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PBC68: a nuclear pore complex protein that associates reversibly with the mitotic spindle

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SUMMARY

Using autoimmune antibodies from a patient with primary biliary cirrhosis we have identified a 68 kDa nuclear envelope protein, termed PBC68. This protein is co-precipitated with a 98 kDa and a 250 kDa polypeptide and is distinct from the nuclear lamins. Immunostaining of digitonin-permeabilized cells indicates that PBC68 is restricted to the inner (nucleoplasmic) face of the nuclear envelope, while indirect immunofluorescence and immunoelectron microscopy show that PBC68 is located on fibrillar structures emanating from the nuclear pore complex. The autoantigen is modified at early prophase and disassembles at prometaphase concurrently with the breakdown of the nuclear envelope. The disassembled material, instead of diffusing throughout the cytoplasm as other nucleoporins, is targeted to the mitotic spindle and remains stably bound to it until anaphase. At telophase PBC68 is released from the mitotic apparatus and reassembles late, after incorporation of LAP2B and B-type lamins, onto the reforming nuclear envelope. The partitioning of PBC68 in dividing cells supports the notion that subsets of nuclear envelope proteins are actively sorted during mitosis by transiently anchoring to spindle microtubules. Furthermore, the data suggest that specific constituents of pore complex are released in a stepwise fashion from their anchorage sites before becoming available for nuclear reassembly.

Key words: Nuclear pore complex, Nucleoporin, Mitosis, Primary biliary cirrhosis, Autoantibodies

INTRODUCTION

The nuclear pore complex (NPC) is a eukaryotic device that allows regulated transport of macromolecules across the nuclear envelope. It is a cylindrical, quasisymmetrical organelle with a mass of 125 MDa and an overall diameter of 120 nm (Reichelt et al., 1990; Hinshaw et al., 1992; Akey and Radermacher, 1993). It is embedded in the nuclear membrane and comprises 50-100 different polypeptides (nucleoporins), which occur in multiple copies and form distinct subcomplexes (for a review see Doye and Hurt, 1997). Topographically, the particle can be separated into various 'territories': the cytoplasmic ring and associated cytoplasmic fibrils, the nucleoplasmic ring and basket structure, the spoke complex and the central channel. Each of these components has a unique protein composition and probably serves different functions or structural roles. The cytoplasmic fibrils and nucleoplasmic basket provide transient docking sites for cargo in transit; the central channel seems to contain the machinery for protein or nucleoprotein translocation; finally, the spoke-ring complex constitutes the basic, 'skeletal' framework of the whole assembly (Ris, 1991; Akey and Radermacher, 1993; Pante and Aebi, 1996, Akey, 1995; Ris, 1997).

The structure of the NPC has been analyzed traditionally using the amphibian oocyte as a model. However, more recently, the yeast NPC has been examined in detail, revealing a conserved architectural plan (Yang et al., 1998; reviewed in Gant et al., 1998). Combined biochemical and genetic approaches have yielded a wealth of information concerning the molecular features and cellular locales of the various NPC subunits (for a recent review see Heese-Peck and Raikhel, 1998). In vertebrates, two integral membrane proteins, gp210 and Pom121, link the complex to a specialized domain of the nuclear envelope, the so-called pore membrane. However, an ever increasing number of nucleoporins have been localized at the cytoplasmic fibrils (e.g., Nup214/CAN, Nup358), the nucleoplasmic basket (e.g., Nup153, Nup265/Trp) and the central channel or the spoke-ring complex (e.g., p62 subcomplex). Most of these proteins possess segments of (predicted) coiled-coil structure, GLFG/FXFG peptide motifs, zinc fingers and distinct post-translational modifications, such as O-linked GlcNAc (Pante and Aebi, 1996). Analogous features occur in yeast nucleoporins many of which are now in the process of being localized.

Considering the huge dimensions and multisubunit composition of the NPC, it is remarkable that such a particle is completely disassembled and precisely reformed during mitotic division. Dissolution of the NPC occurs rather abruptly

and coincides with nuclear envelope breakdown at late prophase-prometaphase. At this point, organized NPCs are no longer detected by electron microscopy, while most nucleoporins diffuse in the cytoplasm. Reassembly of NPC occurs after nuclear envelope precursor vesicles and elements of the endoplasmic reticulum establish an initial contact with segregated chromosomes and start forming a double membranous sheath (Robbins and Gonatas, 1964; Zeligs and Wollman, 1979; Davis and Blobel, 1986; Chaudhary and Courvalin, 1993). Recent studies involving an in vitro reconstituted system have revealed distinct structural intermediates during NPC reformation (Goldberg et al., 1997). Building on this model, it would be interesting to investigate the dynamics of individual nucleoporins and reconstruct the exact mechanism of their mitotic remodelling.

Searching for new proteins of the nuclear envelope, we have systematically screened sera from patients with primary biliary cirrhosis (PBC). These patients are known to develop high titre autoantibodies against components of the nuclear envelope (Courvalin et al., 1990a,b,c). One of our sera reacted exclusively with a non-lamin, 68 kDa autoantigen that we have localized at the periphery of the NPC. Unlike other nucleoporins, the NPC-associated autoantigen is transiently anchored to spindle microtubules during early phases of mitosis. Release of this protein from the mitotic apparatus and incorporation to the NPC do not occur until the nuclear lamins and other nucleoporins have reassembled in the reforming nuclear envelope. These data support a stepwise mechanism of NPC reassembly and reveal regulated sorting during mitosis.

MATERIALS AND METHODS

Cell culture and drug treatments

Human endometrial carcinoma cells (Ishikawa) were cultured in MEM containing 10% serum and antibiotics. Treatment with taxol or taxotere involved incubation with 10 nM of each drug for 20 hours at 37°C. The cells were washed free of drugs and cultured for up to 72 hours in normal media. Under these conditions, pore clustering develops a few hours after release from mitotic blockade, i.e., concurrently with entry in G_1 and well before the advent of apoptosis (Theodoropoulos et al., 1999). To depolymerize the microtubules, the cultures were exposed to 33 mM nocodazole for 1 hour and then chilled on ice for 30 minutes.

Cell fractionation

To isolate nuclei and nuclear envelopes, Ishikawa cells were detached from the culture dishes, centrifuged at 300 ***g*** and washed three times with PBS-1 mM PMSF. The pellet was resuspended in an equal volume of ice-cold buffer H (10 mM Hepes-KOH pH 7.4, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and 2 μg/ml of leupeptin, pepstatin, aprotinin) and Dounce-homogenized under careful phase monitoring. The homogenate was centrifuged at 1200 ***g*** for 10 minutes, yielding a pellet (nuclei) and a supernatant (cytoplasmic fraction). The nuclei were washed in buffer H-150 mM NaCl and used immediately or processed further. Nuclear envelopes were prepared as described previously (Dwyer and Blobel, 1976). Synchronized mitotic cells were fractionated as follows. Interphase cultures (60% confluency) were treated for 18 hours with 100 nM taxol and for 2 hours with 10 nM taxotere. This treatment blocks about 90% of the cells in metaphase. The cells were detached from the substratum and permeabilized with 100 μg/ml digitonin in 20 mM Hepes-KOH, pH 7.4, 155 mM NaCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 10% sucrose, 20 mM NaF, 80 mM β-glycerophosphate, 2 mM ATP, 5 μM taxol, 5 μM taxotere, 1 mM PMSF and protease inhibitors (5 minutes, room temperature). After centrifugation, PBC68 was immunoprecipitated either from the supernatant (cytosolic) fraction, or from the solubilized pellet (cytoskeletal fraction). Solubilization of the latter was achieved by extraction in 33 μM Nocodazole, 2% Triton X-100, 300 mM NaCl, 20 mM Hepes-KOH, pH 7.4, 2 mM $MgCl_2$, 0.1 mM EGTA, 20 mM NaF, 80 mM β-glycerophosphate, 2 mM ATP, 1 mM PMSF and protease inhibitors (0°C).

Microscopy

Indirect immunofluorescence and pre-embedding immunoelectron microscopy were performed exactly as specified in Maison et al. (1997) and Simos et al. (1996). Nuclear lamins and LAP2B were detected by a specific anti-peptide antibodies (for a complete list see Georgatos et al., 1997 and references therein); nucleoporins were immunolocalized using the broad-spectrum monoclonal antibody 414 (Davis and Blobel, 1986). The samples were examined in a conventional epifluorescence, a confocal, or an electron microscope.

Biochemical methods

Immunoprecipitation and western blotting were done as previously described (Simos and Georgatos, 1992; Maison et al., 1997). Attempts to affinity-purify the anti-PBC68 antibodies using nitrocellulose blots of whole nuclei or nuclear envelopes were unsuccessful, because the antigen was scarce and the amount of the recovered antibodies limited. Following an alternative method, autoantibodies recognizing PBC68 were isolated by biochemical fractionation. 1-2 ml samples of No. 27 serum were chromatographed on Protein G-Sepharose and the Igs eluted by glycine-HCl pH 2.3 in a stepwise fashion. Individual fractions from this column were tested by indirect immunofluorescence microscopy and those containing nuclear pore-staining activity were pooled. After an overnight dialysis against 10 mM Na_3PO_4, the pool was further chromatographed on hydroxylapatite using a 10-200 Na_3PO_4 salt gradient. After SDS-PAGE, selected fractions were tested by indirect immunofluorescence microscopy and their reactivity against PBC68 was confirmed by western blotting. The whole serum and the purified IgG fraction stained interphase and mitotic cells in the same way.

RESULTS

Identification of PBC68

To identify new proteins of the nuclear envelope, we have screened 60 sera from patients with primary biliary cirrhosis (PBC). As could be observed by indirect immunofluorescence microscopy, one of these sera, No. 27, contained high titre autoantibodies against components of the nuclear envelope. At the dilutions used, the cross-reaction of this serum with mitochondrial antigens or other cellular constituents was minimal and could be entirely removed by chromatographic purification of the anti-nuclear envelope autoantibodies (for details see Materials and Methods). Immunostaining of interphase cells yielded a ‘spotty’, rim-fluorescence pattern, characteristic of NPC proteins (Fig. 1). The distribution of the autoantigen(s) was not affected by high salt treatment or detergent extraction (data not shown), indicating a tight association with karyoskeletal structures.

Western blotting revealed that No. 27 serum reacted with a single nuclear envelope protein, which we termed PBC68 (Fig. 2a). PBC68 could be specifically immunoprecipitated from total lysates of ^{35}S-labeled cells by using either the whole serum, or a highly purified fraction of the corresponding IgG (Fig. 2b; lanes 27 and 27F). When immunoprecipitation was

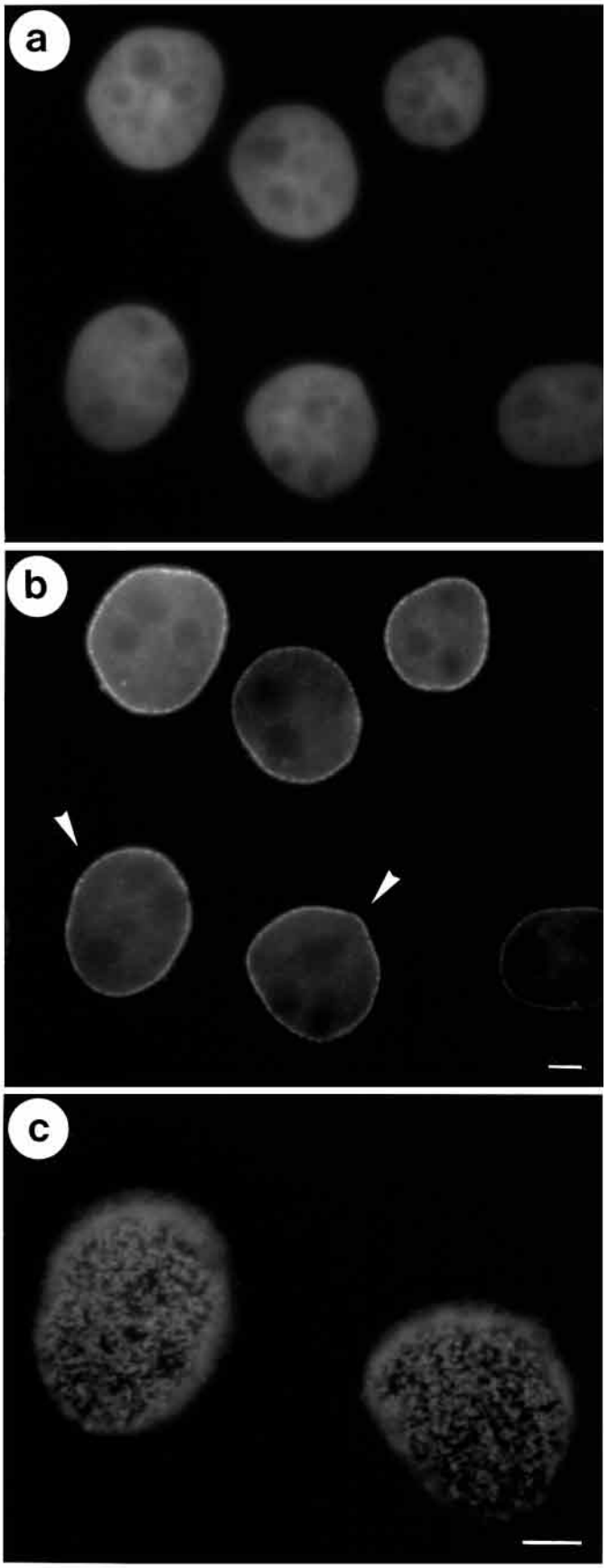


Fig. 1. Immunostaining of interphase cells with autoimmune serum No. 27. Normally growing Ishikawa cells stained with DAPI (a) or No. 27 serum (b,c; dilution 1:80). The two nuclei indicated by arrowheads in b are shown magnified and photographed at a different focal level in c. Note the 'spotty' rim-fluorescence in b and the typical NPC staining pattern in c. Bars, 2 μm.

performed under non-denaturing conditions, the autoantigen was consistently co-isolated with two other polypeptides that had an apparent M_r of 98 000 and 250 000 (Fig. 2b, arrows). The co-precipitated 98 kDa protein band was always more prominent than the 250 kDa band, but this may reflect differences in methionine content.

Inspection of the blots and the autoradiograms shown in Fig. 2a, b revealed that PBC68 had exactly the same electrophoretic mobility with nuclear lamin B. Therefore, to find out whether PBC68 was a B-type lamin, we performed additional immunoprecipitation experiments using several PBC sera that contained anti-nuclear autoantibodies. The material immunoprecipitated by the various sera was further analyzed by SDS-PAGE and the corresponding blots probed with broad spectrum anti-lamin B antibodies. Data depicted in Fig. 2c show that No. 27 serum did not precipitate B-type lamins, while another serum (No. 6), which decorated the nuclear envelope in a continuous rim-fluorescence fashion, did.

Extending the biochemical analysis, we immunoprecipitated material from total cellular extracts using all PBC sera in our collection. Representative blots shown in Fig. 2d indicated that PBC68 is a unique autoantigen not recognizable by other PBC autoantibodies.

PBC68 is associated with the nuclear pore complex

As stated above, the pattern of PBC68 distribution in interphase cells was almost diagnostic for an NPC-associated protein. Nevertheless, to establish the subcellular distribution of PBC68 by a more stringent method, we examined human carcinoma cells that had been treated for 20 hours with 10 nM taxol and then released for several hours into normal media. We have recently found that such a treatment results in extensive clustering of the NPCs as cells exit mitosis and re-enter the cell cycle. NPC clustering occurs well before apoptosis and correlates with nucleo-cytoplasmic transport defects (Theodoropoulos et al., 1999). Consistent with a pore complex localization, PBC68 co-distributed with the nucleoporins recognized by the monoclonal antibody 414 and exhibited a clustered distribution (Fig. 3).

To confirm the localization of PBC68 at the level of the electron microscope, we performed immunogold staining of Triton X-100-permeabilized cells. As shown in Fig. 4, the labeling was restricted to fibrillar elements extending from the periphery of the NPC. However, since the cells were treated with non-ionic detergent before immunogold labeling (pre-embedding immunoelectron microscopy), we could not distinguish by this technique whether the PBC68-bearing fibrils represented the filaments that emanate from the cytoplasmic ring of the NPC or parts of the nucleoplasmic basket.

To differentiate between these possibilities, we performed indirect immunofluorescence experiments using digitonin-permeabilized cells. At a certain range of concentrations, digitonin disrupts the plasma membrane without affecting the

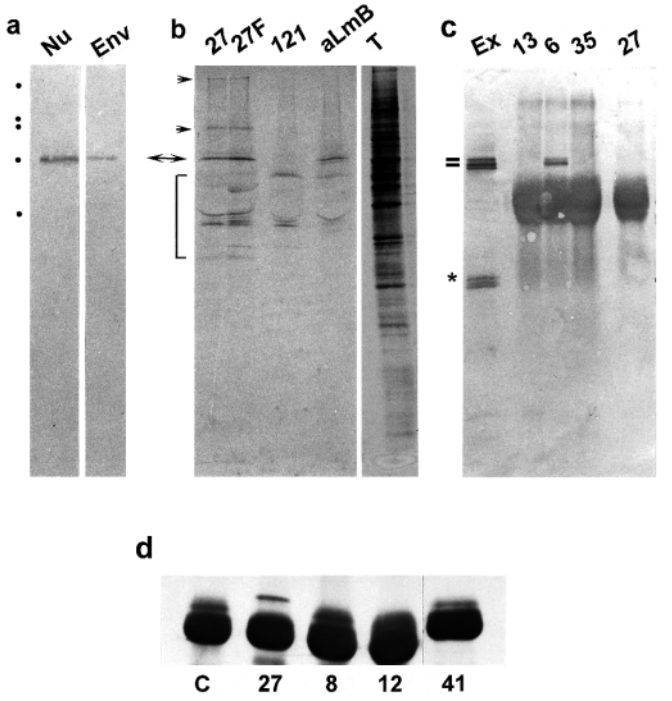


Fig. 2. Biochemical characterization of PBC68. (a) Western blot depicting electrophoretically fractionated nuclei (Nu) and nuclear envelopes (Env) that have been probed with No. 27 serum (dilution 1:300). Dots indicate molecular mass markers at 200, 115, 97, 68 and 42 kDa, while a double arrow shows the position of PBC68. (b) Autoradiogram showing metabolically labeled proteins that are immunoprecipitated by whole No. 27 serum (27), a highly purified fraction of No. 27 IgG (27F), control serum (121) and anti-lamin B antibodies (aLmB). (T) is a sample of the total detergent extract that was used as a starting material. Double arrow indicates PBC68 (notice the comigration with lamin B1); arrows denote two polypeptides with apparent M_r of 98 000 and 250 000 that are reproducibly co-precipitated with the autoantigen; proteins precipitated in a non-specific fashion are included in a bracket. (c) Western blot showing material that has been precipitated by sera No. 13, 6, 35 and 27 and probed by anti-lamin B antibodies. (Ex) is a sample of the detergent/high salt extract used for immunoprecipitation. Double bar indicates the position of intact lamin B (B1 and B2) and asterisk denotes some minor degradation products. The diffuse band present in all immunoprecipitates represents the heavy chains of IgG. (d) Representative western blot showing proteins that have been precipitated by various PBC autoantibodies and probed by No. 27 serum (1:400). Sample (C) represents a control immunoprecipitation using serum from a healthy subject. Only the relevant area of the blot is included here.

nuclear envelope (Adam et al., 1992); thus, nuclear antigens facing the cytoplasm are accessible to exogenously added antibodies, whereas proteins localized along the inner surface of the nucleus are not (as an example see Simos et al., 1996). Similarly to anti-lamin B antibodies, the anti-PBC68 antibodies failed to stain the nuclear periphery of digitonin-permeabilized cells (Fig. 5a′,b,b′). However, anti-vimentin antibodies readily decorated the cytoplasmic intermediate filaments indicating that the plasma membrane had been successfully opened (Fig. 5a). From these data, it can be concluded that PBC68 is localized at the nucleoplasmic face of the NPC.

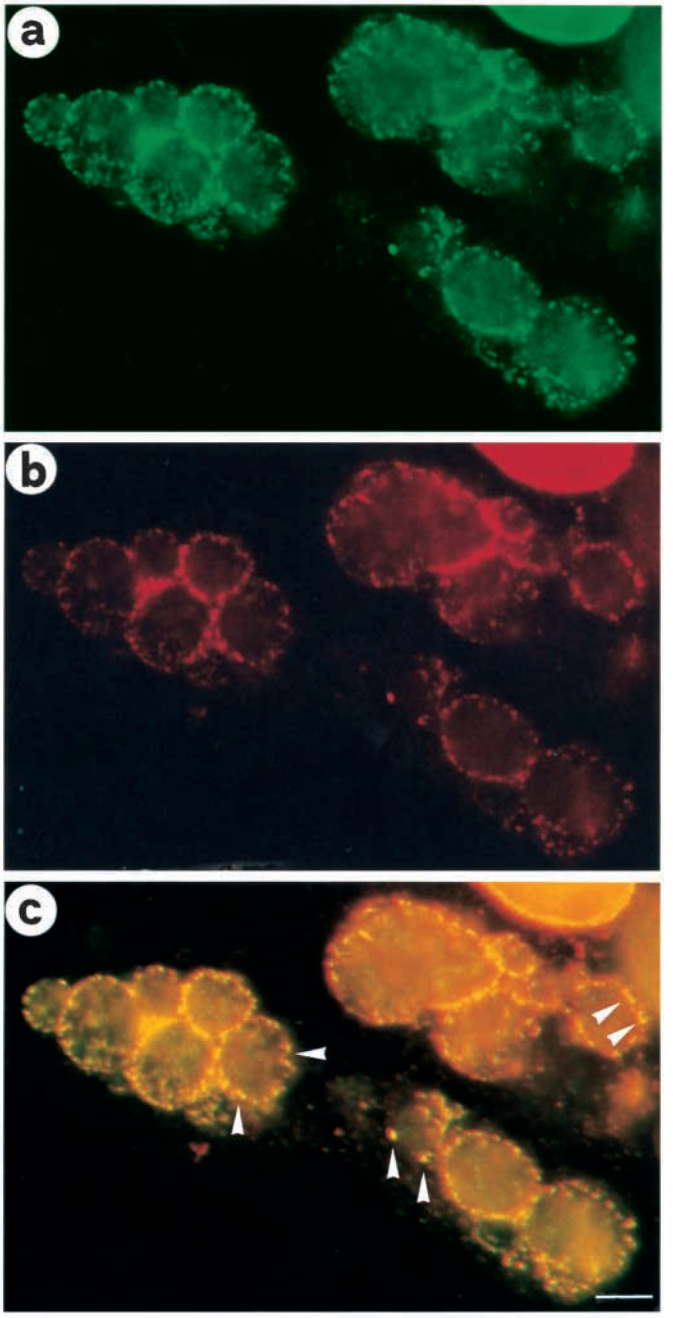


Fig. 3. Co-distribution of PBC68 and ‘classic’ nucleoporins. Ishikawa cells were treated with 10 nM taxol (20 hours, 37°C) and then released into normal media to induce re-entry into the cell cycle and formation of NPC clusters. The specimens were doubly stained with anti-PBC68 antibody (a; FITC) and the monoclonal antibody 414 (b; rhodamine). Co-localization of PBC68 and the nucleoporins recognized by 414 is apparent in the merge (c, arrowheads). Bar, 3 μm.

Partitioning of PBC68 during mitosis

To examine the fate of PBC68 during mitosis, we first studied normally occurring mitotic cells that had not been exposed to cell-cycle-arresting agents. During prophase, when the surface of the nucleus began to deform (for details see Georgatos et al., 1997), the protein was tightly associated with the nuclear envelope (Fig. 6b/b′, c/c′). However, in this phase, major

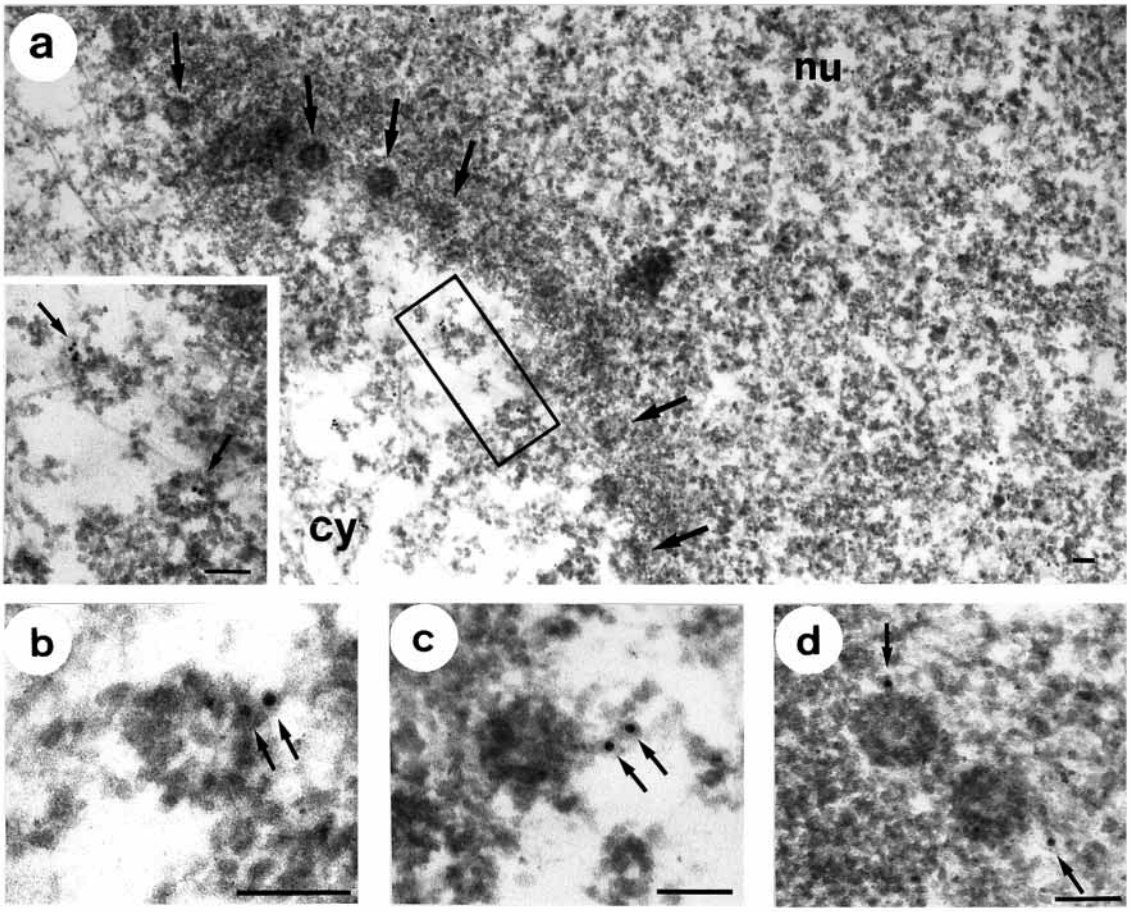


Fig. 4. Sublocalization of PBC68 by pre-embedding immunoelectron microscopy. (a) Part of a (tangential) section showing the nuclear boundary of an Ishikawa cell that has been permeabilized with Triton X-100 and labeled by anti-PBC68 antibodies and protein A-gold. Arrows indicate serially arranged NPCs. The inset represents an enlargement of the area included in the box and depicts two NPCs decorated by the antibodies (arrows). nu, nucleoplasm; cy, cytoplasm, respectively. (b-d) A gallery of immunogold-decorated NPCs at high magnification. Note the labeling of fibrillar elements that project from the periphery of the NPC (arrows). Bars, 100 nm.

modifications or molecular rearrangements in PBC68 must have taken place because the antigen became much more reactive towards the autoantibodies. This could be graphically shown by performing indirect immunofluorescence microscopy with highly diluted No. 27 serum. As shown in the example of Fig. 6a/a′, autoantibodies diluted 1:640 did not decorate at all interphase cells, but stained brightly all prophase cells. The same could be observed using highly enriched anti-PBC68 IgG prepared from No. 27 serum (data not shown). Parallel (Fig. 6b′-e′), or double (Fig. 6f,f′) immunostaining with 414 and anti-PBC antibodies provided hints that mitotic disassembly of p62 and related nucleoporins may occur slightly before disassembly of PBC68 (Fig. 6b-f,f′). However, no major differences in the distribution of PBC68 and the antigens recognized by the 414 monoclonal could be detected at this stage.

The distribution of PBC68 became distinct from that of other nucleoporins during metaphase. As shown in Fig. 7g,h,j, the antigens recognized by 414 were scattered throughout the cytoplasm, consistent with previous observations in rat cell lines (Davis and Blobel, 1986). In contrast, most of PBC68 was localized at the spindle poles in association with the spindle microtubules (Fig. 7a,b,i). The spatial association of PBC68 with the mitotic apparatus and its absence from the surfaces of chromosomes could be better assessed employing confocal microscopy (Fig. 7c-f). The decoration of microtubule-containing structures by anti-PBC68 antibodies was not due to a cross-reaction with tubulin: the autoantibodies did not decorate the cytoskeleton of interphase cells, or the asters of mitotic microtubules at prophase/prometaphase (Figs 1a-d, 3a, 5b, 6a,b,c,f); furthermore, no cross-reaction with tubulin was detected in western blots (data not shown).

Differential sorting of PBC68 and NPC proteins was also observed during anaphase. At this stage, the antigens recognized by the 414 monoclonal were already associated with the surface of chromosomes, while PBC68 was still bound to the spindle poles (Fig. 8a-h).

To examine whether PBC68 was tightly or loosely bound to the microtubules, we permeabilized mitotic cells with digitonin and washed out all soluble elements before fixation and immunostaining with anti-PBC68 antibodies. Images presented in Fig. 9a demonstrate that a substantial amount of the antigen was retained on the mitotic spindle. Moreover, the distribution of mitotic PBC68 was randomized when the cells were exposed to high concentrations of nocodazole (Fig. 9b). In contrast, after treatment with the microtubule-stabilizing agent taxotere, PBC68 was detected along the length of the microtubules (Fig. 9c).

To fully confirm that the spindle-associated material corresponds to PBC68 and not to a cross-reacting species, we analyzed synchronized mitotic cells. The cells were blocked in metaphase by taxol/taxotere and then permeabilized with digitonin in the presence of microtubule-stabilizing agents. Immunoprecipitation with the autoimmune antibodies was carried out either from the cytosolic fraction, or the cytoskeletal ‘residue’ which was subsequently solubilized in

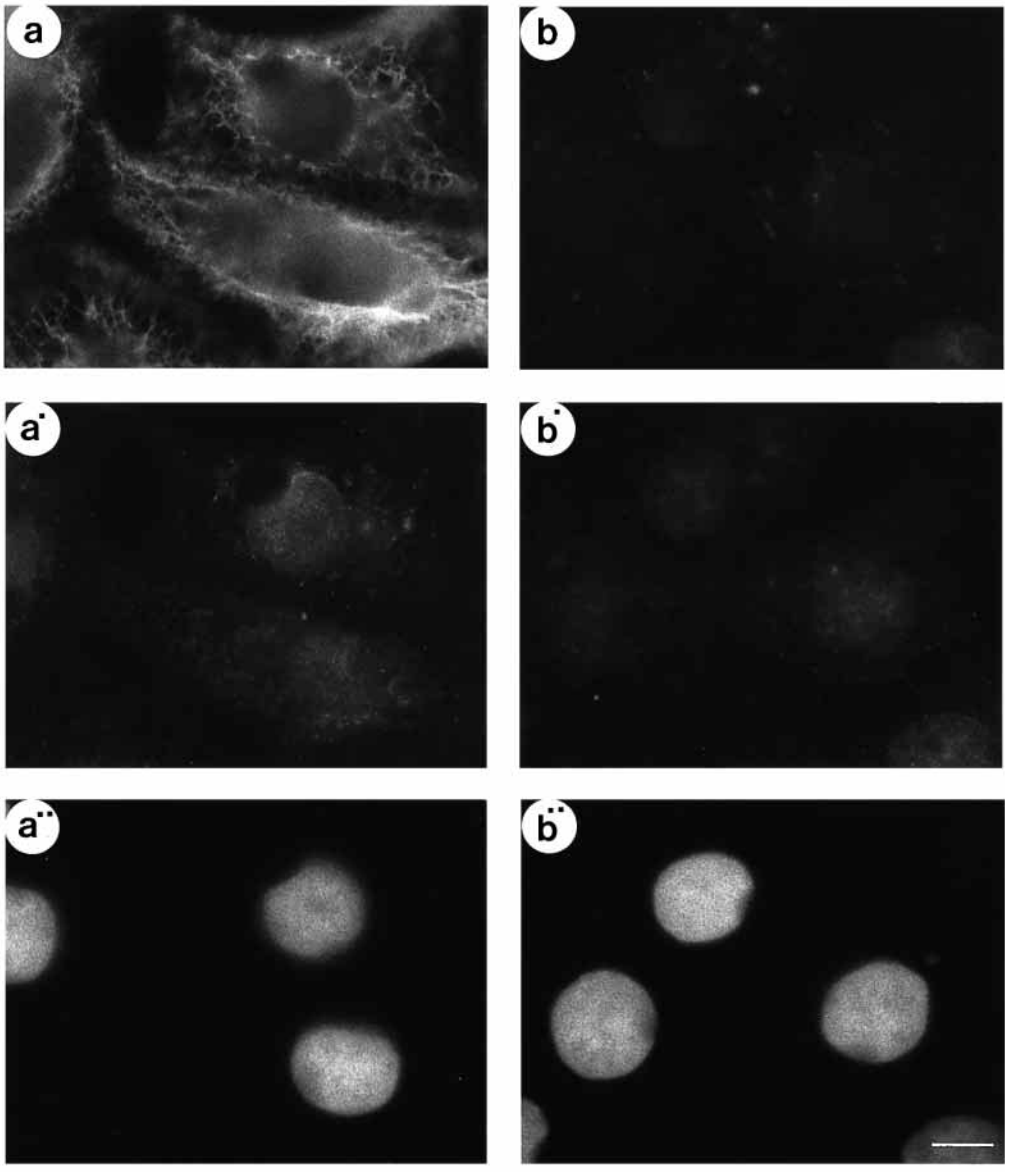


Fig. 5. Immunostaining of digitonin-permeabilized cells. Cells permeabilized with 50 μg/ml digitonin were stained with various combinations of antibodies and examined by indirect immunofluorescence microscopy. Specimens in a,a′,a″ were stained with anti-vimentin antibodies, anti-lamin B antibodies and DAPI, respectively. Specimens in b,b′,b″ were decorated by anti-PBC68 antibodies, anti-lamin B antibodies and DAPI, respectively. Bar, 6 μm.

detergent/high salt (for details see Materials and Methods). As shown in Fig. 10c, PBC68 was abundantly present in the cytoskeletal fraction, but barely detectable in the cytosolic material that was released by digitonin. Immunostaining of synchronized cells that were permeabilized in suspension with digitonin and spun down on glass coverslips revealed intense staining of microtubule asters and complete absence of any diffuse cytoplasmic background (Fig. 10a,b). Taken together, these data suggest that the majority of the NPC protein is stably bound to mitotic microtubules.

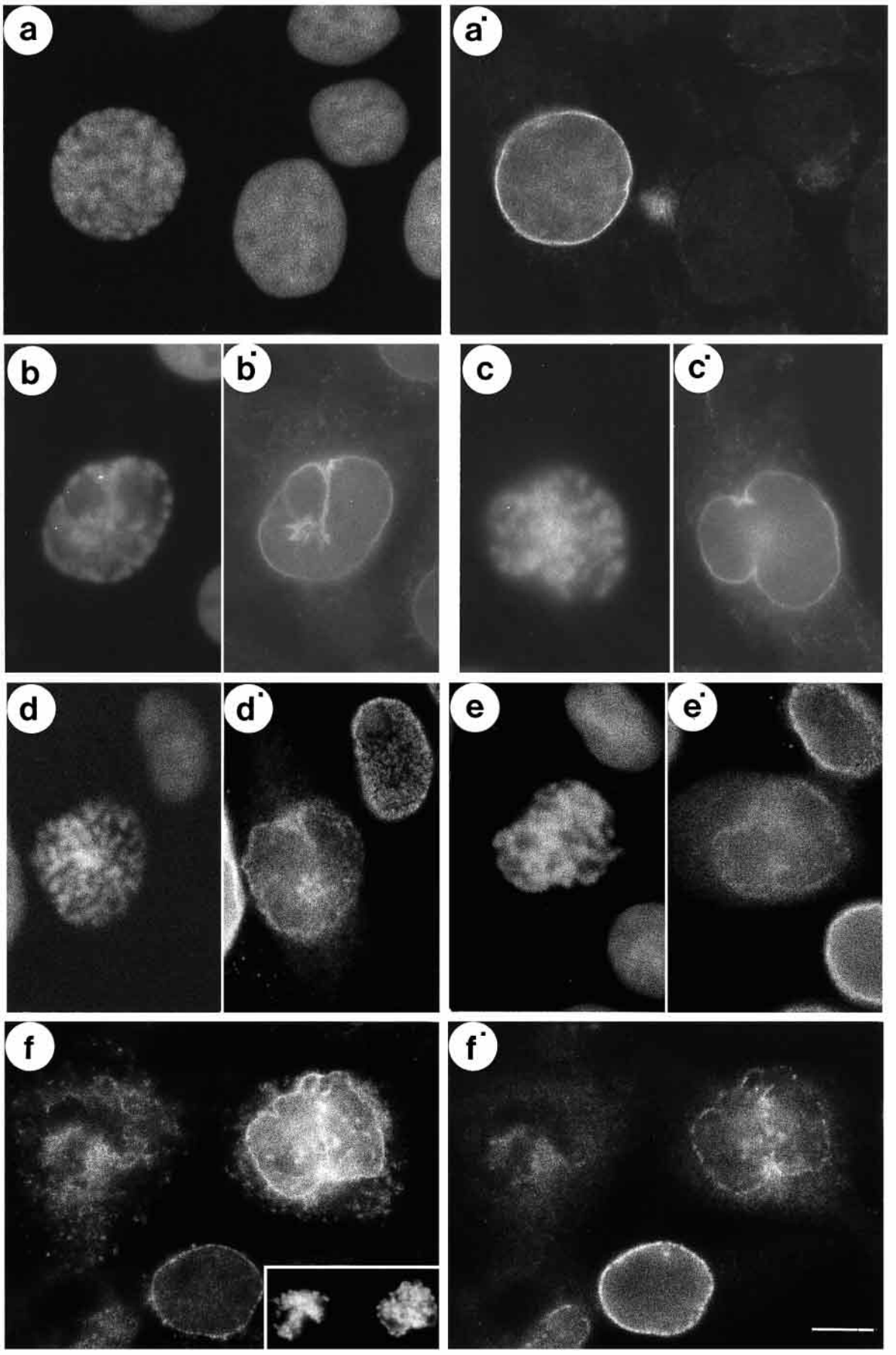
Finally, to compare the fate of PBC68 with that of other nuclear envelope proteins at the end of mitosis, we performed double-staining experiments employing anti-lamin, anti-LAP2B and anti-nucleoporin (414) antibodies. Data shown in Fig. 11 indicated that reassembly of PBC68 occurs after lamin B, lamin A, LAP2B and the 414 recognized nucleoporins have assembled. By this measure, PBC68 is one of the last component to be recruited at the nuclear envelope in late telophase/early G_1.

DISCUSSION

Using human autoantibodies, we have identified PBC68, a 68 kDa protein tightly associated with the nuclear envelope. During interphase, PBC68 is located at the NPC; however, upon mitotic disassembly of the nuclear envelope, this polypeptide associates reversibly with the spindle microtubules. Such a partitioning has not been observed so far with other NPC components.

The exclusive localization of PBC68 at the NPC is supported by several lines of evidence: first, as documented by indirect immunofluorescence microscopy, the interphase distribution of this polypeptide (‘spotty’ staining of the nuclear periphery) is

Fig. 6. Distribution of PBC68 during prophase. (a′-f′) A gallery of prophase cells stained with No. 27 serum (a′, 1:640; b′,c′,f′, 1:160) and the monoclonal antibody 414 (d′,e′,f′). (f,f′) A double immunofluorescence experiment; (a-e, inset in f) corresponding DAPI profiles. Bar, 5 μm.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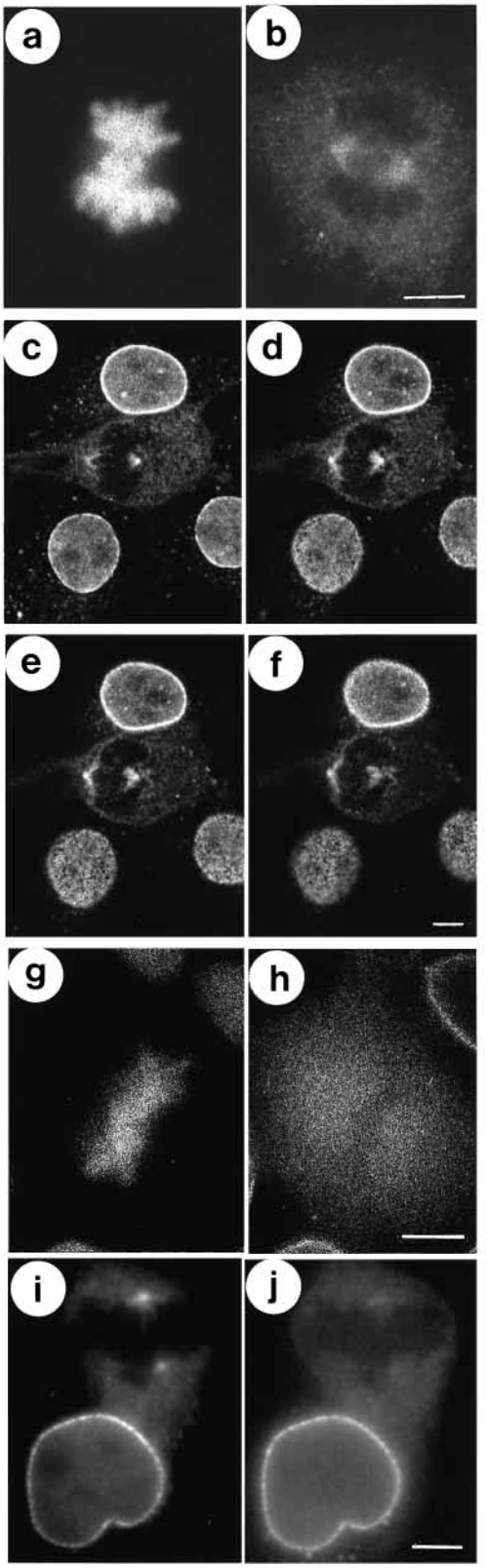

Fig. 7. Distribution of PBC68 during metaphase. Representative examples of metaphase cells stained with (b-f,i) anti-PBC68 antibodies or (h,j) the 414 monoclonal. DAPI profiles corresponding to b and h are shown in a and g, respectively. (i,j) A double immunofluorescence experiment; (a,b,g-j) conventional epifluorescence micrographs; (c-f) consecutive confocal sections. Bars, 3 μm.

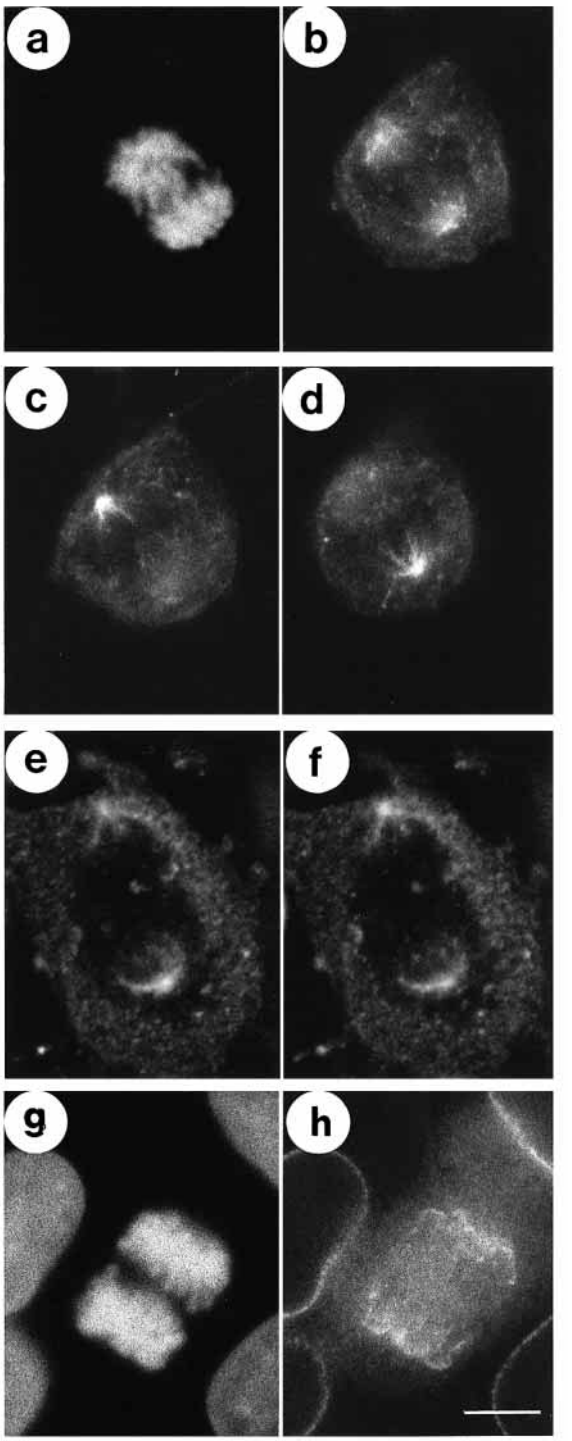

Fig. 8. Distribution of PBC68 during anaphase. Anaphase cells stained with (b-f) anti-PBC68 antibodies or (h) the 414 monoclonal. DAPI profiles corresponding to b and h are shown in a and g, respectively. (b-d) The same cell photographed at different focal levels; (a-d,g,h) conventional epifluorescence captures; (e,f) consecutive confocal sections. Bar, 4 μm.

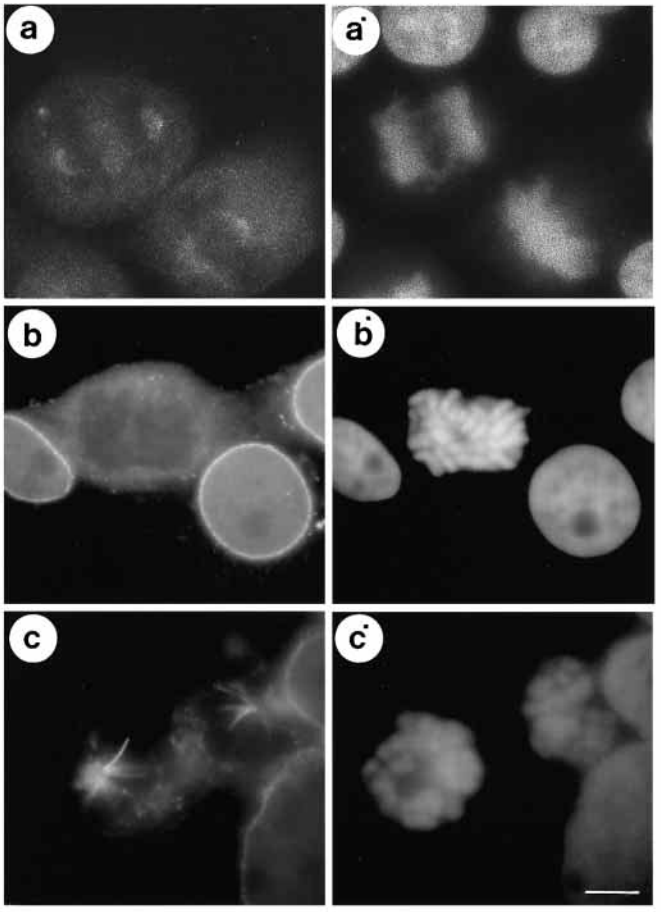

Fig. 9. Distribution of mitotic PBC68 after various treatments. Ishikawa cells stained with anti-PBC68 antibodies after treatment with (a) digitonin, (b) nocodazole and (c) taxotere. (a′,b′,c′) The corresponding DAPI profiles. Note that removal of soluble cytoplasmic constituents does not affect the association of PBC68 with spindle microtubules. Depolymerization of microtubules by nocodazole randomizes PBC68 distribution, whereas stabilization of the microtubules with taxanes strengthens binding to the mitotic apparatus. Bar, 5 μm.

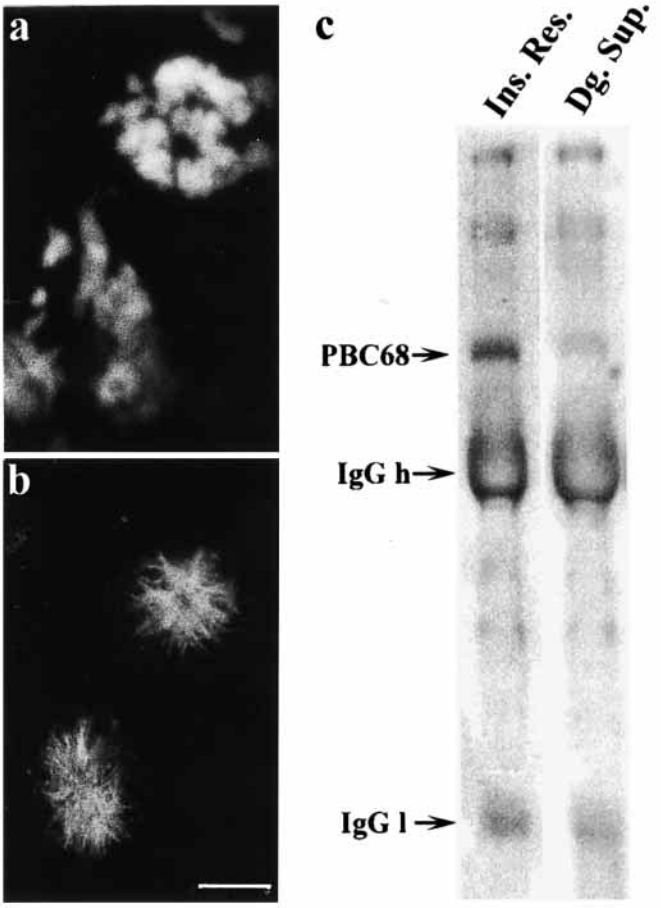

Fig. 10. Distribution of mitotic PBC68 upon fractionation of synchronized cells. Ishikawa cells were blocked in metaphase after a 20 hour treatment with taxol/taxotere and permeabilized with digitonin. (a,b) Staining of the cells, after spinning onto glass coverslips and through a cushion of 20% sucrose, with (a) DAPI and (b) anti-PBC68 antibodies. Note the intense decoration of the microtubule asters and the complete absence of cytoplasmic background. Bar, 3 μm. (c) Immunoprecipitation of mitotic PBC68 from the cytoskeletal fraction (Ins. Res.) and the cytosolic material released by digitonin (Dg.Sup.). The immunoprecipitates were analyzed in SDS gels (overrun on purpose to better separate PBC68 from the heavy chains of IgG) and western blotted. The positions of PBC68 and the IgG heavy (IgG h) and light (IgG l) chains are indicated by arrows.

typical for an NPC-associated protein and does not change upon high salt or detergent treatment; second, upon induction of NPC clustering, PBC68 co-distributes with p62 and other ‘classic’ nucleoporins recognized by the monoclonal antibody 414; finally, immunogold labeling of Triton-extracted cells shows a restricted localization in one of the NPC ‘territories’, i.e., pore-associated fibrils.

At present, the primary structure of PBC68 and its sequence relationship with the known nucleoprins are not clear. However, since no other NPC protein has been reported to have a relative molecular mass value of 68 000 and because the mitotic partitioning of this polypeptide is rather distinct, it is likely that it represents a novel component of the NPC. If this were correct, it would be tempting to speculate that the 98 kDa protein co-precipitated with PBC68 is actually Nup98, a mammalian nucleoporin that localizes at the nucleoplasmic fibrils (Radu et al., 1995).

Considering the currently available information, we would argue that PBC68 has an inherent ability to associate with the mitotic microtubules. As could be seen by conventional and confocal microscopy, a large fraction of PBC68 localizes at the spindle poles during metaphase and anaphase. Furthermore, the association with the spindle apparatus is promoted by microtubule-stabilizing drugs and abolished by depolymerizing agents. Apparently, this is a regulated and largely reversible interaction activated at prometaphase and weakened at late prophase/G_1.

Adopting a recently proposed format of NPC reassembly (Gant et al., 1998), it is interesting to note that PBC68 is released from the microtubules and reassociates with the nuclear envelope at a stage when peripheral structures (i.e., pore-associated filaments) are expected to form. This, together with the differential sorting of PBC68 during metaphase and

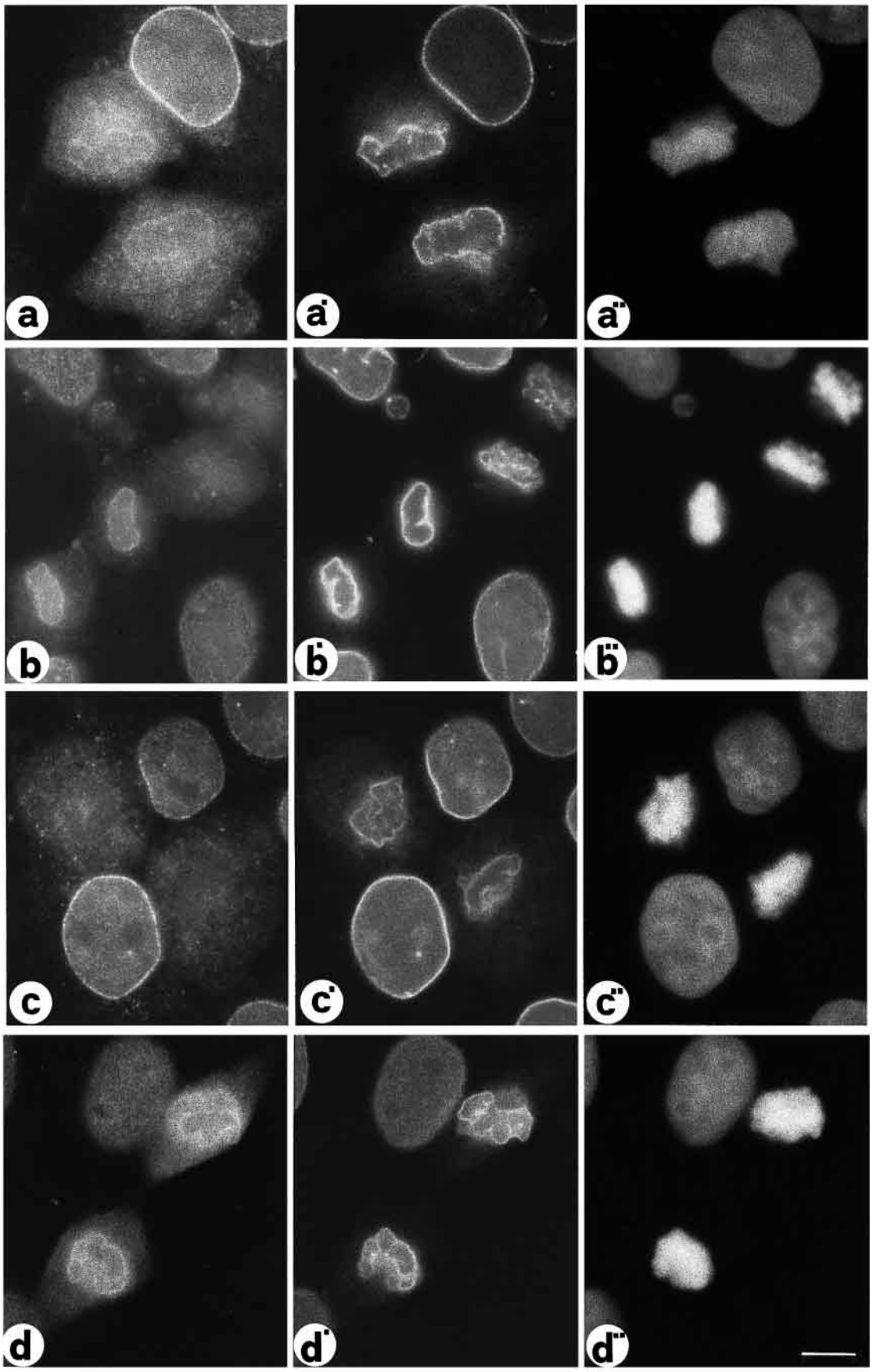

Fig. 11. Distribution of PBC68 in late mitotic cells. Naturally occurring telophase/early G_1 cells stained with (a-d) anti-PBC68 antibodies and either (a′) monoclonal antibody 414, (b′) anti-LAP2B, (c′) anti-lamin B, or (d′) anti-lamin A. (a″-d″) Corresponding DAPI profiles. Notice that PBC68 reassembles after lamins, LAP2B and the 414-recognized nucleoporins have been recruited in the reforming nuclear envelope. Bar, 5 μm.

anaphase, provide convincing evidence for a stepwise reconstruction of the NPC at the end of mitosis.

Apart from PBC68, a number of other nuclear proteins exhibit a transient association with mitotic microtubules. For instance, the nucleoplasmic/nuclear matrix proteins NuMa (Tang et al., 1994), B1C8 (Wan et al., 1994) and LAP2A (Dechat et al., 1998), the nucleolar protein B23 (Zatsepina et al., 1999) and the inner nuclear membrane protein LAP1C (Maison et al., 1997) have been localized in the area of the spindle during mitosis. Binding to elements of the cytoskeleton would provide an efficient means for separating relatively 'promiscuous' or 'multivalent' molecules, which are unleashed into the cytoplasm upon nuclear envelope breakdown. The regulated release of these proteins from their docking sites may allow an orderly, stepwise reassembly of complex nuclear structures at the end of the cell division cycle.

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REFERENCES

Adam, S. A., Sterne-Marr, R. and Gerace, L. (1992). Nuclear protein import using digitonin-permeabilized cells. *Meth. Enzymol.* **219**, 97-110.

Akey, C. W. (1995). Structural plasticity of the nuclear pore complex. *J. Mol. Biol.* **248**, 273-293.

Akey, C. W. and Radermacher, M. (1993). Architecture of the Xenopus nuclear pore complex revealed by three-dimensional cryo-electron microscopy. *J. Cell Biol.* **122**, 1-19.

Chaudhary, N. and Courvalin, J.-C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. *J. Cell Biol.* **122**, 295-306.

Courvalin, J.-C., Lassoued, K., Bartnik, E., Blobel, G. and Wozniak, R. W. (1990a). The 210-kDa nuclear envelope polypeptide recognized by human autoantibodies in primary billiary cirrhosis is the major glycoprotein of the nuclear pore. *J. Clin. Invest.* **86**, 279-285.

Courvalin, J.-C., Lassoued, K., Worman, H. J. and Blobel, G. (1990b). Identification and characterization of autoantibodies against the nuclear envelope lamin B receptor from patients with primary billiary cirrhosis. *J. Exp. Med.* **72**, 961-967.

Courvalin, J.-C., Chaudhary, N., Danon, F, Brouet, J.-C. and Lassoued, K. (1990c). Characterization of human autoantibodies specific for lamin A. *Biol. Cell.* **69**, 93-97.

Davis, L. I. and Blobel, G. (1986). Identification and characterization of a nuclear pore complex protein. *Cell* **45**, 699-709.

Dechat, T., Gotzmann, J., Stockinger, A., Harris, C. A., Talle, M. A., Siekierka, J. J. and Foisner, R. (1998). Detergent-salt resistance of LAP2a in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. *EMBO J.* **17**, 4887-4902.

Doye, V. and Hurt, E. C. (1997). From nucleoporins to nuclear pore complexes. *Curr. Opin. Cell Biol.* **9**, 401-411.

Dwyer, N. and Blobel, G. (1976). A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* **70**, 581-591.

Gant, T. M., Goldberg, M. W. and Allen, T. D. (1998). Nuclear envelope and nuclear pore assembly: analysis of assembly intermediates by electron microscopy. *Curr. Opin. Cell Biol.* **10**, 409-415.

Georgatos, S. D., Pyrpasopoulou, A. and Theodoropoulos, P. A. (1997). Nuclear envelope breakdown in mammalian cells involves stepwise lamina disassembly and microtubule-driven deformation of the nuclear membrane. *J. Cell Sci.* **110**, 2129-2140.

Goldberg, M. W., Wiese, C., Allen, T. D. and Wilson K. L. (1997). Dimples, pores, star-rings and thin rings on growing nuclear envelopes: evidence for structural intermediates in nuclear pore complex assembly. *J. Cell Sci.* **110**, 409-420.

Heese-Peck, A. and Raikhel, N. V. (1998). The nuclear pore complex. *Plant Mol. Biol.* **38**, 145-162.

Hinshaw, J. E., Carragher, B. O. and Milligan, R. A. (1992). Architecture and design of the nuclear pore complex. *Cell* **69**, 1133-1141.

Maison, C., Pyrpasopoulou, A., Theodoropoulos, P. A. and Georgatos, S. D. (1997). The inner nuclear membrane protein LAP1 forms a native complex with B-type lamins and partitions with spindle-associated mitotic vesicles. *EMBO J.* **16**, 4839-4850.

Pante, N. and Aebi, U. (1996). Molecular dissection of the nuclear pore complex. *Crit. Rev. Biochem. Mol. Biol.* **31**, 153-199.

Radu, A., Moore, M. S. and Blobel, G. (1995). The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell* **81**, 215-222.

Reichelt, R., Holzenburg, A., Buhle, E. L., Jarnik, M., Engel, A. and Aebi, U. (1990). Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. *J. Cell Biol.* **110**, 883-894.

Ris, H. (1991). The three dimensional structure of the nuclear pore complex as seen by high voltage electron microscopy and high resolution low voltage scanning electron microscopy. *EMSA Bull.* **21**, 54-56.

Ris, H. (1997). High resolution field-emission scanning electron microscopy of nuclear pore complex. *Scanning* **19**, 368-375.

Robbins, E. and Gonatas, N. K. (1964). The ultrastructure of a mammalian cell during the mitotic cycle. *J. Cell Biol.* **21**, 429-463.

Simos, G. and Georgatos, S. D. (1992). The inner nuclear membrane protein p58 associates in vivo with a p58 kinase and the nuclear lamins. *EMBO J.* **11**, 4027-4036.

Simos, G., Maison, C. and Georgatos, S. D. (1996). Characterization of p18, a component of the lamin B receptor complex and a new integral membrane protein of the avian erythrocyte nuclear envelope. *J. Biol. Chem.* **271**, 12617-12625.

Tang, T. K., Tang, C. J., Chao, Y. J. and Wu, C. W. (1994). Nuclear mitotic apparatus protein (NuMa): spindle association, nuclear targeting and differential subcellular localization of various NuMa isoforms. *J. Cell Sci.* **107**, 1389-1402.

Theodoropoulos, P. A., Polioudaki, H., Kostaki, O., Derdas, S. P., Georgoulias, V., Dargemont, C. and Georgatos, S. D. (1999). Taxol affects nuclear lamina and pore complex organization and inhibits import of karyophilic proteins into the cell nucleus. *Cancer Res.* (in press).

Wan, K. M., Nickerson, J. A., Krockmalnic, G. and Penman, S. (1994). The B1C8 protein is in the dense assemblies of the nuclear matrix and relocates to the spindle and pericentriolar filaments at mitosis. *Proc. Natl. Acad. Sci. USA* **91**, 594-598.

Yang, Q., Rout, M. P. and Akey, C. W. (1998). Three-dimensional architecture of the isolated yeast nuclear pore complex: functional and evolutionary implications. *Mol. Cell* **1**, 223-234.

Zatsepina, O. V., Rousselet, A., Chan, P. K., Olson, M. O., Jordan, E. G. and Bornens, M. (1999). The nucleolar phosphoprotein B23 redistributes in part to the spindle poles during mitosis. *J. Cell Sci.* **112**, 455-466.

Zeligs, J. D. and Wollman, S. H. (1979). Mitosis in rat thyroid epithelial cells in vivo. I: Ultrastructural changes in cytoplasmic organelles during the mitotic cycle. *J. Ultrastruct. Res.* **66**, 53-77.