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NOTES

Immunoreactive Levels of Myosin Light-Chain Kinase in Normal and Virus-Transformed Chicken Embryo Fibroblasts

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Calmodulin, a calcium-modulated effector protein, is an important mediator of the intracellular actions of calcium through its interaction with calmodulin-binding proteins. We report here that the immunoreactive levels of a calmodulin-binding protein, myosin light-chain kinase, are decreased in transformed chicken embryo fibroblasts.

We have previously shown (10, 13) that the levels of calmodulin are two- to fourfold higher in virus-transformed chicken embryo fibroblasts (CEF) than in normal CEF. The molecular mechanism responsible for the higher levels appears to be a preferential increase in the rate of synthesis of calmodulin that results from an increased amount of calmodulin-specific mRNA in transformed CEF (14). Studies on the subcellular distribution of calmodulin and calmodulin-binding proteins in CEF (10) demonstrated the presence of calmodulin-binding proteins in normal and transformed CEF and suggested that there may be quantitative alterations in the levels of certain calmodulin-binding proteins after transformation. We have recently reported (W. H. Burgess, D. M. Watterson, and L. J. Van Eldik, J. Cell. Biol., in press) the identification of several classes of calmodulin-binding proteins in CEF. However, to more completely understand the role of calmodulin in CEF and the perturbations of calmodulin homeostasis in transformed CEF, quantitation of calmodulin-binding proteins in normal and transformed cells is necessary.

Because of the many documented changes in cell shape and cytoskeletal structure which occur upon viral transformation (5, 7), calmodulin-binding proteins that may be involved in nonmuscle contractile processes are obvious potential targets of altered calmodulin regulation in transformed cells. One of these proteins, myosin light-chain kinase (MLCK), is an enzyme which catalyzes the phosphorylation of the regulatory light chain of myosin in a calcium- and calmodulin-dependent manner (3, 6). As part of a previous communication (12), we reported our initial results that indicated that skeletal muscle light-chain phosphorylating activity was higher in transformed CEF than in normal CEF. To determine the levels of MLCK protein in normal and transformed CEF, we prepared antibody against purified chicken gizzard MLCK, established a radioimmunoassay for MLCK, and measured the immunoreactive levels of the protein. We report here that MLCK levels are higher in normal CEF than in transformed CEF by both immunoreactivity and enzyme activity, using cardiac light chain as substrate.

Chicken gizzard MLCK (1) and vertebrate calmodulin (10) were purified as described previously. Mixed myosin light chains were prepared from bovine cardiac muscle or rabbit skeletal muscle as described before (2), and the regulatory light chain was purified as described previously (Burgess et al., in press). MLCK activity was assayed as described in reference 2, except that light chains were used at a concentration of 30 μM and calmodulin was used at a concentration of 12 nM. Under the conditions used, MLCK assays were linear with time, and only values on the linear portion of the activity curves were used to calculate the specific activity. Normal CEF and CEF transformed by the Prague strain of Rous sarcoma virus, subgroup C, were grown and subcellular fractions were prepared as described before (10). In some experiments, a supernatant fraction was prepared directly from the homogenate by centrifugation at 55,000 × *g* for 60 min. Mouse antibodies against chicken gizzard MLCK were prepared as previously described (Burgess et al., in press). Iodinations and competition radioimmunoassays were done by procedures similar to those described for calmodulin (11). Immunoblot analyses were done as described (Burgess et al., in press), except that peroxidase-labeled goat anti-mouse immunoglobulin G was used instead of biotinylated second antibody and avidin-biotinylated peroxidase complex.

We have previously reported (Burgess et al., in press) the detection of MLCK as a calmodulin-binding protein in CEF by enzyme activity, immunoreactivity, and iodinated calmodulin-binding activity, using gel overlay procedures. To quantitate the levels of MLCK, we prepared antibodies against gizzard MLCK and set up a quantitative radioimmunoassay (Burgess et al., in press). Analysis of subcellular fractions of normal and transformed CEF by competition radioimmunoassay showed that, similar to calmodulin distribution (10), 80 to 90% of the MLCK immunoreactivity was in the S fraction in both cell types. Therefore, in all subsequent experiments, only the S subcellular fraction or a 55,000 × *g* supernatant fraction was analyzed. Tabulation of the results of four experiments (Table 1) shows that the immunoreactive levels of MLCK are approximately 2.8-fold higher in normal CEF supernatants than in transformed CEF supernatants.

These radioimmunoassay data are supported by immunoblot analysis of CEF extracts (Fig. 1). When equal amounts of protein from normal and transformed CEF were analyzed after electrophoresis and transfer to nitrocellulose

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TABLE 1. Immunoreactive levels of MLCK in CEF supernatants[a]

Expt	MLCK (μg/mg)	
	Normal	Transformed
1	0.22	0.09
2	0.18	0.04
3	0.21	0.07
4	0.13	0.06
Mean ± SE	0.185 ± 0.040	0.065 ± 0.021

[a] Analyzed by competition radioimmunoassay, using ^{125}I-labeled chicken gizzard MLCK and mouse antiserum against chicken gizzard MLCK.

for reactivity with the antibodies to MLCK, a doublet band that comigrated with the doublet band in purified gizzard MCLK preparations was the major reactive species in the CEF extracts. Based on the intensity of the band, there appears to be more MLCK in normal CEF than in transformed CEF.

Because of these observations, we reinvestigated MLCK enzyme activity in normal and transformed CEF by using several different substrates. We determined that the substrate in our previous studies (12), rabbit skeletal muscle light chain, is a relatively poor substrate for the MLCK from CEF and for purified smooth-muscle MLCK. When cardiac light chain was used as substrate, we detect 15- to 20-fold more MLCK activity in CEF than when skeletal light chain was used. Similar results were obtained with purified chicken gizzard MLCK. The specific activity of chicken gizzard muscle MLCK with cardiac light chains as substrate is similar to that reported (1) for turkey gizzard MLCK with gizzard (smooth-muscle) light chains. However, smooth-muscle light chains are substrates for other protein kinases, including other calmodulin-regulated protein kinase (8, 9). Therefore, we measured MLCK activity in normal and transformed CEF supernatants by using cardiac light chain as substrate. Under these conditions, calcium-calmodulin-dependent MLCK activity was 2.7 ± 0.9-fold higher in normal CEF than in transformed CEF (n = 18). In a representative experiment, the incorporation of ^{32}P into cardiac light chain was 1,148 pmol min^{-1} mg of CEF protein^{-1} for normal CEF supernatant and 327 pmol min^{-1} mg^{-1} for transformed CEF supernatant. These enzyme data are consistent with the analysis of MLCK immunoreactivity and indicate that by both enzyme activity and immunoreactivity there is approximately two- to threefold more MLCK in normal CEF than in transformed CEF.

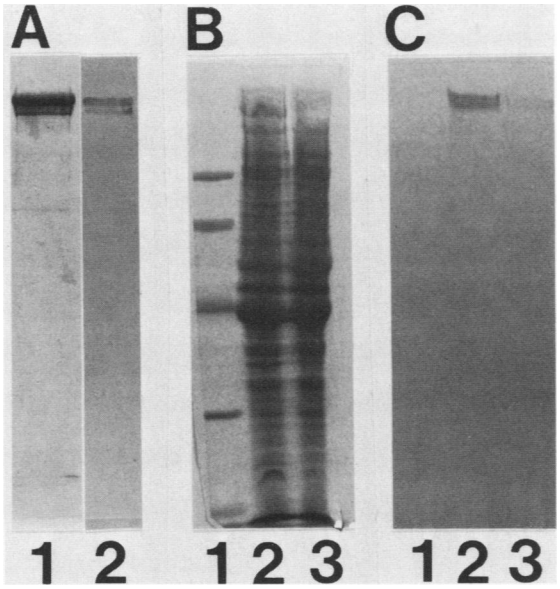

FIG. 1. Immunoblot analysis of purified MLCK and CEF extracts. Samples were subjected to electrophoresis in the presence of sodium dodecyl sulfate on 10% (wt/vol) acrylamide gels as described previously (10; Burgess et al., in press). (A) shows a Coomassie blue-stained gel of 3 μg of purified chicken gizzard MLCK (lane 1). In a separate experiment (lane 2), 0.15 μg of MLCK was subjected to electrophoresis, transferred to nitrocellulose paper, and processed for immunoblot analysis as described before (Burgess et al., in press). After incubation with mouse antiserum to gizzard MLCK (1:250 dilution) and washing with phosphate-buffered saline containing 0.05% (vol/vol) Tween 20, antibody reactivity was visualized by using peroxidase-labeled goat anti-mouse immunoglobulin G as second antibody and 4-chloro-1-naphthol as substrate. (B and C) Molecular weight standards (lane 1) (top to bottom: 94,000; 67,000; 43,000; 30,000; 20,000), 90 μg of a supernatant fraction from normal CEF (lane 2), and 90 μg of a supernatant fraction from transformed CEF (lane 3) were subjected to electrophoresis, transferred to nitrocellulose, and either stained with amido black (B) or incubated with antiserum to MLCK (1:100 dilution) (C).

It should be emphasized that, although MLCK and calmodulin levels are perturbed in transformed CEF, this does not mean that increasing calmodulin levels or decreasing MLCK activity will bring about the transformed phenotype. Whether the decrease in MLCK levels is related to transformation per se or to some other cellular parameter is not known. Related to the latter point, we have examined in preliminary experiments the relationship between MLCK activity and cell density or proliferation of normal CEF. Our results suggest that MLCK activity in normal CEF at low cell densities or during proliferation is intermediate between that found in confluent normal CEF and that found in transformed CEF. Thus, MLCK activity appears to decrease under conditions in which CEF are undergoing rapid growth. Further studies will be required to address the molecular mechanism of the decrease in MLCK levels in transformed CEF.

The physiological significance of decreased levels of MLCK in transformed CEF is not known. The effects of calmodulin on MLCK activity and the role of MLCK in cell motility have been the subject of numerous studies by several investigators (for review, see reference 4). Even with this extensive history, there is still controversy surrounding the exact role of light-chain phosphorylation in muscle function. However, because of the potential involvement of MLCK in regulation of the ATPase activity and the aggregation state of myosin in CEF, alterations in MLCK activity may be related to the changes in cytoskeletal structure and cellular architecture which occur upon transformation.

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