Association of Creatine Phosphokinase with the Cytoskeleton of Cultured Mammalian Cells

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ABSTRACT. Using an antibody specific for creatine phosphokinase (CPK), we have discovered an association between that enzyme and the cytoskeleton. Immunofluorescence observations show that CPK is associated with intermediate filaments in PTK cells and BALB/3T3 cells. The CPK distribution also follows intermediate filaments when cells are treated with colchicine.

Intermediate (10-nm) filaments, microtubules, and actin filaments in the cytoplasm of cells contribute to a fibrous network known as the cytoskeleton. The role of microtubules in maintaining cell shape (10) and in organizing components of the cytoplasm (3, 7, 23) has been demonstrated in many systems (see also reference 28 for review). Intermediate filaments have been receiving increasing attention recently and are considered to be an integral part of the cytoskeleton as well. The distribution of these filaments has been determined in cultured cells by immunocytochemical methods (19) as well as in cytoskeleton models prepared by extraction with Triton X-100 (2, 25). The configuration of these filaments throughout the cytoplasm of many cells suggests a structural or supporting role (9). Desmin, a structural protein in skeletal muscle (15), is found as a component of intermediate filaments in embryonic chick cells (14), suggesting a structural role for these filaments.

Although the configuration of these structural elements, as well as the characterization of their proteins, is well documented, less is known about the source of energy that may be used by contractile activities requiring cytoskeletal support. In muscle, it is well known that ATP is supplied by mitochondria. In addition, creatine phosphokinase (CPK) catalyzes the generation of ATP from creatine phosphate and ADP, maintaining an equilibrium. Thus, creatine phosphate serves as a energy source, readily converted into ATP. CPK has long been known as a mitochondrial constituent (24) where it likely catalyzes the formation of creatine phosphate from ATP and creatine. Much of the CPK is soluble, and recent evidence has shown that CPK may be found in the M-line of striated muscle (31, 33) as well as in mitochondria (24). Immunological analysis has shown that antibodies specific for CPK will react with purified M-line protein, verifying the presence of CPK on the M-line (30).

Three isoenzymes of CPK have been demonstrated. Muscle, or M-type, is characteristic of skeletal muscle (8). Brain, or B-type, has been demonstrated in brain, nonmuscle tissue, and embryonic myoblasts (1, 18, 29). Transition from B-type to M-type CPK in cultured myoblasts has been shown to reflect the differentiation of the cells (4, 18, 29). In addition, a hybrid MB isoenzyme has been demonstrated (8), as has a mitochondrial isoenzyme (12).

A large number of contractile and control proteins have been demonstrated to be important in nonmuscle cell motility (22). For this reason, we have investigated the possibility that creatine phosphokinase may be found in nonmuscle cells. This paper reports our findings on the localization of this enzyme in cultured mammalian cells.

MATERIALS AND METHODS

Antibody Preparation

Creatine phosphokinase was isolated from chicken breast muscle by a modification of the method of Morimoto and Harrington (17). During the wash procedure, centrifugation of the muscle mince was replaced by filtration through cheesecloth, to allow the use of very large volumes of wash solution. Batch treatment of the extracted protein with DEAE-cellulose was eliminated. The sample was further purified on a DE-52 column. Protein purity was assayed by sodium dodecyl sulfate–polyacrylamide gel (SDS-gel) electrophoresis (34). CPK had a minimum specific activity of 35,000 U/mg (determined by Sigma kit 45uv, Sigma Chemical Co., St. Louis, Mo.). We emulsified 1 mg of purified protein with complete Freund's adjuvant (Grand Island Biological Co., Grand Island, N.Y.) and injected it subcutaneously into each rabbit. Booster injections of 1 mg in incomplete Freund's adjuvant (GIBCO) were administered every 14 d. Blood was collected from the ear every 14 d beginning with day 28. Antiserum collected after three injections was used in this study. The immunoglobulin fraction was collected from the serum by precipitation at 50% saturation of ammonium sulfate (Schwartz-Mann). Antibody specificity was tested by double immunodiffusion in 0.75% agarose in phosphate-buffered saline (PBS) according to Ouchterlony (20).

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Antibodies specific for intermediate filaments were obtained as an autoimmune rabbit antiserum (11, 19) and were shown to be specific by immunodiffusion and absorption (B. Eckert, manuscript in preparation).

To further characterize the antibody reacton in PTK cells, a 1:10 dilution of the IgG fraction was mixed with a low salt extract of PTK cells. This extract was prepared in essentially the same way as the low salt extract of muscle (17) used for CPK isolation. Material precipitated by the antibody was collected by centrifugation and analysed by SDS-gel electrophoresis.

Immunofluorescence

Cells (lines PTK_1 and BALB/3T3) were grown on coverslips. The coverslips were washed with PBS and treated for 20 min either in 3.7% formaldehyde in PBS or in absolute acetone at −20°C. Some preparations were immersed in liquid nitrogen for 1 min and thawed in one of the fixatives. Formaldehyde-fixed cells were made permeable to antibodies by treatment for 10 min in 95% ethanol. Cells were then treated for 45 min at 37°C with 1:50 dilution of the IgG fraction from immunized rabbits. After washing in PBS, cells were treated for 45 min with goat anti-rabbit IgG conjugated with tetramethylrhodamine (N. L. Cappel Laboratories Inc., Cochranville, Pa.). Coverslips were washed, mounted in elvanol (DuPont Co., Wilmington, Del.), and observed on a Zeiss Ultraphot IIIB equipped with epifluorescence optics. Micrographs were taken on Kodak Plus-X film and developed in Diafine.

RESULTS

Immunodiffusion

CPK (M-type) used for immunization is shown in lane *2* of Fig. 1 *a*. Lane *1* shows a preparation of B-type CPK from rabbit (obtained from Sigma Chemical Co.) for comparison. The difference in mobility of these isoenzymes is apparent. Double immunodiffusion of the IgG fraction of serum from immunized rabbits shows specificity of the antibody (Fig. 1 *b*). A precipitin band forms between the antibody and purified CPK. No reaction occurs between the antibody and actin (purified according to reference 26), tubulin (purified according to reference 36), cycle-1 desmin (purified according to reference 16), or intermediate filament protein from 3T3 cells (isolated according to reference 9). Fig. 1 *c* shows immunodiffusion of the antiserum against M-type and B-type CPK. The antibody reacts only with M-type CPK. Lane *3* of Fig. 1 *a* shows gel electrophoresis of a pellet obtained by reaction of CPK antibody with a low salt extract of PTK_1 cells. A band having the same mobility as CPK is found in this sample and is not observed in the antibody fraction alone (lane *4*).

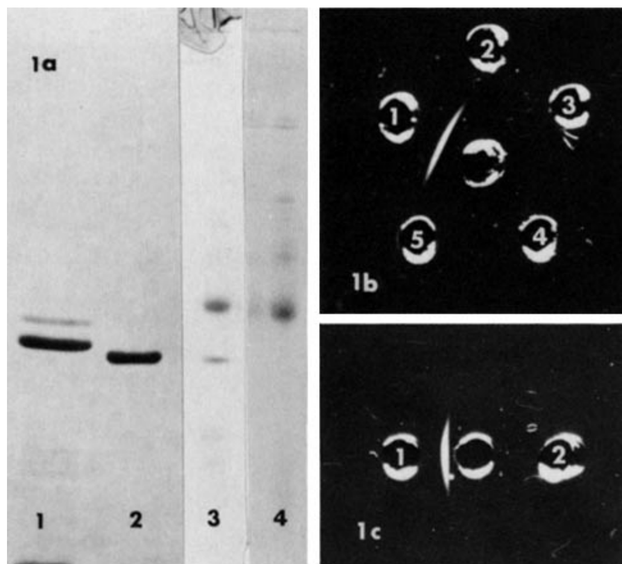

FIGURE 1 (*a*) SDS-gel electrophoresis. Lane *1*, B-type CPK obtained commercially. Several impurities are visible. Lane *2*, CPK purified from chicken breast muscle; Lane *3*, precipitate obtained by mixing anti-CPK with low salt extract of PTK_1 cells; Lane *4*, anti-CPK sample alone. (*b*) Precipitin reaction of antibody against CPK. Center well, IgG fraction of serum from immunized rabbit; well *1*, CPK; well *2*, actin; well *3*, tubulin; well *4*, 3T3 intermediate filaments; well *5*, cycle-1 desmin. (*c*) Precipitin reaction of antibody against CPK. Center well, IgG fraction of serum from immunized rabbit; well *1*, M-type CPK; well *2*, B-type CPK.

Immunofluorescence

Immunofluorescence observations of PTK_1 and 3T3 cells, treated with antibody specific for CPK, reveal fluorescent fibers in the cytoplasm. Their patterns in PTK_1 (Fig. 2) and 3T3 (Fig. 3) cells have some basic similarities. The fibers appear to emanate from a ring around the nucleus and extend into the cytoplasm with a coarse, sometimes wavy, appearance. The distribution is quite similar to that of intermediate filaments in PTK_2 cells (9) and in 3T3 cells (11). Fig. 4 shows a PTK_1 cell treated with an antibody specific for intermediate filaments, using conditions identical to those used for anti-CPK. This staining pattern is consistent when either acetone or formaldehyde fixation is used. Rapid freezing in liquid nitrogen followed by thawing into chemical fixatives also produced similar results. No reaction was obtained with IgG fraction from preimmune serum (Fig. 7).

Figs. 5 and 6 show PTK_1 cells reacted with antibody against actin (see reference 5 for characterization) and against tubulin (6). Clearly, the pattern of anti-CPK staining is most similar to that of intermediate filaments.

Cells were also treated with 2×10^{-5} M colchicine for 15 h to see how the loss of microtubules might affect the distribution

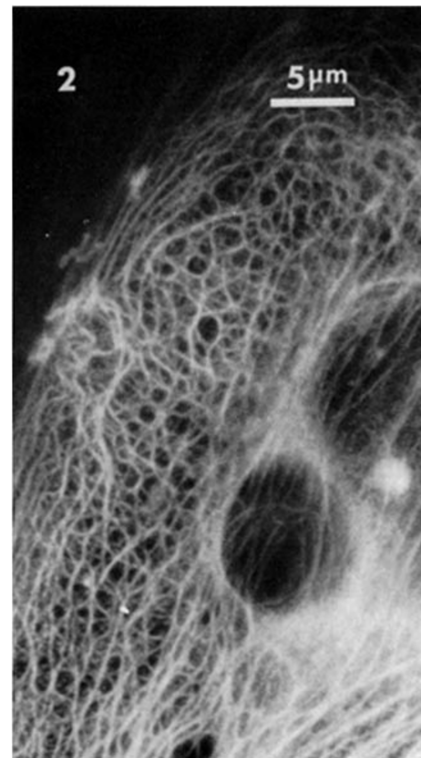

FIGURE 2 Indirect immunofluorescence of a PTK_1 cell treated with antibody specific for creatine phosphokinase. Cytoplasmic fibers show labeling and have a pattern characteristic of PTK_1 intermediate filaments.

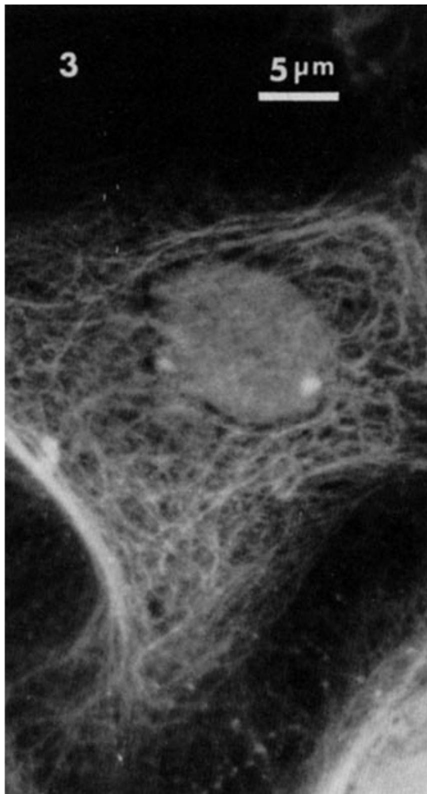

FIGURE 3 Indirect immunofluorescence of a 3T3 cell treated with antibody specific for creatine phosphokinase. Cytoplasmic fibers label with antibody.

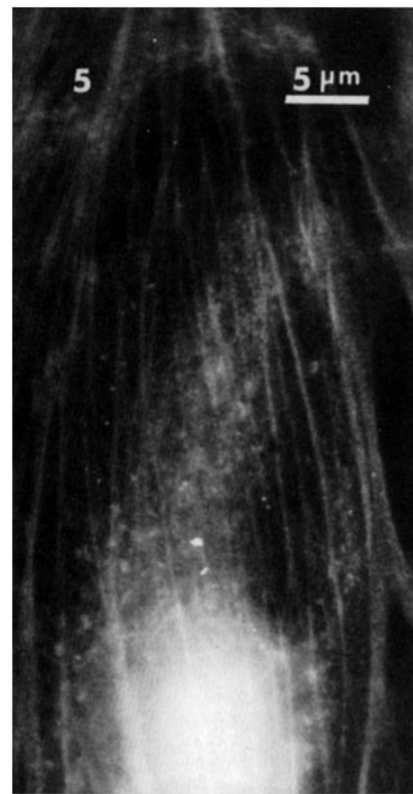

FIGURE 5 Indirect immunofluorescence of a PTK_1 cell after treatment with antibody specific for actin. Stress fibers are clearly labeled.

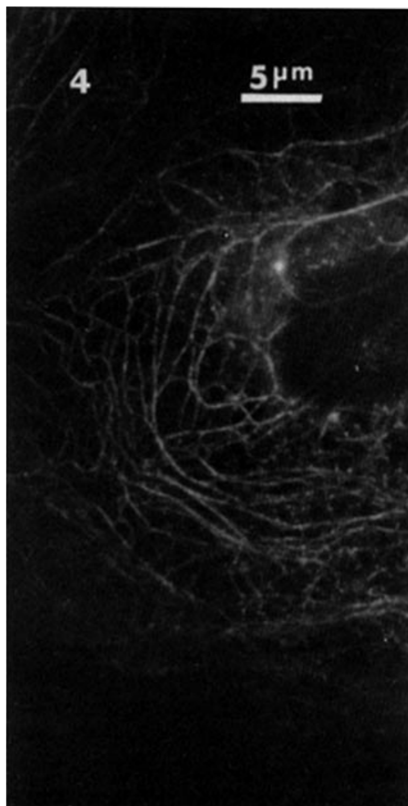

FIGURE 4 Indirect immunofluorescence of a PTK_1 cell after labeling with antibody against intermediate filaments. The pattern is the same as in Fig. 3.

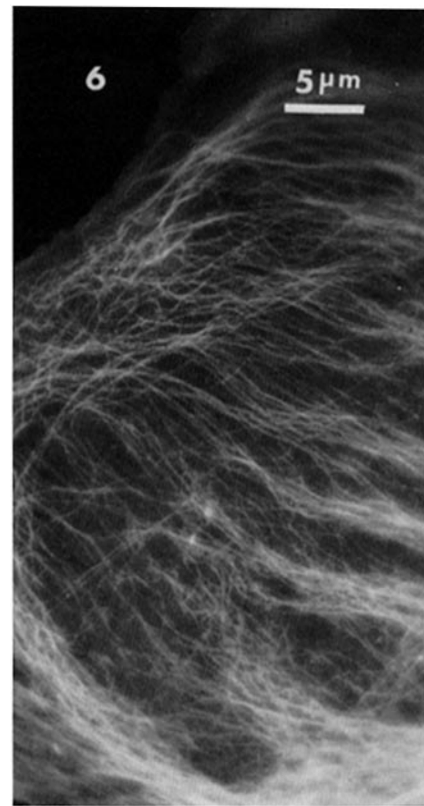

FIGURE 6 Indirect immunofluorescence of a PTK_1 cell after treatment with antibody specific for tubulin. Microtubules are clearly labeled.

FIGURE 7 PTK$_1$ cell treated with preimmune IgG and observed by indirect immunofluorescence. No labeling is observed.

FIGURE 9 Indirect immunofluorescence of a PTK$_1$ cell after treatment with anti-CPK antibody pre-absorbed with purified CPK (sample shown in Fig. 1 *a*, lane *2*). Labeling of filaments is abolished.

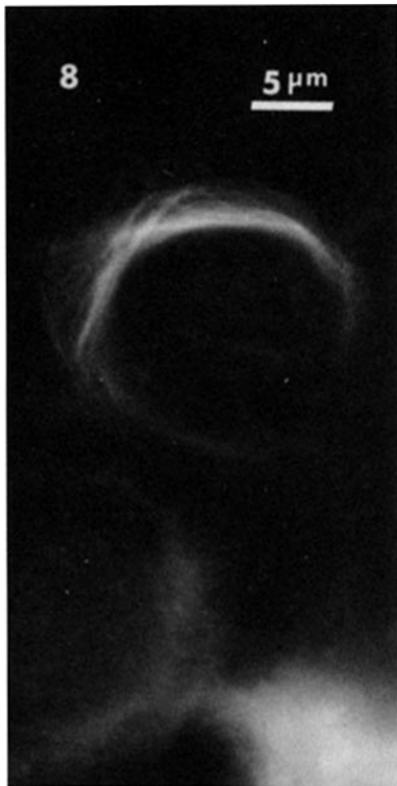

FIGURE 8 Indirect immunofluoresence of a 3T3 cell treated with 2 $\times 10^{-5}$ M colchicine before fixation. CPK labeling appears in fibers coiled around the nucleus.

of CPK. As seen in Fig. 8, the configuration of CPK labeling appears to change in the same way that the distribution of intermediate filaments changes under these conditions (11, 19). These filaments form a cap or ring near the nucleus, and the CPK labeling appears with the same pattern.

Preabsorption of antisera with purified intermediate filaments or CPK further establishes the specificity of the staining. When antisera are reacted for 1 h at 37°C with purified CPK, the labeling pattern observed above is eliminated from the cells (Fig. 9). If a similar experiment is done with intermediate filament protein purified from PTK$_1$ cells, the labeling is not removed.

DISCUSSION

The observations presented here indicate that creatine phosphokinase of the M-type is associated with intermediate (10-nm) filaments in cultured mammalian cells. The images of CPK antibody staining are comparable with the distribution of intermediate filaments in both cell types studied. Comparison of anti-CPK immunofluorescence with images showing antibody against actin (13) or antibody against tubulin (6, 35) shows less similarity and suggests that CPK is probably not associated with these elements. Furthermore, the CPK labeling appears to follow intermediate filaments when cells respond to colchicine. Coiling of intermediate filaments around the nucleus after colchicine treatment has been demonstrated (11, 19, 27), and our observations on colchicine treated cells show a similar distribution of CPK. Reproducible association with intermediate filaments under different fixation conditions, as well as the response to colchicine, makes it unlikely that this association is an artifact of fixation.

The nature of the association between CPK and intermediate filaments is not yet understood. The present data show a similarity of immunocytochemical staining pattern between antibody specific for CPK and antibody specific for intermediate filaments. Although this does not demonstrate binding of CPK to the filaments, it does indicate an association of CPK with cytoskeletal fibers. The association of CPK with a particular cytoskeletal element suggests that the enzyme may have a functional role in contractile or cytoskeletal activities. In skeletal muscle, CPK functions by catalyzing ATP generation by transferring a phosphate group from creatine phosphate to ADP. This generation of ATP, more rapid than oxidative phosphorylation, is essential for maintenance of adequate ATP levels for contraction. This enzyme may serve as part of an energy-generating system in nonmuscle cells in the same way that it does in skeletal muscle.

Characterization of the antibody indicates that it is specific for M-type CPK. The antiserum reacts only with M-type CPK and not with B-type CPK in double immunodiffusion tests. The antibody is also capable of precipitating a protein from PtK_1 extracts that comigrates with CPK on SDS-gel electrophoresis. A few faint bands appear in the upper region of Fig. 1 *a*, lane *3*: these may be precipitated by the antibody either (*a*) because the antibody has reacted with them or (*b*) because they are associated with CPK. We have not distinguished between these possibilities, although preabsorption studies clearly show that the staining pattern observed is only the result of CPK and not of these other proteins. Tests of the antibody against cytoskeletal proteins, potentially able to give spurious immunofluorescence results, were negative. Tests of preimmune IgG, designed to check for the presence of autoimmune antibodies against intermediate filaments (11, 19), were negative for rabbits that were immunized with CPK.

It is interesting to find such a clear localization of M-type CPK in nonmuscle cells when one considers reports on B-type CPK. This isoenzyme has been demonstrated in young embryonic muscle (21), and the appearance of M-type CPK in these cells has been used as a marker for muscle differentiation (18, 29). It has, therefore, been suggested that M-type CPK is a characteristic protein of differentiated skeletal muscle (29). Because we have shown M-type specificity for our antibody and have carefully controlled for nonspecific staining, our results show that this muscle-type protein is in nonmuscle cells. The interpretation of these observations certainly requires further investigation.

Association of CPK with intermediate filaments in nonmuscle cells is an interesting comparison with CPK localization in the sarcomere. Wallimann et al. (31, 32, 33) have shown localization of the muscle form of CPK in the M-line, presumably in association with the thick filament. This portion of the filament, however, is not directly involved with use of ATP. Rather, the central zone of the thick filament may be thought of as a supporting structure. The active ATPase may be from 0.1 to 0.5 μm in distance from the M-line-bound CPK. Intermediate filaments are thought of as structural components that organize cytoplasmic events or organelles (9, 25). The cytoskeleton may serve as a structural support for a CPK-catalyzed energy-generating system, some distance from the point of ATP use. In this way, organization of the intermediate filament network might influence local contractile activity by influencing local availability of ATP.

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