

Regulated Docking of Nuclear Membrane Vesicles to Vimentin Filaments during Mitosis

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Abstract. During mitosis, several types of intermediate-sized filaments (IFs) undergo an extensive remodelling in response to phosphorylation by cdc 2 and other protein kinases. However, unlike the nuclear lamins, the cytoplasmic IFs do not seem to follow a fixed disassembly stereotype and often retain their physical continuity without depolymerizing into soluble subunits. To investigate potential interactions between mitotically modified IFs and other cellular structures, we have examined prometaphase-arrested cells expressing the IF protein vimentin. We demonstrate here that vimentin filaments associate *in situ* and co-fractionate with a distinct population of mitotic vesicles. These vesicles carry on their surfaces nuclear lamin B, the inner nuclear membrane protein p58, and wheat germ agglutinin (WGA)-binding proteins. Con-

sistent with a tight interaction between the IFs and the mitotic membranes, vimentin, nuclear lamin B, and a 180-kD WGA-binding protein are co-isolated when whole mitotic homogenates are incubated with anti-vimentin or anti-lamin B antibodies immobilized on magnetic beads. The vimentin-associated vesicles are essentially depleted of ER, Golgi and endosomal membrane proteins. The interaction of vimentin with lamin B-carrying membranes depends on phosphorylation and is weakened by dephosphorylation during nuclear reassembly *in vitro*. These observations reveal a novel interaction between IFs and cellular membranes and further suggest that the vimentin filaments may serve as a transient docking site for inner nuclear membrane vesicles during mitosis.

CELL division in higher eukaryotes involves a coordinated series of disassembly and reassembly events. In early prophase, the spatial organization of microtubules and actin microfilaments is dramatically changed. The disassembly of interphase microtubules is thought to provide building blocks required for the subsequent assembly of the mitotic spindle. At the prophase/prometaphase transition, the nuclear membrane is fragmented and the nuclear lamina is completely depolymerized (Gerace and Blobel, 1980). The disassembly of the nuclear lamina is triggered by the hyperphosphorylation of the lamin proteins, a process mediated by cdc 2 and possibly other kinases (Gerace and Blobel, 1980; Peter et al., 1990; Heald and McKeon, 1990; Ward and Kirschner, 1990). At the end of mitosis, the polymerization competence of lamins is restored by specific dephosphorylation (Burke and Gerace, 1986). Concomitant with the fragmentation of the nuclear membrane, the ER and the Golgi apparatus are broken down and disperse in the cytoplasm (Zeligs and Wollman, 1979). Although the extent of fragmentation of the cytoplasmic membranes seems to vary in the different cell types (Zeligs

and Wollman, 1979), it is becoming increasingly clear that this process also involves cdc 2-dependent phosphorylation of membrane proteins and inhibition of vesicular transport from one membrane compartment to the other (Tuomikoski et al., 1989; Warren, 1989).

Unlike the nuclear lamins, the cytoplasmic intermediate filaments (IFs)¹ do not appear to follow a fixed disassembly stereotype during mitosis. In some cells they spatially reorganize but maintain their physical continuity (Aubin et al., 1980; Blose, 1979; Blose and Bushnell, 1982; Hynes and Destree, 1978; Zieve et al., 1980a), whereas in other cells they partially disassemble into fibrogranular or fibrillar aggregates (Horwitz et al., 1981; Franke et al., 1982, 1984; Klymkowsky et al., 1991; Rosevear et al., 1990). Interestingly, in cells possessing two independent IF systems (e.g., vimentin and cytokeratins) one of the two networks is often disassembled whereas the other remains intact during mitosis (as an example see Jones et al., 1985).

Because the cytoplasmic IFs are not universally disassembled during cell division, it is conceivable that they may play a role in mitotic events. This idea is supported by several ob-

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1. *Abbreviations used in this paper:* IF, intermediate filaments; LRV, large round vesicles; MRV, medium size round vesicles; SRV, small round vesicles; TME, tubular membrane elements; WGA, wheat germ agglutinin.

servations. For example, vimentin IFs are known to form during prometaphase a "cage" around the chromosomes and the mitotic spindle (Aubin et al., 1980; Zieve et al., 1980a). This structure may protect sensitive mitotic devices from mechanical perturbations or facilitate the translocation of sister chromatids by generating an organelle-free zone around the elongating spindle. Vimentin filaments may also act in cooperation with microtubule motors (Gyoeva and Gelfand, 1991), or serve as "reservoirs" for nuclear matrix proteins released into the cytoplasm during mitosis (Marugg, 1992).

To explore such possibilities, we have made a systematic effort to identify IF-interacting components in mitotic cells. We show here that vimentin filaments of prometaphasic cells are associated with vesicles which carry on their surface nuclear membrane markers and wheat germ agglutinin (WGA)-binding proteins. We further show that these vesicles are distinct from ER, Golgi, and other cytoplasmic membranes and that their binding to vimentin is modulated by phosphorylation and dephosphorylation.

Materials and Methods

Synchronization of Cultured Cells and Subcellular Fractionation

CHO, BHK-21, and DU 249 cultures were synchronized by 2 mM thymidine and 20 ng/ml nocodazole, as described in Zieve et al. (1980b); 3T3 cells were synchronized with 10 mM thymidine and 40 ng/ml nocodazole; HeLa cells were synchronized by 5 mM thymidine and 40 ng/ml nocodazole. The mitotic cells were detached by a gentle shake-off and the mitotic index was routinely checked by DAPI staining. The cells were homogenized at 0°C in KHM buffer (78 mM KCl, 50 mM Hepes-KOH, pH 7.0, 4 mM MgCl₂, 10 mM EGTA, 8.37 mM CaCl₂, 1 mM DTT, and 20 mM cytochalasin B), as previously described (Burke and Gerace, 1986). In some experiments, the homogenate was spun at 12,000 g for 20 min at 4°C and the pellet fraction (P1) was thoroughly resuspended in ice-cold KHM buffer for further analysis. In other experiments, the whole homogenate was used without previous fractionation. In both cases the material was analyzed fresh. To prepare mitotic or interphasic cytosol, the same homogenization method was used and the 12,000 g supernatants were further centrifuged at 163,000 g for 1 h at 4°C. Aliquots of these preparations were stored at -70°C for a maximum of 7 d. The mitotic cytosol was supplemented with 1 mM ATP and an ATP-regeneration system (20 mM phosphocreatine, 400 µg/ml creatine kinase) before use. Microsomes were prepared from rat liver according to standard procedures (Blobel and Dobberstein, 1975). Lamin-depleted nuclear membranes were prepared by 8 M urea extraction of rat liver nuclear envelopes, as previously described (Georgatos and Blobel, 1987).

For flotation experiments, a small amount of a concentrated sucrose solution (95% wt/vol in KHM buffer) was added to the resuspended P1 fraction, or to whole mitotic homogenates, to reach a final sucrose concentration of 75%. Each sample was placed at the bottom of a SW 50.1 centrifuge tube (Beckman Instruments Inc., Palo Alto, CA) and overlaid sequentially with 1.5 ml of 50% sucrose, 1.5 ml of 30% sucrose, and 1.525 ml of 20% sucrose (in KHM buffer). The gradients were centrifuged for 20 h at 4°C and at 243,356 g and fractionated into 27 fractions using an AutoDensi Flow fractionator. For fractionating DU 249 cell homogenates, the protocol described by Wilson et al. (1988) was used instead of the above method. This involved application of the sample in 45.5% sucrose and flotation through steps of 37.6%, 30.8%, and 23.9% sucrose for 2 h at 4°C and at 200,000 g.

Protein Chemical and Immunochemical Procedures

Vimentin was purified from bovine lens tissue as specified by Georgatos et al. (1985). Nuclear lamins were isolated from rat liver nuclear envelopes as previously described (Georgatos and Blobel, 1987; Georgatos et al., 1988). Normal mouse and rabbit IgG and ATPγS were obtained from Sigma Immunochemicals (Deisenhofen, F. R. G.). WGA-biotin, anti-biotin, and protein A-gold were gifts from G. Griffiths (EMBL). Potato acid phosphatase was provided by A. Papavasiljoiu (EMBL). The synthetic peptides used as immunogens were made at the Protein Sequencing and Peptide Synthesis

Facility of EMBL and at the Biopolymer Facility of The Rockefeller University (New York). Coupling of synthetic peptides to derivatized agarose and affinity purification of antibodies were performed as specified in Djabali et al. (1991). Western blotting was as specified by Georgatos et al. (1987).

To monitor vimentin we used an anti-peptide antibody (av2) made against the COOH-terminal peptide V2 (KTVETRDGQVINETSEHHDDL; for sequences see Quax et al., 1983) and a previously characterized mAb (mAb 7A3, see Papamarcaki et al., 1991). Affinity-purified av2 IgG (prepared by chromatography in an Affigel-V2 column), av2 serum and 7A3 IgG gave indistinguishable results in all assays tested. In some indirect immunofluorescence experiments we also used a polyclonal anti-vimentin antibody raised against electrophoretically pure mouse vimentin (generous gift of P. Traub, Max Planck Institute for Cell Biology, Ladenburg, F. R. G.). To monitor nuclear lamin B we used primarily two specific antisera: the anti-peptide antibody #16, developed against the COOH-terminal sequence LIWKNQNSWGTGEDVK of mouse lamin B1 and another anti-peptide antibody (aNLS; provided by N. Chaudhary, The Rockefeller University) against mouse lamin B1 (for sequences see Höger et al., 1988). These antibodies reacted exclusively with type B lamins and were used without further purification in most of the experiments (except in immunoelectron microscopy, where we also used affinity-purified aNLS antibodies to confirm the results). For other confirmatory work we also used a previously characterized antibody (aPI) specific for mammalian and avian lamins B1 and B2 (Djabali et al., 1991). To identify A and C lamins we used an anti-peptide antibody (aLI) recognizing common determinants in the NH₂-terminal domains of lamins A, B, and C (for characterization see Djabali et al., 1991; Simos and Georgatos, 1992). To immunolocalize p58, we used an anti-peptide antibody (aR7) against the sequence (C)TERNDTSSKLLQQLKPDVE in the NH₂-terminal domain of this protein (Worman et al., 1990). The aR7 antibody was used exclusively in its affinity-purified form. A rabbit antiserum against the α subunit of the "signal sequence receptor" (SSR) and an organelle-specific antiserum recognizing four different ER and one Golgi membrane protein (Louvard et al., 1982) were provided by D. Louvard (Inst. Pasteur, Paris, France), B. Dobberstein (ZMBH, Heidelberg, F. R. G.) and B. Sodeik (EMBL). A mAb against the "docking protein" (signal recognition particle receptor) was supplied by D. Meyer (University of California, Los Angeles, CA). Two rabbit antisera developed against the mannose-6-phosphate receptor were provided by B. Hoflack (EMBL). All antibodies were tested by immunoblotting and immunofluorescence assays on mitotic and interphasic cells before use.

Immunoisolation of vimentin-membrane complexes by antibodies immobilized on magnetic beads was performed as follows. av2 serum (10 µl), affinity-purified av2 IgG (10 µg), anti-lamin B serum (aNLS, 10 µl), or normal rabbit IgG (10 µg) were mixed with 50 µl of magnetic beads coated with sheep anti-rabbit IgG (Dynabeads, Dynal, Oslo, Norway). After an overnight incubation, the beads were washed with PBS and mixed with mitotic homogenates (the equivalent of one culture plate). After an incubation for 2 h at 4°C, the beads were separated from the liquid phase with the aid of a magnet applied at the wall of a test tube and then washed three times with PBS.

In Vitro Assays

To examine the binding of vimentin to ER membranes and lamin-depleted nuclear envelopes, the following assay method was used. The membranes (78 µg of salt-washed rat liver microsomes and 15 µg of urea-extracted rat liver nuclear envelopes) were resuspended in KHM buffer, 1 mM ATP, 20 mM phosphocreatine and 400 µg/ml creatine kinase, and preincubated with 1/10 vol of mitotic or interphasic CHO cytosol (2.5 mg/ml) for 1 h at 0°C. In parallel, purified vimentin (42 µg) reconstituted into filaments and equilibrated in the same media was pre-incubated with 1/10 vol of mitotic or interphasic cytosol as above. The membrane suspension was mixed with vimentin and further incubated at 0°C for 1 h. At the end of the incubation, the reaction mixtures (typically 100 µl containing 10 µl of cytosol) were made 75% in sucrose, loaded at the bottom of a sucrose step gradient, and floated as described above. Fractions from each gradient were analyzed by SDS-PAGE and immunoblotting. Exactly the same procedure was followed to assay binding of lamin B to microsomes and lamin-depleted nuclear envelopes.

For in vitro nuclear reassembly, mitotic CHO homogenates were incubated in KHM buffer, for 2 h at 4°C, or at 33°C. When needed, 2.5 µl of potato acid phosphatase (stock 100 U/ml), ATPγS (to 5 mM), or 6 µl ³²P ATP (5,000 C/mmol) were added in the beginning of the incubation per 50 µl of assay mixture. Samples taken from these reaction mixtures were either analyzed by flotation in sucrose gradients, or fixed and examined by thin-sectioning and transmission EM.

Microscopy

Indirect immunofluorescence microscopy was performed as previously described (Kouklis et al., 1991; Merdes et al., 1991), except that in some cases (double-staining) where the cells were permeabilized with 0.05% digitonin. For whole-mount EM, samples were applied to carbon-coated grids, for 3 min, at room temperature. The samples were stained for 2 min with 2% uranyl acetate. For whole-mount immunoelectron microscopy, unfixed or paraformaldehyde-fixed samples were first incubated for 15 min with 0.5% (wt/vol) cold fish gelatin in KHM buffer (buffer G) and then for 30 min with the appropriate antibody solution (diluted 1:10 in buffer G). After washing with buffer G (three times for 5 min), protein A-gold (diluted 1:100-1:150) was added and the specimens were incubated for 20 min. After another washing (three times for 5 min with KHM buffer and 1 s with distilled water), the samples were stained with 2% uranyl acetate. In a variation of this protocol, the specimens were incubated with a secondary goat anti-rabbit antibody (diluted 1:7 in buffer G) after incubation with the primary rabbit antibodies. For controls in such cases the samples were incubated with the same reagents except the primary antibodies. For double-immunostaining, the specimens were incubated first with one antibody and then with protein A-gold (9 nm). After blocking unsaturated protein A sites with 0.1-1% buffered glutaraldehyde (3 min), the samples were washed with 20 mM glycine/KHM buffer, and incubated with the second antibody. Protein A-gold (14 nm) was added next and, after appropriate washings, the samples were stained with 2% uranyl acetate. Staining of ultrathin frozen sections with anti-vimentin and anti-lamin B antibodies (diluted 1:7-1:30) was performed

as previously described (Griffiths et al., 1984). The specimens were visualized in a Philips 301 or a Philips 400 microscope operated at 80 kV.

Other Methods

SDS-PAGE was performed as described (Laemmli, 1970). Protein concentrations were measured using a Bio-Rad kit (Bio-Rad Labs., Richmond, CA).

Results

In Situ Studies

To monitor the distribution of vimentin filaments in mitotic cells, we performed indirect immunofluorescence experiments with several cell lines (CHO, DU 249, BHK-21, 3T3, and HeLa cells). Since CHO cells could be much more efficiently synchronized (up to 99% prometaphasic cells after thymidine-nocodazole treatment and shake-off), and since the results were similar in all cases, we decided to use this cell line as our principal model system.

The organization of vimentin filaments in mitotic CHO cells has been previously examined by Zieve et al. (1980a)

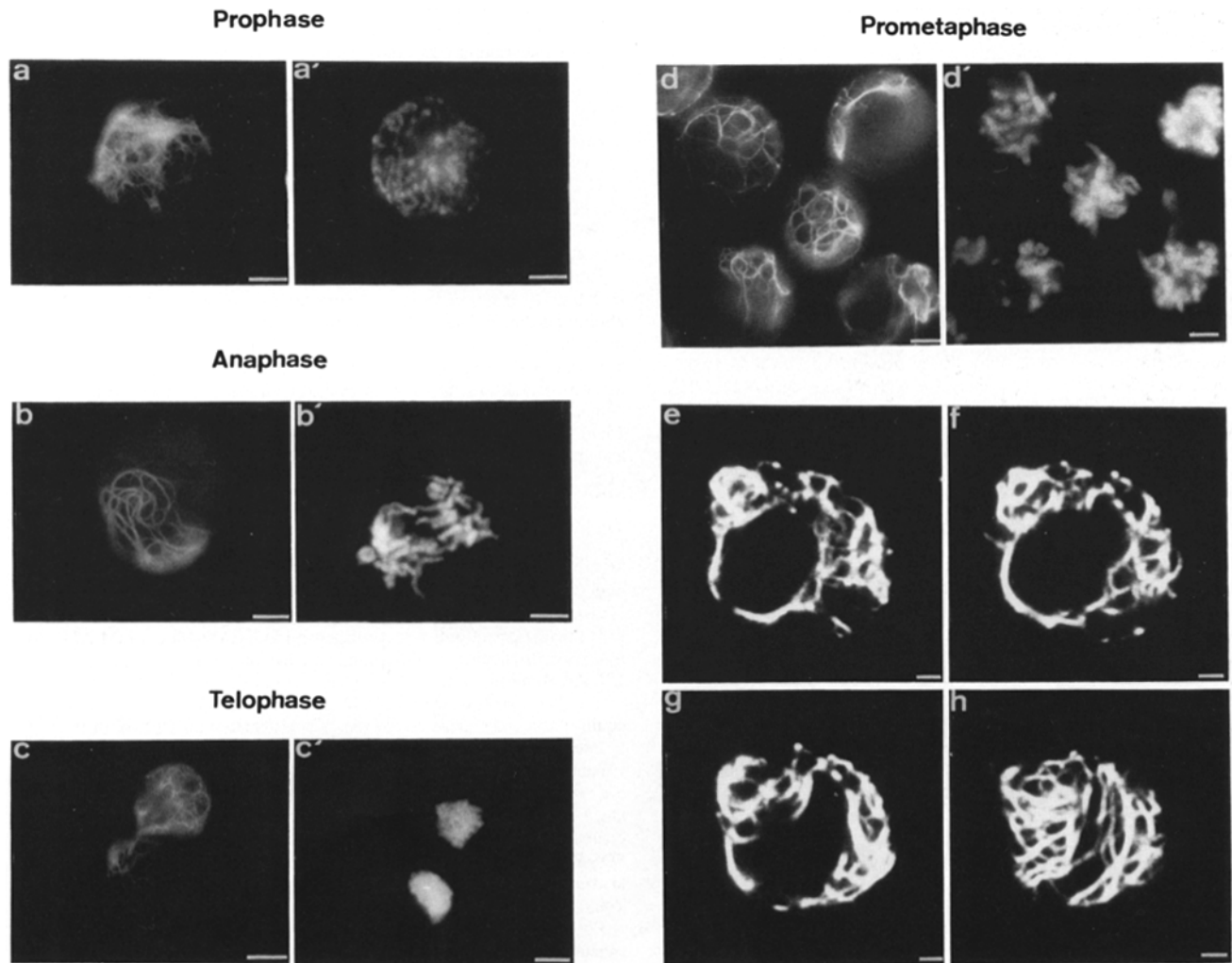


Figure 1. Distribution of vimentin filaments in mitotic CHO cells as detected by indirect immunofluorescence microscopy. CHO cells at different phases of mitosis were decorated with the anti-vimentin mAb 7A3 (a-h) and counterstained with DAPI (a'-d'). (a and a') Late prophase; (b and b') anaphase; (c and c') telophase; (d and d', e-h) prometaphase. The images shown in e-h are optical sections of a prometaphasic cell, similar to the cells shown in d, visualized by confocal microscopy. Bars, 2 μ m.

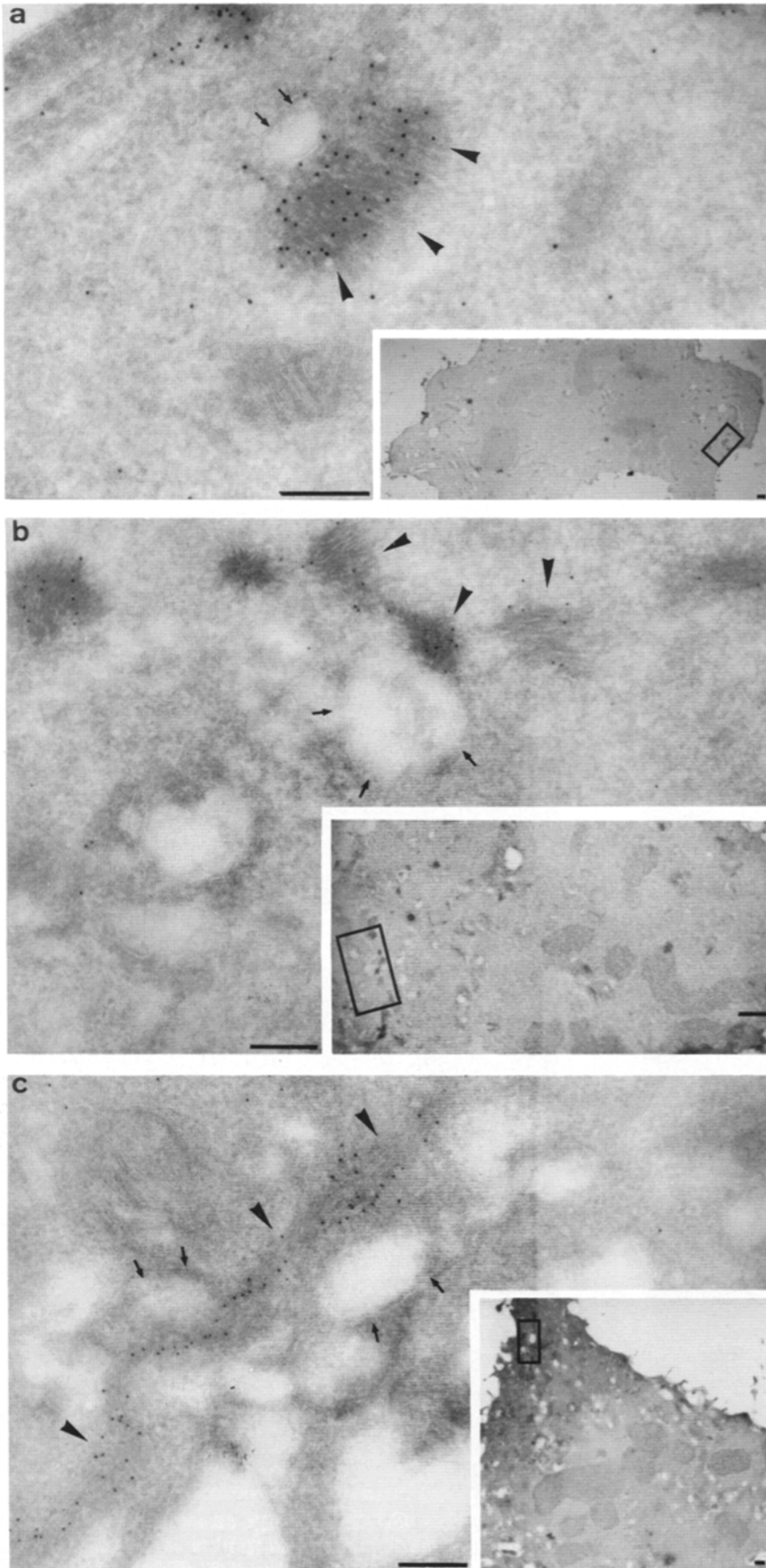


Figure 2. Association of vimentin filaments with membrane vesicles in prometaphasic CHO cells as revealed by immunoelectron microscopy. Three characteristic profiles of ultra-thin frozen sections of mitotic CHO cells decorated with the mAb 7A3 and showing the close spatial proximity of the IFs (*arrowheads*) and round vesicular structures (*small arrows*). In *a*, arrays of IFs form a partial "cap" around a small membrane vesicle (~ 150 nm in diameter). In *b*, a large vesicle (~ 390 nm in diameter) appears to form a "focal" contact with vimentin filaments. In *c*, a long vimentin bundle forms multiple lateral associations with various vesicular structures along its length. The insets in each panel show parts of the corresponding mitotic cells containing the chromosomes (lower magnification). Rectangles denote the regions from which the images shown in *a*, *b*, and *c* were taken. Bars, 200 nm.

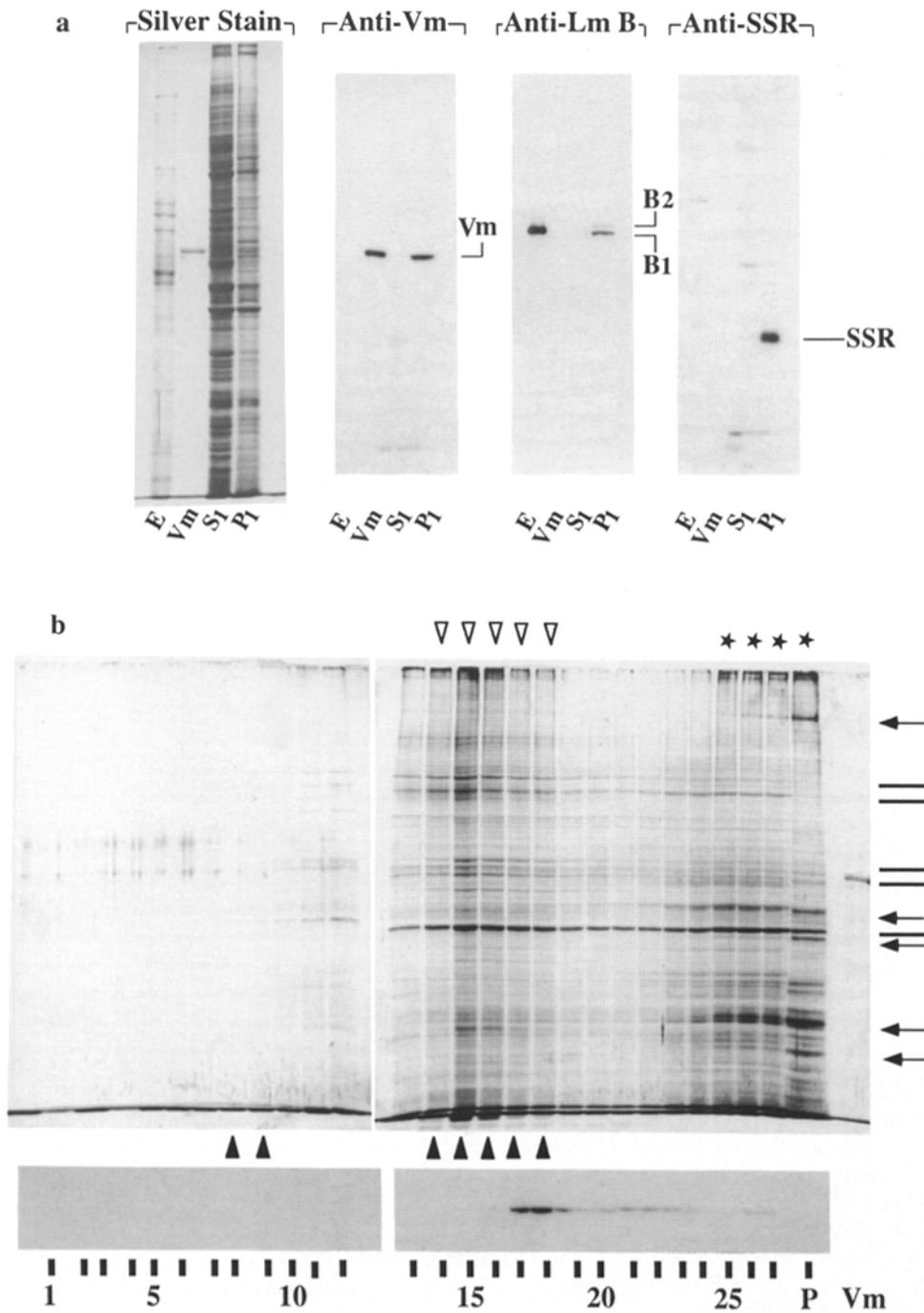


Figure 3. Subcellular fractionation of mitotic CHO cells. (a) Mitotic cells, prepared as described in Materials and Methods, were homogenized at 0°C and spun at 12,000 *g* for 20 min at 4°C. Equivalent portions of the supernatant (SI) and the pellet (PI) fraction were analyzed by SDS-PAGE, in parallel with isolated rat liver nuclear envelopes (*E*) and purified calf lens vimentin (*Vm*). The gels were stained with silver or transferred to nitrocellulose and probed with specific antibodies, as indicated. The position of vimentin, nuclear lamins B1 and B2, and the α subunit of SSR ("signal sequence receptor") are marked by bars. (b) The PI fraction was fully resuspended in KHM buffer, adjusted to 75% (wt/vol) in sucrose, loaded at the bottom of a sucrose step gradient and centrifuged (for details see Materials and Methods). The upper panel shows the electrophoretic profile of the gradient fractions after SDS-PAGE and staining with silver. *P* is the pellet obtained after centrifugation, whereas *Vm* denotes vimentin (run as a marker). The fractions containing SSR and lamin B (as determined by immunoblotting) are indicated by filled and open arrowheads, respectively. Stars correspond to lamin A and C material as detected by immunoblotting. Asterisks show proteins which did not float, whereas bars indicate proteins which migrated at the 30–50% sucrose interface. The bottom panel depicts an immunoblot of the gradient fractions with anti-vimentin antibodies. (Only the relevant area is shown.) Note that the bulk of vimentin floats at the 30–50% sucrose interphase.

using detergent-extracted, prometaphasic cell models. This study suggested that the IFs retain their physical continuity and form a fibrous cage around the chromosomes (see Introduction). To examine whether this pattern of distribution reflects the natural arrangement of the filaments in intact cells and to rule out the possibility that the filaments transiently disassemble, we examined mitotic cells at different stages of cell division. As shown in Fig. 1, *a–d*, from

prophase to late telophase, the IFs had the appearance of a highly anastomosed network and there was no indication of transient disassembly or physical breakage. In late prophase/prometaphase vimentin formed incomplete (open) cages surrounding the chromosomes. Conventional and confocal microscopy showed that arrays of fibers emanating from the cases extended to the mitotic cytoplasm and formed a complex IF network (Fig. 1, *d*, *d'* and *e–h*).

To examine the *in situ* arrangement of vimentin filaments at a higher resolution, we used immunoelectron microscopy. Although intermediate filaments could be better visualized after removal of the soluble cellular elements by detergent extraction or after fixation with methanol and pre-embedding immunolabeling, we generally avoided these manipulations to maintain membrane integrity and minimize antigen redistribution during sample processing. Thus, we performed most of the analysis on ultrathin frozen sections of intact, paraformaldehyde-fixed CHO cells (Griffiths et al., 1984).

Consistent with the indirect immunofluorescence data, we found that the vimentin filaments of prometaphasic cells were organized as parallel arrays of 10 nm fibers (Fig. 2 *c*, *arrowheads*). Upon a closer inspection, we noticed that vimentin filaments extended to the periphery of the cells and closely associated with round vesicular structures (Fig. 2, *a-c*, *small arrows*). These vesicles ranged in diameter from 100 to 400 nm and could be morphologically differentiated from flat membrane cisternae and tubular elements representing fragments of the ER or the Golgi apparatus (see below and in Lucoq et al., 1987; Pypaert et al., 1993; Zelig and Wollman, 1979). The close proximity of IFs and the membrane vesicles suggested an interaction between these two structures. However, since we could not distinguish by these methods between spatial juxtaposition and physical contact, we proceeded with subcellular fractionation.

Analysis of Mitotic Cell Homogenates and Subcellular Fractions

To separate the IFs from soluble cytosolic elements, nocodazole-arrested cells were homogenized and the lysates centrifuged at 12,000 *g*. SDS-PAGE and immunoblotting analysis of the resulting fractions (Fig. 3 *a*) showed that vimentin partitions with the pellet (P1), consistent with the idea that the IFs do not depolymerize during cell division. Most of the cellular membranes were also pelleted during the 12,000 *g* centrifugation, as demonstrated by the partitioning of the α subunit of the SSR and the type B lamins (Fig. 3 *a*).

To examine whether the vimentin filaments were bound to mitotic membranes, we resuspended the P1 fraction in homogenization buffer and subjected it to flotation through a sucrose step gradient (for details see Materials and Methods). When the gradient fractions were examined by SDS-PAGE and immunoblotting, we observed that the bulk of vimentin migrated at the 30–50% sucrose interface, overlapping with ER membranes and lamin B-associated vesicles (Fig. 3 *b*). This suggested that vimentin was strongly bound to mitotic membranes. A smaller percentage of vimentin was found within the 50% sucrose phase, whereas only traces of this protein were detected in the pellet fraction

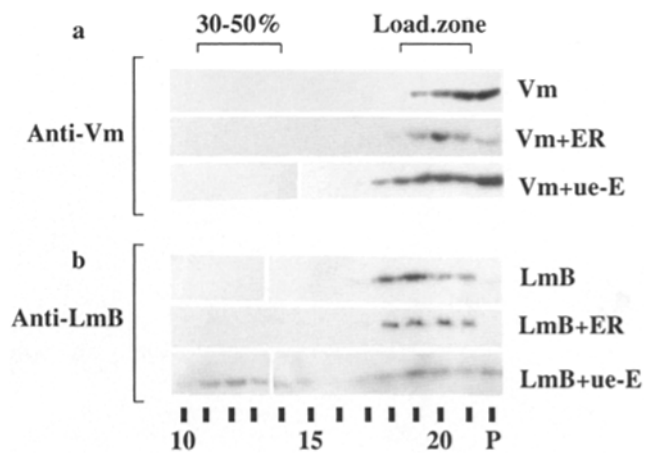
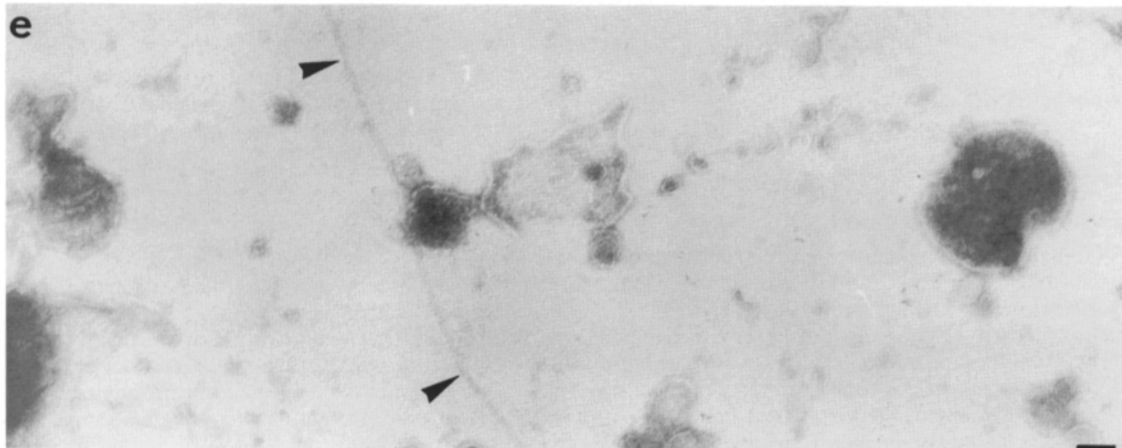
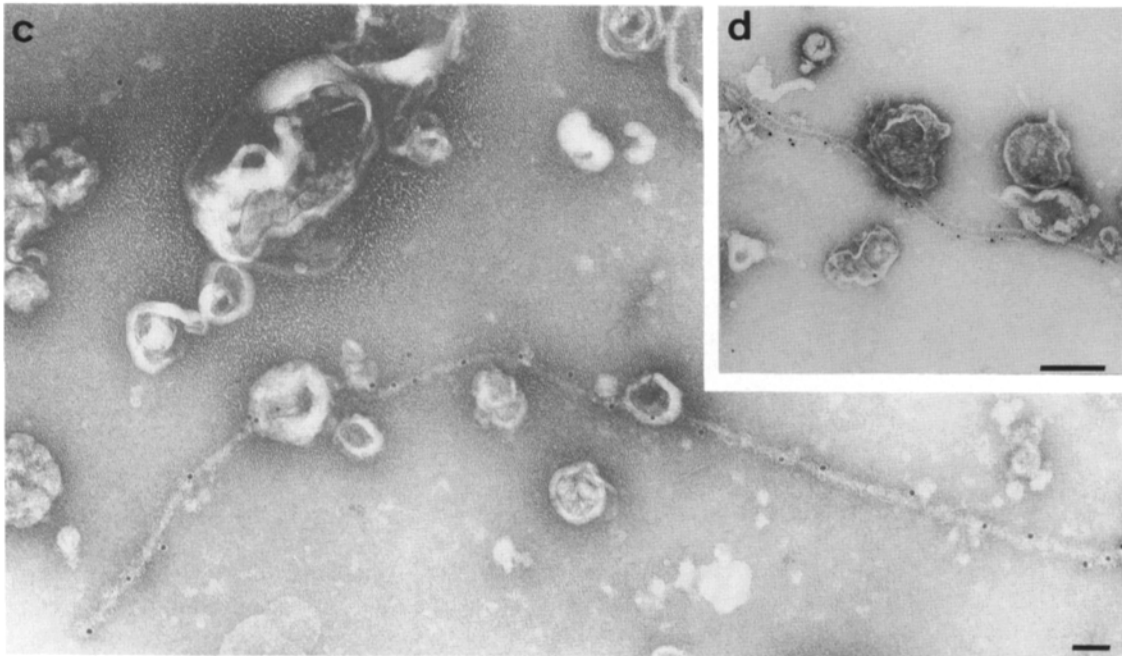
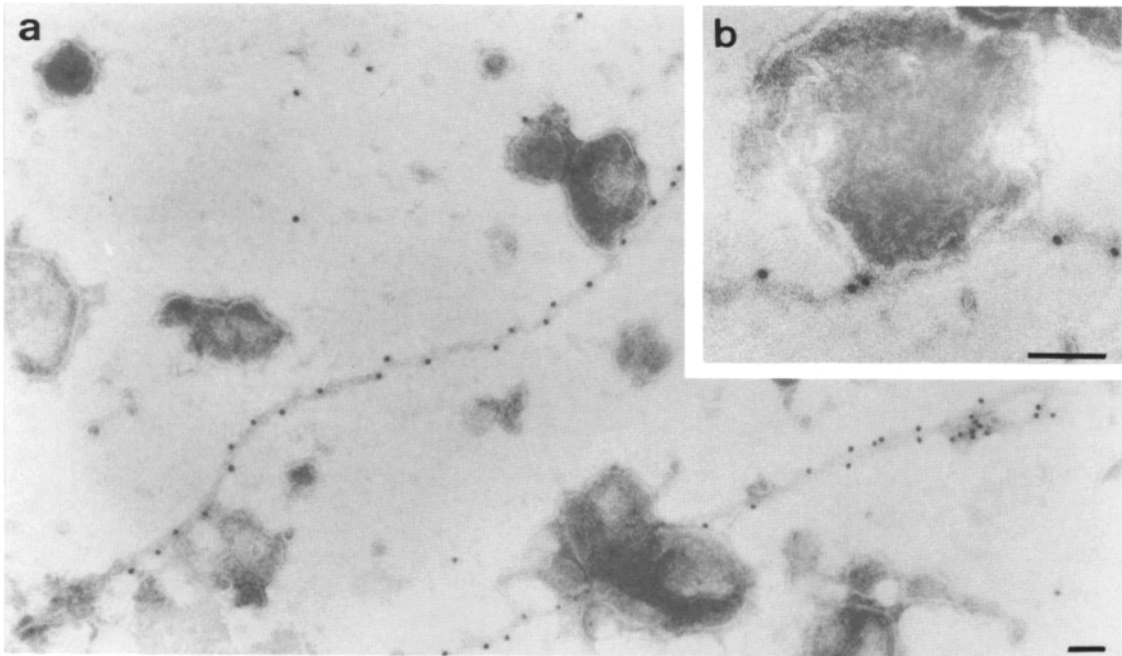


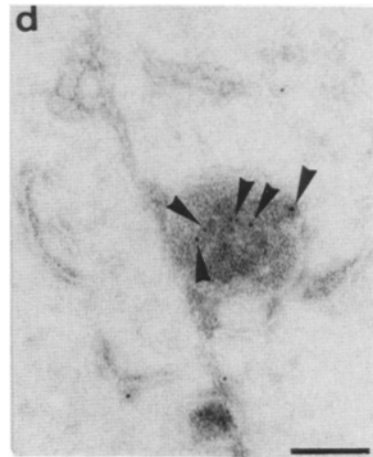
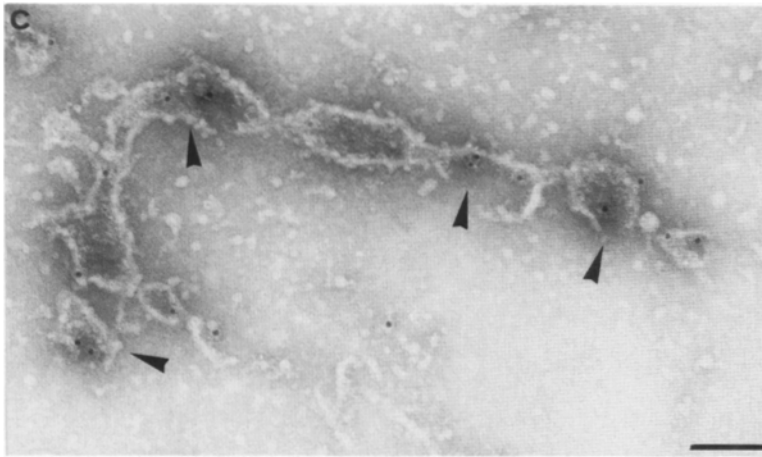
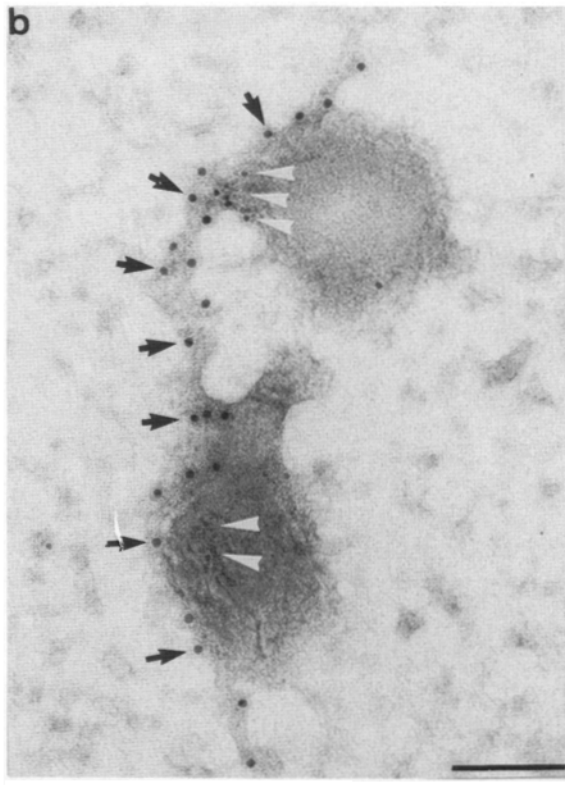
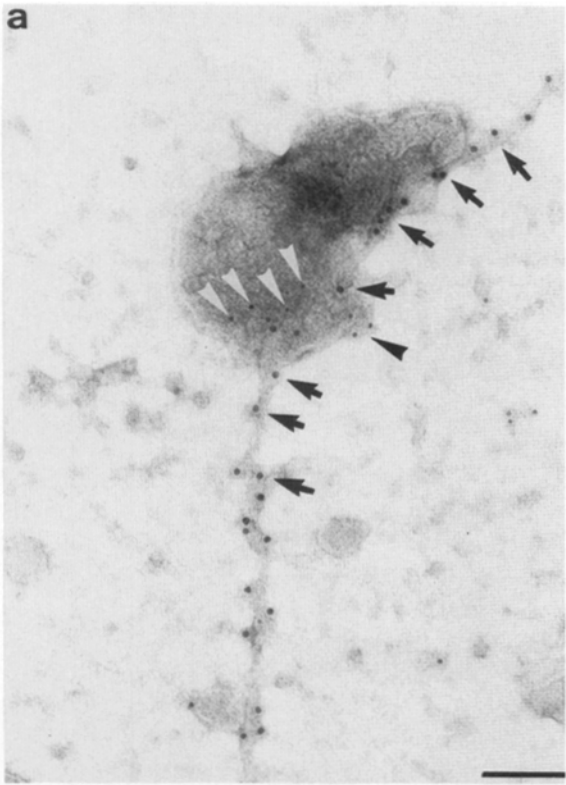
Figure 4. Assessment of the binding of purified vimentin and lamin B to membrane preparations in the presence of mitotic cytosol. Purified vimentin (*a*) or lamin B (*b*) were incubated, as indicated, with salt-washed rat liver microsomes or lamin-depleted nuclear envelopes in the presence of mitotic cytosol (for details see Materials and Methods). The samples were subjected to flotation in sucrose step gradients (as in Fig. 3) and the resulting fractions were analyzed by SDS-PAGE and immunoblotting. *Vm*, vimentin; *LmB*, nuclear lamin B1; *ER*, microsomes; *ue-E*, nuclear envelopes. The fraction numbers are indicated by bars and the pellet fraction by *P*. The area of the 30–50% sucrose interface, as well as the loading zone, are marked by brackets. (Only the relevant parts of each gradient are shown.)

(Fig. 3 *b*). In contrast to vimentin, several other proteins (including some lamin A and C material present in the P1 fraction) were found exclusively in the loading zone or the pellet fraction (Fig. 3 *b*, *arrowheads* and *asterisks*). Analysis of whole mitotic homogenates instead of the P1 fraction yielded the same results, except that the trailing of vimentin in the 50% phase was more pronounced (not shown).

To explore the possibility of nonspecific binding due to hydrophobic interactions between the membrane lipids and the IFs, we performed a series of *in vitro* binding assays. These experiments involved coincubation of salt-washed microsomes or urea-extracted nuclear envelopes with preassembled vimentin filaments in the presence of mitotic cytosol which carries enzymes and factors sufficient to induce mitotic disassembly *in vitro*. Analysis of the reaction mixtures by flotation in sucrose gradients clearly showed that vimentin does not bind to microsomal membranes or urea-extracted nuclear envelopes (Fig. 4 *a*) (for relevant data also see Georgatos and Blobel, 1987). The same was observed when purified rat liver lamin B was coincubated with microsomes and mitotic cytosol (Fig. 4 *b*). However, in agreement

Figure 5. Identification of IF-membrane complexes by whole-mount immunoelectron microscopy. (*a*) Representative electron microscopic profile of a specimen taken from the 30–50% interface of a flotation gradient (see Fig. 3) and decorated with anti-vimentin antibodies (*aV2*) and protein A-gold. Note the presence of long vimentin filaments and their multiple associations with membrane vesicles. (*b*) A detail of a filament-vesicle contact in a different sample of the same preparation. Note the close apposition and the extensive contact between the labeled filament and the surface of the vesicle. (*c*) Profile of a specimen taken from a fresh, unfractionated homogenate of mitotic CHO cells and processed as in *a*. (*d*) A different example from a preparation similar to this shown in *c* where a pair of parallel vimentin filaments contacts two vesicles. (*e*) A control showing a field from a specimen incubated only with Protein A-gold. Note the complete absence of labeling. Arrowheads indicate a vimentin filament associated with a vesicle. Bars, 100 nm.





to previous studies (Worman et al., 1988), lamin B was able to associate and float with the urea-stripped and lamin-depleted nuclear envelopes (Fig. 4 b), probably by virtue of binding to "lamin B receptors" (Worman et al., 1988; Foissner and Gerace, 1993). These data confirm that the comigration of IFs and lamin B with membrane vesicles is not the result of "sticking" to membrane lipids.

To assess the physical state of vimentin in the mitotic cell fractions and to visualize its associations with other organelles, we performed whole mount EM. In survey experiments we noticed that the material floating in the 30–50% sucrose interface contained some distinct membranous structures. Based on size or shape criteria, we could distinguish the following types of membranes: (a) small, round vesicles (SRVs) with diameters in the order of 100 nm; (b) medium size, round vesicles (MRVs) with diameters in the range of 100 to 300 nm; (c) large, round vesicles (LRVs) with a size equal to or greater than 400 nm; and (d) flat, or tubular membrane elements (TMEs) possessing various sizes. Immunostaining with anti-vimentin antibodies and protein A-gold showed that the fractions from the 30–50% sucrose interface contained long vimentin filaments laterally associated with SRVs and MRVs (Fig. 5 a). (The specificity of the immunodecoration of the vimentin filaments is demonstrated in Fig. 5 e, where a specimen similar to the above has been incubated with protein A-gold alone.) The area of contact between the filaments and the vesicles was extensive (Fig. 5 b). The same vimentin-membrane associations could be detected in material obtained from whole mitotic cell homogenates processed for negative staining within minutes from preparation (Fig. 5 c). Thus, we could rule out fortuitous binding of the filaments to the membranes during flotation. Analyzing unfractionated mitotic homogenates, we sometimes encountered pairs of intertwined vimentin filaments laterally associated with the vesicles (an example is shown in Fig. 5 d). These structures probably represented remnants of IF bundles which had been mechanically disrupted during homogenization.

Characterization of the IF-associated Vesicles by Whole Mount Immunoelectron Microscopy

To characterize the vimentin-associated vesicles, we used immunocytochemical methods. Double-staining with anti-lamin B and anti-vimentin antibodies showed that the vimentin-associated membranes carry on their surfaces nuclear lamin B (Fig. 6, a and b). Most of the lamin B-bearing vesicles could be classified either as SRVs, or as MRVs. Interestingly, in the majority of the vesicles, the bulk of lamin

Table I. Presence of Various Markers in IF-associated and -free Mitotic Vesicles

Vimentin-associated vesicles		Free vesicles
Lamin B	67% (n = 523)	9% (n = 410)
p58	58% (n = 69)	20% (n = 200)
WGA	70% (n = 80)	66% (n = 230)
SRP Rc	12% (n = 127)	37% (n = 555)
ER/Golgi proteins	6% (n = 123)	50% (n = 207)
Mannose 6P Rc	2% (n = 79)	12% (n = 331)
Control (No Ab)	1% (n = 151)	2% (n = 411)

Total cell membranes from homogenized mitotic CHO cells were decorated by specific antibodies and protein A-gold as explained in Materials and Methods. (n) equals the number of vesicles counted in each immunolabeling experiment. For WGA labeling, mitotic membranes isolated by flotation were used and the specimens were sequentially incubated with biotin-WGA, anti-biotin antibodies, and protein A-gold. For staining with anti-p58 antibodies, homogenates of mitotic chicken hepatoma cells (DU 249) were used. Controls involved incubation of the specimens with protein A-gold alone (EM profile shown in Fig. 5 e).

B was found near the region where filaments contacted the membranes. Antibodies recognizing ER/Golgi membrane markers (Louvard et al., 1982) did not decorate the filament-associated vesicles, but heavily labeled flat membrane cisternae which possessed a characteristic morphology (Fig. 6 c).

To examine whether the IF-associated vesicles contain markers of the inner nuclear membrane, we also used antibodies directed against p58, an integral protein of the inner nuclear membrane (Worman et al., 1988, 1990). Because our anti-p58 antibodies did not recognize the mammalian form of p58, in this case we immunodecorated material obtained from chicken mitotic (DU 249) cells. As shown in Fig. 6 d, the IF-associated vesicles were positively stained by the anti-p58 antibodies.

Morphometric analysis presented in Table I showed that the majority of the vesicles which associated with vimentin filaments contained nuclear lamin B and p58. Only a small percentage of the IF-bound vesicles possessed the "docking protein" (SRP receptor), other ER/Golgi proteins, or the mannose 6-phosphate receptor. This contrasted the relative abundance of the same markers among "free" vesicles where the ER/Golgi proteins and the "docking protein" (SRP receptor) were much more frequently seen than lamin B and p58.

The majority of the IF-bound vesicles could be decorated with WGA (Table I), a lectin known to react with proteins carrying O-linked N-acetylglucosamine or sialic acid resi-

Figure 6. Characterization of the vimentin-associated vesicles by whole-mount immunoelectron microscopy. (a and b) Two representative examples showing contacts between lamin B-carrying vesicles and vimentin filaments. The specimens were taken from a fresh mitotic homogenate and decorated with anti-vimentin antibodies (mAb 7A3)/protein A-gold (large gold, arrows) as well as with anti-lamin B antibodies (aNLS)/protein A-gold (small gold, black or white arrowheads). Notice that most of the lamin B is localized at the region where the filaments contact the vesicles. (c) Immunostaining of a preparation similar to the one shown above using specific antibodies that recognize a group of Golgi and ER membrane proteins (Louvard et al., 1982) and protein A-gold. Note that the decorated TME are morphologically distinct from the IF-associated vesicles. (d) Staining of the IF-associated vesicles with the anti-p58 antibody (aR7) and protein A-gold (arrowheads). This specimen has been taken from a homogenate of mitotic DU 249 cells (see text). (e) Double staining of a (grazing) ultrathin frozen section of a mitotic CHO cell with anti-vimentin antibodies (large gold, large arrowheads) and anti-lamin B antibodies (small gold, small arrowheads). Note the "cap" of vimentin around the lamin B-carrying vesicle. Although the degree of labeling is low, measurements of the density of gold particles/unit area in this and other specimens suggest a specific immunostaining. Bars, 100 nm.

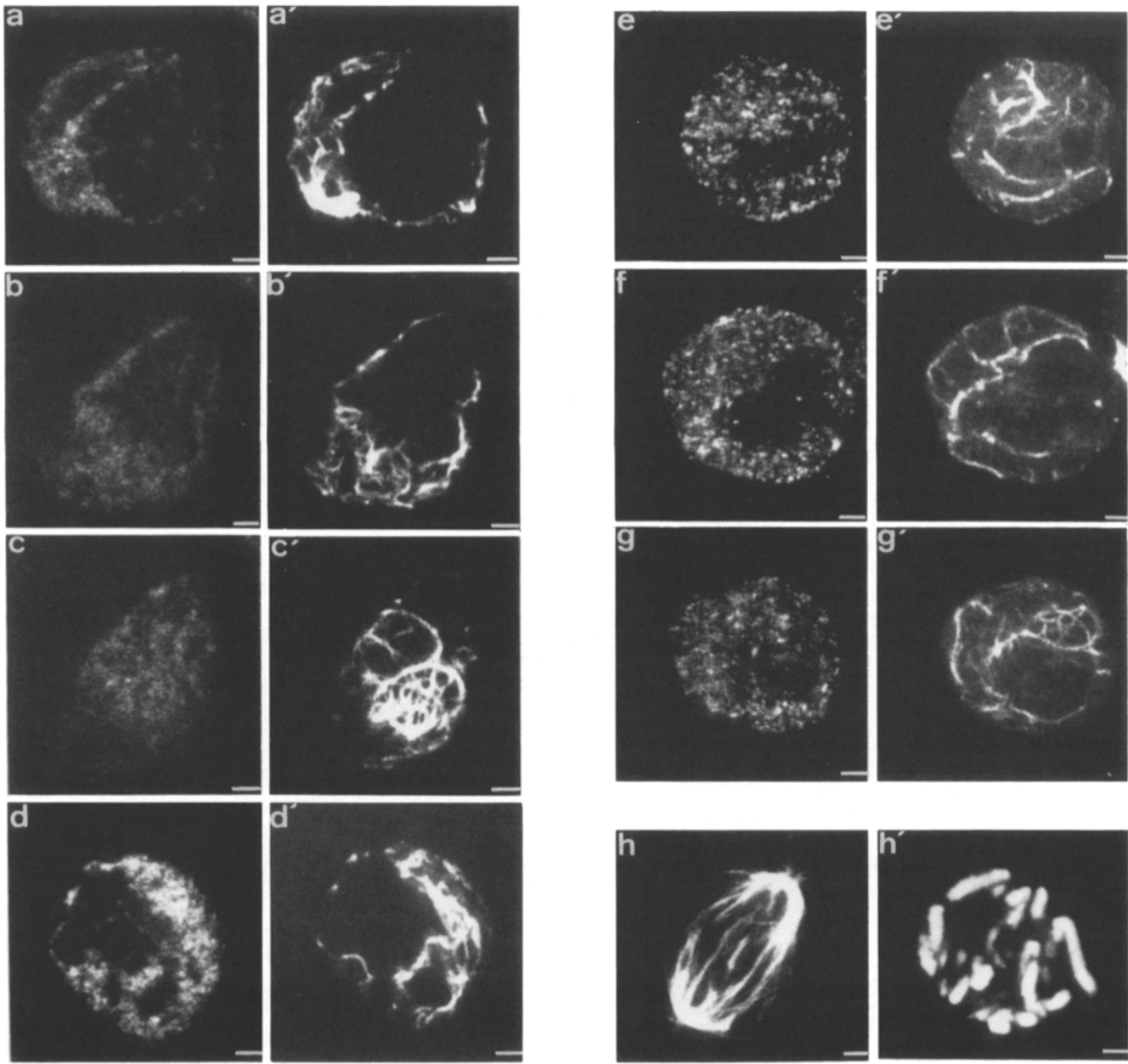


Figure 7. Colocalization of vimentin filaments and lamin B-carrying vesicles as detected by confocal microscopy. Prometaphasic CHO cells were doubly stained and processed for indirect immunofluorescence as specified in Materials and Methods. The samples were first screened in a conventional microscope to ensure that all of the cells showed the same distribution of the marker proteins. Subsequently, four to five cells from each coverslip were subjected to analysis by confocal microscopy and 6 to 15 optical sections of each cell ($0.8\text{-}\mu\text{m}$ apart, from the bottom to the top of the cell) were recorded, depending on the thickness of the specimen. The cells shown here have been decorated as follows. Panels on the left handside: (a' - d') labeling with anti-vimentin antibodies (mAb 7A3, Rhodamine); (a - d) labeling with anti-lamin B antibodies (FITC). The first three pairs of micrographs (a , a' - c , and c') show different optical sections of the same cell, whereas the last pair (d and d') shows a single section of a different cell. Panels on the right handside: (e' - g') labeling with anti-vimentin antibodies (mAb 7A3, Rhodamine); (e - g) labeling with anti-mannose 6-phosphate receptor antibodies (FITC); (h') labeling with Propidium Iodide (a DNA stain); (h) staining with anti-tubulin antibodies (FITC). The first three pairs of micrographs (e , e - g , and g') show different optical sections of the same cell, whereas the last pair (h and h') shows a single section of a different cell. The distances between the different focal planes are as follows: (a and a') to (b and b') = $1.6\ \mu\text{m}$ (b and b' to c and c') = $0.8\ \mu\text{m}$; (e and e' to f and f') = $3.2\ \mu\text{m}$; (f and f' to g , and g') = $4.8\ \mu\text{m}$. Of note is that in some cases adjacent optical sections of the same cell show a distinctly different pattern of IF distribution (e.g., sections b' and c'), whereas in other cases the IF pattern starts to change after 5 or 6 sections (e.g., sections f' and g'). This is due to differences in thickness and to heterogeneity in the three-dimensional distribution of the IFs. Bars, $2\ \mu\text{m}$.

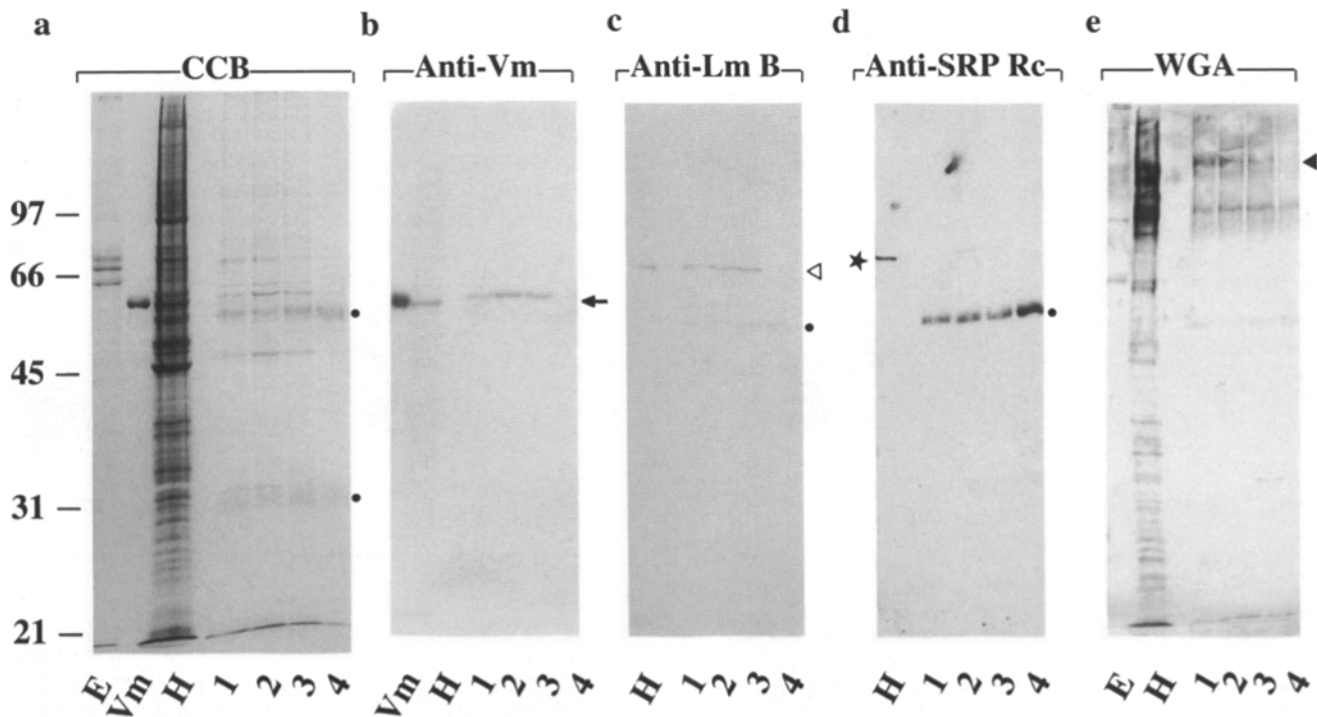


Figure 8. Coisolation of vimentin, lamin B, and a 180-kD WGA-binding protein from whole mitotic CHO cell homogenates. Equal amounts of a mitotic CHO homogenate were incubated with anti-lamin B antibodies (aNLS) (lanes 1), affinity-purified aV2 IgG (lanes 2), aV2 antiserum (lanes 3), and normal rabbit IgG (lanes 4). The antibodies were pre-reacted with magnetic beads carrying anti-rabbit antibodies as specified in Materials and Methods. The immune complexes were harvested by a magnet and resolved by SDS-PAGE (Coomassie blue profile shown in *a*). Replica gels were blotted onto nitrocellulose filters and probed with the anti-vimentin antibody 7A3 (*b*), with the #16 anti-lamin B antibody (*c*), with the anti-SRP receptor antibody (*d*), or with WGA-biotin and anti-biotin antibodies (*e*). The blot shown in *e* has been pre-blotted with anti-lamin B antibodies. The position of vimentin is indicated by an arrow, lamin B is indicated by an open arrowhead, the SRP receptor protein is indicated by an asterisk, and the 180-kD WGA-binding protein is indicated by a closed arrowhead. Dots denote the IgG heavy and light chains, whereas E, Vm and H correspond to rat liver nuclear envelopes, purified lens vimentin, and the mitotic CHO homogenate used for the immunoisolation. Molecular weight values (in kD) are indicated on the left. Note that the anti-SRP receptor antibody readily detects the corresponding protein in the starting material, but yields a very faint band in one of the immunisolates.

dues. However, the same lectin decorated a high percentage of free vesicles, as it could be expected from the fact that it binds to numerous glycoproteins residing in different compartments. Due to the lack of monospecificity, we could not distinguish whether the staining was specific for the IF-associated vesicles; however, data shown below suggest that WGA specifically reacts with a 180-kD glycoprotein which resides in the IF-associated membranes.

Colocalization of Lamin B-carrying Vesicles and Vimentin Filaments in Whole Mitotic Cells

Associations between lamin B-carrying vesicles and vimentin IFs could also be demonstrated by immunoelectron microscopy on paraformaldehyde-fixed ultrathin frozen sections of prometaphasic cells (Fig. 6 *e*). However, in general the immunostaining in these doubly decorated preparations was weak. Therefore, to better examine the interaction of lamin B-containing vesicles with IFs in situ, we performed double-immunofluorescence assays and analyzed the specimens by confocal microscopy. These data are presented in Fig. 7 (*a-d* and *a'-d'*). Staining with anti-lamin B antibodies yielded a dense punctate pattern, in agreement with the fact that the corresponding antigen is located in membrane vesicles. Labeling with anti-vimentin produced a clearly fila-

mentous pattern consisting of anastomosed filaments and filament bundles. Consistent with the previous results, the lamin B-carrying vesicles colocalized with vimentin filaments, especially in areas where well-dispersed fibers could be discerned. The apparent colocalization of lamin B and vimentin was not due to "cross-talk" between the two fluorochromes. This could be confirmed by varying the relative intensities of the fluorescence, by experimenting under conditions of low laser power, and by performing other double-labeling experiments. For example, double-immunofluorescence for vimentin and the mannose 6-phosphate receptor protein yielded distinct immunostaining patterns (Fig. 7, *e-g* and *e'-g'*) and only occasionally individual vesicles were seen in the neighborhood of vimentin filaments. Another control in which microtubules and chromosomes were visualized under the same staining conditions also showed the complete lack of "cross-talk" between different fluorochromes (Fig. 7, *h* and *h'*).

Identification of Vimentin-associated Proteins

To confirm the in situ data by biochemical methods, we attempted to immunoisolate native vimentin en bloc with its partners. To accomplish this, we mechanically disrupted synchronized CHO cells and used specific anti-vimentin an-

tibodies immobilized on magnetic beads to absorb the vimentin filaments (for details see Materials and Methods). Based on semi-quantitative assays, we calculated that 30–50% of the input vimentin could be immunisolated by this method, depending on the amount of antibody used. Analysis of the antibody-bound material by SDS-PAGE revealed that vimentin was associated with a distinct group of cellular proteins (Fig. 8 *a*). In agreement to the immunoelectron microscopy data, the immunisolates were essentially depleted of ER membrane elements such as the “docking protein” (SRP receptor) (Fig. 8 *d*). However, by probing blots of the same material with specific antibodies, we could readily identify nuclear lamin B as one of the components (directly

or indirectly) associated with the IFs (Fig. 8 *c*). To confirm this observation, we performed the reverse experiment using magnetic beads coated with anti-lamin B antibodies. As shown in Fig. 8 (*b* and *c*), the anti-lamin B antibodies precipitated both vimentin and lamin B.

Since most of the vimentin-associated vesicles could be decorated by WGA (see above), we probed blots of the immunisolated material with this lectin. (As a routine, these blots were first probed by anti-lamin B antibodies to ensure that the immunisolation had worked.) We found that the vimentin-associated proteins included a 180-kD polypeptide (Fig. 8 *e*) which may account for the binding of the lectin to the vesicles.

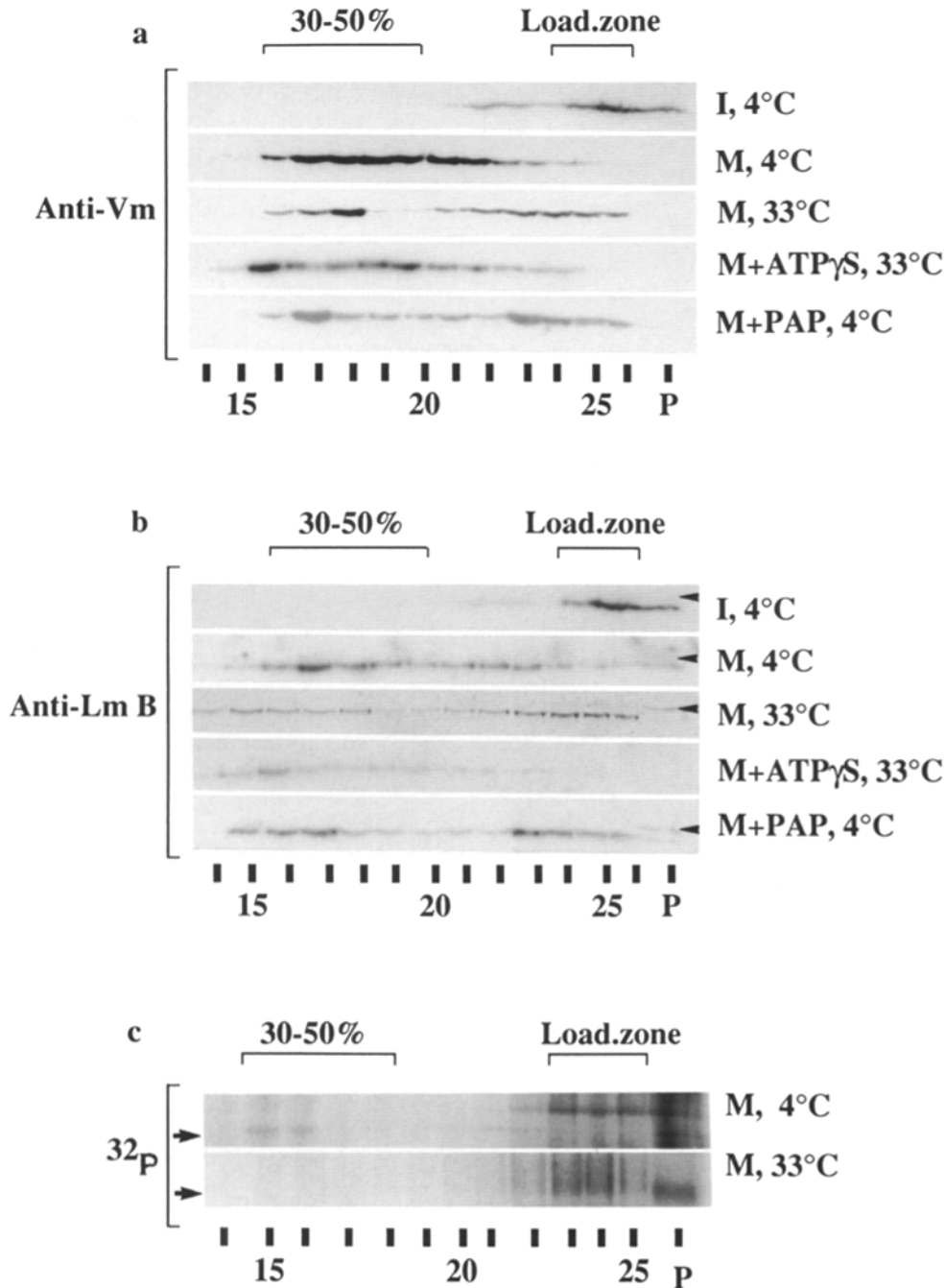


Figure 9. Modulation of vimentin-membrane interactions during postmitotic reassembly in a CHO cell-free system, as detected by flotation in sucrose gradients. Homogenates from interphasic (*I*) or mitotic (*M*) cells were prepared as specified in Materials and Methods. The mitotic homogenates were incubated for 2 h at 4°C or 33°C, in the presence or absence of ATP γ S, with or without pretreatment with potato acid phosphatase (*PAP*), as indicated. After analysis in sucrose step gradients (see Fig. 3), the fractions were processed for Western blotting using anti-vimentin antibodies (*a*), or anti-lamin B antibodies (*b*). Only the relevant parts of the gradients are shown (fractions 13–27), because fractions 1–12 were free of vimentin and lamin B reactivity (see Fig. 3). *P* represents the material pelleted in each case and the bars underneath each panel correspond to fraction numbers. Arrowheads denote the position of the (quantitatively) minor lamin B2. *c* shows fractions of mitotic cell homogenates analyzed in sucrose gradients after SDS-PAGE and autoradiography. In this case the homogenates have been supplemented with a trace quantity of 32 P-ATP at 4°C and further incubated at the indicated temperatures. The position of vimentin (as detected by parallel immunoblotting) is indicated by an arrowhead.

Effects of Phosphorylation and Dephosphorylation

Knowing that vimentin and lamin B undergo reversible hyperphosphorylation during mitosis (Gerace and Blobel, 1980; Evans and Fink, 1982; Chou et al., 1990), we sought to examine whether the interaction of vimentin with the lamin B-carrying membranes are affected by such a modification. To answer this question in a physiological context, we compared the flotation characteristics of vimentin and lamin B when homogenates of mitotic CHO cells are incubated either at 4°C (a condition maintaining the mitotic state) or, at 33°C (a condition promoting postmitotic dephosphorylation and allowing nuclear reassembly to proceed; see Burke and Gerace, 1986). As a control, we also analyzed by flotation interphasic CHO cell homogenates. The results of this analysis can be summarized as follows. First, "interphasic" vimentin did not float (Fig. 9 a, top), confirming the previous results and showing that broken IFs do not "stick" to cytoplasmic membranes. Second, the vimentin filaments of mitotic cells floated up to the 30–50% interface when the homogenates were incubated at 4°C (Fig. 9 a, second panel from top). Third, when the mitotic homogenates were incubated at 33°C and then subjected to flotation, nearly half of the vimentin material migrated to the 30–50% interface, whereas the other half remained in the 50% sucrose phase and the loading zone (Fig. 9 a, third panel from top). This biphasic profile could be reproduced by treating mitotic homogenates (kept at 4°C) with exogenous phosphatase (Fig. 9 a, bottom). On the contrary, when ATP γ S was added to the homogenates before raising the temperature to 33°C, most of vimentin floated at the 30–50% sucrose interface (Fig. 9 a, second panel from bottom).

The opposing effects of ATP γ S and phosphatase strongly suggested that the vimentin-membrane interactions are modulated by phosphorylation and dephosphorylation. To test this interpretation further, mitotic homogenates were supplemented with ³²P-ATP at low temperature and then incubated either at 4°C (to maintain the "mitotic" state), or at 33°C (to induce postmitotic reassembly). Analysis in sucrose gradients followed by SDS-PAGE and autoradiography confirmed that the "mitotic" vimentin loses all the phosphate that it acquired under "mitotic" conditions as the cell-free system progresses to nuclear reassembly (Fig. 9 c).

In parallel to vimentin, we also examined the partitioning of lamin B under the same conditions. As it could be expected, "interphasic" lamin B did not float (Fig. 9 b, top). However, most of the lamin B (B1) migrated at the 30–50% interface when the mitotic homogenates were incubated at 4°C (Fig. 9 b, second panel from top), consistent with the fact this protein is associated with membrane vesicles during mitosis. After an incubation of the homogenates at 33°C, lamin B (B1) showed exactly the same biphasic profile as vimentin (Fig. 9 b, third panel from top). Finally, treatment with acid phosphatase at 4°C and addition of ATP γ S resulted in flotation profiles which were almost identical to those of vimentin's (Fig. 9 b, first and second panel from bottom).

Interestingly, nuclear lamin B2, a quantitatively minor lamin barely detectable in CHO cells, was consistently found in the pellet fraction and did not float with membranes (Fig. 9 b, lanes P). However, similar experiments with mitotic DU 249 cells, in which lamin B2 represents the major lamin form, showed that the avian analogue of this protein does

float with membranes (Meier, J., and S. D. Georgatos, manuscript in preparation). We do not have at present a clear explanation for this phenomenon.

Discussion

In Vivo and In Vitro Associations between Vimentin Filaments and Membrane Vesicles

Using immunoelectron and confocal microscopy, we have shown that the vimentin filaments of prometaphasic cells are associated with a distinct population of membrane vesicles. Lateral associations between IFs and membrane vesicles have also been documented in mitotic cell homogenates and in material isolated by flotation in sucrose gradients.

Since vesicle binding to vimentin filaments does not seem to require an intact filament network, associations between IFs and membrane vesicles may also occur in cells which disassemble their IFs during mitosis. The complete disassembly of cytoplasmic IFs during mitosis does not seem to be a common phenomenon. Although indirect immunofluorescence observations sometimes create the impression that the entire IF system of certain mitotic cells breaks down into non-filamentous "spots", more careful analysis by EM reveals that the "spots" include numerous filamentous forms and that the cytoplasm of these cells contains abundant, micrometer-long IFs (see Franke et al., 1982; and Rosevear et al., 1990 for illustrative paradigms).

It has been previously reported that vimentin filaments bind under *in vitro* conditions to vesicles reconstituted from total cell lipids and phospholipid mixtures (Traub et al., 1986). Apparently, such hydrophobic interactions do not account for the findings reported here: under our assaying conditions, vimentin does not bind to microsomal membranes and to lamin-depleted nuclear envelopes. Furthermore, as discussed below, the IF-associated vesicles carry specific markers and appear to dissociate from the IFs in response to postmitotic dephosphorylation. Vimentin is also known to form a filament basket around lipid globules present in differentiating adipocytes. The IF basket is "sandwiched" between the surface of the globule and a flat, fenestrated membrane cisterna, suggested to represent ER (Franke et al., 1987). The lipid globule-vimentin association does not seem to be relevant to the interactions we have studied in this work.

Origin of the Vimentin-associated Vesicles

All the data we have obtained so far point to the conclusion that the lamin B-carrying and vimentin-associated vesicles are derived from the inner nuclear membrane. This interpretation is further supported by recent studies which show that lamin B remains (at least in part) associated with the inner nuclear membrane protein p58 during mitosis (Meier, J., and S. D. Georgatos, manuscript in preparation). Interestingly, the IF-bound vesicles do not seem to contain ER and Golgi membrane proteins. Consistently, most of the membranes labeled with anti-ER and anti-Golgi antibodies are ultrastructurally different from the lamin B-carrying vesicles.

Our data are in line with previous observations showing that the majority of vesicles which assemble around the chromosomes at the end of mitosis, and which presumably carry inner nuclear membrane proteins, are distinct from the bulk

of the ER membranes (Wilson et al., 1988). However, these findings differ from the results obtained in a previous study with mitotic chicken erythroid cells (Stick et al., 1988). In the latter case, lamin B2 was found to partition with membranes which (on the basis of their morphology) were suggested to represent "ER". We believe that part of this difference is due to the fact that the disassembly of the nuclear envelope yields vesicles with different morphologies in different cell types. This becomes evident when one compares the flotation characteristics of mitotic membranes obtained from CHO and DU 249 cells (Meier, J., and S. D. Georgatos, unpublished observations). Another complication in directly comparing these previous results with ours is that lamin B2 partitions with membrane vesicles in chicken cells, where it constitutes the major lamin B species, but behaves differently in CHO cells where it represents a small fraction of the lamin B complement (see Results). Further studies with isotype-specific or species-specific antibodies may shed more light on this problem.

Components Involved in the Vimentin-membrane Interaction

Because nuclear lamin B and lamin B-related proteins bind to vimentin and other type III IF proteins *in vitro* (Cartaud et al., 1989, 1990; Djabali et al., 1991; Georgatos and Blobel, 1987; Georgatos et al., 1987; Papamarcaki et al., 1991) and since lamin B is located primarily at the sites of filament-membrane contact (this report), it is tempting to speculate that the two proteins interact during mitosis. Supporting this interpretation is the fact that vimentin and lamin B can be coimmunoprecipitated by specific antibodies from whole cell lysates. However, against the idea of a direct interaction is the fact that 70% of the lamin B can be extracted from the membranes with Triton X-100, whereas vimentin remains insoluble under these conditions (Maison, C., and S. D. Georgatos, unpublished observations; see also Gerace and Blobel, 1980). It is possible that the Triton-resistant fraction of lamin B (a reproducible 30% in our hands) resides in vesicles tightly bound to IFs, whereas the Triton-soluble lamin B resides in non-associated and loosely bound vesicles. Another possibility could be that the non-ionic detergent lowers the stability of the lamin B-vimentin complexes by dispersing the clusters of lamin B. The clustering of lamin B may facilitate binding to the filaments in a cooperative fashion. Future studies directed to this problem may enable us to distinguish whether lamin B is indeed the factor which links the filaments to the membranes, or whether it constitutes a mere "passenger" of the vimentin-associated vesicles.

Regulation of the Vimentin-Membrane Interactions during Postmitotic Reassembly

When homogenates of mitotic CHO cells are incubated at temperatures non-permissive for postmitotic reassembly and then analyzed in sucrose gradients, a significant amount of vimentin floats in the 30-50% interface together with the lamin B-carrying vesicles. However, the relative amount of floating vimentin is reduced when the homogenates are analyzed after an incubation at temperatures allowing postmitotic reassembly to proceed. This difference can be explained in two ways. First, concomitant with postmitotic

reassembly, some of the filaments may dissociate from the membranes in response to dephosphorylation. Alternatively, vimentin filaments may remain bound to vesicles which start assembling around the (fast-sedimenting) chromosomes. We favor the first interpretation because when we examined *in vitro* reassembled nuclei by EM we did not observe an accumulation of IFs in the vicinity of the nascent nuclear envelope (Pyrpasopoulou, A., and S. D. Georgatos, unpublished observations), as one would expect if the second scenario were correct. The absence of IFs around reassembled nuclei is also apparent in several previously published images (Newport, 1987; Newport et al., 1990; Wilson and Newport, 1988; Burke and Gerace, 1986; Boman et al., 1992).

Vimentin and lamin B are known to be reversibly hyperphosphorylated during mitosis in many cells including the CHO cells (Chou et al., 1990; Evans and Fink, 1982; Gerace and Blobel, 1980; Burke and Gerace, 1986; Peter et al., 1990). It is therefore reasonable to suspect that this modification affects the protein-protein interactions involved in the association between the filaments and the lamin B-carrying membranes. Our data strongly support that phosphorylation/dephosphorylation plays a key role in the regulation of vimentin-membrane interactions because addition of ATP γ S to mitotic homogenates increases the relative amount of floating vimentin, whereas treatment with phosphatase decreases it.

Potential Functions

A straightforward interpretation of our results could be that vimentin filaments "dock" nuclear envelope-derived vesicles during mitosis. Such a docking may facilitate the sorting of these vesicles away from other membranes derived from the ER and the Golgi apparatus (see Introduction). Alternatively, the segregation of nuclear envelope vesicles onto IFs may prevent the premature association of these membranes with the surfaces of the chromosomes. Finally, another interesting possibility is that the segregation of vesicles away from the mitotic apparatus facilitates the movement and translocation of the sister chromatids in an "organelle-free" zone. It is conceivable that the generation of such a zone in the area of the spindle may involve transient binding of different kinds of vesicles to cytoskeletal elements (such as the IFs and the actin microfilaments), or to anchorage sites located at the plasma membrane.

This work is dedicated to Lukia and Artemis on the occasion of their 3rd birthday.

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