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Visualization of the Ca^{2+}-transport system of the mitotic apparatus of sea urchin eggs with a monoclonal antibody

(Ca^{2+} regulation/mitosis/*in vitro* immunization)

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ABSTRACT **Monoclonal antibodies have been obtained to components of Ca^{2+}-sequestering vesicles from the endoplasmic reticulum of HeLa cells by isolating hybridomas that were generated by the *in vitro* immunization of lymphocytes followed by fusion with plasmocytoma cells. One of these monoclonal antibodies specifically labels punctate structures which appear in the mitotic apparatus of sea urchin eggs at the beginning of prophase and disappear upon the completion of cytokinesis. The antibody inhibits the Ca^{2+} uptake of the membrane system *in vitro*. It reacts with one 46-kDa protein out of the complex protein mixture from the membrane fraction. We take all this as evidence that in fact a specific Ca^{2+}-transport system is part of the mitotic apparatus, that such a system is very conserved, and that it is most probably derived from the endoplasmic reticulum.**

One of the main obstacles to an analytical understanding of mitosis is the fact that nearly all components involved are also used for other processes by the cell. So far, no "mitotic" proteins have been conclusively described. However, a few proteins such as the cyclins do appear to function specifically during mitosis (1). Components used at other times of the cell cycle for a variety of functions are assembled to form the mitotic apparatus. This holds true for the most conspicuous structural component of the mitotic apparatus, the microtubules. An elaborate interphase network of microtubules, disassembled just prior to the onset of mitosis, is reassembled as the spindle fibers of the mitotic apparatus (2–6). A similar case is represented by the lamins (7–10), components of the nuclear envelope, that are found after the breakdown of the nuclear envelope in the mitotic apparatus.

Experiments on live sea urchin eggs using fluorescent calcium chelators have directly shown fluctuations in free Ca^{2+} at mitosis (11). Functional evidence for the existence of an intracellular Ca^{2+}-transport system active at mitosis was given in elegant studies by Suprynowicz and Mazia (12). Similar findings were obtained by Petzelt and Wülfroth (13) in a study of Ca^{2+} transport by a purified membrane fraction isolated at various stages of the cell cycle. That system was shown to be resistant to the inhibitors of the mitochondrial Ca^{2+} uptake ruthenium red and azide in concentrations up to 0.1 mM. Vanadate treatment did not block Ca^{2+} uptake in this system thus excluding contamination by plasma membrane vesicles. Therefore, the fraction was tentatively identified as part of the endoplasmic reticulum. The system described occurs in a very similar form in mammalian cells with almost identical physiological properties. Only the temperature optimum differs—i.e., 25°C for sea urchin eggs and 37°C for mammalian cells. These reports and others (14–22) sustain the hypothesis that Ca^{2+} controls the course of mitosis. There is every reason to think that Ca^{2+} is also involved in the fine tuning of other processes throughout the cell cycle (23–25). In this study, we consider whether a special Ca^{2+}-regulating system is assigned to mitosis or whether a system operating at other times in the cell cycle is used and ask whether the Ca^{2+}-controlling system for mitosis is located in the mitotic apparatus itself.

We use the purified vesicle system whose Ca^{2+}-transport activity during mitosis has been identified (13) as the immunogen to generate monoclonal antibodies for an immunocytochemical study of the localization of the Ca^{2+}-transport system in mitotic cells. The cells selected for analysis were sea urchin eggs since (*i*) their synchronous development makes them suitable for studies of Ca^{2+}-dependent processes such as mitosis, (*ii*) their size allows sufficient discrimination between parts of the mitotic apparatus and the rest of the cell, and (*iii*) much of the evidence for the role of Ca^{2+} in the regulation of mitosis has been obtained by using sea urchin eggs.

MATERIALS AND METHODS

Isolation of the Ca^{2+}-Transport System. HeLa cells growing in suspension and sea urchin eggs were used to prepare the Ca^{2+}-uptake system. Purification of the vesicles was performed essentially as described (13).

Preparation and Characterization of Monoclonal Antibodies. The *in vitro* immunization procedure followed the protocol given by Pardue *et al.* (26), except that the incubation period of the lymphocytes with the antigens (derived from HeLa cells) was extended up to 8 hr. The lymphocytes derived from BALB/c mice were fused with the nonsecreting plasmocytoma cell line P3X63Ag8.653 (27) at day 5 after the immunization, and the antibody-producing hybridomas were cloned twice by limited dilution. The resulting antibody was classified by using double immunodiffusion techniques (28). It was further purified by three cycles of precipitation with saturated ammonium sulfate, finally taken up in 50 mM Mops, pH 7.0, and dialyzed extensively against the same buffer.

Polyacrylamide gel electrophoresis was performed according to ref. 29, and the immunoblotting procedure followed the protocol described in ref. 30. The antigen–antibody complex was visualized (31) using goat anti-mouse antibody coupled to alkaline phosphatase (Boehringer Mannheim) as the second antibody.

Inhibition of the Ca^{2+} Transport by the Antibodies. The system was isolated from sea urchin eggs as described in ref. 13. Inhibition of Ca^{2+} transport was performed by adding various amounts of the purified antibody to the membrane preparation. The system was incubated at 25°C for 15 min, and then the Ca^{2+} uptake was stimulated by the addition of adenosine triphosphate as described (13).

Immunofluorescence. For the immunofluorescence experiments the fertilization membranes of the eggs were removed as described in ref. 32, and the eggs at the selected mitotic stage were attached to polylysine-coated coverslips [1 mg of polylysine/ml (33)]. Cells were fixed in 2.5% (vol/vol)

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formaldehyde in sea water for 30 min, permeabilized in acetone at −20°C for 60 min, and quenched with 1M glycine. Nonspecific binding sites were blocked with 5% (wt/vol) bovine serum albumin in 50 mM Mops, pH 7.0, and cells were incubated with the antibody for at least 12 hr. After several washings the second antibody, fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgG (The Jackson Laboratory) was applied for 45 min. Finally, the eggs were embedded in 25 mg of 1,4-diazabicyclo[2,2,2]octane/ml (Aldrich) in 90% (vol/vol) glycerol, 10% (vol/vol) phosphate-buffered saline, pH 7.1, and viewed using an inverted microscope (Zeiss). Photographs were taken by using Kodak Tri-X-Pan film.

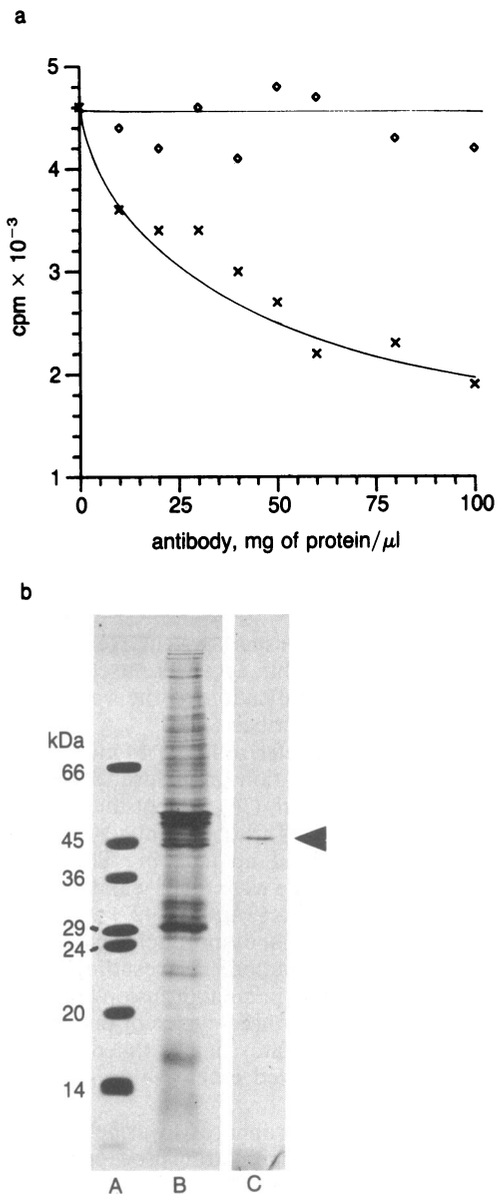

FIG. 1. Identification of the antigen of the monoclonal antibody 7/13. (*a*) The Ca^{2+}-uptake system of sea urchin eggs (13), consisting mainly of membranes of the endoplasmic reticulum, was used in order to determine the inhibiting capacity of antibody 7/13 (×). As a control (◇) another antibody, which did not block Ca^{2+} uptake (an IgM) was used. Increasing amounts of antibody purified by ammonium sulfate were added, and the uptake of $^{45}Ca^{2+}$ was determined. Since the protein concentration was kept constant for all preparations, the capacity of the antibodies to inhibit the Ca^{2+} transport could be compared directly. (*b*) Lane A, several molecular size standards. Lane B, proteins of the isolated Ca^{2+}-uptake system from the Hawaiian sea urchin *Tripneustes gratilla*, the pattern of the European species *Paracentrotus lividus* being virtually the same. Lane C, immunoblot of a gel identical to that in lane B. Only one protein, which has a molecular size of 46 kDa, reacts with the antibody.

RESULTS

The antibodies for further study were selected for their ability to inhibit Ca^{2+} uptake in our *in vitro* system (13). Ca^{2+}-transport vesicles from HeLa cells and from sea urchin eggs were used in these tests. We obtained three antibodies that inhibited Ca^{2+} transport. When these antibodies were tested on sea urchin eggs, we found that one of them (7/13) reacted well with the mitotic apparatus *in situ*. The others gave rise to a rather diffuse labeling of the whole cell.

Characterization of Antibody 7/13. Fig. 1 shows that the antibody inhibits Ca^{2+} uptake of isolated membrane fractions in a concentration-dependent manner. The same concentration of other, unrelated antibodies was used as control. Inhibition of Ca^{2+} uptake by the antibody occurs in membrane systems from HeLa cells as well as from sea urchin eggs (Table 1). Fig. 1*b*, lane B, shows the complex protein pattern of the Ca^{2+}-uptake system of sea urchin eggs; immunoblotting procedures reveal that the antibody reacts with only one protein that has an apparent molecular size of 46 kDa. Similar results were obtained with the membrane preparation from HeLa cells. The antibody reacts also in this case with a 46-kDa protein (data not shown). By double immunodiffusion techniques the antibody was identified as an IgM.

Distribution of Antibody 7/13 During Mitosis. In early prophase the antibody binds to structures around the nucleus in a punctate pattern (Fig. 2*a*). After the breakdown of the nuclear envelope more of these structures appear and are found throughout the assembling spindle and the asters (Fig. 2*b* and *c*). No preferential accumulation in the mitotic spindle itself can be observed, indicating that these labeled structures are not a constitutive part of the microtubular arrangement of the mitotic apparatus. In anaphase, these components are concentrated in the huge asters although they never follow the astral rays to the cell periphery (Fig. 2*d*). In telophase, when the nuclei are being re-formed, the maximum labeling is found on the distal side of the nuclear envelope (Fig. 2*e* and *f*). Immediately after the completion of mitosis the labeled punctate structures disappear. They can be found again at the next prophase (Fig. 2*h*). During interphase no distinct structure can be seen that reacts with the antibody; the labeling pattern resembles that obtained with the other two antibodies against the Ca^{2+}-transport system.

Table 1. Inhibition of the Ca^{2+} uptake by the antibody 7/13 of membrane preparations derived from sea urchin eggs and HeLa cells

Antibody, μl/mg of transport membrane	$^{45}Ca^{2+}$, cpm × 10^{-2}	
	Sea urchin eggs	HeLa cells
0	46	56
20	34	45
40	30	39
60	22	33
80	23	27
100	20	23
A23187*	12	13

*For comparison the effect of the Ca ionophore A23187 (20 μM) on the membrane preparations is shown. As described in ref. 13, a high proportion of calcium is thereby released from the transport system allowing the discrimination between Ca^{2+} bound to proteins and Ca^{2+} actively taken up by the membranes.

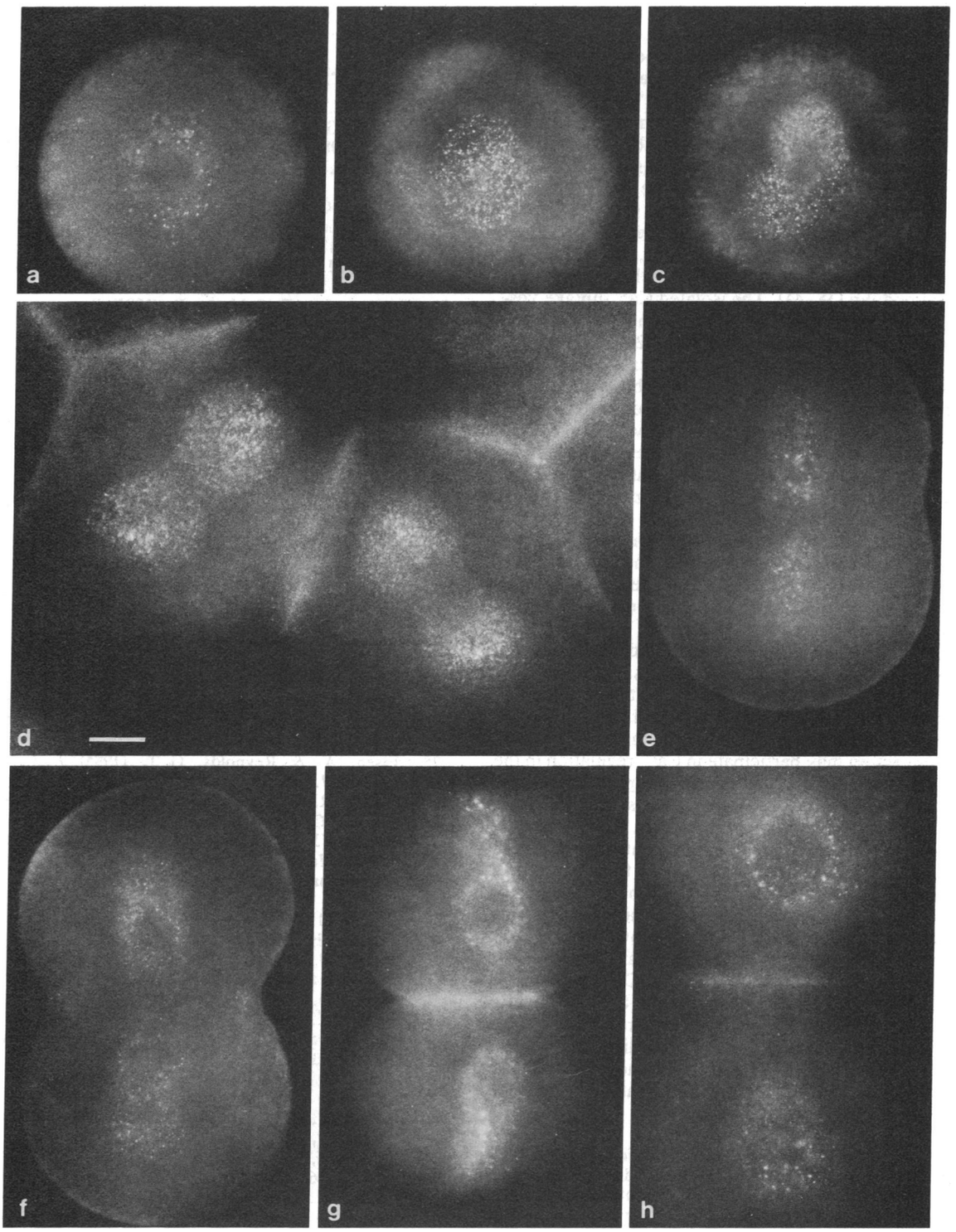

FIG. 2. Distribution of the 46-kDa protein in mitotic sea urchin eggs as shown by immunofluorescence with antibody 7/13. Whereas the unfertilized egg as well as the interphase egg does not show any conspicuous pattern besides a diffuse labeling of the whole cell (data not shown), vesicle-like structures begin to appear at prophase around the nucleus (*a*). As prophase progresses, the number of these vesicles increases at the breakdown of the nuclear envelope towards the asters (*b* and *c*). The distribution in metaphase and anaphase is characterized by their preferential localization in the asters whereas the interzonal region seems to be depleted of them (*d*). At telophase the number of vesicles is reduced beginning at the proximal site of the daughter nuclei (*e*); as the cell progresses through cytokinesis they become fewer and fewer (*f* and *g*) and disappear completely in interphase. At the second prophase they reappear (*h*) in a pattern similar to that of the first prophase—compare *a* with *h*. (Bar = 20 μm.)

DISCUSSION

These results show clearly that at mitosis a membraneous Ca^{2+}-uptake system is assembled and maintained throughout. Given the evidence that all three antibodies interfere with the Ca^{2+}-uptake system, one must conclude that the specific labeling of the mitotic apparatus obtained with monoclonal antibody 7/13 is the result of stronger binding which persists throughout the procedures used. Such differentiation of the Ca^{2+}-sequestering system present throughout

the cell cycle is visualized, then, by selective decoration with the one antibody.

The close correlation between the appearance of the punctate structures and the mitotic apparatus suggests strongly a tight coordination between the Ca^{2+} regulation and the course of mitosis. A hypothesis has been proposed that lowering Ca^{2+} levels stimulates the assembly of microtubules, and elevation of Ca^{2+} stimulates disassembly (14). Since microtubules have been shown to be Ca^{2+} sensitive (34), and calmodulin is known to occur in the spindle (35–37), this hypothesis seems to be applicable to the mitotic apparatus. The range of Ca^{2+} concentrations, to which the vesicles are sensitive, is comparable to that of systems requiring calmodulin (38–41). The isolated Ca^{2+}-uptake vesicles are susceptible to all known calmodulin inhibitors even though they do not respond to calmodulin (42). This suggests that the vesicular system may contain a "calmodulin-like" activity as an integral protein. The results obtained with the immunofluorescence techniques are not completely sufficient for the identification of the reactive sites of the Ca^{2+}-transport system with the 46-kDa protein. On the basis of the inhibitor studies (13) and the purification procedures for the membrane system used for immunization, we place them tentatively as part of the endoplasmic reticulum.

If Ca^{2+} regulates mitosis, why then are the mitotic asters decorated with the antibody and not the spindle itself? For a long time the mitotic poles have been neglected, and attention has been focused on the site of actual chromosomal movement, the spindle. Only recently has the governing and determining function of the poles been studied experimentally (43, 44). Results of these experiments support the view that the poles are the mitotic centers that determine the course of mitosis. We have shown here that the 46-kDa protein of the isolated vesicles is located in the mitotic asters. This 46-kDa protein may participate in Ca^{2+} regulation in the mitotic apparatus. This conclusion is supported by the abundance of vesicles found electron microscopically in the astral regions of the mitotic apparatus of sea urchin eggs (14, 43). The question is now whether this control results in a direct change of the Ca^{2+} concentration in these structures conveying such a change by an as yet unknown cascade of events to the spindle, or whether these Ca^{2+}-sequestering vesicles have a long-range effect, directly lowering the Ca^{2+} concentration in the spindle itself. Preliminary experiments, using Quin2 as an intracellular indicator for Ca^{2+}, support the former possibility. At least in anaphase the Ca^{2+} concentration is reduced markedly in the asters compared to the spindle and the residual cytoplasm (C.P., R. Nobiling, and M. H., unpublished results). Further work will show how such a compartmentalization of the mitotic apparatus is related to the progress through mitosis.

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