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ARTICLES

Peripheral Nuclear Matrix Actin Forms Perinuclear Shells

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Abstract Perinuclear actin shells have been reported in a variety of organisms. The shells have been identified by staining perinuclear material with fluorescently-labelled phalloidin, but have not been localized to a specific subcellular compartment at the ultrastructural level. We show here that the shells of 3T3 cells lie in the peripheral nuclear matrix. Nuclear shells and matrix actin in other parts of the nucleus are not usually detected by immunohistochemical staining because they are inaccessible to antibodies or to phalloidin. Immunohistochemical detection of nuclear actin is only possible during its deposition at the end of mitosis, or in interphase nuclei that have been extracted with detergent, digested with nucleases and washed with high salt buffers. J. Cell. Biochem. 70:240–251, 1998.

Key words: actin; actin-like proteins; lamin; nuclear matrix; perinuclear actin shells

The presence of intranuclear actin is well established. Ultrastructural and immunohistochemical studies have demonstrated that actin is present in aggregates or diffuse patterns throughout nuclei of various cell types (dorsal root ganglion neurons and PC12 cells [Milankov and DeBoni, 1993; Sahlaset al., 1993]; rat oocyte [Funakiet al., 1995]; *Rana* oocyte [Parfenovet al., 1995]). Cell fractionation and immunoblot analysis has shown that actin is associated with nuclei (DRG neurons and PC12 cells [Milankov and DeBoni, 1993; Sahlaset al., 1993]; frog oocytes [Clark and Merriam, 1977; Parfenovet al., 1995]; rat hepatocytes [Bachset al., 1990]; and various tissue cell lines [Almaset al., 1992; Fey et al., 1984; Mattern et al., 1996; Nakayasu and Ueda, 1985a,b; Nakayasu and Ueda, 1986; Valkov et al., 1989]. Biochemical analysis of nuclear-associated actin suggests it may be a distinct nuclear isoform in hepatoma ascites [Bremer et al., 1981] and *Calpodes* silk gland epithelial cells [Henderson and Locke, 1992].

The functional significance of nuclear actin is less well-defined. However, perturbation of actin alters nuclear metabolism. Microinjection of anti-actin antibodies or actin binding proteins into nuclei blocks chromosome condensation in *Xenopus* oocytes [Rungger et al., 1979] and transcription in *Pleurodeles* oocytes [Scheer et al., 1984]. Cytochalasins, drugs that disrupt actin filaments, compromise the structural integrity of *Drosophila* polytene chromosomes [Sauman and Berry, 1994], induce DNA fragmentation in cell culture [Kolberet al., 1990], induce specific changes in HeLa cell gene expression [Zambettiet al., 1991], and inhibit protein translocation across the nuclear envelope of lymphocytes [Polet, 1990].

The nuclear matrix is a three-dimensional nuclear scaffold that resists extraction with detergent, digestion with nucleases and solubilization with moderate to high salt buffer washes [Berezney and Coffey, 1974, 1977]. It contains actin. Immunoblot analysis of isolated nuclear matrices show that actin is a component of this scaffold [Bachs et al., 1990; Fey et al., 1984; Mattern et al., 1996, 1997; Nakayasu and Ueda, 1985a,b, 1986; Valkovet al., 1989]. Immunofluorescent microscopy and immunogold electron microscopy have shown actin throughout the nuclear matrix of HeLa cells [Fey et al., 1984], mouse leukemia L5178Y cells [Nakayasu and Ueda, 1985b], and Guerin ascites tumour cells [Valkov et al., 1989]. The function of nuclear matrix actin is unknown, but it probably has a structural role [Nakayasu and

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Ueda, 1983] and may serve as a substrate for the attachment of pre-mRNA molecules [Nakayasu and Ueda, 1985a; Schroder et al., 1987].

Perinuclear actin shells have been identified in vertebrate cells (3T3 and NRK-49F [Clubb and Locke, 1996]), insect cells (*Calpodes* silk gland epithelial [Henderson and Locke, 1992], and *Calpodes* epidermal cells [Clubb and Locke, 1996; Jeun and Locke, 1993]), a protist (*Amoeba* [Pomorski and Grebecka, 1993, 1995]), fungi (*Neocallimastix, Orpinomyces* [Li and Heath, 1994], *Neozygites* [Butt and Heath, 1988]), plant cells (*Nicotiana* [Kengenet al., 1995; Kengenet al., 1993], and *Micrasterias* [Meindlet al., 1994]). Shells of phalloidin-stainable material have been associated with the nuclear envelope, leaving open the question whether it has a cytoplasmic or nuclear location. Perinuclear shells were associated with antigens found at the nuclear periphery or lamina in *Calpodes* silk glands [Henderson and Locke, 1992]. Ultrastructural studies showed a thin layer of cytoplasm devoid of organelles around the silk gland nuclei [Henderson and Locke, 1992]. Since this 'zone of exclusion' appeared to contain cytoskeletal elements, including microfilaments and microtubules, it was putatively identified as the perinuclear actin shell [Henderson and Locke, 1992]. In *Amoeba*, microfibrils with a size similar to filamentous-actin, associate with both the cytoplasmic and nucleoplasmic faces of the nuclear envelope [Leeson and Bhatnagar, 1975]. The only example of perinuclear actin shells whose location is known is in the fungus *Neozygites*, where the diameter of actin shells is consistently larger than the diameter of the associated nucleus, and must be cytoplasmic [Butt and Heath, 1988].

Perinuclear actin shells may be present within nuclei. Electron microscopy of *Amoeba* nuclei showed parallel bands of thin filaments next to the nuclear lamina [Leeson and Bhatnagar, 1975]. Nuclear-associated fractions prepared from *Calpodes* silk gland cells contain two distinct fibrous networks [Henderson and Locke, 1992]. One fraction contained a nuclear matrix antigen while the second contained actin [Henderson and Locke, 1992]. Isolated nuclear matrices of Guerin ascites tumour cells contain actin in the nuclear lamina [Valkovet al., 1989].

Transient perinuclear actin shells have been observed in 3T3 cells during cytokinesis and early interphase [Clubb and Locke, 1996]. The objective of this study has been to determine the precise location of these shells in relation to the nuclear envelope, whether they are in the nucleoplasm or the cytoplasm.

MATERIALS AND METHODS

Cell Cultures

3T3 (CCL 92) cell cultures were purchased from the American Type Culture Collection (ATCC; Rockville, MD). Cultures were maintained at 37°C and 5% CO_2 in alpha-Minimal Essential Media (α-MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco), 1% glucose (BDH, Poole, England), and 0.1 M Hepes buffer (pH 7.5; Gibco). Cultures were routinely subcultured every 4 or 5 days using a solution of 0.25% trypsin (Gibco), 0.5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS; pH 7.5). Immunohistochemical staining was performed on subconfluent cultures of 3T3 cells grown on 18 or 22-mm square coverslips maintained in 35-mm petri dishes (Falcon, Becton Dickinson, Lincoln Park, NJ).

Immunohistochemistry

Cells were rinsed in PBS, fixed for 15 min in 2% formaldehyde, and extracted with 0.2% Triton X-100 for 5 min. Alternatively, coverslips were immersed in −20°C methanol for 5 min followed by three rinses of PBS. Prior to incubation with primary antibodies, nonspecific binding was blocked with 1% bovine serum albumin (BSA; Sigma) in PBS containing 0.1% Tween 20 for 15–45 min. Blocking for longer times did not alter the staining patterns of any antigen studied. Once blocked, coverslips were placed onto a drop of polyclonal anti-actin antibody (1:40; Sigma, A-2668), monoclonal anti-actin antibody (1:400; ICN, C4) or anti-lamin antibody (1:20; a generous gift from Dr. Moir and Dr. Goldman, Northwestern University) solution and incubated at room temperature. All antibodies were prepared in blocking solution. After 1 h, coverslips were rinsed with three-5 minute changes of PBS containing 0.005% Tween 20 and blocked again for 15 min in 1% BSA in PBS containing 0.1% Tween 20. Fluorescein isothiocyanate labelled goat-anti-rabbit (1:20; Sigma) or goat-anti-mouse (1:20; Caltag Laboratories, San Francisco, CA) antibodies were applied to the cells. F-actin was labelled by including 1 µg/ml phalloidin-rhodamine (Sigma) in the sec-

ondary antibody solution. After 1 h the cells were rinsed with PBS for 15 min and mounted in glycerol: PBS (9:1; pH 9.0). Specimens were stored at −20°C.

Nuclear Matrix Preparation

Nuclear matrices of 3T3 cells were prepared from cells grown on glass coverslips. Cells were rinsed with PBS prior to extracting cytoplasmic and nuclear soluble proteins with 0.5% Triton X-100 in CSK buffer (10 mM Pipes [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM ethyleneglycol-bis (B-aminoethyl) N, N, N′, N′-tetraacetic acid [EGTA], 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). Chromatin was removed by incubating the extracted cells with 100 units/ ml DNase I (Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) in CSK buffer for 30 minutes and rinsing with 0.25 M $(NH_4)_2SO_4$ in CSK buffer for 5 min. The coverslips were placed in fresh CSK buffer. Residual proteins were removed by gradually adding an equal volume of 4 M NaCl in CSK buffer to the coverslips. The resultant matrix was rinsed in CSK buffer, fixed with 2% formaldehyde or −20°C methanol, and processed for immunohistochemistry as outlined above. The effectiveness of nuclease digestion was determined by Hœchst 33342 (20 μg/ml in PBS; Calbiochem, La Jolla, CA) staining and fluorescent microscopy before and after DNase I treatment.

Nuclear matrix proteins were prepared from isolated 3T3 cell nuclei. Cell suspensions were centrifuged at 300 Xg for 5 min, resuspended in isolation buffer (5 mM Pipes [pH 8.0], 85 mM KCl, 1 mM $CaCl_2$, and 5% sucrose) containing 0.5% Nonidet P40 and set on ice for 10 min. This suspension was centrifuged at 700 *g* for 5 min, resuspended by trituration in ice cold isolation buffer, and centrifuged at 700 *g* for 5 min. All solutions were ice-cold and all centrifuge steps were carried out at 4°C. Pelleted nuclei were extracted as outlined above and suspended in SDS sample buffer (80 mM Tris [pH 6.8], 2% SDS, 1 mM PMSF, 5% β-mercaptoethanol, 0.001% bromophenol blue) at a concentration of 5.0×10^4 matrices per microlitre.

Electrophoresis and Western Blot Analysis

3T3 cell lysate was prepared from a cell suspension. Cells were removed from T75 flasks by treatment with 0.25% trypsin, 0.5 mM EDTA in PBS, or by scraping cells from the substrate. The resultant suspension was centrifuged at 300 *g* for 5 min, resuspended in SDS sample buffer, and boiled for 5 min. This lysate was cooled on ice and triturated four times with a 23-gauge needle to shear DNA. Lysate was stored at −20°C

Proteins were separated using standard PAGE techniques [Laemmli, 1970] on a Bio-Rad Minigel apparatus (Richmond, CA). Gels and nitrocellulose membranes were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Proteins were transferred to the membrane at constant voltage (50 V) for 3.5–4 hours. The membrane was blocked using 5% skim milk powder in PBS containing 0.1% Tween 20 for 30 min. The membrane was incubated overnight at 4°C in fresh blocking solution containing polyclonal anti-actin (A-2668; 1:500), monoclonal anti-actin (C4; 1:5,000), or without antibody. The next day the membranes were washed with three changes of PBS containing 0.1% Tween 20 and incubated for 1 h at room temperature in alkaline phosphatase conjugated anti-rabbit or anti-mouse antibodies (1:3,000; Bio-Rad) in fresh blocking buffer. After three washes in PBS containing 0.1% Tween 20 and a 5 min wash in TRIS colour development buffer (0.1 M Tris [pH 9.5], 0.5 mM $MgCl_2$), the bound antibodies were visualized using the Bio-Rad BCIP/NBT Immuno-Blot Alkaline Phosphatase assay kit.

Transmission Electron Microscopy

Electron microscope observations were made on the perinuclear region of 3T3 cells derived from synchronous and asynchronous cultures and suspensions of mitotic cells prepared as previously reported [Clubb and Locke, 1996]. Pellets of mitotic cells and cells cultured in 35-mm polystyrene petri dishes were fixed in 1% EM-grade glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 4% sucrose for 1 h. After fixation, pellets and cells were washed three times (10 min each) in 0.1 M phosphate buffer containing 4% sucrose and postfixed in 1% osmium tetroxide in phosphate buffer for 1 h. They were then rinsed in two changes of phosphate buffer containing sucrose and four changes of distilled water before staining overnight in half-saturated aqueous uranyl acetate at 60°C [Locke and Huie, 1980] and dehydrated through an ethanol series. Pellets were washed in propylene oxide, 1:1 propylene oxide: araldite and embedded in araldite. Cells cultured on petri dishes were removed from their substrate

by gently flooding propylene oxide over the cells to dissolve the bottom of the dish. The resultant suspension of fixed and stained cells was pelleted (14,000 *g* for 5 min) and the pellet immersed in 1:1 propylene oxide: araldite before embedding in araldite. Silver sections of embedded cells were cut using a diamond knife on a Riechert OMU 3 ultramicrotome. Sections were stained with lead citrate and uranyl acetate and examined on a Philips 300 TEM at an accelerating voltage of 80 kV.

Microscopy and Image Preparation

Stained cultures were photographed using a Zeiss Photomicroscope (Thornwood, NY) or a Bio-Rad MRC600 confocal laser-scanning microscope using a Nikon Planapo X60 oil immersion objective (NA = 1.4) on a Nikon Diaphot-TMD inverted microscope.

Immunofluorescent and electron microscopy negatives were scanned using a Howtek D4000 drum scanner connected to a Macintosh 8500 PowerPC. Images and plates were prepared using Adobe Photoshop v4.01 and Adobe Illustrator v7.01. Pseudocolouring and superimposition of images was done using Northern Exposure v2.9c (Empix Imaging, Mississauga, ON) running on a 486 PC. Images were printed using a Phaser 450 dye sublimation printer.

RESULTS

Actin in the Perinuclear Shells of Recently Divided Cells Colocalizes With Lamin

Earlier work used rhodamine labelled phalloidin to describe transient actin perinuclear shells in recently divided 3T3, NRK-49F, and *Calpodes* epidermal cells [Clubb and Locke, 1996]. When anti-lamin labelling was combined with the rhodamine labelled phalloidin, newly divided 3T3 cells showed perinuclear shell actin colocalized with the nuclear lamina (Fig. 1). In double-labelled cells phalloidin-rhodamine stained F-actin (Fig. 1A) appeared to be in the position of the anti-lamin-fluorescein stained nuclear lamina (Fig. 1B). It was not immediately clear that these images of the shells were resolved at a high enough resolution to discriminate between actin inside and outside the nuclear envelope. Scale drawings of actin overlapping the lamin within the nucleus (500 nm apparent thickness, Fig. 1C, bottom diagram), and outside the envelope (500 nm apparent thickness of the actin shell and 60 nm average thickness of the nuclear envelope; Fig. 1C, top diagram) showed that immunofluorescent images should easily resolve the location of the actin with respect to the nuclear envelope. Merging images of the F-actin and anti-lamin staining images showed the actin shell to be on the inside, co-existing spatially with lamin (Fig. 1D).

Lamina Assembly Precedes Actin Shell Formation

Actin shells were not observed before a completed rim of lamin staining was present at the nuclear periphery. Lamin protein began to associate with condensed chromosomes prior to actin shell formation, showing that lamina assembly after mitosis precedes actin shell formation. The difference in timing of appearance of the two shell components suggests that they play different roles in nuclear reformation.

The absence of an actin shell outside of the nuclear envelope has been confirmed by ultrastructural studies.

Electron Microscopy of the Perinuclear Shell Region

Nuclei are surrounded by a thin layer of cytoplasm that is devoid of organelles in *Calpodes* silk gland epithelial cells [Henderson and Locke, 1992]. If the perinuclear shell were in this location in 3T3 cells, electron microscopy might be expected to show some fibrous, actin-like, structure on the cytoplasmic face of the nuclear envelope. A 'zone of exclusion' was not present in 3T3 cells; transmission electron microscopy showed no distinct cytoplasmic structure that could be interpreted as a perinuclear shell (Fig. 2). The composition and density of perinuclear regions was no different from nearby regions of the cytoplasm. Lipid droplets (Fig. 2A), Golgi complexes (Fig. 2B), mitochondria (Fig. 2C), and endoplasmic reticulum were juxtaposed to the nuclear envelope. The perinuclear cytoplasm contained cytoskeletal elements, such as microtubules but no actin filaments (Fig. 2E). Fixation was adequate to show actin filaments in subcortical actin networks (Fig. 2D), making it unlikely that the inability to find them in cytoplasmic perinuclear actin shells could be the result of inadequate structural preservation. A thin band of heterchromatin was always present in the nucleoplasm adjacent to the nuclear envelope in interphase and newly divided cells.

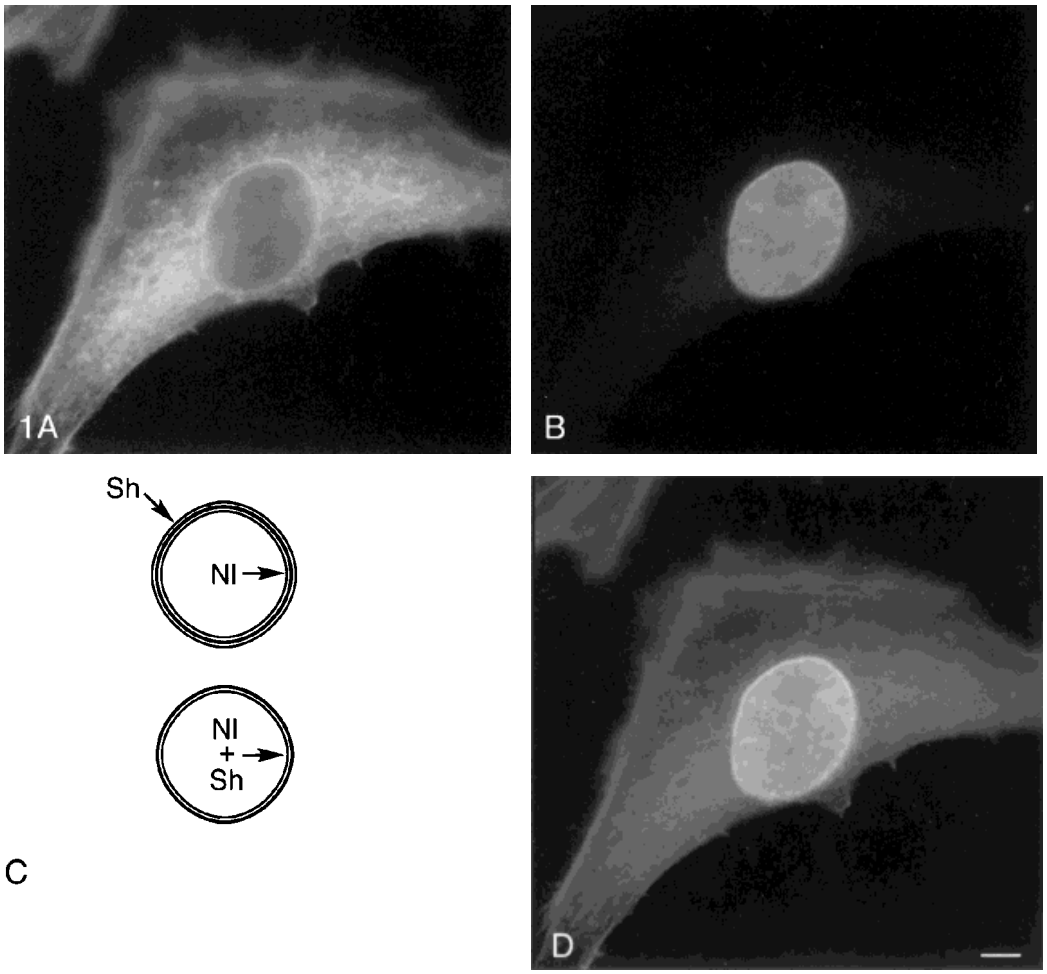

Fig. 1. Immunofluorescent microscopy shows that actin colocalizes with lamin in the perinuclear shells of newly divided cells. Lamin labelled with anti-lamin antibodies together with rhodamine labelled phalloidin were used to localize lamin and F-actin in nuclei of recently divided 3T3 cells. The perinuclear shell stained for both actin and lamin. **A**: Rhodamine-labelled phalloidin stained the nuclear periphery and cytoskeletal components. **B**: Fluorescein-labelled anti-lamin antibodies stained only the nuclear periphery. **C**, top: A scale drawing of an actin shell (Sh; 500 nm apparent thickness) if it were to be positioned outside the envelope in the cytoplasm (nuclear lamin [Nl] has an apparent thickness of 500 nm and the nuclear envelope has an average thickness of 60 nm). Bottom: Scale drawing of an actin shell positioned within the nucleus overlapping the lamin inside the envelope. The drawings show that immunofluorescent images would be expected to resolve differences in position of the actin shell, whether it coincides with the lamina or the cytoplasmic surface. **D:** Merging images of the F-actin and anti-lamin staining showed the actin shell to be on the inside, co-existing spatially with the lamin as in C, bottom. Scale bar = 5 μm.

Localization by Antibodies Confirms the Presence of Actin in the Perinuclear Shell

Previous reports of perinuclear actin shells have relied on phalloidin-rhodamine staining for their identification. Since phalloidin-rhodamine has the potential for binding to actin-related proteins [Bingham and Schroer, 1995] 3T3 cells were stained with anti-actin antibodies to confirm the presence of actin in the perinuclear shells. A polyclonal antibody (A-2668) was used to stain 3T3 cells (Fig. 3). This antibody stained stress fibres, amorphous perinuclear material, diffuse intranuclear material that was excluded from nucleoli, and perinuclear shells of recently divided cells. The shells only stained in cells at the end of mitosis as in the pair of mitotically-related cells (Fig. 3A) or during early interphase (Fig. 3B,C). The formation of the actin shell is not dependent on proper cytokinesis. Mitotic cells sometimes failed to separate, leaving them binucleate. Both nuclei in such cells had actin perinuclear shells (Fig. 3C). Most cells in culture did not have shells with actin demonstrable by phalloidin

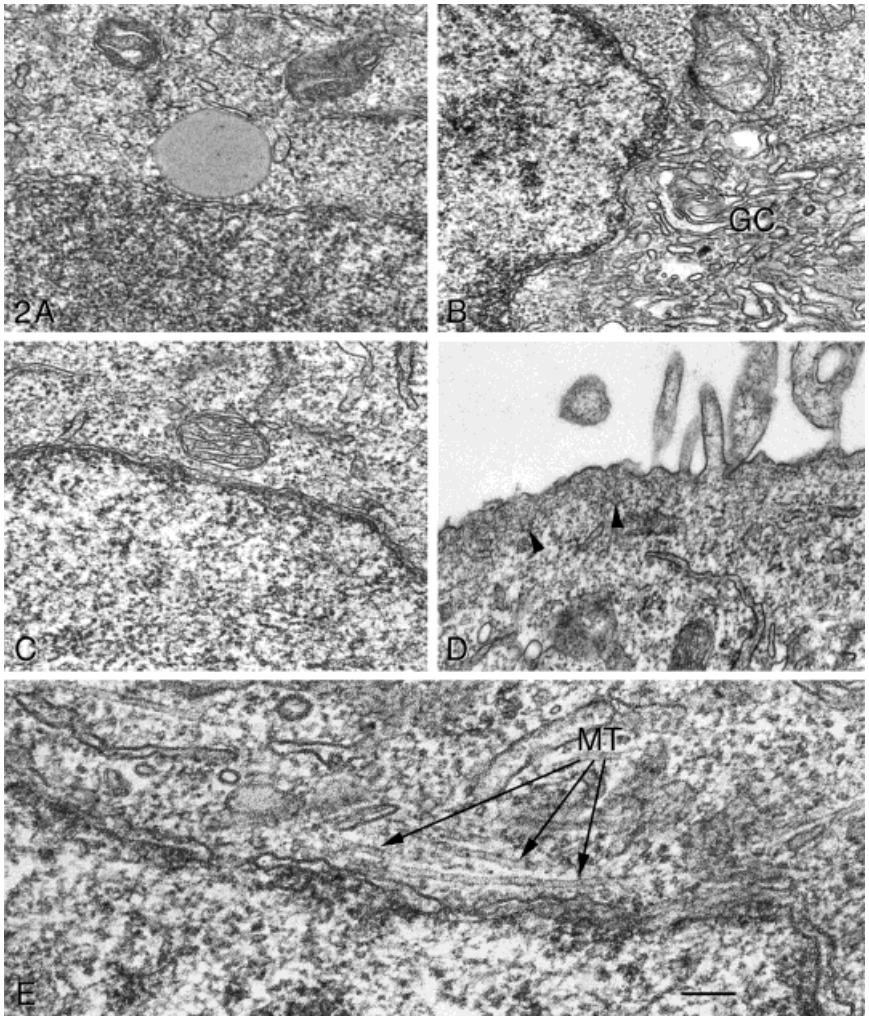

Fig. 2. Transmission electron microscopy showed no distinct actin network on the cytoplasmic side of the nuclear envelope of newly divided 3T3 cells. The composition and density of perinuclear cytoplasm was no different from that of nearby regions. Microtubules (**E;** MT) were occasionally present adjacent to the nuclear envelope. Lipid droplets (**A**), Golgi complexes (**B**; GC), and mitochondria (**C**) lay near the cytoplasmic face of the envelope showing that cytoskeletal elements do not form a zone of exclusion. Subcortical actin networks were present in these cells (**D**; arrowheads), showing that the procedures used in this study preserved actin (Scale bar = 250 nm for A-D or 100 nm for E).

staining, raising the question whether the actin component of the shell that is present at the conclusion of division is lost or merely masked during interphase.

Perinuclear Shell During Interphase

Anti-actin antibody distribution confirmed the unavailability of perinuclear shell actin during interphase. In most interphase cells phalloidin-rhodamine did not stain perinuclear shells [Clubb and Locke, 1996]. Polyclonal anti-actin antibodies stained stress fibres and a slight, more diffuse perinuclear component than in newly divided cells (Fig. 4B). Similar results were obtained using a monoclonal antibody (C4) that recognizes α, B, and γ isoforms of actin in many different organisms [Lessard, 1988; Oteyet al., 1987]. No perinuclear shells were stained in any interphase cells (Fig. 4A). These results confirmed earlier observations that most nuclei lack perinuclear actin shells, the actin shells ceasing to be demonstrable as the cells enter interphase [Clubb and Locke, 1996].

Perinuclear actin shells are presumed to be intranuclear, since transmission electron microscopy showed no cytoplasmic perinuclear shell and actin distribution overlapped with lamin. If actin is stably associated with the nuclear matrix, removal of nuclear material might therefore unmask actin. The sequential

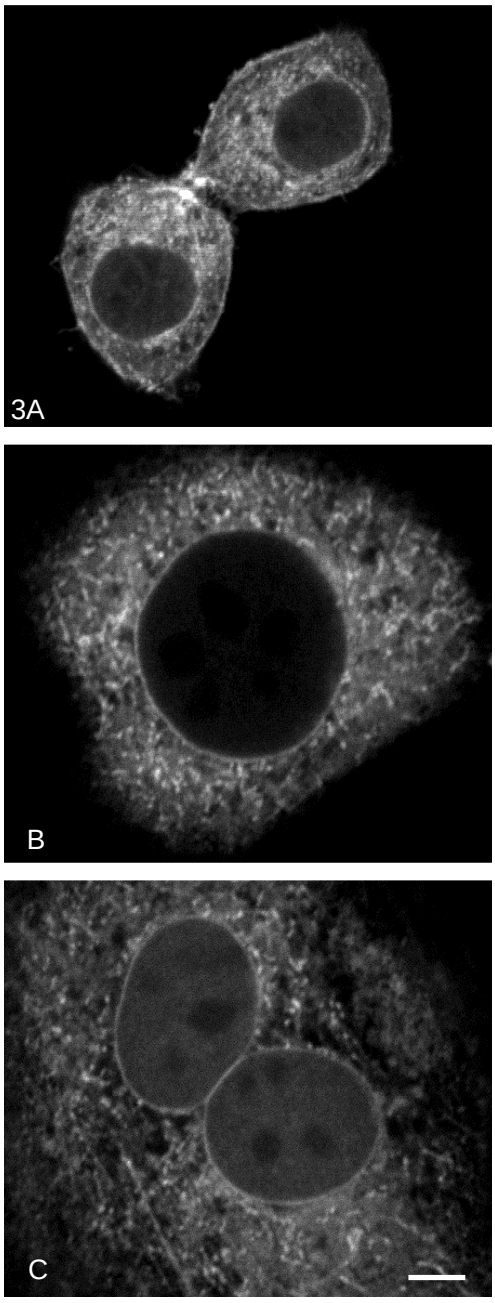

Fig. 3. Confocal images of anti-actin antibodies confirm the perinuclear shell localization of actin shown by rhodamine conjugated phalloidin. Newly divided 3T3 cells were stained with polyclonal anti-actin antibody (A-2668). **A**: Actin in the newly formed perinuclear shells of a pair of mitotically-related cells during cytokinesis. **B**: The perinuclear shell continues to react with anti-actin antibodies during early interphase. **C:** Failed cytokinesis leaves some interphase cells binucleated. In this cell both nuclei have actin perinuclear shells, showing that the formation of the actin shell is independent of cytokinesis (Scale bar = 5 μm).

extraction of cells with detergent, digestion of DNA with DNase I followed by washing with salt buffers, removes most of the nuclear contents [Berezney and Coffey, 1974, 1977]. Such extractions of cell cultures allowed anti-actin antibodies to stain components in interphase nuclei (Fig. 4C,D). Actin was present through the nucleus, most noticeably in aggregates, a faint shell, and an even fainter background. Extraction was essential to detect nuclear matrix actin. The harshness of this extraction protocol was demonstrated by the removal of cytoplasmic and nucleoplasmic material and complete- (Fig. 4C) or partial-solubilization (Fig. 4D) of stress fibres.

Optical sections obtained with a confocal microscope localized actin in the nuclear periphery, with similar staining patterns with both anti-actin antibodies (Fig. 4E,F). Fluorescence in control cells treated with goat anti-mouse-FITC (Fig. 4G) or goat anti-rabbit-FITC (Fig. 4H) antibodies alone were negligible, confirming that the staining in Figure 4E,F is caused by the specificity of the primary antibody.

Except for the Perinuclear Shell, Actin, and Lamin Have Different Staining Patterns in the Nucleus

Nuclear actin and lamins are both closely associated with the nuclear periphery, suggesting that the masking of shell actin in interphase might be due to interaction with lamin, an idea that would be supported if nuclear extraction were to remove lamin from the shell, and if lamin colocalized with all nuclear actin. However, the pattern of lamin distribution was not altered by extraction, nor did it exclusively colocalize with nuclear actin. Anti-lamin antibodies produced similar staining patterns in extracted (Fig. 5B) and in unextracted cells (Fig. 1B). Actin, but not lamin, occurred in nuclear aggregates after extraction (Fig. 5A). The lamin distribution determined by anti-lamin antibodies differs from the actin distribution determined by anti-actin antibodies, further strengthening the reliability of the actin localization.

Immunoblot Analysis Shows That Actin Is Present in the Nuclear Matrix of 3T3 Cells

The specificity of the anti-actin antibodies was tested by immunoblotting 3T3 cell lysate with the two anti-actin antibodies. The monoclonal anti-actin recognized a single band with an M_r of 40×10^3 (Fig. 6A, C4). The polyclonal

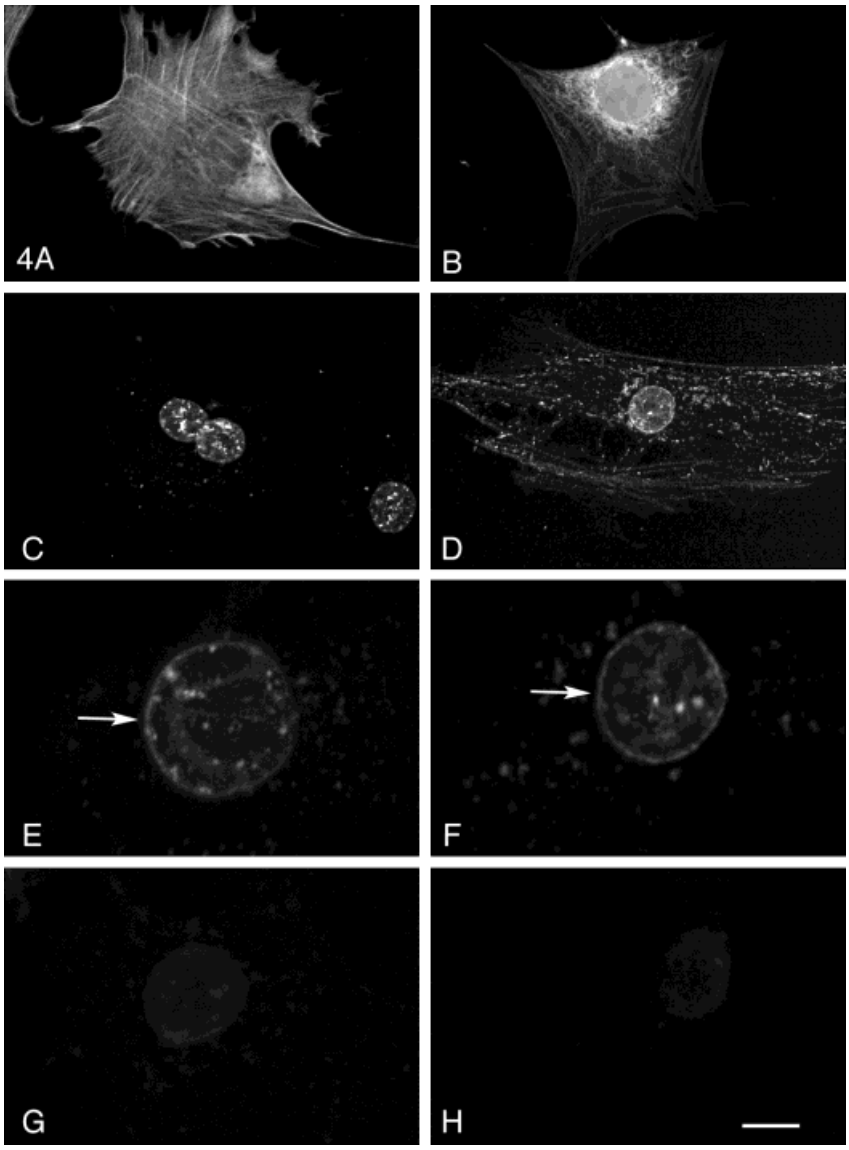

Fig. 4. Removal of chromatin from interphase 3T3 cell nuclei unmasks actin in nuclear matrices. In unextracted interphase cells, anti-actin antibodies stained stress fibres and a slight, more diffuse perinuclear component than in newly divided cells. **A**: Monoclonal and (**B**) polyclonal antibodies to actin. Extraction to remove chromatin (see Materials and Methods), allowed the anti-actin antibodies to stain nuclear components. **C**: Monoclonal and (**D**) polyclonal antibodies recognized punctate intranuclear aggregates and the nuclear periphery. Chromatin extraction also resulted in the complete (C) or partial (D) solubilization of stress fibres. Anti-actin antibodies stain the nuclear periphery in interphase cells after chromatin has been extracted (see Materials and Methods). **E:** Monoclonal and (**F**) polyclonal. Actin occurs at the nuclear periphery (arrows) and in intranuclear aggregates. The position of the peripheral staining is similar to that of the perinuclear shell in newly divided cells. Control cells treated with goat anti-mouse-FITC (**G)** or goat anti-rabbit-FITC (**H**) antibodies alone showed negligible nuclear staining in images recorded under identical optical conditions to E and F (Scale bar = 25 μm for A-D or 5 μm for E-H).

antibody reacted with many more proteins with M_r ranging from 35×10^3 to greater than 165×10^3 (Fig. 6A, A-2668). Some of the cell localization with the polyclonal antibody may therefore be due to actin-like proteins as well as actin. For this study an actin-like protein is any protein that shares antigenic determinants with actin.

Both antibodies also reacted with nuclear matrix preparations (Fig. 6B). The monoclonal anti-actin antibody recognized a single band with a M_r of 40×10^3 (Fig. 6B, C4). The poly-

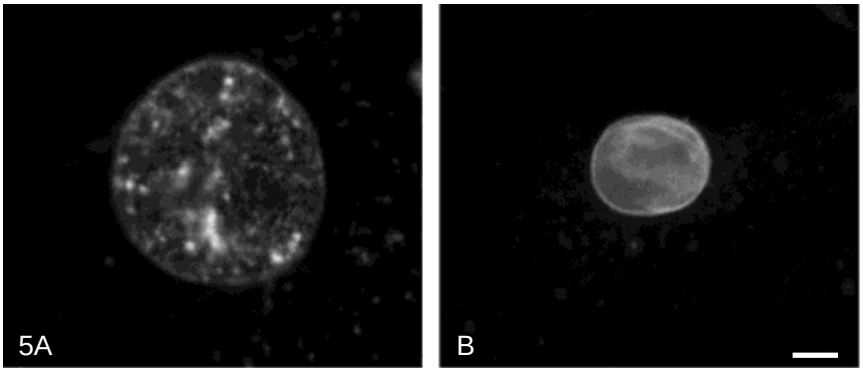

Fig. 5. Nuclear actin and lamin have different distributions, with actin, but not lamin, occurring in nuclear aggregates. **A**: Extracted interphase 3T3 cell nuclei stained with a monoclonal anti-actin antibody. Actin occurs at the nuclear periphery and within intranuclear aggregates. **B**: Extracted interphase 3T3 cell nuclei stained with anti-lamin antibodies. Lamin occurs at the nuclear periphery and diffusely in the inner nuclear matrix (Scale bar = 5 μm).

clonal anti-actin antibody recognized three proteins including a 40 × 10^3 protein that appeared to be actin, and two actin-like proteins of M_r 50 × 10^3 and 65 × 10^3 (Fig. 6B, A-2668). The detection of nuclear matrix proteins that react with anti-actin antibodies confirms our interpretation of the visual images - actin and actin-like proteins are found in the nucleoplasm of 3T3 cells.

DISCUSSION

The main finding is that perinuclear actin shells in 3T3 cells are intranuclear, part of the peripheral nuclear matrix. Perinuclear actin shells have been reported in a range of organisms, from plants [Kengen et al., 1993, 1995; Meindl et al., 1994], fungi [Butt and Heath, 1988; Li and Heath, 1994], protists [Pomorski and Grebecka, 1993, 1995], insects [Clubb and Locke, 1996; Henderson and Locke, 1992; Jeun and Locke, 1993], to vertebrates [Clubb and Locke, 1996]. The common feature is that shells are associated with the nuclear envelope, some inside, others outside. With such different kinds of cell, a varying location is perhaps not surprising, but it suggests that shells, in the broad sense, may be of more than one kind. Perinuclear actin shells in 3T3 cells colocalize with lamin proteins in a single nucleoplasmic structure, part of a nuclear matrix that may be the norm for vertebrates. Nuclear matrix actin is probably involved in nuclear reassembly. We observed that lamin appears before shell actin in 3T3 cells. In agreement with this, actin is required for nuclear assembly in *Xenopus* cell-free extracts where it accumulates after the lamina forms [Zhang et al., 1996]. The appear-

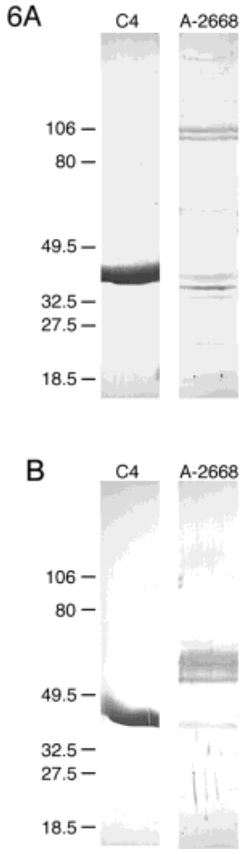

Fig. 6. Immunoblot analysis shows that actin is present within the nuclear matrix of 3T3 cells. **A:** A monoclonal antibody (C4) recognized a single protein in 3T3 cell lysate while a polyclonal antibody (A-2668) recognized actin and many actin-like proteins. **B**: Immunoblot analysis of proteins from isolated nuclei whose contents were extracted (see Materials and Methods) showed that nuclear matrices contained actin. Isolation and extraction of nuclei removed many of the actin-like proteins identified by the polyclonal antibody in whole cell lysate. A: Lanes were loaded with the equivalent of 1 × 10^4 cells. B: Lanes were loaded with 1 × 10^6 nuclear matrices. Standards were Bio-Rad prestained low molecular weight standards.

ance of peripheral nuclear matrix actin in *Xenopus* nuclear reconstitution experiments parallels the observation that perinuclear actin shells are transient structures at the end of mitosis [Clubb and Locke, 1996].

A second finding is that removal of chromatin exposes nuclear actin. Masking of nuclear actin from antibody and phalloidin staining has been previously reported in *Hyalophora* ovarian follicle cells, *Drosophila* polytene chromosomes and *Xenopus* embryos [Sauman and Berry, 1994]. The process of masking is not unique to nuclear actin. Nuclear proteins are masked in HeLa and L6E9 cells [Chaly et al., 1996; Nickerson et al., 1992]. In 3T3 cells, nuclear proteins mask actin in interphase nuclei, making it inaccessible to antibodies and phalloidin and undetectable by routine immunohistochemistry. After the removal of soluble proteins and chromatin, immunofluorescent staining of 3T3 cells and immunoblot analysis of nuclear matrices show actin in the shells and in granular aggregates as a stable, insoluble element of the nuclear matrix.

The distribution of actin in the nuclear matrix resembles the spatial organization of nuclear metabolism. Transcription [Ciejeket al., 1983; Jackson et al., 1993; Wansink et al., 1993], DNA replication [Dijkwel et al., 1986; Ferreira and Carmo-Fonseca, 1997; O'Keefe et al., 1992], and DNA repair [Jackson et al., 1994; Mullenders et al., 1988] occur in domains associated with the nuclear matrix. Specific DNA sequences termed "matrix-associated-regions" or MARs attach chromosomal DNA to the nuclear matrix [Boulikas, 1992]. Nuclear actin is a matrix protein that interacts with MAR sequences in vitro [Ivanchenko and Avramova, 1992]. If it does the same in vivo, it could be a matrix attachment to DNA. The pattern of nuclear matrix actin in 3T3 cells might then reflect the organization of DNA into transcription or replication domains.

Nuclear matrix actin is involved in RNA nuclear metabolism and mRNA metabolism is also organized into subnuclear domains [Spector, 1996; Visa et al., 1993]. More than 90% of mRNA-precursors remain tightly bound to the nuclear matrix during the preparation of matrices from leukemia cells [Nakayasu and Ueda, 1985a]. This interaction relies on actin, since mRNA-precursors can be extracted under conditions promoting filamentous-actin depolymerization [Nakayasu and Ueda, 1985a; Schroder et al., 1987]. Actin is one of several matrix proteins that form multiprotein complexes with mRNA-precursors [Nakayasu and Ueda, 1985a]. Since mRNA processing relies on the presence of nuclear actin, nuclear matrix actin distribution may reflect the spatial organization of mRNA processing, for example in differentiated neurons, aggregates of nuclear actin are associated with aggregates of mRNA processing factors [Sahlas et al., 1993]. Our study shows 3T3 cell nuclei have matrix actin organized into distinct aggregates or domains.

In addition to actin, the nuclear matrix of 3T3 cells also contains actin-like proteins. Actin-related proteins (Arps) are a superfamily of proteins that would be expected to react with polyclonal anti-actin antibodies. Amino acid sequence comparisons between actin and Arps show shared regions of structural similarity [Frankel and Mooseker, 1996]. Conserved sequences would serve as shared epitopes and permit antibodies raised specifically against actin to recognize actin-related proteins. Arps are found in nuclei. Arp4 (*Drosophila* Arp 13E) occurs in the nuclei of *Drosophila* tissues with a punctate staining pattern [Frankel et al., 1994, 1997]. Arp6 (*S. cerevisiae* Act3p) occurs diffusely through nuclei [Weber et al., 1995]. Neither Arp4 nor Arp6 required chromatin extraction for their detection [Frankel et al., 1994, 1997; Weber et al., 1995]. In 3T3 nuclear matrices the polyclonal anti-actin antibody recognizes two actin-like proteins. Both proteins from 3T3 nuclei have an M_r greater than 50×10^3. Since Arp4 has a molecular mass of 45 kD and Arp6 is extracted upon DNase I digestion, the 3T3 actin-like proteins may be new members of the growing family of actin-related proteins. Since the nuclear matrix staining patterns of the polyclonal and monoclonal anti-actin antibodies are similar, we suppose that the peripheral and intranuclear aggregates are actin, and that the actin-like proteins are associated with the nuclear matrix actin.

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