

A 62-kD Protein Required for Mitotic Progression Is Associated with the Mitotic Apparatus during M-Phase and with the Nucleus during Interphase

Jennifer A. Johnston and Roger D. Sloboda

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755; and The Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Abstract. A protein of 62 kD is a substrate of a calcium/calmodulin-dependent protein kinase, and both proteins copurify with isolated mitotic apparatuses (Dinsmore, J. H., and R. D. Sloboda. 1988. *Cell*. 53:769-780). Phosphorylation of the 62-kD protein increases after fertilization; maximum incorporation of phosphate occurs during late metaphase and anaphase and correlates directly with microtubule disassembly as determined by *in vitro* experiments with isolated mitotic apparatuses. Because 62-kD protein phosphorylation occurs in a pattern similar to the accumulation of the mitotic cyclin proteins, experiments were performed to determine the relationship between cyclin and the 62-kD protein. Continuous labeling of marine embryos with [³⁵S]methionine, as well as immunoblots of marine embryo proteins using specific antibodies, were used to identify both cyclin and the 62-kD pro-

tein. These results clearly demonstrate that the 62-kD protein is distinct from cyclin and, unlike cyclin, is a constant member of the cellular protein pool during the first two cell cycles in sea urchin and surf clam embryos. Similar results were obtained using immunofluorescence microscopy of intact eggs and embryos. In addition, immunogold electron microscopy reveals that the 62-kD protein associates with the microtubules of the mitotic apparatus in dividing cells. Interestingly, the protein changes its subcellular distribution with respect to microtubules during the cell cycle. Specifically, during mitosis the 62-kD protein associates with the mitotic apparatus; before nuclear envelope breakdown, however, the 62-kD protein is confined to the nucleus. After anaphase, the 62-kD protein returns to the nucleus, where it resides until nuclear envelope disassembly of the next cell cycle.

OUR understanding of the cell cycle and the molecules involved in its regulation has increased dramatically in the last few years (for reviews see Pardee, 1989; Laskey et al., 1989; Murray and Kirschner, 1989a; McIntosh and Koonce, 1989; Hartwell and Weinert, 1989; O'Farrell et al., 1989). It is now known that maturation-promoting factor (MPF)¹ is a protein kinase consisting of two proteins, p34 and p45 (for review see Masui, 1991). The p34 subunit is homologous to the *cdc2* gene product of yeast (Lohka, 1989), whereas the p45 subunit is a member of a family of proteins called cyclins (Evans et al., 1983). The cyclins are proteins that oscillate in abundance and whose synthesis has been shown to be required for progression through the cell cycle (Hutchinson et al., 1988; Murray and Kirschner, 1989b; Minshull et al., 1989). The kinase activity of MPF can be regulated by the association of p34 with different cyclin molecules at different times in the cell cycle (Nurse, 1990). G1

cyclins are proteins that are required to begin the DNA synthesis phase of the cell cycle (Hadwinger et al., 1989; Richardson et al., 1989; Wittenburg et al., 1990; Girard et al., 1991), whereas G2 cyclins are required for the cell to enter mitosis (Swenson et al., 1986; Pines and Hunt, 1987; Minshull et al., 1989; Murray and Kirschner, 1989b; Murray et al., 1989). G2 cyclins are synthesized and accumulate until metaphase (Kobayashi et al., 1991), and for this reason G2 cyclins have become known as mitotic cyclins. At anaphase onset, mitotic cyclins are specifically degraded (Evans et al., 1983; Murray et al., 1989), and their degradation is required for the cell to exit from mitosis. The kinase activity of the MPF/cyclin complex is believed to be a master control that will act upon a large number of cellular factors to allow the proper coordination of cell cycle events. The specific factors that are acted upon by the complex are as yet largely unknown; however, if cyclin/MPF is indeed a master control, it could act upon cytoplasmic structural elements, nuclear structural elements, DNA replication enzymes, metabolic controls, protein synthesis, and/or transcriptional control factors. Therefore, the identification of cyclin-like molecules is important as these are the molecules that allow the cascade of events to occur that lead to cell division.

Microtubule-associated proteins (MAPs) are proteins that

J. A. Johnston's present address is Division of Biology, 147-75, California Institute of Technology, Pasadena, California 91125.

1. *Abbreviations used in this paper:* Ca/Cam, calcium/calmodulin; kMT, kinetochore MT; MA, mitotic apparatus; MAP, microtubule-associated protein; MBLSW, Marine Biological Laboratory seawater; MPF, maturation-promoting factor; MT, microtubule.

interact with microtubules (MTs), and brain MAPs decorate the walls of MTs as determined by electron microscopy (Amos and Baker, 1979; Kim et al., 1979; Sloboda and Rosenbaum, 1979). MAPs have been shown to stimulate the assembly of tubulin into MTs and to stabilize assembled tubulin in vitro (Sloboda et al., 1975; Sloboda and Rosenbaum, 1979). In vivo, the assembly of tubulin into MTs may also be affected by MAPs (Bre and Karsenti, 1990). It has been established that MTs are more stable and longer during interphase than the MTs that are present during mitosis (Saxton et al., 1984). Interestingly, in vitro studies using the cdc2 kinase in *Xenopus* extracts have demonstrated that the mean length of MTs in interphase and metaphase is directly correlated to the level of cdc2 kinase activity (Verde et al., 1990). Because the level of cdc2 kinase activity is regulated by its association with cyclin, MT dynamics throughout the cell cycle may be (at least in part) due to the association of cyclin with the cdc2 gene product. It has also been suggested that there are cell cycle stage-specific MAPs that associate with MTs only at certain times in the cell cycle (Lydersen and Pettijohn, 1980; Izant et al., 1982; Pettijohn et al., 1984; Nislow et al., 1990; Wright et al., 1991), and this transient association may be responsible for cell cycle-specific changes in MT dynamics.

MT assembly is sensitive to changes in calcium concentration in vitro (Weisenberg, 1972). Note also that the intracellular concentration of free calcium changes throughout the cell cycle (for a comprehensive review see Hepler, 1989). In particular, it has been reported that calcium levels rise at metaphase (Peonie et al., 1985, 1986), a time in the cell cycle that correlates with changes in MT stability before chromosome movement at the beginning of anaphase. Moreover, perturbation of intracellular calcium levels by a variety of techniques blocks mitosis in sand dollar embryos (Silver, 1986, 1989). Calmodulin is a protein that will bind calcium ions (Teo and Wang, 1973) and activate specific cellular protein kinases. Calmodulin has been shown to be involved in the mediation of the effects of calcium on MTs in the presence of MAPs (Marcum et al., 1978; Schliwa et al., 1981). For example, mitotic apparatus (MA) MTs will depolymerize in the region of microinjection when calcium-saturated calmodulin is injected into mitotic cells (Keith, 1987). Therefore, MA MTs are sensitive to calcium/calmodulin (Ca/Cam). The addition of calcium, calmodulin, and ATP to isolated MAs results in the phosphorylation of a single protein having a relative molecular mass of 62 kD (Dinsmore and Sloboda, 1988). Phosphorylation of this protein correlates with MT disassembly (Dinsmore and Sloboda, 1988) as measured either by a decrease in birefringence of the isolated MA or by solubilization of tubulin subunits. Therefore, the Ca/Cam-dependent phosphorylation of the 62-kD protein may control MT stability at the metaphase to anaphase transition. Interestingly, phosphorylation of the 62-kD protein is cell cycle dependent (Dinsmore and Sloboda, 1988); the incorporation of phosphate begins at the time of nuclear envelope breakdown, and increases until metaphase, whereupon the level of incorporated phosphate decreases after anaphase. This apparent cyclic phosphorylation of the 62-kD protein may result either from the action of a kinase/phosphatase pair, or from the synthesis and degradation of the 62-kD protein in a manner similar to the mitotic cyclins, or from some combination of these activities.

As described above, MT dynamics throughout the cell cycle may be due to the association of cyclin with the cdc2 gene product. Because the 62-kD protein is involved in the stability of MTs and is phosphorylated in a manner similar to the pattern of cyclin synthesis and destruction, it is important to determine if the 62-kD protein is a cyclin. Here, the 62-kD protein is examined for its presence and localization throughout the cell cycle. The protein is shown to be distinct from cyclin because [³⁵S]methionine labeling of newly synthesized proteins reveals the characteristic cyclic nature of cyclin synthesis and degradation, whereas the synthesis of the 62-kD protein is not cyclical. Densitometric analysis of immunoblots specific for the 62-kD protein shows the protein is present in the sea urchin egg from before fertilization through at least the four-cell stage. The subcellular localization of the 62-kD protein confirms the distinction between this protein and cyclin, and reveals that the 62-kD protein associates with MTs in a stage-specific manner. Immunogold electron microscopy of isolated MAs confirms the association of the protein with MTs. Most interestingly, the 62-kD protein resides in the nucleus before nuclear envelope breakdown, and associates with the MTs of the forming MA only after nuclear envelope breakdown. At telophase, the 62-kD protein is sequestered to the forming daughter nuclei. The relationship of the 62-kD protein with respect to the known cyclin molecules, and known stage-specific MAPs, is discussed.

Materials and Methods

Reagents and Organisms

Lytechinus pictus and *Strongylocentrotus purpuratus* were obtained from Marinus, Inc. (Westchester, CA). *Spisula solidissima* were obtained from the Department of Marine Resources, Marine Biological Laboratory (Woods Hole, MA). Prestained standards for SDS-PAGE were purchased from Sigma Chemical Co. (St. Louis, MO). ¹²⁵I-Donkey anti-rabbit IgG, 750–3,000 Ci/mmol, and [³⁵S]methionine, 3,000 Ci/mmol, were purchased from Amersham Corp. (Arlington Heights, IL). Fluoresceinated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Kirkegaard and Perry (Gaithersburg, MD). Nitrocellulose, 0.22- μ m pore size, was from Schleicher and Schuell (Keene, NH). All other chemicals were purchased from Sigma Chemical Co.

Cell Cycle Experiments

Lytechinus pictus or *Strongylocentrotus purpuratus* were induced to spawn by intracoelomic injection of 0.5 M KCl. Eggs were collected by inverting spawning females over a beaker containing artificial Marine Biological Laboratory seawater (MBLSW; Cavanaugh, 1956); sperm were collected "dry" by inverting males over watch glasses. For all experiments, embryos were maintained at 15–18°C for *L. pictus* and 12°C for *S. purpuratus* in a large volume with gentle agitation to allow proper aeration. Eggs were washed three times with fresh MBLSW and then fertilized with 50 μ l of a 1:1,000 dilution of dry sperm into 100 ml of egg suspension (~3 ml of settled eggs in 100 ml MBLSW). 10 ml of the fertilized egg suspension was then immediately transferred to a scintillation vial that contained 50 μ Ci of [³⁵S]methionine. Both the radioisotope-labeled and unlabeled embryo cultures were stirred continuously, and aliquots were removed from the unlabeled culture and fixed for later determination of cell synchrony. 15 s later, an equal aliquot was removed from the [³⁵S]methionine culture to be analyzed by SDS-PAGE. Protein concentrations were determined by the method of Bradford (1976) as modified by Pierce Chem. Co. (Rockford, IL).

To determine cell synchrony, 200- μ l aliquots of cells were removed at specified times after fertilization and prepared for lacto-orcein staining (Westendorf et al., 1989). For each time point, 8.5 μ l of embryos was counted in quadruplicate using phase-contrast microscopy. The percentage of embryos at metaphase for each 8.5- μ l aliquot was calculated, and the av-

erage percentage for each time point was determined. Proteins from the cell cycle experiment were prepared for electrophoresis by placing a 200- μ l aliquot of labeled embryos into ice-cold calcium free MBLSW, sedimenting in a microfuge for 5 s, followed by resuspension of the pellet in 2 \times Laemmli (1970) sample buffer.

Cell Fractionation Experiments

Female gonads from *Spisula solidissima* were excised and placed into 0.45- μ m millipore-filtered seawater. The gonads were forced through three layers of cheesecloth to dissociate the oocytes, and then allowed to settle in fresh filtered seawater. The oocytes were washed by repeated settling in filtered seawater. Nuclei were prepared with membranes intact as follows: Washed oocytes were sedimented in a hand centrifuge and resuspended in soaking buffer (25 mM Pipes, 2 mM CaCl₂, 1 M glycerol, pH 9.0) for 1 h. Oocytes were sedimented as before, and then resuspended in 8 vol of H buffer (5 mM Pipes, pH 6.9, 0.7 mM MgCl₂, 6% hexylene glycol). Oocytes were swirled gently, underlaid with a sucrose cushion (0.4 M sucrose, 5 mM Pipes, pH 6.9, 0.35 mM MgCl₂), and centrifuged at 300 g at 4°C for 5 min. The nuclear supernatant was removed and concentrated with Aquacide (Calbiochem Corp., La Jolla, CA) to a 50% reduction in volume. The pellet of nuclei was resuspended in 10 ml of H buffer, underlaid with the sucrose cushion, and centrifuged as before. Nuclei were sedimented three times through sucrose, and then resuspended in 3 ml of H buffer. Each preparation of nuclei was examined microscopically to confirm the presence of intact nuclei.

Nuclei with disrupted membranes were prepared as follows: Washed oocytes were placed in soaking buffer for 1 h. Soaking buffer was removed by aspiration, and the oocytes were resuspended in eight times the oocyte volume of breaker buffer (0.25 M sucrose, 5 mM Pipes, pH 6.9, 0.7 mM MgCl₂, 0.5% NP-40). Nuclei were then prepared as per H buffer nuclei. Protein concentrations were determined by the method of Bradford (1976) as modified by Pierce Chem. Co.

Electrophoresis, Immunoblotting, Autoradiography, and Densitometry

Proteins were analyzed on either 4–10% acrylamide, 2–8 M urea linear gradient gels, or 8% polyacrylamide gels with 3% stacking gels according to the buffer conditions of Laemmli (1970), and stained with Commaassie brilliant blue R250. All gels contained the following prestained molecular mass standards: triosephosphate isomerase (26.6 kD), lactate dehydrogenase (36.5 kD), fumarase (48.5 kD), pyruvate kinase (58 kD), fructose-6-phosphate kinase (84 kD), β -galactosidase (116 kD), α_2 -macroglobulin (180 kD). Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975) as modified by Dessev et al. (1988). The pH gradient for the first dimension IEF was established with ampholines from Sigma Chemical Co. using a 4:1 mix of pH 5–7 and 3–10, respectively.

Electrophoretic transfer of protein to nitrocellulose paper (0.22 μ m) was performed according to the method of Towbin et al. (1979). The transfer was carried out at 400 mA constant current for 1 h in a tank buffer that contained 25 mM Tris and 192 mM glycine (pH 8.3). After completion of the transfer, the nitrocellulose was blocked in 50 ml PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) containing 5% nonfat dried milk, 2.5% FCS, 0.02% NaN₃ for 1 h. The nitrocellulose was then rinsed in PBS and incubated in anti-62-kD antibody affinity purified by the method of Talian et al. (1983), without subsequent conjugation to a fluorochrome, for 3–8 h in the presence of 0.02% NaN₃ to inhibit bacterial growth. The initial production and characterization of these antibodies to the 62-kD polypeptide has been described previously (Dinsmore and Sloboda, 1989). The primary antibody was removed and the blots were washed three times in PBS + 0.05% Tween 20 (PBST), and then processed with either a 1:1,000 dilution of ¹²⁵I-donkey anti-rabbit IgG (750–3,000 Ci/mmol, Amersham Corp.) for 1 h, or a 1:7,500 dilution of alkaline phosphatase-labeled secondary antibodies (Promega, Madison, WI) for 30 min. The ¹²⁵I-labeled blots were washed in PBST as before, allowed to air dry, and exposed at –80°C using X-Omat AR film (Eastman Kodak Co., Rochester, NY) and a Du Pont Cronex Lighting Plus intensifying screen. After 24–72 h, the films were developed in a Kodak RP X-Omat processor model M7B. Alkaline phosphatase-labeled blots were developed as directed by Promega.

Densitometry was performed on a MasterScan Densitometer (CSPI, Billerica, MA). To scan the immunoblots, a reference point on a nonspecific portion of a given autoradiograph was selected as a control for background.

Each alkaline phosphatase- (or ¹²⁵I-) labeled band on the immunoblot was then scanned in reference to this point. Triplicate experiments were scanned, the data were normalized, and plotted \pm SEM using Cricket Graph software on a Macintosh IIfx.

Immunofluorescence

Lytechinus pictus were induced to spawn by intracoelomic injection of 0.5 M KCl as described above. Eggs were washed three times with fresh MBLSW and fertilized in the presence of 1 mM 3-amino-1,2,4 triazole to prevent hardening of the fertilization envelope. The softened fertilization envelopes were removed by passing the eggs through a nytex mesh 10 μ m smaller than the egg diameter. Eggs were then washed three times in MBLSW and allowed to develop with gentle stirring to provide proper aeration and aid synchronous development. Aliquots of embryos were then removed and allowed to adhere to poly-L-lysine (0.1%)–coated coverslips for 5 min, and then plunged into 90% methanol–20 mM EGTA (Harris, 1986) to fix and permeabilize the eggs. HOECHST 33342 at 1 μ g/ml (Calbiochem Corp.) was included in the fixative to stain the chromatin. Coverslips were then washed three times in PBS and 25 μ l of primary antibody (see immunoblotting section for details of antibody production) were applied for 30–90 min. Coverslips were then washed in PBST three times, for 2 min each wash. 25 μ l of a 1:10 dilution of the appropriate fluorescein-labeled secondary antibody was applied for 30 min; coverslips were washed in PBST as before, and mounted in 1% phenylene diamine (Johnson and de C. Nогiera Araujo, 1981) to reduce photobleaching.

Microscopy

Light and Fluorescence Microscopy. Phase-contrast images of lacto-orcein stained embryos were counted using an Optiphot microscope (Nikon, Inc., Garden City, NY) equipped with a 40 \times /1.0 NA oil immersion objective and a 100-W mercury arc lamp for illumination. Epifluorescence specimens were viewed with an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) illuminated with a 100-W mercury lamp. The microscope was equipped with a 63 \times /1.4 NA plan-apochromatic objective and the appropriate Quantifying Imaging Filters (Omega Corp., Brattleboro, VT) for fluorescein and HOECHST.

Electron Microscopy. Mitotic apparatuses were isolated from *Lytechinus pictus* or *Strongylocentrotus purpuratus* as described (Dinsmore and Sloboda, 1988) and fixed for 10 min at room temperature in 3.7% formaldehyde in 50 mM Pipes, pH 6.9, or 4% paraformaldehyde in 50 mM Pipes, pH 6.9. Fixed MAs were centrifuged at 1,000 g for 5 min and resuspended in 1 ml of primary antibody. Primary antibody was either affinity-purified anti-62-kD antibodies or a 1:100 dilution of Sigma monoclonal anti- β -tubulin antibody in PBS (137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4). The primary antibody/MA solution was allowed to incubate with gentle rocking at 4°C for 2–12 h. MAs were then sedimented at 1,000 g for 3 min, and resuspended in 1 ml of PBST. This wash was repeated three times. After the last wash step, the sedimented MAs were resuspended in 1 ml of a 1:10 dilution of gold-conjugated secondary antibody in PBS (goat anti-rabbit antibodies were conjugated to 15-nm gold particles, whereas goat anti-mouse antibodies were conjugated to 10 nm gold) and rocked at 4°C for 1 h. MAs were washed as before and sedimented at 10,000 g for 10 min. The resulting pellet was overlaid with 2% glutaraldehyde in PBS for 30 min. The pellets were then washed four times with PBS without disturbing the pellet. After the fourth wash, 2% OsO₄ in 0.1 M cacadolate buffer (pH 7.2) was added for 20 min at 4°C. The osmium solution was removed and the pellets were washed as before. After the fourth wash, the material was dehydrated through a series of ethanol washes (25–100%) and infiltrated with LR White Medium resin in a 2:1 ratio to 100% ethanol and finally polymerized in 100% LR White resin at 60°C for 20–22 h. Thin sections (40–60 nm) were cut, floated onto formvar- and carbon-coated copper grids, and subsequently stained with 2% uranyl acetate followed by 4% lead citrate. Grids were then viewed in a 100CX electron microscope (JEOL U.S.A. Inc., Peabody, MA).

Results

Characterization of Affinity-purified Antibodies to the 62-kD Protein

For the experiments described in this work, it was essential to have antibodies that were highly specific and reacted only

with the 62-kD protein. To obtain these antibodies, rabbit polyclonal antiserum (Dinsmore and Sloboda, 1989) was affinity purified using immobilized 62-kD protein according to the technique described by Talian et al. (1983) but without subsequent direct conjugation of the affinity-purified antibodies to a fluorochrome. From previous work the 62-kD protein had been determined to focus at an approximate pI of 6.7 in two-dimensional gel electrophoresis (Dinsmore and Sloboda, 1988). On a two-dimensional immunoblot of total *S. purpuratus* protein (Fig. 1) the affinity-purified antibodies recognize only one protein at 62 kD that focuses at a pI of 6.7. Moreover, when the experiment is performed with radio-labeled phosphorylated 62-kD protein, the antibody reacts with the only phosphorylated protein in the preparation (data not shown). It was important to confirm the identity of the affinity-purified antibodies via two-dimensional gel electrophoresis and immunoblotting because the 62-kD protein migrates very closely to keratins in one-dimensional SDS-PAGE. Thus it is common to have antibodies to keratin, which often are present in rabbit serum, contaminating affinity-purified 62-kD antibodies prepared by this method. Two-dimensional immunoblotting allowed the 62-kD protein to be identified and distinguished from the keratins, which focus at a pI of 7.4–8.0 for the basic keratins and a pI of 4.5–6.0 for the acidic keratins (O'Guin et al., 1990). The results shown in Fig. 1 demonstrate the specificity of the affinity-purified 62-kD antibodies. Only affinity-purified antibodies that yielded one spot at a pI of 6.7 and at a molecular weight of 62-kD on two-dimensional immunoblots were used for the experiments described here.

62-kD Protein Is Present throughout the Cell Cycle

To determine the synthesis and abundance of the 62-kD protein as a function of the cell division cycle, *Strongylocentrotus purpuratus* or *Lytechinus pictus* embryos were incubated continuously in [³⁵S]methionine and aliquots were removed at various time points during the first two cell cycles after fertilization. These aliquots were fixed for lacto-orcein staining (Westendorf et al., 1989) to assess the synchrony of the embryos in culture, or analyzed by SDS-PAGE. For cell synchrony determination, the percentage of embryos at metaphase ($n = 100$) was calculated for each time point and

plotted as a function of time after fertilization. Such analyses revealed that the synchrony of a given culture was generally somewhat reduced after the first division; however, the population of developing embryos remained highly synchronous. For example, 95% of the embryos in a given culture reached metaphase of the first division at the same time, whereas by the second cell division 80% of the embryos reached metaphase at the same time.

Aliquots of embryos analyzed by SDS-PAGE were either stained with Coomassie blue, dried, and subjected to autoradiography (Fig. 2) or the proteins were transferred to nitrocellulose and probed with affinity-purified antibodies to the 62-kD protein (Fig. 3 a). The [³⁵S]methionine profile shown in the autoradiograph in Fig. 2 allows the visual determination of proteins which undergo oscillations in synthesis throughout the cell cycle. The 56-kD sea urchin cyclin, which was initially identified using similar procedures (Evans et al., 1983), can be seen to oscillate in abundance throughout the two cell cycles shown here (*asterisk*, Fig. 2). The location of the 62-kD protein as determined by immunoblotting is identified by the small arrow (Fig. 2). A distinction between the 62-kD protein and the 56-kD cyclin protein can be made on the basis of the visible difference in relative molecular mass between the two proteins, and, more importantly, on the obvious oscillation in abundance of cyclin but stable presence of the 62-kD protein. For comparison, a protein that continuously incorporates [³⁵S]methionine is shown by the arrowhead (Fig. 2). The 62-kD protein does not oscillate in abundance and, therefore, by this criterion does not represent a previously undetected form of cyclin. A similar analysis of embryo proteins at time points taken more frequently than 10 min did not reveal a short interval of time when the 62-kD protein was not present.

Immunoblots of gels of the type shown in the autoradiograph in Fig. 2 were probed with affinity-purified antibodies to the 62-kD protein (Fig. 3 a). Clearly, the 62-kD protein is present in the unfertilized egg, as well as throughout the two cell cycles shown here. However, from visual examination of such immunoblots, the level of the 62-kD protein does not appear to be completely constant (Fig. 3 a). Therefore, densitometry was used to quantify the presence of the protein throughout the first two cell cycles in the sea urchin embryo (Fig. 3 b). The graph in Fig. 3 b demonstrates the

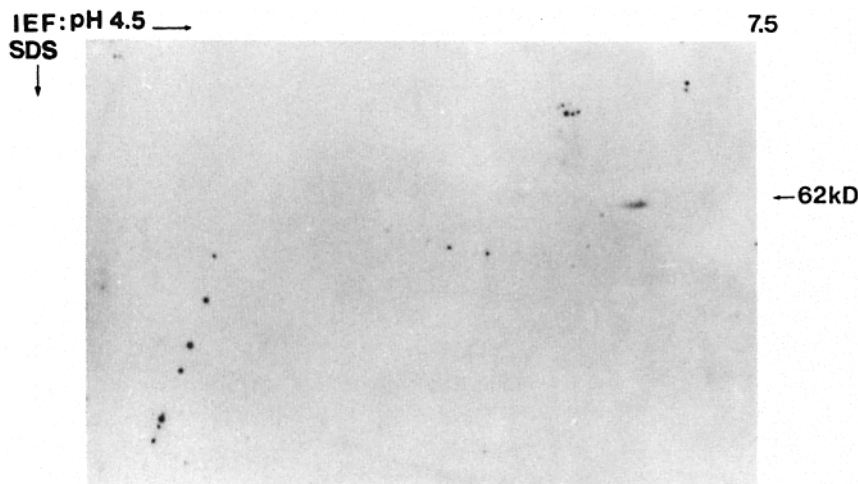


Figure 1. Two-dimensional immunoblot of a homogenate of *Strongylocentrotus purpuratus* eggs. Proteins in a homogenate of *S. purpuratus* eggs were resolved by two-dimensional gel electrophoresis and transferred to nitrocellulose. The nitrocellulose was then probed with affinity-purified antibodies to the 62-kD protein, followed by donkey anti-rabbit ¹²⁵I-labeled secondary antibodies. Shown here is an autoradiograph resulting from exposure of the blot to X-ray film. The position of the 62-kD protein is as indicated in the figure.

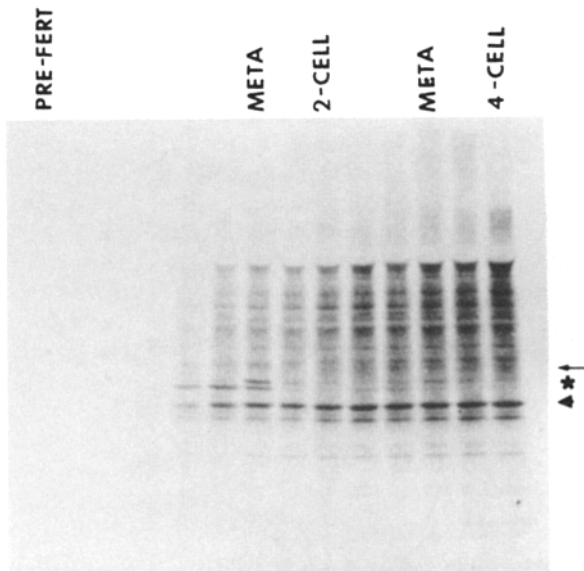


Figure 2. An autoradiograph revealing the proteins that are newly synthesized in the first two cell cycles of the sea urchin, *Lytechinus pictus*. *L. pictus* eggs were fertilized, and the embryos were allowed to develop in the continuous presence of [³⁵S]methionine; aliquots were removed before fertilization and every 5 min thereafter and processed for SDS-PAGE and autoradiography (shown here are representative time points). The 56-kD cyclin protein is marked with an asterisk, and the location of the 62-kD protein as determined by immunoblotting (see Fig. 3 a) is indicated by the arrow. A protein that continuously incorporates [³⁵S]methionine is indicated with an arrowhead. Each lane contained the same amount of protein (~10 μg). For comparison, time points of note are indicated above the lanes: *PRE-FERT*, embryos before fertilization; *META*, metaphase embryos; *2-CELL*, embryos at the two-cell stage; *4-CELL*, embryos at the 4-cell stage.

results obtained with immunoblots using ¹²⁵I-labeled secondary antibodies. The 62-kD protein is present before fertilization, and the relative band intensity of the 62-kD protein increases as the cell goes through the first two cell cycles. There appears to be a slight but reproducible decrease in the detectable level of the 62-kD protein just before each metaphase (see the 75-min time point in Fig. 3 b). This could be caused by antibody epitope occlusion due to the phosphorylation of the protein which would prevent a subpopulation of the affinity-purified antibodies from binding to the 62-kD protein and result in a consistent slight decrease in the apparent abundance of the protein as detected on immunoblots at the sensitivity of the densitometer. However, the overall increase in the quantity of the 62-kD protein measured by immunoblotting is consistent with the densitometric analysis of the 62-kD protein in autoradiographs of gels of [³⁵S]methionine-labeled proteins (data not shown). The 62-kD protein is synthesized at a low rate throughout two cell cycles, and immunoblots (Fig. 3) revealed the protein is present before fertilization and increases in amount throughout the two cell cycles assayed here. Taken together, the data in Figs. 2 and 3 argue strongly that the 62-kD protein is not a member of the cyclin family of proteins.

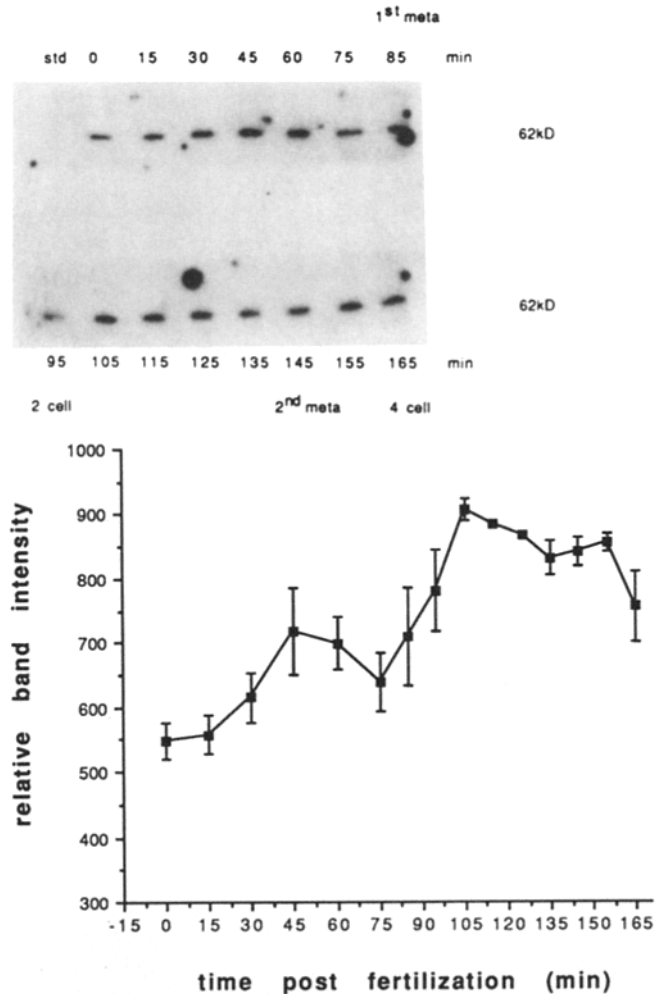


Figure 3. (Top) Immunoblot analysis of the 62-kD protein throughout two cell cycles. Aliquots of embryos from a developing culture of *S. purpuratus* were removed from the culture every 5 min and prepared for SDS-PAGE and immunoblotting. Shown here is an autoradiograph of the 62-kD region of an immunoblot probed with affinity-purified antibodies to the 62-kD protein, followed by ¹²⁵I-labeled donkey anti-rabbit secondary antibodies. Each lane contained a total of ~10 μg protein. Time in minutes after fertilization is indicated for each lane, and transition points in the cell cycle are indicated as follows: *1st meta*, metaphase of the first cell division; *2 cell*, two-cell stage; *2nd meta*, metaphase of the second cell division; *4 cell*, 4-cell stage; *std*, lane containing molecular weight standards. (Bottom) Densitometric analysis of the 62-kD protein on immunoblots using affinity-purified antibodies to the 62-kD protein. The relative band intensity on immunoblots of the 62-kD protein throughout two cell cycles was determined by densitometry as described in Materials and Methods. The relative amount of 62-kD protein increases as a function of time after fertilization for the two cell cycles shown here. Key stages in the cell cycle are as follows: 0 min, unfertilized eggs; 85 min, first metaphase; 95 min, two-cell stage; 145 min, second metaphase; 155 min, four-cell stage. Bar, ±SEM. *n* = 3 for each time point shown.

The 62-kD Protein Is Present When Cyclin A Is Absent

Because of the slight but consistent decrease in the quantity of the 62-kD protein detectable by immunoblotting just before metaphase, experiments that directly compared the level

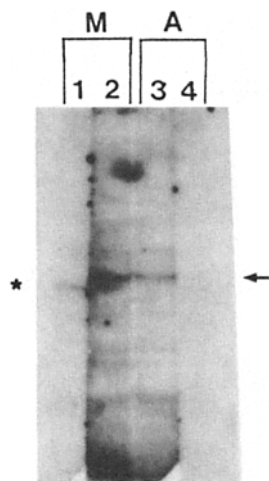


Figure 4. The 62-kD protein is present after anaphase onset and is distinct from cyclin A. Proteins in homogenates of *Spisula solidissima* embryos at metaphase (M) and anaphase (A) were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was then probed with either antibodies to *Spisula* cyclin A (lanes 1 and 4) or antibodies to the 62-kD protein (lanes 2 and 3), followed by incubation in ^{125}I -labeled donkey anti-rabbit secondary antibodies, followed by autoradiography. Shown here is the resulting autoradiograph. The asterisk indicates cyclin A, and the arrow indicates the 62-kD protein.

of the 62-kD protein with the level of a known mitotic cyclin, cyclin A of the surf clam (Swenson et al., 1986), were performed. Whole egg homogenates of the surf clam at metaphase and at anaphase of the first cell cycle were analyzed by immunoblotting. Fig. 4 shows the result of an immunoblot

in which the presence of the two proteins at two discrete time points can be compared. Fig. 4, lane 1, shows a metaphase homogenate probed with antibodies to cyclin A, whereas lane 2 shows the same metaphase homogenate probed with antibodies to the 62-kD protein. Both proteins are clearly present at metaphase. Fig. 4, lane 3, shows an anaphase homogenate probed with antibodies to the 62-kD protein, and lane 4 shows the same anaphase homogenate, probed with antibodies to cyclin A. A comparison of lanes 3 and 4 reveals that the 62-kD protein is present in the anaphase homogenate, but that cyclin A is not. These experiments demonstrate that the 62-kD protein is not degraded after metaphase in a manner similar to cyclin A.

62-kD Protein Is Located in Association with Microtubules in Isolated Mitotic Apparatuses

Previously, indirect immunofluorescence experiments of isolated MAs viewed with confocal microscopy have revealed coincident staining of the 62-kD protein and tubulin (Dinsmore and Sloboda, 1989). Interestingly, the 62-kD protein was shown to sediment with the insoluble material of the MA (the MA remnant) after phosphorylation of the 62-kD protein solubilized the MTs of the MA (Dinsmore and Sloboda,

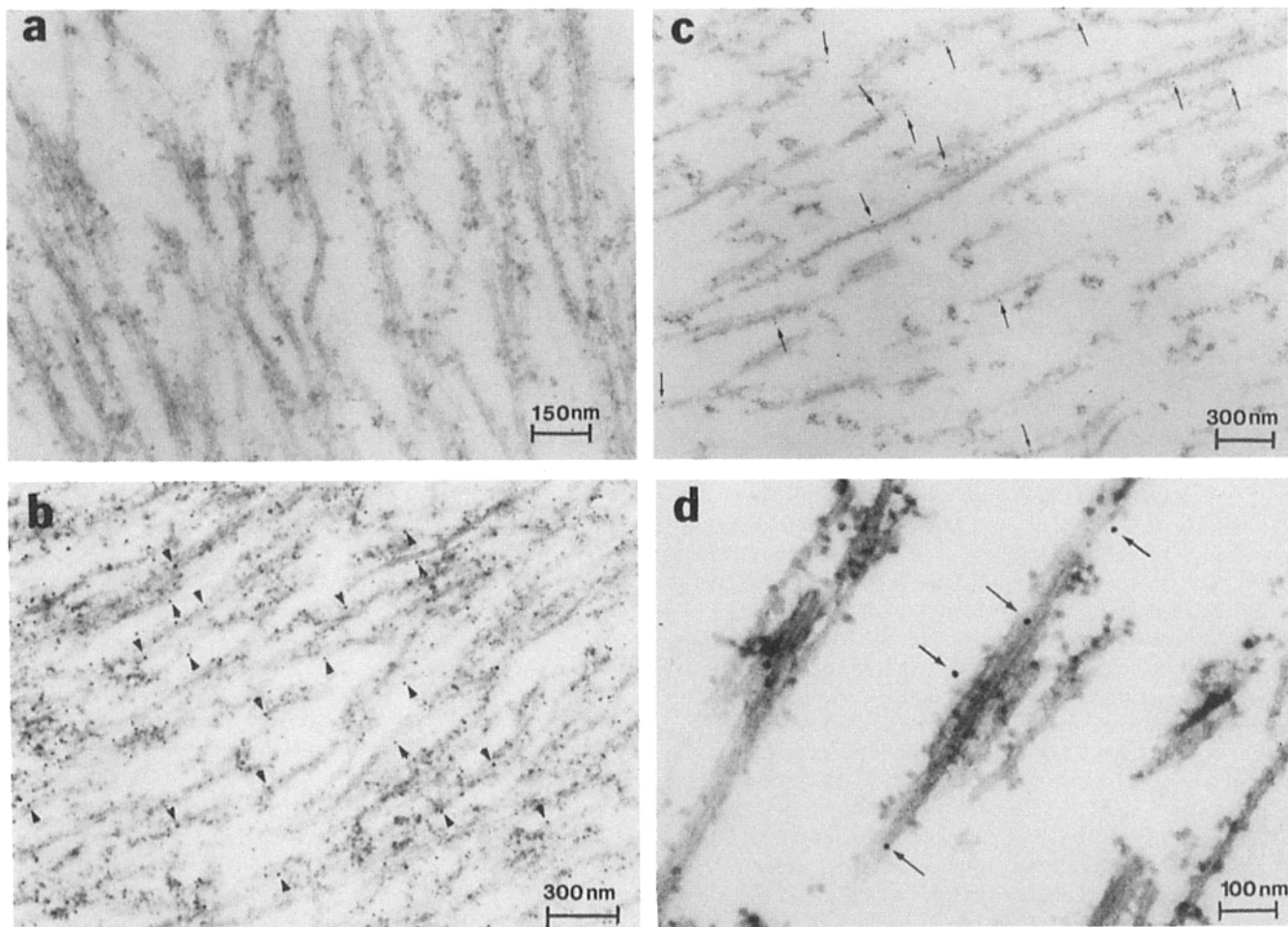


Figure 5. Immunoelectron microscopy of microtubules from isolated mitotic apparatuses. (a) Control MA incubated with gold-labeled secondary antibodies alone. (b) MTs of isolated MAs labeled with antibodies to β -tubulin, followed by secondary antibodies conjugated to 10-nm colloidal gold (arrowheads). (c and d) MTs of isolated MAs labeled with antibodies to the 62-kD protein followed by secondary antibodies conjugated to 15-nm colloidal gold (arrows).

1989). Therefore, the 62-kD protein may be associated with both MTs and an as yet unidentified component of the MA remnant. To determine if the colocalization seen with confocal microscopy was due to an association of the 62-kD protein with MTs, or if the 62-kD protein was associated with an alternative component of the MA remnant, immunoelectron microscopy of isolated MAs was used to examine the ultrastructural relationship between tubulin and the 62-kD protein. To do this, isolated MAs were prepared for immunogold electron microscopy. Little, if any, gold label is present in MAs treated with preimmune serum or with gold-labeled secondary antibodies alone (Fig. 5 a). When isolated MAs were incubated in antibodies specific to tubulin (Fig. 5 b) or antibodies specific to the 62-kD protein (Fig. 5, c and d), followed by the appropriate gold-labeled secondary antibody, specific labeling was obtained. As can be seen in Fig. 5 b, the MTs of the MA densely label with antibodies to tubulin (*arrowheads*). The 62-kD protein, which is present stoichiometrically with respect to tubulin (Dinsmore and Sloboda, 1989), is located intermittently along the MTs (*arrows*, Fig. 5 c). Fig. 5 d is an enlargement of a region of the MA shown in Fig. 5 c, and shows the 62-kD protein is located at defined positions along the MTs of the MA (*arrows*). In these MA preparations, there does not appear to be a fibrous component other than MTs present. Thus, Fig. 5 clearly shows that the 62-kD protein is associated with the MTs of the MA, and this result at the higher resolution afforded by immunoelectron microscopy confirms and reinforces the initial confocal microscopic observations of Dinsmore and Sloboda (1989).

Immunolocalization of the 62-kD Protein in Sea Urchin Eggs and Embryos

The unfertilized sea urchin egg does not contain polymerized MTs (Schatten and Schatten, 1981), yet the 62-kD protein was found to be present in unfertilized eggs of the sea urchin (Fig. 3). Because the 62-kD protein is located close to the MTs of the isolated MA (Dinsmore and Sloboda, 1989; Fig. 5), it was of interest to determine the subcellular localization of the protein in whole eggs and embryos. At a variety of time points in the cell cycle, sea urchin eggs and embryos were fixed and processed for indirect immunofluorescence using a monoclonal antibody to β -tubulin (Fig. 6) or affinity-purified antibodies to the 62-kD protein (Fig. 7). HOECHST 33342 was included in the fixation step to identify the DNA. As seen in Fig. 6, c, e, and g, and Fig. 7, a, c, e, and g, HOECHST 33342 can be used to determine the stage of the cell cycle of the developing embryo as indicated by chromatin condensation and chromosome position. The immunofluorescence patterns in Figs. 6 (tubulin) and 7 (62-kD protein) allow a comparison of the localization of the MTs and the 62-kD protein in the developing embryo. As previously reported (Schatten and Schatten, 1981), no MTs can be detected in the unfertilized egg (Fig. 6 b). At pronuclear fusion, the tubulin has begun to polymerize (Fig. 6 d), and the fully formed mitotic apparatus microtubules are clearly visible at metaphase (Fig. 6 f).

In contrast to the lack of MT staining in the unfertilized egg, the 62-kD protein can be seen to localize to the female pronucleus (Fig. 7, a and b). At pronuclear fusion/early prophase, even though the tubulin has begun to polymerize

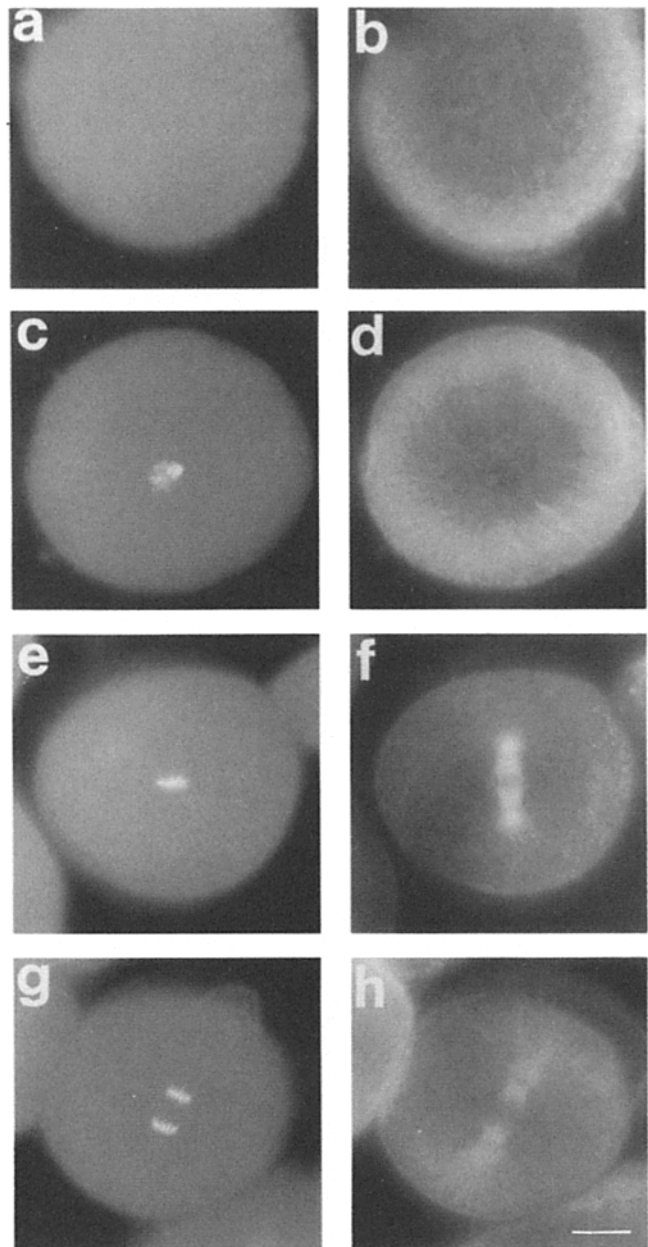


Figure 6. Immunolocalization of β -tubulin in *Strongylocentrotus purpuratus*. Eggs and embryos of *S. purpuratus* were processed for indirect immunofluorescence at a variety of time points during the first cell cycle. The extent of chromatin condensation is visible in c, e, and g with HOECHST 33342. The distribution of microtubules throughout the first cell cycle is revealed by monoclonal antibodies to β -tubulin in b, d, f, and h. Stages shown are (a and b) unfertilized egg; (c and d) pronuclear fusion; (e and f) metaphase; (g and h) late anaphase. Bar, 20 μ m.

(Fig. 6 d), the 62-kD protein remains localized to the nucleus (Fig. 7 d). However, by metaphase, the localization of the 62-kD protein is now coincident with the MA and in the same region as the MTs (compare Fig. 6 f with Fig. 7 f). From this data it can be emphasized once again that the protein is present not only in the unfertilized egg but also throughout the cell cycle. As seen in Fig. 7 h, at mid-anaphase the 62-kD protein is still present in the embryo and

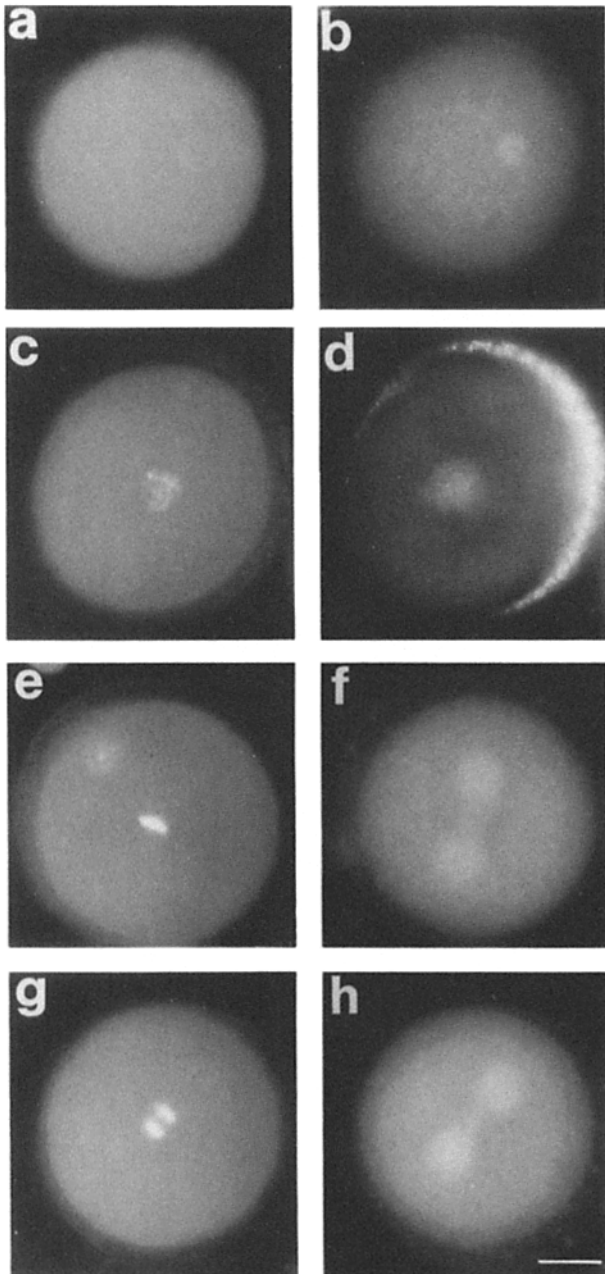


Figure 7. Immunolocalization of the 62-kD protein in *Strongylocentrotus purpuratus*. Eggs and embryos of *S. purpuratus* were processed for immunofluorescence at a variety of time points during the first cell cycle. The extent of chromatin condensation is visible in *a*, *c*, *e*, and *g* with HOECHST 33342. The distribution of the 62-kD protein throughout the first cell cycle is shown using affinity-purified antibodies to the 62-kD protein in *b*, *d*, *f*, and *h*. Stages shown are the same as for Fig. 6. The background fluorescence along the right edge of the cell shown in *d* is due to secondary antibodies trapped by a portion of the fertilization envelope that was not removed completely from the embryo shown here. The fluorescent signal at roughly 11 o'clock in the embryo shown in *e* is from an out-of-focus sperm head attached to a remaining portion of the fertilization envelope. Bar, 20 μ m.

has not undergone an apparent specific degradation or change in localization. Moreover, the protein is again present in the nuclei that form in the daughter cells at telophase (see Fig. 8 and next section). These experiments show that the 62-kD protein is associated with the nucleus or with microtubules in a stage-specific manner.

62-kD Protein Returns to the Nucleus after Anaphase

The 62-kD protein is located in the female pronucleus in unfertilized eggs and in the nucleus of embryos before nuclear envelope breakdown of the first cell division (Fig. 7, *b* and *d*). To determine if the 62-kD protein returns to the nucleus after the first cleavage division, indirect immunofluorescence experiments of embryos at telophase were performed to determine the localization of the 62-kD protein after mitosis (Fig. 8). Such experiments show that antibodies to the 62-kD protein distinctly label the newly formed nuclei (Fig. 8 *d*). The possibility that the fluorescence seen in Fig. 8 *d* is due to bleed through of fluorescence from the HOECHST 33342 can be eliminated, as the embryo shown in Fig. 8 *b* stained for tubulin does not show a fluorescent signal in the fluorescein channel emanating from the region of the nucleus. Both embryos have been labeled with fluorescein-conjugated secondary antibodies, and thus any fluorescence in the fluorescein channel due to the HOECHST 33342 would be visible independently of the primary antibody used. Therefore, it is clear from these data that the 62-kD protein is partitioned to the forming daughter nuclei during telophase.

Cell Fractionation Confirms the Nuclear Localization of the 62-kD Protein

Because fixation of sea urchin embryos for immunofluorescence experiments can be susceptible to artifacts because of the type of fixation used (K. Swenson, personal communication), cell fractionation was performed as an independent test of the subcellular localization of the 62-kD protein. To determine if the 62-kD protein was indeed in the nucleus before fertilization, nuclei were isolated from unfertilized eggs using two different approaches. The first approach was to prepare nuclei and nuclear supernatant in which the nuclear envelope remained intact. The second approach was to prepare nuclei and nuclear supernatant in which the nuclei had been permeabilized by treatment with the detergent NP-40. Immunoblotting of the intact and permeabilized nuclear preparations confirmed the nuclear localization of the protein. The 62-kD protein was found in the nuclei whether the nuclear membrane was intact or permeabilized (Fig. 9, *left*, compare *I* with *D*).

In addition to the microscopic examination of subcellular fractions for the presence of nuclei, the distribution of the 67-kD nuclear lamina protein was determined as yet another independent assessment of the fractionation. Immunoblots confirm the presence of the 67-kD lamina protein in only the nuclear fractions (Fig. 9, *right*, compare *Sup* with *Nuc*). This indicates a complete fractionation of cellular components and purification of nuclei. It is also worthwhile to note the obvious difference in molecular mass between the 67-kD nuclear lamina protein and the 62-kD MA phosphoprotein. This difference indicates that the lamins, which are also phosphorylated in a stage-specific manner during the cell cy-

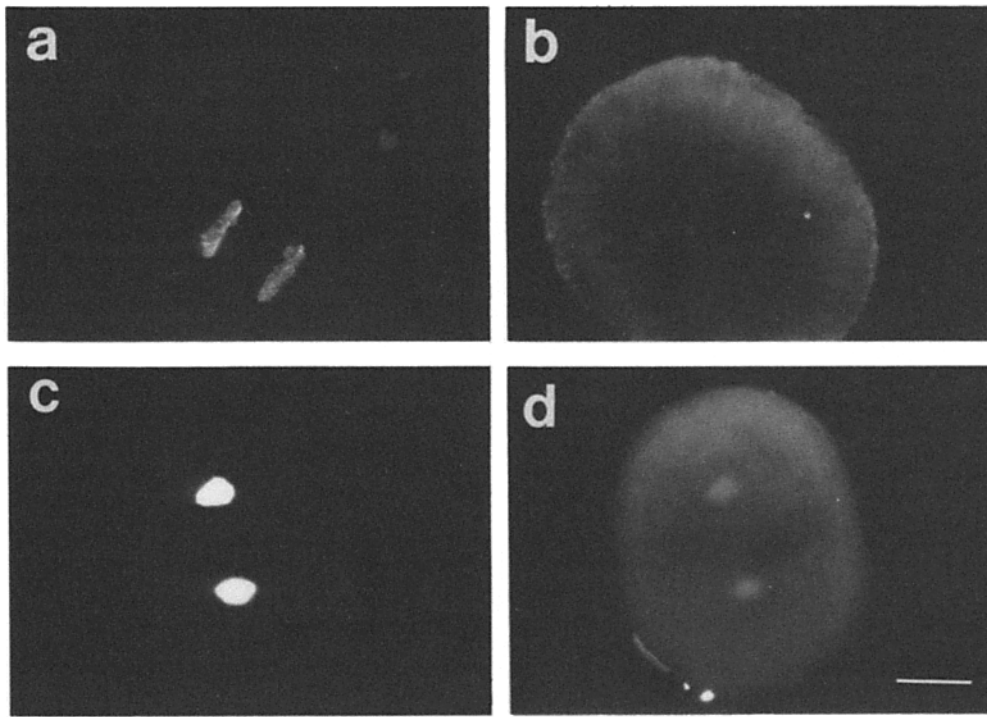


Figure 8. Immunolocalization of α -tubulin and the 62-kD protein after telophase in *Lytechinus pictus* embryos. The extent of chromatin condensation is visible with HOECHST 33342 (a and c) and thus identifies the daughter nuclei resulting from the first mitosis. The embryos were processed for indirect immunofluorescence with antibodies to α -tubulin or antibodies to the 62-kD protein, followed by incubation in the appropriate fluorescein-conjugated secondary antibodies. (a) HOECHST 33342 and (b) antitubulin indirect fluorescence; (c) HOECHST 33342 and (d) anti-62 kD indirect fluorescence. Bar, 20 μ m.

cle (Dessev et al., 1989), are not related to the 62-kD protein. In addition, as seen in Figs. 7 b and 8 d, the immunofluorescence pattern of the 62-kD protein does not appear as a ring around the periphery of the nucleus, as has been observed for the lamins (Gallant and Nigg, 1992) be-

cause of their location subjacent to the nuclear envelope. Rather, the 62-kD protein can be found throughout the nucleus. The results shown in Figs. 7-9 indicate the 62-kD protein is a bona fide resident of the nucleus before nuclear envelope breakdown and that the 62-kDa protein returns to the daughter nuclei during or soon after they reform at telophase.

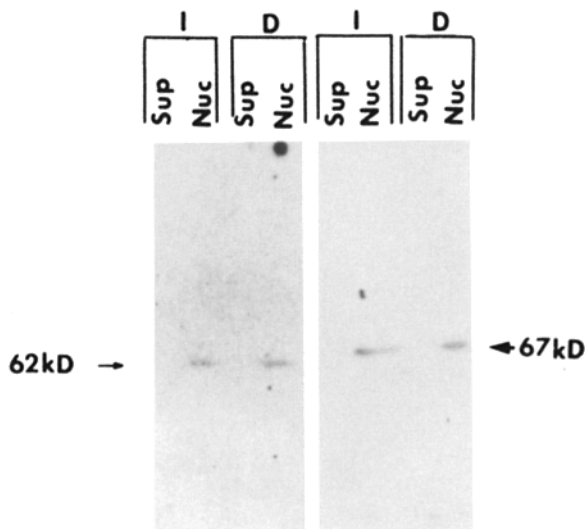


Figure 9. Subcellular fractionation confirms the nuclear location of the 62-kD protein. *Spisula solidissima* nuclei were prepared intact (I) or with the nuclear membranes disrupted with detergent (D). The resulting preparations were resolved by SDS-PAGE and the location of the 62-kD protein was revealed by affinity-purified antibodies in the blot shown on the left. An identical blot probed with antibodies to a 67-kD nuclear lamina protein is shown on the right. The nuclear lamina is not solubilized by the permeabilization, and this is demonstrated by the presence of the 67-kD lamina protein in only the nuclear fractions. This same result is true for the 62-kD protein. Sup, supernatant; Nuc, nuclei.

Discussion

Previously, using in vivo radiolabeling experiments, Dinsmore and Sloboda (1988) identified a 62-kD protein that incorporated labeled phosphate from the time of nuclear envelope breakdown until the start of anaphase. After anaphase, radiolabeled phosphate was not visible in the 62-kD region of the gel until mitosis of the next cell cycle, when the protein again incorporated phosphate. This phenomenon could have been due to cyclic phosphorylation/dephosphorylation of the 62-kD protein, or to synthesis and degradation of the protein, perhaps in combination with phosphorylation. In the data reported here, various approaches have been used to determine the relative amount of the 62-kD protein throughout the first two cell cycles of the sea urchin embryo to distinguish between these possibilities. To do this, autoradiographs of [³⁵S]methionine-labeled proteins (Fig. 2) and immunoblots (Fig. 3 a) specific for the 62-kD protein were compared. Cyclin clearly oscillates in abundance (Fig. 2). In contrast, the 62-kD protein does not oscillate in abundance (Fig. 3); instead it increases in abundance as the embryos develop from one cell to four cells. Most likely, newly synthesized 62-kD protein is required to maintain appropriate levels of the protein in each of the resulting daughter cells. The constant presence of the 62-kD protein is in contrast to the mitotic cyclins which are characterized by their destruction specifically at metaphase (Draetta and Beach,

1988; Evans et al., 1983; Solomon et al., 1988; Standart et al., 1987). From the immunoblotting (Figs. 3 *a* and 4) and densitometry (Fig. 3 *b*) experiments reported here, it is clear the 62-kD protein does not represent a previously undetected member of the cyclin family of proteins.

The presence of the 62-kD protein during anaphase was confirmed with immunofluorescence microscopy. As described in Fig. 7, the 62-kD protein is visible in the region of the MA with immunofluorescence at anaphase, and in Fig. 8 can be seen to be present in the forming nuclei at telophase. Immunofluorescence experiments with antibody to clam cyclin (Westendorf et al., 1989), *Drosophila* cyclin (Lehner and O'Farrell, 1989), human cyclin (Pines and Hunter, 1991), and most recently chicken cyclin (Gallant and Nigg, 1992) reported diffuse cytoplasmic immunofluorescence of cyclin before metaphase, but by anaphase cyclin was not detected in these cells. The ability to detect the 62-kD protein in sea urchin embryos at times after metaphase confirms the persistent presence of the protein. Furthermore, comparative immunoblots of *Spisula* embryos at anaphase (Fig. 4) demonstrate the presence of the 62-kD protein when cyclin A has been degraded. Therefore, the decrease in phosphorylated 62-kD protein that has been reported to occur at the end of anaphase (Dinsmore and Sloboda, 1988) is not due to a specific destruction of the protein, but rather most likely to the action of a specific phosphatase. In fact, recent studies have shown that protein phosphatase type 1 is essential for mitosis in *Drosophila* (Axton et al., 1990) and is required for the exit from mitosis in *Aspergillus nidulans* (Doonan and Morris, 1989) and *Schizosaccharomyces pombe* (Booher and Beach, 1989; Ohkura et al., 1989; Ohkura and Yanagida, 1991). The dephosphorylation of the 62-kD protein by a phosphatase of this type may be involved in the regulation of the function of the 62-kD protein in mitosis, but this has not yet been determined.

Subcellular fractionation experiments (Fig. 9) demonstrate that the 62-kD protein is located in the nucleus during interphase. This data confirms the nuclear localization of the protein at interphase (Fig. 7), and again at telophase (Fig. 8), as revealed by indirect immunofluorescence. By contrast, the cyclins are located in the cytoplasm, at least until prometaphase (Pines and Hunter, 1991; Gallant and Nigg, 1992). This difference in immunofluorescent localization is further evidence that the 62-kD protein is not a member of the cyclin family of proteins.

The nuclear localization of the 62-kD protein reported here (Figs. 7-9) emphasizes the specific function of the protein. Using microinjection experiments, Dinsmore and Sloboda (1989) demonstrated that functional 62-kD protein is required at the metaphase-to-anaphase transition. In these studies it was shown that microinjection of affinity-purified antibodies to the 62-kD protein resulted in an arrest of the cell cycle at metaphase. Injection of antibodies after the onset of anaphase resulted in the completion of mitosis and the normal assembly of the MA in preparation for the second cell division. At this time the cell arrested at metaphase of the second cell cycle and developed no further. This data defines a specific requirement for the protein at the metaphase-to-anaphase transition. Thus, the sequestration of the 62-kD protein to the nucleus before metaphase (Fig. 7, *b* and *d*) and again after telophase (Fig. 8 *d*) may be necessary to prevent the protein from inappropriate interaction with MTs

during interphase, and supports the conclusion that the protein is required at the metaphase-to-anaphase transition. The localization of proteins to the MA during mitosis, and to the nucleus during interphase, has been reported previously (Izant et al., 1982; Lydersen and Pettijohn, 1980); however, no functions for these proteins (either nuclear or MA) have been defined. The 62-kD protein described in this report has been shown to induce MT disassembly when phosphorylated in vitro (Dinsmore and Sloboda, 1988) and to change its subcellular location during each cell cycle.

Cam kinase II, an enzyme with broad substrate specificity (MAP-2, tau, vimentin, synapsin I; for review see Schulman, 1988), has been implicated as a mediator of the effects of elevated calcium (Baitinger et al., 1990) in many cell types (Schulman, 1988). Cam kinase II from rat brain has been localized to the nucleus in interphase and to the MA at metaphase (Ohta et al., 1990). It has also been shown that an MA-associated kinase will phosphorylate synapsin I (Dinsmore and Sloboda, 1990); presumably this is the same kinase that phosphorylates the 62-kD protein. In these experiments, purified synapsin I was added to purified MAs and the action of the MA-associated kinase was stimulated by the addition of calcium, calmodulin, and ATP. Phosphorylation of synapsin I in addition to phosphorylation of the 62-kD protein was observed under these conditions. Subsequent digestion of synapsin I with V8 protease and analysis of the resulting peptides revealed that synapsin I was phosphorylated on a 30-kD fragment (Dinsmore and Sloboda, 1990), which is characteristic of Cam kinase type II activity (Kennedy and Greengard, 1981). Therefore, the similar cell cycle pattern of localization of Cam kinase type II and the 62-kD Ca/Cam-dependent protein kinase may be more than coincidence, and this question is currently being examined.

A 46-kD component of the calcium transport system of the sea urchin has been localized throughout the cell cycle (Petzelt and Hafner, 1986) and in isolated MAs (Petzelt et al., 1987). Because the function of the 62-kD protein is dependent on calcium, it is interesting to compare the localization of the 62-kD protein with a protein involved in calcium transport. During interphase the 46-kD protein is located in the cytoplasm surrounding the nucleus, whereas the 62-kD protein is distinctly nuclear. However, by metaphase, both proteins display a diffuse fluorescence in the region of the MA; the patterns are almost identical. In isolated MAs, however, the proteins are not localized in identical regions. The 46-kD component is concentrated around the poles, whereas the 62-kD protein is co-linear with all of the MTs of the MA (Dinsmore and Sloboda, 1989).

The phosphorylation of the 62-kD protein only occurs in the presence of calcium and calmodulin (Dinsmore and Sloboda, 1988), and calmodulin is localized only to the kinetochore MTs (kMTs) of the MA (Welsh et al., 1979; Vantard et al., 1985; Stemple et al., 1988). These immunofluorescence data suggest that when calcium is released from MA membranes (Silver et al., 1980) that are concentrated around the spindle poles (Petzelt et al., 1987), calcium can bind to calmodulin on the kMTs and activate the calmodulin-dependent kinase. The 62-kD protein that is associated with the kMTs of the MA (Dinsmore and Sloboda, 1989) can then be specifically phosphorylated to destabilize the kMTs at the metaphase-to-anaphase transition, allowing the events of anaphase to proceed.

The mechanism that provides the motive force for the movements of the chromosomes to the poles along the kMTs during anaphase A remains elusive. Although there is a slow flux of subunits of kMT subunits toward the poles because of disassembly of these MTs at their minus (i.e., pole) ends (Mitchison, 1989), it is generally accepted that chromosome movement occurs through active kMT depolymerization from the kinetochore (plus) end (Gorbsky et al., 1988; Nicklas, 1989; Coue et al., 1991). This movement may be facilitated by a dynein-like, minus end-directed mechanoenzyme at the kinetochore which serves to couple the chromosomes to the MTs while at the same time translocating the chromosomes along the kMTs to the poles (Gorbsky et al., 1987, 1988; Mitchison, 1989; Nicklas, 1989; Pfarr et al., 1990; Steuer et al., 1990; Hyman and Mitchison, 1991; Wordeman et al., 1991; Zinkowski et al., 1991). Presumably, at the start of anaphase, the kinetochore mechanoenzyme is activated and the MTs are destabilized via the phosphorylation of the 62-kD protein (Dinsmore and Sloboda, 1988), thus allowing movement of the chromosomes to the poles along the now destabilized and thus depolymerizing kMTs.

From the results of Dinsmore and Sloboda (1988, 1989) it is clear a protein of 62 kD is involved in MT depolymerization at the metaphase-to-anaphase transition. This 62-kD protein is located close to the MTs of the MA (Dinsmore and Sloboda, 1989; compare also Figs. 5 c and d, 6, and 7). However, it will only act to destabilize MTs in the region of the MA where the 62-kD protein has been phosphorylated because of its interaction with a calcium/calmodulin-dependent kinase. Therefore, the 62-kD protein may function to destabilize the kinetochore MTs of the MA at the onset of anaphase A, a time when an increase in the intracellular concentration of calcium is presumed to occur, and at a location where calcium-sequestering membranes (Silver et al., 1980; Petzelt and Hafner, 1986; Petzelt et al., 1987) and calmodulin (Welsh et al., 1979; Vantard et al., 1985; Stemple et al., 1988) are located. The destabilization of the kMTs may then allow a motor localized at the kinetochore to translocate the chromosomes to the poles. After telophase, the 62-kD protein does not undergo specific degradation, but rather is sequestered to the forming daughter nuclei until nuclear envelope breakdown of the next cell cycle.

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