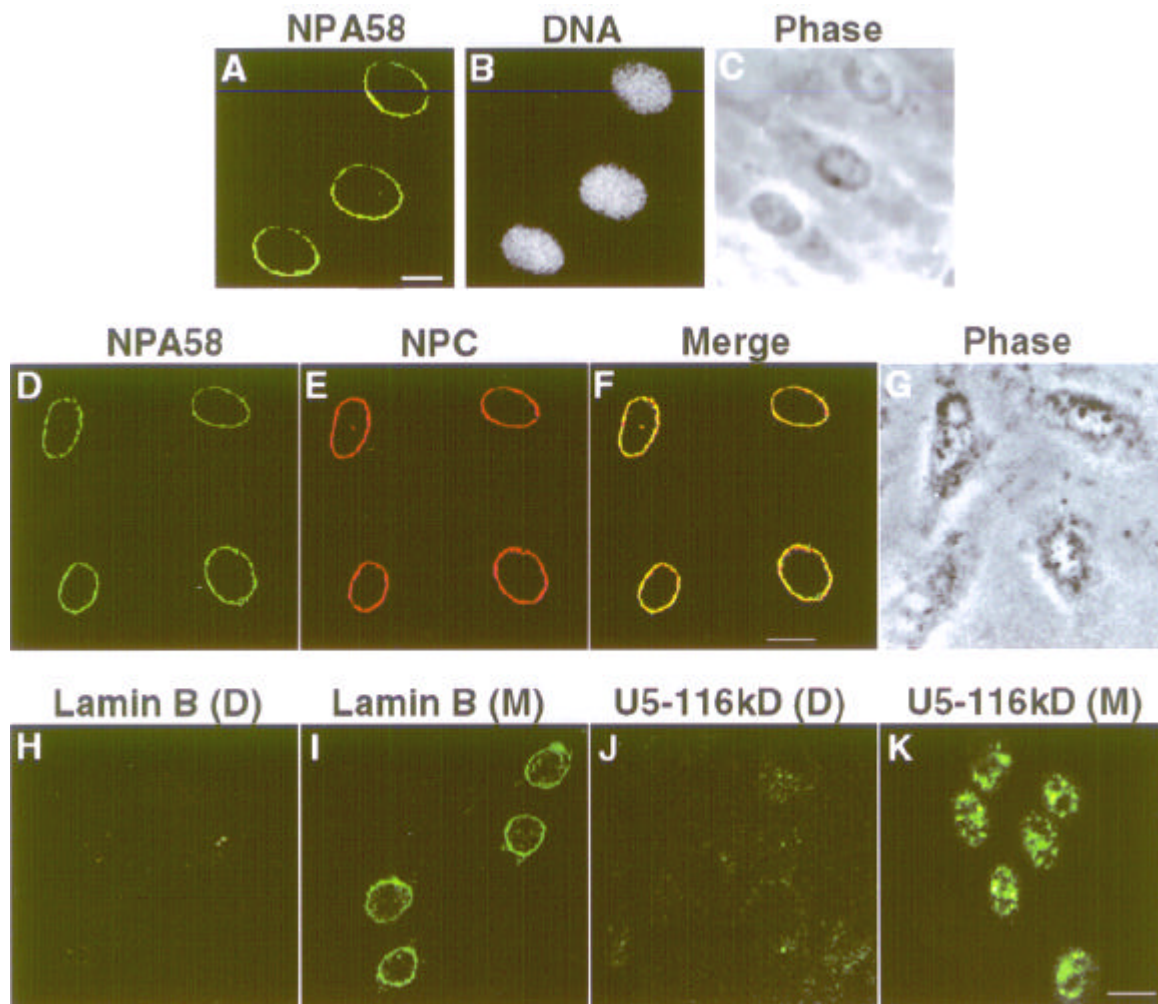


Figure 1. Immunolocalization of NPA58 and mAb 414-reactive NPC proteins in F-111 cells. (A–C) F-111 cells stained with mAb E2 (NPA58) and DAPI (DNA) after fixing with formaldehyde. (D–F) F-111 cells were permeabilized by digitonin treatment and immunostained with mAb E2 (NPA58) and mAb 414 (NPC) and colocalization of the antigens in a single optical section of 0.2μ is indicated (Merge). (G) Phase contrast picture of the digitonin-treated cells. (H–K) Control immunostaining reactions with antibodies to lamin B1 and U5-116 kDa in cells permeabilised with digitonin alone (H and J) or further permeabilized with methanol (I and K). Bar, 10μ m.

3.3 Localization of nuclear marker proteins in mitotic cells

We subsequently used antibodies to lamin A and lamin B1 to mark the temporal differences of their incorporation into the nuclear envelope during the progress of mitosis. In addition, antibodies against PCNA and U5-116 kDa were also used to localize these proteins during mitosis and study their import into the nucleus following formation of the nuclear envelope. As seen from figure 3, in interphase cells the nuclear lamins were localized at the periphery of the nucleus as a smooth rim. The nuclear lamina was depolymerized at the beginning of mitosis and the lamin proteins were dispersed in the cytoplasm of the prometaphase and metaphase cells. The cytoplasmic staining persisted in anaphase but by late telophase lamin B1 was targeted to the nuclear envelope and appeared at the nuclear rim while lamin A was still cytoplasmic. The lamin A proteins did not relocalize to the nucleus till cytokinesis was complete. This data is consistent with earlier reports where lamin B1 has been observed to reassociate with the nuclei in telophase and lamin A protein is

imported and incorporated into the lamina as a late event (Newport *et al* 1990; Chaudhary and Courvalin 1993; Meier and Georgatos 1994). During interphase PCNA, which is an important factor for regulating DNA replication, was mostly localized within the nucleus and could be visualized as large speckles in S-phase nuclei as reported previously (Kill *et al* 1991). U5-116 kDa is a splicing

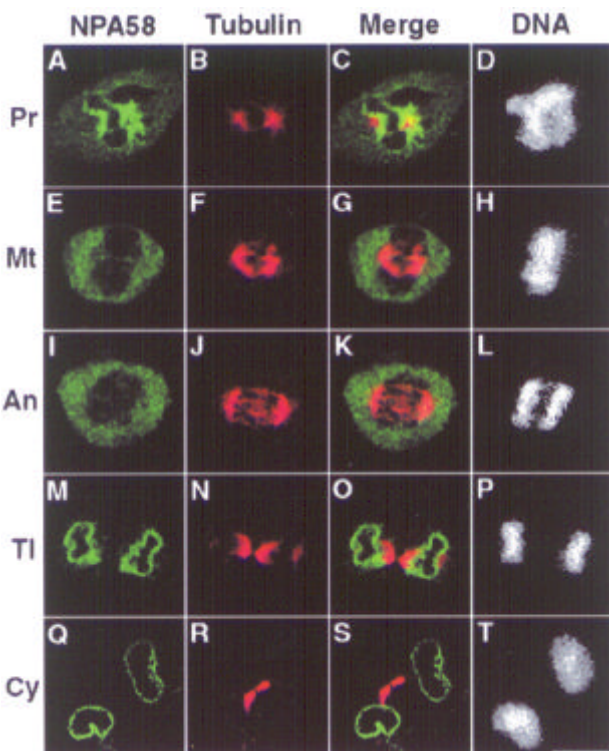


Figure 2. Distribution of NPA58 and tubulin during mitosis. Formaldehyde-fixed F-111 cells were immunolabelled with antibodies to NPA58 and tubulin and counterstained for DNA. Cells in different stages of mitosis were analysed. (A–D) Late prophase/early prometaphase, Pr; (E–H) metaphase, Mt; (I–L) anaphase, An; (M–P) telophase, TI; (Q–T) cytokinesis, Cy. Bar, 10 μ M.

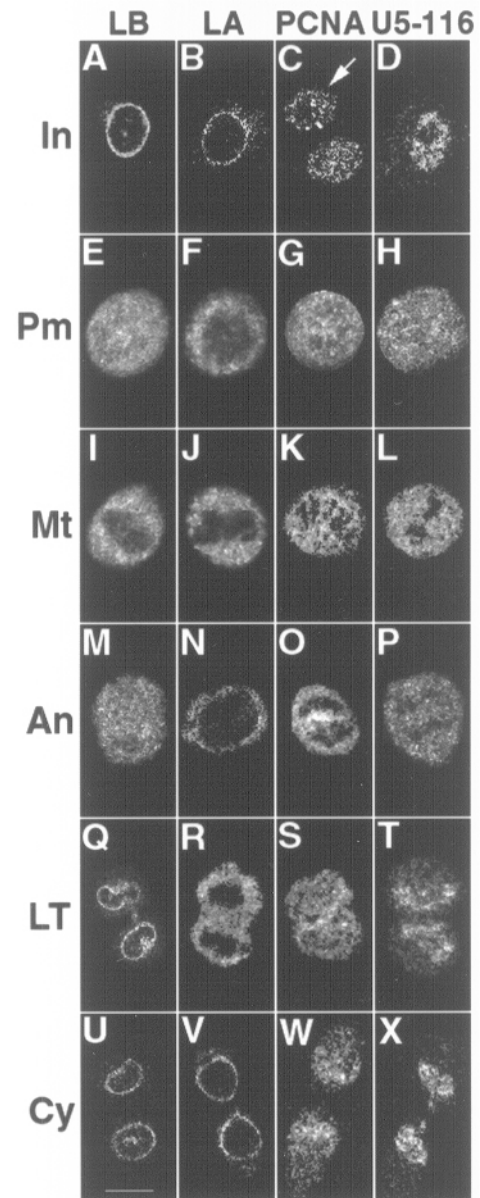


Figure 3. Differential localization of nuclear marker proteins during mitosis. F-111 cells were fixed and immunostained with antibodies to lamin B1 (LB), lamin A (LA), PCNA and U5-116 kDa (U5-116) to study their distribution at different stages of mitosis. (A–D) Interphase, In; (E–H) prometaphase, Pm; (I–L) metaphase, Mt; (M–P) anaphase, An; (Q–T) late telophase, LT; (U–X) cytokinesis, Cy. Arrow in C indicates cell in S-phase. Bar, 10 μ M.

factor associated with U5 snRNPs and is localized in speckles and perichromatin fibrils within the nucleus (Fabrizio *et al* 1997). As cells enter mitosis, replication and transcriptional events are downregulated and these proteins became dispersed in the cytoplasm of the cell and remained in the cytoplasm through the early phases of mitosis. But by telophase U5-116 kDa was localized in the nucleus while PCNA remained in the cytoplasm and appeared in the nucleus only at the end of cytokinesis. These observations suggested that the nuclear localization of U5-116 kDa occurred prior to PCNA and would there-

fore be a useful marker to study the competence of the newly-formed nuclear envelope for transport during nuclear assembly following mitosis.

3.4 Immunolocalization of NPA58, lamin B1 and NPC proteins in mitotic cells

From the experiment described in the previous section it was observed that there was a temporal difference in the appearance of lamin B1 and lamin A at the nuclear

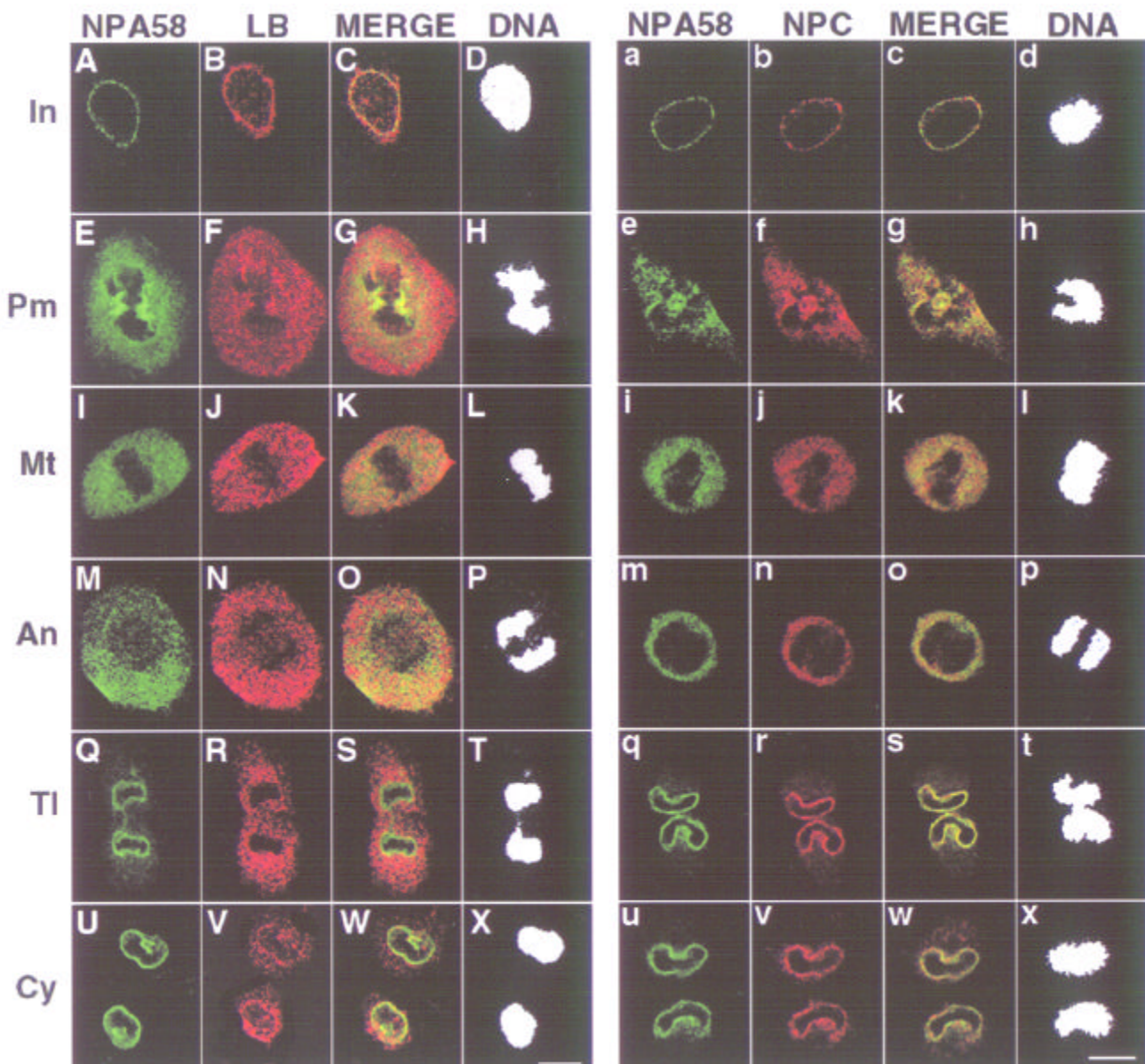


Figure 4. Distribution of NPA58, lamin B1 and NPC proteins during mitosis. Formaldehyde-fixed F-111 cells were stained with antibodies to NPA58 and lamin B1 (LB) or NPA58 and mAb 414-reactive NPC proteins (NPC) and counterstained for DNA to examine their distribution in various stages of mitosis. (A–D, a–d) Interphase, In; (E–H, e–h) prometaphase, Pm; (I–L, i–l) metaphase, Mt; (M–P, m–p) anaphase, An; (Q–T, q–t) telophase, Tl; (U–X, u–x) cytokinesis, Cy. Bar 10 μ M.

periphery during mitosis. The comparatively early association of lamin B1 with the reforming nuclear envelope made it a suitable marker to examine the assembly of the nuclear lamina during the progress of mitosis. We carried out dual labelling experiments with F-111 cells using antibodies to lamin B1 and NPA58 to examine the sequence in which the two proteins associated with the nascent envelope, as shown in figure 4A–X. The peripheral localization of NPA58 and lamin B1 in the interphase cells was disrupted in late prophase. There was progressive increase in the cytoplasmic localization of the two proteins at metaphase that persisted in anaphase. By telophase NPA58 was associated with the nascent nuclear envelope. At this stage although lamin B1 was also largely localized at the periphery of the nucleus, some of the protein was still visible in the cytoplasm and was targeted to the reforming envelope during late telophase and cytokinesis.

Further, in order to examine the localization of NPA58 with respect to NPC proteins during mitosis we performed dual labelling immunofluorescence assays with mAb E2 and mAb 414, as illustrated in figure 4a–x. During interphase both the antibodies stained the intact nuclear periphery in a punctate manner. By prometaphase both NPA58 as well as the mAb 414-reactive NPC proteins were partly dissociated from the nuclear envelope and localized largely in the cytoplasm. The cytoplasmic staining was observed in all the early stages of mitosis till anaphase, and there was no appearance of either protein in association with the chromosomes till this stage. However, by telophase, the NPC proteins as well as NPA58 were completely incorporated within the nuclear envelope as seen in figure 4q–t. There is thus a distinct overlap between the relocalization of NPA58 and mAb 414-reactive NPC proteins, which corroborates the evidence for a close association of NPA58 with the nuclear pores.

In further experiments, F-111 cells were synchronized in metaphase by treatment with nocodazole to enrich for mitotic cells (Jordan and Wilson 1999), and the immunolocalization of a soluble nuclear protein was examined by doubly staining the cells with mAb E2 and antibodies to the intranuclear protein, U5-116 kDa as the cells progressed out of mitosis. As illustrated in figure 5, U5-116 kDa was largely cytoplasmic in its distribution in metaphase-arrested cells where NPA58 was also dispersed in the cytoplasm. As the cells progressed towards late anaphase or early telophase, NPA58 began associating with the chromatin, but U5-116 kDa remained cytoplasmic. However, in cells that had entered telophase, NPA58 was completely associated with the nuclear envelope whereas U5-116 kDa appeared concentrated around the nuclear region. U5-116 kDa was mostly localized inside the nucleus (figure 5N, O) towards the later stages of mitosis. Nuclear localization of U5-116 kDa was obser-

ved in > 90% of the cells scanned in this stage of mitosis, thus confirming the competence of the nuclear envelope for import.

4. Discussion

NPA58 is a 58 kDa rat nuclear pore-associated protein that we have identified recently using a monoclonal antibody mAb E2 (Ganeshan and Parnaik 2000). MAb E2 was earlier shown to block the signal-mediated active transport of nuclear proteins into permeabilized F-111 cells (Pandey *et al* 1994). We have previously demonstrated that NPA58 is phosphorylated in metaphase cells

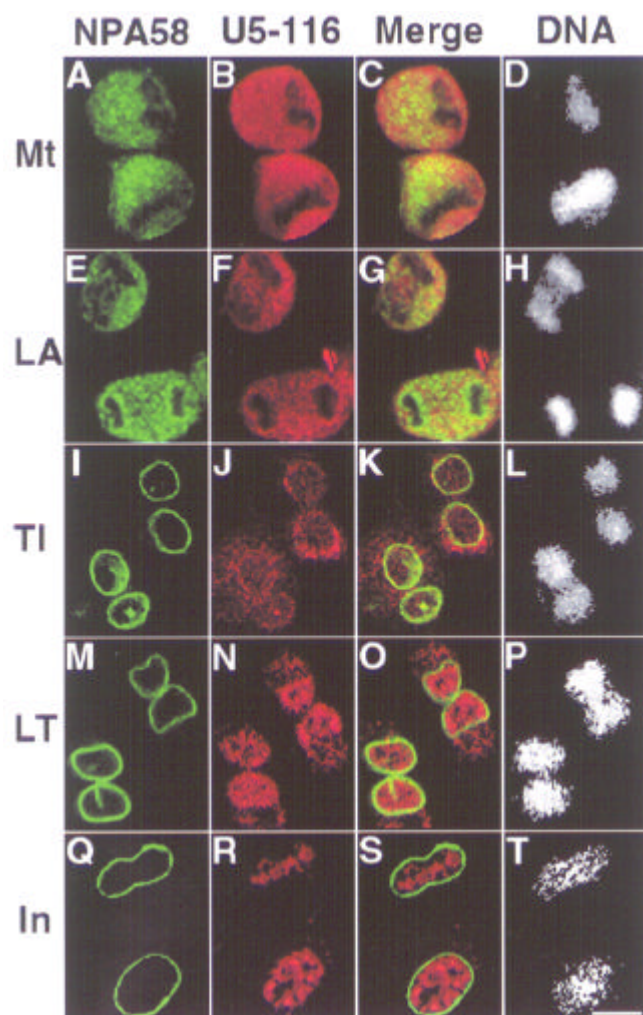


Figure 5. Localization of U5-116 kDa in mitotic cells. F-111 cells arrested in metaphase by treatment with 0.01% nocodazole in the culture medium for 12–14 h were released, fixed over 1–2 h and immunostained with mAb E2 (NPA58) and polyclonal antibodies to U5-116 kDa (U5-116). (A–D) Metaphase, Mt; (E–H) late anaphase, LA; (I–L) telophase, TI; (M–P) late telophase, LT; (Q–T) interphase, In. Bar, 10 μ m.

when the nuclear envelope and NPCs are disassembled and dephosphorylated in early telophase when NPCs and the envelope reassemble, indicating that phosphorylation/dephosphorylation of the protein are important for its association with the envelope (Ganeshan and Parnaik 2000). In the present study we have localized NPA58 to the cytoplasmic face of the envelope, thereby excluding the possibility that it is an inner membrane protein, and provided evidence for its significant colocalization with bona fide NPC proteins.

The assembly of the nuclear envelope starts at late anaphase or very early telophase just after the contractile ring pinches the plasma membrane, and incorporation of inner membrane proteins such as the LAPs, LBR and emerin into the reforming envelope is one of the earliest events in membrane targeting to the chromosomes (Ellenberg *et al* 1997; Chaudhary and Courvalin 1993; Buendia and Courvalin 1997; Yang *et al* 1997; Haraguchi *et al* 2000). Several components of the NPCs such as p62, RanBP2, Nup153, POM121 and Nup214 associate with the reforming envelope concomitantly with the inner membrane proteins, though other NPC proteins such as Tpr270 which is in the nuclear basket, and gp210 a membrane-anchoring protein are recruited to the envelope in late telophase (Bodoor *et al* 1999; Haraguchi *et al* 2000). We have observed that NPA58 associates with the reforming envelope in early telophase overlapping with the relocation of mAb 414-reactive proteins (which include p62, Nup153 and Nup214). Consistent with earlier findings that the lamina is mostly assembled after formation of functional nuclear pores (Gant and Wilson 1997) lamin B1 is localized in the envelope subsequent to NPA58. Haraguchi *et al* (2000) have recently determined the precise timing of nuclear events in telophase and shown that nuclear import activity begins just after the assembly of most NPC components. Their data supports the view that nuclear import function is required for further nuclear organization (Benavente *et al* 1989). Our results on the early recruitment of NPA58 to the reforming nuclear envelope prior to detection of nuclear protein import is consistent with these findings and strengthens our premise that NPA58 is an important constituent associated with functional nuclear pores.

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