

Cytostellin: a novel, highly conserved protein that undergoes continuous redistribution during the cell cycle

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Summary

Cytostellin, a 240 kDa protein, has been purified from mammalian cells by immunoaffinity chromatography using monoclonal antibody H5. Immunofluorescence microscopy shows diffuse and punctate cytotellin immunoreactivity in interphase nuclei. Nuclease digestion and salt extraction are not required to expose the epitope. The onset of prophase is marked by the appearance of multiple intensely immunofluorescent cytotellin-containing 'bodies' within the nucleus. Nuclear disassembly is heralded by the movement of cytotellin bodies from the nucleus to multiple positions throughout the cell. Cytostellin bodies in metaphase, anaphase and telophase cells are widely dispersed, including some in cell processes far removed from the

mitotic spindle apparatus. However, a distinct subset of larger, more intensely staining bodies surrounds the mitotic spindle apparatus. Cytostellin bodies remain in the cytoplasm of the daughter cells and disappear *after* the appearance of nascent nuclei. Cytostellin is immunologically distinct from other nuclear and cytoplasmic proteins, and it has been detected by immunoblot analysis in all species tested from yeast to humans. Based upon these findings, we postulate that cytotellin has a cell cycle-dependent function which is conserved in higher and lower eukaryotic cells.

Key words: mitosis, cell division, cell cycle, nuclear structure, nuclear protein.

Introduction

Animal cell division is achieved by a highly ordered sequence of events, including chromosomal DNA replication, condensation and segregation by the mitotic spindle apparatus, which forms during prophase (Inoue, 1981; Pickett-Heaps et al., 1982; Borisy et al., 1984; Baserga, 1985). During prophase the nuclear envelope disassembles (Newport and Forbes, 1987; Newport and Spann, 1987; Gerace and Burke, 1988), and simultaneously, cytoplasmic and subcortical cytoskeletal structures reorganize (Borisy et al., 1984; Kilmartin and Adams, 1984). Several membrane-associated proteins redistribute from the periphery to a diffuse distribution throughout the cell, actin filaments and microtubules reorganize and the cells assume a more rounded shape. The transition from metaphase to anaphase is characterized by separation of the daughter chromatids and the appearance of a cleavage furrow. Telophase cells elongate in a direction perpendicular to the metaphase plate, and their nuclear lamina and envelope reassemble. Cytoplasmic fission at the midbody partitions nuclear structures, cytoplasmic membranes, cytoskeletal structures and soluble proteins approximately equally between daughter cells.

The partitioning of cytoplasmic structures into daughter cells appears to be coordinated with the segregation of the

chromosomes. The spindle microtubules between the centrioles specify the direction of chromosome movements, and may play a role in establishing the position of the cleavage plane (Inoue, 1981; Pickett-Heaps et al., 1982; Borisy et al., 1984; Vallee and Shpetner 1990). However, the mechanisms by which the spindle apparatus is linked to more peripheral cytoplasmic structures are poorly understood. Proteins which establish mitosis-specific interactions involving the spindle apparatus and more peripheral cell structures, such as the subcortical cytoskeleton, need to be identified to understand how the partitioning of cytoplasmic and nuclear structures is coordinated.

The present study describes a 240 kDa protein that moves from the interphase nucleus to multiple discrete sites (bodies) throughout dividing cells. This protein, which appears to be immunologically distinct from other known proteins, has been called "cytotellin" to describe its "starry sky" appearance in postmitotic daughter cells. A group of cytotellin-bodies is arranged in a ring that surrounds the mitotic spindle apparatus, while the other cytotellin-containing bodies are located in peripheral sites which are far-removed from the mitotic spindle. The cytotellin bodies move during mitosis to positions from which they have the potential to interact with both the spindle apparatus and more peripheral cell structures.

Materials and methods

Preparation of immunogen, immunization and hybridoma production

The immunogen consisted of a mixture of phosphotyrosine-containing proteins extracted from Madin Darby Canine Kidney (MDCK) cells, which express elevated levels of the pp60^{c-src} tyrosine kinase (Warren et al., 1988), and was purified by anti-phosphotyrosine-affinity chromatography (Glenney et al., 1988; Kanner et al., 1989). MDCK cells were grown to confluence on microcarrier beads (Cytodex 3, Pharmacia Inc.) in 2 l spinner culture flasks. The cell-coated beads were washed three times with cold 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (TBS), and then extracted for 15 min in ice-cold "PiTyr extraction buffer," composed of: 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, 2 mM NaN₃ and 0.2 mM Na₃VO₄. The insoluble material was pelleted by centrifugation at 20,000 g for 30 min. The clarified extract was passed twice over a 1 ml anti-phosphotyrosine affinity column composed of 4 mg of monoclonal antibody Py20 covalently bound to 1 ml of agarose gel (Py20-agarose; ICN immunobiologicals). The column was washed with cold 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 0.2 mM Na₃VO₄ and 1 mM phenylmethylsulfonyl fluoride (PiTyr wash buffer) and then eluted with 5 mM phosphotyrosine in PiTyr wash buffer. The eluate was lyophilized and frozen at -80°C until used for immunization.

Approximately 50 µg of phosphotyrosine-containing MDCK cell protein were homogenized with complete Freund's adjuvant and injected intraperitoneally into female BALB/c mice (Harlow and Lane, 1988). Three weeks later, secondary immunizations were performed using incomplete Freund's adjuvant. Sera obtained by tail bleeds was assayed for the presence of antibodies directed against MDCK cell proteins by immunoblot analysis 5-7 days after secondary and tertiary immunizations. Immune splenocytes were fused with X63Ag8.653 myeloma cells using standard polyethylene glycol-mediated fusion methods (Harlow and Lane, 1988), and plated into 96-well plates at limiting dilution in hypoxanthine-aminopterin-thymidine selection medium.

Screening of monoclonal antibodies

Hybridomas producing antibodies directed at MDCK cell proteins of the desired molecular weight were identified by immunoblot analysis (Towbin et al., 1979), performed in a 45-lane multiblot chamber (Immunitics, Cambridge MA). Primary screens were done on pooled supernatants (4 supernatants per lane), and later screens on single hybridoma supernatants. Positive clones were subcloned twice, expanded and frozen. The subclass of each mAb was determined by enzyme-linked immunoassay (Mouse Hybridoma Subtyping Kit, Boehringer-Mannheim). Hybridoma ascites was generated using standard techniques (Harlow and Lane, 1988).

Sample preparation, immunoprecipitation, gel electrophoresis

Proteins were prepared for gel electrophoresis by boiling for 5 min in 25 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 20% glycerol and 0.004% bromophenol blue (2× sample buffer). Protein concentrations were determined by the bicinchoninic acid assay (BCA, Pierce Chemical Co, Rockford, Ill), and where indicated, the amount of protein loaded to each lane was normalized. The cells and tissues came from the following sources: human, CACO2 colonic adenocarcinoma cell line; dog, Madin Darby Canine Kidney cell line; mouse, murine erythroleukemia stem cells; chicken, platelets (a gift of Dr. William Horne); newt (*Triturus viridescens* tissue);

fish (*Lesbistes reticularis* tissue); insect (*D. melanogaster* cells); nematode (*C. elegans* tissue, a gift of Dr. Michael Stern) and yeast (*S. pombe*, a gift of Dr. Tom Tupi). Solubilized proteins were separated on 7% SDS polyacrylamide gels as described (Laemmli, 1970).

MDCK cell protein for immunoprecipitations was prepared by boiling the cells in hot "SDS lysis buffer": 1% SDS, 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.2, 150 mM NaCl, 2 mM NaN₃, 0.2 mM Na₃VO₄, 5 mM EDTA and 2 mM EGTA. The viscous material was sheared 5 times through a 23 gauge needle and diluted with 6 volumes of "Triton/DOC dilution buffer": 10 mM Na₂HPO₄/NaH₂PO₄, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, 2 mM NaN₃, 0.2 mM Na₃VO₄, 5 mM EDTA and 2 mM EGTA. The diluted extracts contained 0.14% SDS. Immunoprecipitations were performed using the anti-cytostellin mAbs H5 or H14, which were covalently bound to agarose beads, or bound non-covalently via a goat anti-mouse IgM antibody to *Staphylococcus aureus* protein G beads (Sigma, Chemical Co, St. Louis, Mo). Negative controls consisted of monoclonal IgMs (TEPC 183 or MOPC 104E, obtained from Sigma Chemical Co, St. Louis, Mo). Immunoprecipitation was carried out for 2-4 h at 4°C, and the precipitates washed 3-5 times in cold "IP wash buffer" (6 parts of "Triton/DOC dilution buffer":1 part "SDS lysis buffer"). The washed immunoprecipitates were analyzed by immunoblotting after SDS-PAGE.

The extractability of cytoestellin was determined on MDCK cells grown to confluency in cell culture dishes (150 mm diameter). The cell monolayers were washed twice with cold TBS and rocked for 15 min at 4°C in 5 ml Triton/DOC dilution buffer. The insoluble material was pelleted by centrifugation at 48,000 g for 30 min 4°C, and then boiled in 2× sample buffer. The solubilized cytoestellin was immunoprecipitated with an excess of mAb H14 as described above, boiled in 2× sample buffer, and run onto a 7% polyacrylamide gel. The amount of cytoestellin in the soluble and insoluble fractions was determined by immunoblotting with anti-cytoestellin mAb H14 (H5 gives same result, not shown here).

Immunoblot analysis

Proteins separated on acrylamide gels were transferred to nitrocellulose membranes overnight at 500 mA (Towbin et al., 1979). The membranes were blocked in 5% bovine serum albumin in TBS overnight at room temperature. The primary antibody (i.e. H5, H14 or control IgM, at 1-5 µg/ml in TBS-5% BSA) was incubated with the bound proteins for 2 h at room temperature, then washed three times (15 min/wash) in TBS-0.05% Tween-20. The secondary antibody (either goat anti-mouse IgM linked to alkaline phosphatase or goat anti-mouse IgM linked to biotin) was incubated with the membrane for 1 h, and washed as before.

For blots incubated with goat anti-mouse IgM-biotin, avidin-alkaline phosphatase was used as the tertiary reagent. Bands were developed using the alkaline phosphatase substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co, St. Louis, Mo). In some cases ¹²⁵I-avidin (New England Nuclear/DuPont) was used as the tertiary reagent for autoradiography.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (Warren and Nelson, 1987), with minor modifications. Cells were grown on glass coverslips, and fixed in 1.7% paraformaldehyde (weight/volume) in PBS (10 mM Na₂HPO₄/NaH₂PO₄, pH 7.2, 150 mM NaCl). The fixed cells were washed in PBS for 15 min and permeabilized with PBS containing 0.5% Triton X-100 and 2 mM MgCl₂ for 15 min at room temperature. Cells were incubated in mAb H5 or control IgM (10

$\mu\text{g/ml}$) for 60 min at room temperature. The cells were washed extensively with PBS and then incubated in biotinylated goat anti-mouse IgM antibody for 60 min at room temperature. The cells were washed again with PBS and incubated with avidin-rhodamine or avidin-fluorescein. Some experiments included rabbit antisera directed against the Nuclear Mitotic Apparatus (NuMA) protein (Yang et al., 1992), provided by Dr. Michael Snyder. In this case, nonimmune rabbit serum was used as a control. To visualize the state of chromosomal condensation, cells were incubated with the DNA binding fluor, 4,6-diamidino-2-phenylindole (DAPI) at 5 $\mu\text{g/ml}$ as described previously (Baron et al., 1991). Following extensive PBS washes, the coverslips were mounted with Aquamount, viewed under oil immersion with the 63 \times objective of a Zeiss Axophot microscope equipped with epifluorescence illumination, and photographed on Ektachrome EES film.

Results

Monoclonal antibodies that bind to a novel 240 kDa MDCK cell protein

Monoclonal antibodies (mAbs) were generated against multiple phosphotyrosine (PiTyr)-containing proteins purified from MDCK cells that express elevated levels of the cellular tyrosine kinase pp60^{c-src}. Some of these PiTyr-containing proteins are potential substrates of c-src. The subject of the present report is a 240 kDa protein that reacts with two of the mAbs.

The immunogen was prepared by extracting MDCK cells with a detergent-containing buffer (0.5% Triton X-100, 0.5% sodium deoxycholate) and affinity purifying the PiTyr-containing proteins on a column composed of agarose-linked anti-PiTyr mAbs (see Materials and methods). The immunogen was a complex mixture of PiTyr-containing proteins, as indicated by immunoblot analysis with anti-PiTyr antibodies (data not shown). The preimmune

mouse serum had no reactivity, but the immune sera contained antibodies reacting with multiple proteins, including a prominent band at ~ 240 kDa (not shown).

Approximately 1500 clones were screened by immunoblot analysis, which yielded hybridomas, H5 and H14, which produced monoclonal IgMs that bind specifically to a 240 kDa protein band (Fig. 1, lanes A and B, solid triangle, left margin). The 240 kDa protein immunoprecipitated with mAb H5 reacts with mAb H5 antibody on blots (Fig. 1E, solid triangle, right margin); however, an additional ~ 210 kDa band (Fig. 1E, open triangle, right margin) is present if the protein is immunoprecipitated before immunoblot analysis. The ~ 210 kDa band is usually absent when samples are boiled immediately in sample buffer, suggesting that it is a proteolytic breakdown product. The protein is immunoprecipitable with H5 and mAb H14, and it is detectable by immunoblotting with either mAb, indicating that mAbs react to the same protein (data not shown). Finally, the epitope recognized by mAb H5 seems to be more sensitive to proteolysis than the H14 epitope (unpublished results).

The relationship of this 240 kDa protein to other known proteins of this approximate size was explored by immunoblotting with antibodies to fodrin, erythrocyte ankyrin, filamin, tensin, talin, ZO-1, and myosin headpiece domain. All of the antibodies reacted with MDCK cell proteins, but none reacted with the p240 molecule (cytotellin) immunoprecipitated with mAb H5 (data not shown).

Cytostellin is inside the nucleus of non-dividing cells, but appears outside of the nucleus in daughter cells

The 240 kDa protein has an unusual subcellular distribution in MDCK cells. mAb H5 immunoreactivity is diffuse, with a variable degree of punctate staining, and it is restricted to the nucleus of nondividing MDCK cells (Fig. 2, panel A). The distribution of p240 is striking in daughter cell pairs

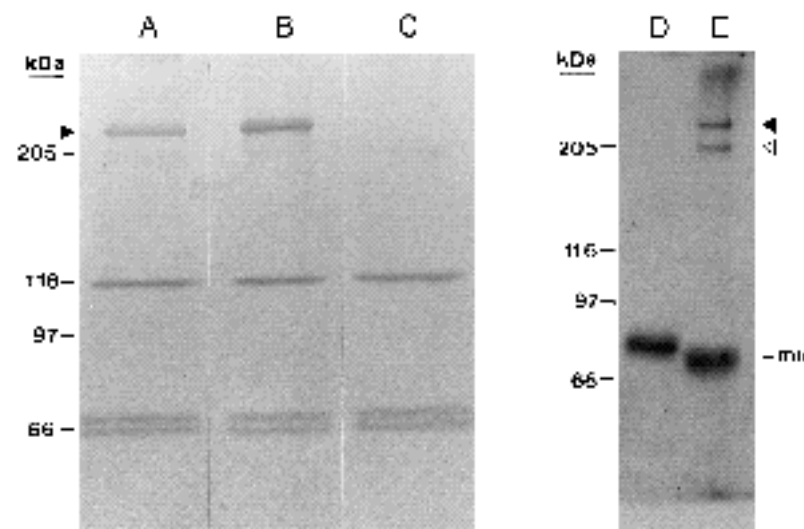


Fig. 1. Monoclonal antibodies bind to a 240 kDa MDCK cell protein. Total MDCK cell protein was electrophoresed on a 7% polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with monoclonal antibodies H5 (lane A), H14 (lane B) and control IgM (lane C). Hybridomas H5 and H14 produce IgM antibodies that react to a 240 kDa protein (solid triangle, left margin). The immunoblots in lanes A-C were developed using biotinylated goat anti-mouse IgM, followed by an avidin-alkaline phosphatase conjugate. The conjugate binds directly to proteins at 116 kDa, 70 kDa and 65 kDa, and these bands are visible in all three lanes. Detergent extracts of MDCK cells were incubated with control IgM, TEPC 183 (lane D) or mAb H5 (lane E) bound covalently to agarose beads. The immunoprecipitates were washed, subjected to 7% PAGE, transferred to nitrocellulose and blotted with mAb H5. A 240 kDa band (solid triangle, right margin), and a 210 kDa band (open triangle

right margin) are visualized. The blot was developed with a biotinylated goat anti-mouse IgM, followed by an ¹²⁵I-avidin conjugate, exposed to X-ray film for 5 h and then developed. The same result is obtained when H14 is substituted in the immunoprecipitation and/or the immunoblotting step of this experiment (data not shown). Molecular mass standards: 205 kDa, myosin; 116 kDa, -galactosidase; 97 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin. mu, heavy chain of IgM.

which were at various stages of cytokinesis (Fig. 2C-F). Postmitotic daughter cell pairs are symmetrical and have multiple discrete, immunoreactive "bodies" scattered throughout the cytoplasm. The fact that p240 is *intranuclear* in non-dividing cells, but forms *extranuclear* bodies in daughter cells, indicates that p240 moves from sites in the interphase nucleus to new locations throughout dividing cells.

Each phase of the cell cycle is marked by altered distribution and intensity of cytotellin immunoreactivity

To characterize the subcellular localization of cytotellin at each phase of the cell cycle, MDCK cells were grown on coverslips at a subconfluent density and were stained simultaneously with the DNA-binding fluor, 4,6-diamidino-2-phenylindole (DAPI) and the H5 mAb. The state of chromosomal condensation, and the contours of the nuclear periphery revealed by DAPI staining, indicate the phase of the cell cycle for each cell in a microscopic field. MDCK cells in the left panels of Fig. 3 reveal the chromosomal DNA, and the same cells are shown in the right panels, which reveal cytotellin immunoreactivity.

Cytotellin immunoreactivity in interphase nuclei is mostly diffuse with a variable degree of fine punctate staining (Fig. 3A, right panel; arrows indicate punctate "dots"). The cytotellin "dots" are separated from the nuclear periphery by a continuous, submembranous zone which appears to follow the contours of the nuclear envelope (Fig. 3A, right panel, bracket). The areas of weak H5 staining in the nucleus, best visualized in Fig. 2A (small arrows), are probably nucleolar regions.

Early prophase is identified by an intense, beaded appearance of the DAPI-stained nuclei, reflecting the onset of chromosomal condensation (Fig. 3B, left panel, arrowheads). Coincident with this change, cytotellin immunoreactivity intensifies and appears as intranuclear "bodies" (Fig. 3B, right panel, arrows). The outermost bodies are arranged in a line separated from the nuclear periphery by a continuous zone lacking discrete immunofluorescent bodies (Fig. 3B, right panel, bracket). Mid-prophase cells are identified by increasing condensation of the chromosomes, and a loss of the smooth edges at the nuclear periphery indicating early disassembly of the envelope (Fig. 3C, left panel, arrowheads). Coincident with these changes, a few cytotellin bodies begin to appear in positions *outside* the disassembling nucleus (Fig. 3C, right panel, arrowheads). By late prophase, multiple cytotellin bodies are widely dispersed to positions remote from the chromosomes (Fig. 3D, right panel, arrows).

The cytotellin bodies remain widely dispersed throughout the cell during metaphase, anaphase and telophase (Fig. 3E-H, right panels, arrows). Metaphase and anaphase chromosomes and the spindle apparatus per se do not stain appreciably with mAb H5, an observation that is best appreciated when the cells are *not* stained simultaneously with DAPI, which is intensely fluorescent and "bleeds" through the rhodamine filter used in the experiments shown in Fig. 3. The lack of *chromosome* staining by mAb H5 is best shown in single-fluor experiments (Fig. 4e).

The degree of nuclear reassembly varies among telophase cells, as indicated by the contour of the nuclear periphery:

it is well-defined in some telophase cells (Figs 2F and 3H, arrowheads), and poorly defined ("fuzzy") in others (Fig. 2C-E, arrowheads). The H5 mAb stained nascent nuclei in early and late stages of reassembly, in addition to the cytoplasmic bodies. (Fig. 2C-F, small arrows). This interpretation is supported by double immunofluorescence with mAb H5 and anti-lamin A, C antibodies: the nuclear lamina reassembles before the cytotellin bodies disappear from the cytoplasm (unpublished results). The cytotellin bodies were widely distributed throughout the cytoplasm, including the tips of cell processes, far removed from the nucleus (Fig. 2D, arrowheads). The number of cytotellin bodies is reduced in telophase cells compared to metaphase and anaphase cells (compare Fig. 3H with 3E, F and G). Significantly, the cytoplasmic cytotellin bodies are present even in daughter cells that have completed cytokinesis. H5 mAb did not stain the midbodies between separating daughter cells (e.g. Fig. 2C).

Cytotellin bodies surround the mitotic spindle apparatus in metaphase and anaphase cells

The pattern of the cytotellin immunoreactivity in metaphase and anaphase cells is variable: most cells are studded with discrete bodies throughout (Figs 4c; 3E-G), but some cytotellin-bodies are smaller, and cytotellin immunofluorescence appears almost homogeneous in other cells (Fig. 4a,b,d,e and f). The variation may represent a transitional state in the cell cycle marked by more finely separated cytotellin structures, or it may be related to technical factors, such as variable fixation and permeabilization. However, metaphase and anaphase cells with either staining pattern contain a distinct group of cytotellin bodies that are positioned in an approximately ring-like structure surrounding the mitotic spindle apparatus (Fig. 4a-f, arrowheads). The peri-spindle cytotellin bodies are larger and more intensely immunoreactive than the more peripheral bodies.

To determine more precisely the spatial relationship between the spindle apparatus and the peri-spindle cytotellin bodies, double immunofluorescence was performed using mAb H5 and polyclonal antibodies directed at the nuclear mitotic antigen (NuMA), a 240 kDa nuclear protein that distributes to the polar regions of the spindle apparatus (Price and Pettijohn, 1986; Yang et al., 1992). Anti-NuMA antibodies stain the crescent-shaped poles of the spindle (the apex of each crescent co-localizes with the centrosome) and thus, NuMA staining marks the ends of the spindle apparatus.

The relative positions of the cytotellin bodies and the NuMA-reactive spindle structures are evident by comparing panels f and g in Fig. 4. The FITC-stained, NuMA-reactive spindle structures are shown (Fig. 4g, arrows). The rhodamine fluorescence of the cytotellin bodies (Fig. 4, arrowheads) was much more intense than the FITC-fluorescence, and was not entirely blocked by the filter, so faint images of the cytotellin-containing bodies are visible in the FITC photomicrograph (Fig. 4g, dots). These cytotellin-containing bodies were not visible *prior* to the bleaching of the FITC by ultraviolet light; moreover, anti-NuMA antibodies do not stain these structures (Yang et al., 1992; Price and Pettijohn, 1986; and data not shown). This

experiment shows that cytotestellin bodies are aligned in a ring-like arrangement, *surrounding* the region of the spindle apparatus, but cytotestellin does not co-localize with the mitotic spindle.

The majority of cytotestellin is readily solubilized from MDCK cells

Nuclear proteins can be classified by function, subcellular localization, and biochemical properties (Berezney and Coffey, 1974, 1977; Capco et al., 1982; Chaly et al., 1984; Fey and Penman, 1988; Cook, 1988; Verheijen et al., 1988; Jackson and Cook, 1988; He et al., 1990; Nickerson et al., 1992). To define cytotestellin's solubility properties in MDCK cells, a simple fractionation experiment was performed on confluent cultures. The majority of the cytotestellin is likely to be *intranuclear* under these conditions (see immunolocalization studies above). Cell monolayers were incubated in 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate (DOC) for 15 min at 4°C and scraped from the dish. Soluble and insoluble fractions were separated by centrifugation. The majority of cytotestellin is solubilized by this protocol (Fig. 5; soluble, "s"; insoluble pellet, "p"), indicating that harsh chaotropic agents, such as sodium dodecyl sulfate, are not needed.

Cytotestellin in the soluble and insoluble fractions exhibits qualitative differences. Firstly, the soluble fraction of cytotestellin is composed of two bands, as shown before (Fig. 5, left margin: solid triangle, 240 kDa band; open triangle, 210 kDa band). Interestingly, the mobility of both cytotestellin bands is retarded slightly in the insoluble fraction and the lower cytotestellin band is relatively more intense than the upper band (Fig. 5, right margin: single dot, upper band; double dot, lower band). Differences between the soluble and insoluble fractions of cytotestellin may reflect multiple isoforms, post-translational modification, or proteolytic breakdown; alternatively, differences may have a

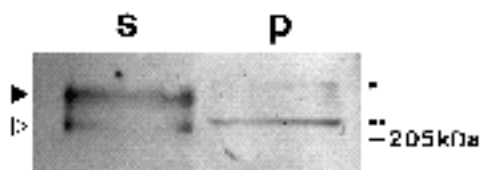


Fig. 5. Detergent solubility of cytotestellin in MDCK cells. Confluent MDCK cells were extracted 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM PMSF, 2 mM NaN_3 , 0.2 mM Na_3VO_4 , 5 mM EDTA and 2 mM EGTA, for 15 min at 4°C. Soluble (s) and insoluble (p) fractions were separated by centrifugation. Detergent-insoluble cytotestellin was solubilized by boiling in SDS sample buffer. Detergent-soluble cytotestellin was immunoprecipitated with mAb H14 and boiled in SDS sample buffer. Both fractions were run onto a 7% polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with mAb H14. The majority of cytotestellin is extracted under these mild conditions. S, supernatant (soluble) fraction; P, pellet (insoluble) fraction; solid triangle (left margin) and single dot (right margin), ~240 kDa cytotestellin species; open triangle (left margin) and double dot (right margin), ~210 kDa cytotestellin species; The molecular mass standard is myosin, 205 kDa.

technical basis. More detailed biochemical extractions, including a comparative analysis of nuclear proteins that have already been characterized in sequential extraction protocols (Berezney and Coffey, 1974, 1977; Capco et al., 1982; Fey and Penman, 1988; Cook, 1988; Verheijen et al., 1988) are required to assign cytotestellin to a defined nucleoskeletal fraction.

Immunoaffinity purification of canine cytotestellin

Cytotestellin has been purified by affinity chromatography using agarose-bound mAb H5. MDCK cell extracts were incubated with mAb H5-agarose beads, washed extensively, boiled in 2×sample buffer and resolved on a 7% polyacrylamide gel. The amount of cytotestellin purified from approximately 50 mg of total MDCK cell protein is visible as a 240 kDa band stained with Coomassie brilliant blue (Fig. 6).

Cytotestellin is conserved evolutionarily

To test the evolutionary conservation of cytotestellin, cells and tissues of a wide variety of eukaryotic species were solubilized for total protein: human, dog, mouse, chicken, newt, fish, insect (*D. melanogaster*), nematode (*C. elegans*) and yeast (*S. pombe*). The extracts were immunoblotted with

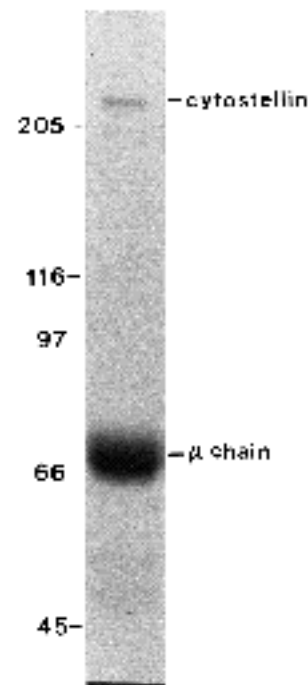


Fig. 6. Immunoaffinity purification of canine cytotestellin. MDCK cell extracts were incubated with mAb H5 - agarose, washed extensively and boiled in SDS sample buffer with 5% - mercaptoethanol. The proteins were resolved on a 7% polyacrylamide gel, which was stained with Coomassie brilliant blue, dried and photographed. The cytotestellin protein band at 240 kDa was immunoaffinity purified from less than 50 mg of total MDCK cell protein. The mu (μ) heavy chain released from agarose-bound mAb H5 is indicated. Molecular mass standards are indicated: 205 kDa, myosin; 116 kDa, -galactosidase; 97 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin. μ , heavy chain of IgM.

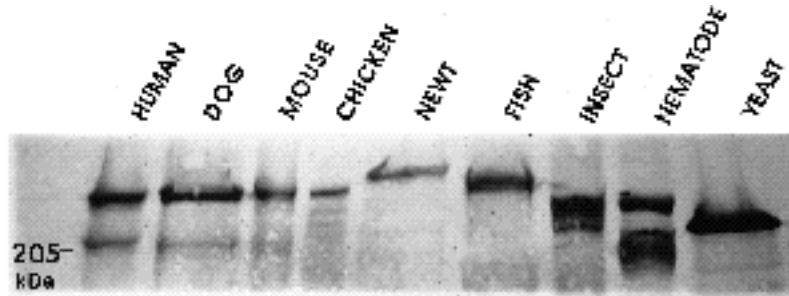


Fig. 7. Evolutionary conservation of cytochrome c. Immunoblot analysis of proteins derived from multiple eukaryotic organisms. Cells and tissues were solubilized by boiling in SDS sample buffer with 5% β -mercaptoethanol. The proteins were resolved on a 7% polyacrylamide gel, transferred to a nitrocellulose membrane and immunoblotted with mAb H5. The anti-cytochrome c mAb H14 gave similar results (data not shown). The ~210 kDa band present in the mammalian species is believed to be a proteolytic breakdown product (see text). The multiple bands in the insect and nematode specimens may represent proteolytic degradation fragments, isoforms of cytochrome c or cross-reactive proteins. The relative molecular mass standard is myosin, which migrates at 205 kDa.

the mAb H5 (Fig. 7), which binds to a protein in all species tested, from yeast to humans. The molecules in amphibian and fish are greater than 240 kDa, those in mammals, birds, insects and nematodes are approximately 240 kDa, and the yeast protein is approximately 210 kDa. All of these eukaryotic homologues are detected by anti-cytochrome c mAb H14, which binds to a separate epitope on cytochrome c (our unpublished results). The demonstration that two cytochrome c epitopes are conserved across a wide phylogenetic spectrum implies that this protein has an evolutionarily conserved function in eukaryotic cells.

Discussion

Nuclear and cytoplasmic processes are physically separated in non-dividing eukaryotic cells by the nuclear membrane (Newport and Forbes, 1987; Gerace and Burke, 1988). Mitotic cells of higher eukaryotes transiently lose this barrier, so there is a potential for interaction between nucleoskeletal and peripheral cytoskeletal molecules. It is possible that the orderly partitioning of nuclear and cytoplasmic structures into daughter cells depends upon mitosis-specific interactions between nuclear and cytoplasmic molecules, but little is known about this subject. Cytochrome c is a nuclear protein during interphase, but during cell division it forms multiple dot-like structures located in subcellular locations far removed from the chromosomes and spindle apparatus. There, cytochrome c has the potential to interact with cytoplasmic and cytoskeletal proteins. The orderly, ring-like arrangement of cytochrome c bodies surrounding the mitotic spindle in MDCK cells (Fig. 4) suggests that cytochrome c may be part of a highly organized network encompassing the spindle apparatus. We speculate that the peripheral cytochrome c bodies might interact with the subcortical cytoskeleton, and the proximal cytochrome c bodies may interact with the mitotic spindle; from these positions, cytochrome c-containing bodies might help to coordinate the partitioning of cytoplasmic structures with the segregation of the chromosomes into daughter cells.

The cytochrome c dots in interphase nuclei are evenly spaced, and arranged in a row which is clearly inside the peripheral rim of the nucleus. It will be important to inves-

tigate the spatial relationship between cytochrome c, chromatin and the nuclear lamina in interphase cells. We do not know whether the increased intensity of cytochrome c staining in prophase nuclei is due to an increase in cytochrome c expression, enhanced epitope availability, or recruitment of cytochrome c to these nucleoskeletal sites. In future studies, we plan to determine whether or not the level of cytochrome c fluctuates during the cell cycle. Finally, the structure of the cytochrome c bodies is not known. They may be nucleoskeletal protein complexes or membrane-bound vesicles that disperse to positions throughout the cell during mitosis.

The numbers of cytochrome c bodies in the cytoplasm of each daughter cell are approximately equal at all phases of mitosis, and diminishes during telophase, when intranuclear cytochrome c immunoreactivity increases. If cytochrome c is degraded in the cell cytoplasm, cyclic resynthesis of cytochrome c must be invoked to explain the increased intranuclear immunoreactivity after telophase. Alternatively, the extranuclear cytochrome c bodies may be recruited back to the nucleus during telophase.

The subcellular localizations of several membrane-associated and soluble proteins in the nucleus and cytoplasm are regulated by protein phosphorylation (Evans and Fink, 1982; Gerace and Burke, 1988; Gurley et al., 1978; Heald and McKeown, 1990; Newport and Forbes, 1987; Peter et al., 1990; Vandre et al., 1991; Ward and Kirschner, 1990; Koch et al., 1991). Cytochrome c is a phosphoprotein (data not shown), and its exodus coincides with nuclear envelope disassembly. The possibility that cytochrome c's cell cycle redistribution is controlled by cyclic phosphorylation reactions is currently under investigation.

Many nuclear proteins undergo a transient redistribution during mitosis, but very few have been shown to localize to multiple discrete bodies or "dots" dispersed throughout mitotic cells (Chaly et al., 1984; Newport and Forbes, 1987; Gerace and Burke, 1988; Benevento, 1991). Examples of this general pattern are the nucleoporins. It has been suggested that the appearance of nucleoporin-staining dots is an indication of molecular complexes that disperse, but fail to disintegrate completely in mitotic cells (Gerace et al., 1978). At interphase, the pattern of nucleoporin immunofluorescence is different from that of cytochrome c, since the nucleoporins form the pore complexes in the

nuclear membrane, which appear as discrete spots arranged in a rim-like pattern on the envelope at the periphery of the nucleus (Gerace et al., 1978).

Antibodies directed at erythrocyte ankyrin also stained discrete "dots" throughout dividing mammalian cells, but in addition, they stained the spindle poles and the midregion of the cell, near the cleavage furrow (Bennett and Davis, 1981). This pattern differs from that of cytochellin. Antibodies directed at erythrocyte ankyrin do not bind to cytochellin which has been immunoprecipitated from MDCK cells (unpublished results), but these antibodies do bind to ankyrin in MDCK cells (Morrow et al., 1989).

Antibodies directed at two nuclear matrix proteins, p107 (Smith et al., 1985) and p240 (H1B2) (Nickerson et al., 1992), stain discrete bodies throughout mitotic cells. The sizes and the immunofluorescence patterns of H1B2 (Nickerson et al., 1992) and cytochellin are similar. However, there are significant differences in their immunofluorescent patterns at each stage of the cell cycle. Firstly, markedly different treatments are required to expose the epitopes of these proteins in interphase nuclei. A brief permeabilization with 0.5% Triton X-100 following fixation revealed the cytochellin immunoreactivity, which was diffuse with multiple punctate dots distributed throughout the nucleus. In contrast, Triton X-100 extraction was insufficient to unmask H1B2 antigen; visualization of this nuclear matrix antigen required DNAase I digestion followed by 0.25 M ammonium sulfate extraction prior to fixation. In early prophase cells, cytochellin appears as multiple punctate foci arranged in a row parallel to the nuclear periphery. In contrast, H1B2 immunoreactivity appeared as a single small spot in each nucleus. In addition, mAb H5, but not H1B2, stained a group of large bodies arranged in a ring-like structure surrounding the region of the spindle apparatus. Finally, the H1B2 antibody stained the centrioles and the midbody, structures that do not stain with mAb H5.

The relatively mild conditions required to expose the H5 epitope of cytochellin in immunofluorescence experiments correlates with its solubility properties determined biochemically (Fig. 5). Most of the cytochellin in MDCK cells is readily extracted with nonionic detergents, indicating that cytochellin is not a nuclear matrix protein, at least by operational biochemical criteria (Berezney and Coffey, 1974, 1977; Capco et al., 1982; Fey and Penman, 1988; Cook, 1988; Verheijen et al., 1988); however, it is possible that the fraction of cytochellin which partitions with the Triton X-100/DOC pellet (Fig. 5) is a component of the nuclear matrix. Despite the biochemical differences between cytochellin and nuclear matrix proteins, the similar appearance of p107 (Smith et al., 1985), H1B2 (Nickerson et al., 1992) and cytochellin bodies in dividing cells suggests the possibility that cytochellin may co-distribute and/or associate with these or other nuclear matrix proteins during the cell cycle.

Cell cycle control mechanisms and many of the proteins that have a structural or mechanical role in mitotic chromosome segregation are highly conserved in evolution (Beach, 1988; Cross et al., 1989; Nurse, 1990; Moudjou et al., 1991; Borisy et al., 1984; Vallee and Shpetner, 1990). However, there are significant differences in the mitotic processes of higher and lower eukaryotes (Kubai, 1975;

Heath, 1980). For example, yeast cells divide without disassembling their nuclear membrane (Byers, 1981; King and Hyams, 1982; McCully and Robinow, 1971). The spindle pole bodies anchor the mitotic spindle to the nuclear membrane, and the nucleus splits into daughter nuclei during cytokinesis.

Two epitopes (H5 and H14) are conserved in the ~210 kDa *S. pombe* molecule, suggesting that this protein is a cytochellin homologue. The presence of cytochellin homologues in lower eukaryotes which maintain nuclear envelope structure during cell division, and in higher eukaryotes which disassemble the nuclear envelope, raises intriguing questions about the function and subcellular distribution of cytochellin during mitosis in these evolutionarily divergent cells. Insight into cytochellin's function may be gleaned from studying its distribution in dividing yeast cells. If a ring-like arrangement of cytochellin bodies surrounds the yeast mitotic spindle, one might predict that it assembles *within* the dividing nucleus, since yeast cells maintain the nuclear envelope during cell division.

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Fig. 2. Indirect immunofluorescence of cytotellin in MDCK cells. Indirect immunofluorescence was performed on MDCK cells that had been fixed with 1.7% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 in PBS. The first antibody was either mAb H5 (A,C-F) or a control IgM (B). The second antibody was biotinylated goat anti-mouse IgM and the tertiary reagent was an avidin-fluorescein isothiocyanate (FITC) conjugate. (A and B) non-dividing cells; (C-F) daughter cell pairs. Arrowheads indicate the nuclear periphery, short arrows indicate nucleolar areas and the long arrows indicate the cytotellin-containing bodies outside the nascent nuclei. All panels are presented at the same magnification. Bar, 25 μ m.

Fig. 3. MDCK cells stained simultaneously with DNA-binding dye, DAPI, and anti-cytostellin monoclonal antibody, H5. Indirect immunofluorescence was performed on MDCK cells that had been fixed with 1.7% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 in PBS. First, the cells were reacted with mAb H5 (right panels), followed by a biotinylated goat anti-mouse IgM and then an avidin-rhodamine conjugate. Next, the cells were stained with DAPI (left panels). (A) nondividing cells; (B) early prophase; (C) mid prophase; (D), late prophase; (E) metaphase; (F) early anaphase; (G) late anaphase; (H) telophase. Arrowheads indicate periphery of nucleus. Small arrows indicate cytotellin bodies. Long arrows indicate the plane of metaphase plate. Brackets indicate margin under nuclear periphery which lacks discrete cytotellin immunoreactivity. All panels are presented at the same magnification. Bar, 25 μ m.

Fig. 4. Indirect immunofluorescence showing cytotellin-containing bodies that surround the mitotic spindle in dividing MDCK cells. Indirect immunofluorescence was performed on MDCK cells that had been fixed with 1.7% paraformaldehyde and permeabilized with 0.5% (v/v) Triton X-100 in PBS. (a-d) Dividing cells were reacted with anti-cytostellin mAb H5, followed by a biotinylated goat anti-mouse IgM, and avidin-rhodamine. The cells were also stained with DAPI, which is not revealed here. The dividing cell in (e) was stained with anti-cytostellin mAb H5, followed by a biotinylated goat-anti-mouse IgM, and avidin-FITC. (f and g) The dividing cell was reacted with anti-cytostellin mAb H5 (f), and rabbit anti-NuMA (g) (Yang et al., 1992). The mAb H5 staining is indicated by rhodamine fluorescence and anti-NuMA is indicated by FITC fluorescence. Arrowheads indicate large intensely immunofluorescent cytotellin-containing bodies. Dots indicate approximate position of metaphase plate. Arrows in g indicate NuMA-reactive regions of mitotic spindle. Light dots in g indicate position of cytotellin-immunoreactive bodies which were not blocked by the FITC filter. All panels are presented at the same magnification. Bar, 25 μ m.