Changes in Contractile Proteins during Differentiation of Myeloid Leukemia Cells. II. Purification and Characterization of Actin

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ABSTRACT A myeloid leukemia cell line, M1, differentiates to macrophage and gains locomotive and phagocytic activity when incubated with conditioned medium (CM) from a fibroblast culture and bacterial endotoxin. To characterize the actin molecules before and after differentiation, the actin was purified through three sequential steps: DEAE-Sephadex A-50, polymerization/depolymerization, and Sephadex G-150 chromatography.

There were no essential differences between the inhibitory activity of actins from control M1 cells and CM-treated M1 cells on both DNase I and heavy meromyosin (HMM) K^+-EDTA-ATPase; the same dose response as with skeletal muscle actin took place. After the treatment with CM, however, the specific activity for the activation of HMM Mg^{2+}-ATPase by actin became two-fold that of untreated M1 actin, which was one third of the value for skeletal muscle actin. The V_{max} for the control and the CM-treated M1 cell, as well as the skeletal muscle actins, proved to be the same. By contrast, the K_{app} values for the control and CM-treated M1-cell actins were 3- and 1.5-fold the value for skeletal-muscle actin. This means that CM treatment of the M1 actin produced a twofold affinity for the Mg^{2+}-ATPase of skeletal-muscle myosin.

The critical concentrations for polymerization were compared under different salt concentrations and temperatures. Although no marked difference was found for the presence of 2 mM $MgCl_2$, 0.1 M KCl in place of $MgCl_2$ at 5°C gave the following values: 0.1 mg/ml for skeletal-muscle actin, 0.7 mg/ml for control M1 actin, 0.5 mg/ml for CM-treated M1 actin, and 1.0 mg/ml for the D^- subline that is insensitive to CM. Although the critical concentration of D^- actin is extraordinarily high, this actin showed normal polymerization above the critical concentration. This together with the data presented in our previous paper, that the D^- actin in the crude extract did not polymerize, suggests that an inhibitor for actin polymerization is present in this subline.

The kinetics experiment at 0.1 M KCl and 25°C revealed a slower polymerization of untreated M1- and D^--cell actins as compared with CM-treated M1 actin. This delayed polymerization was due to a delay during the nucleation stage, not during the elongation stage.

By isoelectric focusing, the ratios of β- to γ-actin showed a marked difference depending on the states of cells: about 4.9 for control M1, 2.8 for CM-treated M1, and 7.6 for D^--subline actins. Tryptic peptide maps also revealed the presence of different peptides. Thus, the functional differences of actin before and after the differentiation was accompanied by some chemical changes in actin molecules.

Locomotive and phagocytic activities of cells are controlled by the actomyosin system that includes actin, myosin, and a number of regulatory proteins. Although these contractile proteins in muscle cells are organized in a concrete structure, those in nonmuscle cells are not so strictly organized and must change their form, localization, and relation to each other,

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responding to various external stimuli. In nonmuscle cells, rapid polymerization and depolymerization of actin molecules may be essential for phagocytosis and locomotion, because they control the deformability of cell shape (7, 20, 49, 50). Profilin (6, 32, 39), isolated from calf spleen and human platelets, and DNase I (1, 21, 30) as well as cytochalasin D (4, 5) inhibit the polymerization of actin by different mechanisms, whereas phalloidin (51) has been reported to stabilize the polymerized actin filaments. The actin in nonmuscle cells must be exposed to various regulatory proteins and can be kept in the depolymerized state even when the intracellular concentration is above the critical concentration for polymerization and when the ionic concentration and temperature are high enough to promote polymerization.

Korn and his colleagues (13, 14) developed a purification procedure applicable to nonmuscle cell actins, which made it possible to recover enough of the nonmuscle actins to compare their chemical and functional properties with those of skeletal-muscle actin. They pointed out marked differences in the critical concentration for polymerization of actins in 0.1 M KCl at 5°C, and the activation of skeletal-muscle heavy meromyosin (HMM) Mg^{2+}-ATPase by actins from *Acanthamoeba*, vertebrate nonmuscle cells (human platelet, embryonic chick brain, rat liver) and skeletal muscle (13). Stossel and his colleagues (9, 16, 17, 56) isolated an actin-binding protein from rabbit alveolar macrophages and identified its localization in the protruding cellular periphery and its definite role in the gelation of the cytoplasm. Local gelation and solation in the cytoplasm and their rapid interchangeability constitute one of the most important mechanisms by which nonmuscle cells carry out their locomotive and phagocytic activities (2, 8, 18, 48). Gelation factors other than the actin-binding protein were identified in chicken gizzard (54, 59), *Acanthamoeba castellanii* (35, 40), and Ehrlich ascites tumor cells (41); these factors must play an important role in the actomyosin system of nonmuscle cells. The macrophage, like the amoeba, is a motile cell in nature. The mechanism of the transition from the non-motile to the motile state was studied by Tilney (57, 58) using echinoderm sperm actin, the only report that has focused on the transition mechanism.

A tissue culture system would enable us to carefully control the environmental condition of the cultured cells, and to study the reactions of the motile apparatus when a number of stimulants were inoculated into the culture medium. However, this characteristic of tissue culture has not been used for biochemical studies of contractile proteins from nonmuscle cells.

We established a cultured cell line of murine myeloid leukemia cells, named Ml (24, 25), which can be induced to differentiate to macrophages when cultured with various differentiation-stimulating factors (D factors) (22, 23, 37, 43). Before differentiation, the Ml cells are neither motile nor phagocytic, but the differentiated cells show these activities (25, 42) (Fig. 1) as well as induction of the Fc receptor (33), elevation of lysosomal enzymes (27, 28, 46), changes in cell morphology (19, 24), and loss of leukemogenicity (25). In a previous paper (44), we reported that the actin content, its rate of synthesis, and the F-actin ratio in the total actin increased after differentiation of the Ml cells and that a prominent change was found in the polymerizability of G-actin. The actin in the D factor (conditioned medium from fibroblast culture)–treated Ml cell showed greater ability to sediment, depending on the increased concentration of $MgCl_2$ and/or KCl and proteins, as compared with the actin from untreated Ml cells.

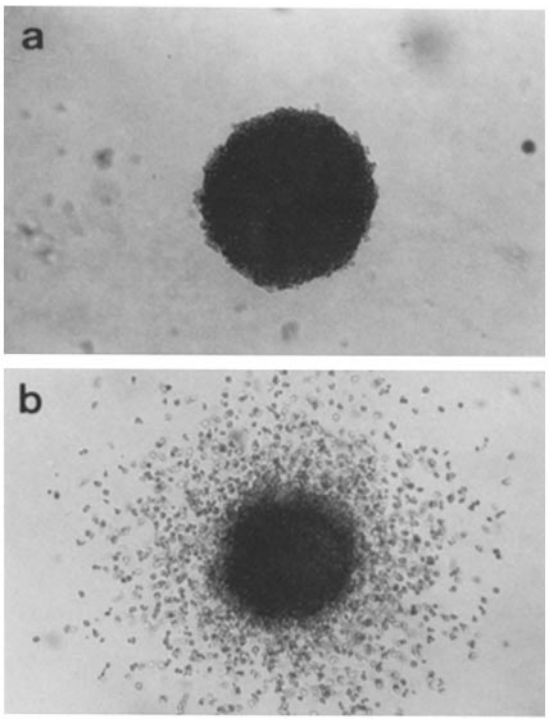

FIGURE 1 Induction of cell motility. The Ml cells were seeded in a soft agar (0.3%), and 10-d-old colonies were poured with MEM (*a*) or CM (*b*) (3 ml/6-cm plate) to be observed 4 d later. Cells exposed to CM are beginning to migrate into agar. Unstained, × 60.

The actin from the D^- subline, which is insensitive to the D factor, showed almost no polymerization.

We here present several experimental results and a discussion as to whether the differences found for actin polymerization before and after differentiation are due to changes in the properties of the actin molecules or to interference with polymerization by regulatory proteins. Purified actins from untreated and treated Ml cells were compared for their activation of skeletal-muscle HMM Mg^{2+}-ATPase, for their polymerization at different temperatures and salt concentrations, and for the ratios of β- to γ-isoactin. The relation between the changes found in the contractile protein and the induction of differentiated functions, cell motility, and phagocytosis also are discussed.

MATERIALS AND METHODS

Cell Line and Culture

The Ml cell line was isolated from a myeloid leukemia of an SL strain mouse and has been maintained in vitro since 1969 (24). The cells of this line are morphologically myeloblasts, neither motile nor phagocytic. When incubated for several days with various D factors, including the glycoproteins present in the conditioned medium (CM) from a fibroblast culture, ascites, and serum, glucocorticoids, lipopolysaccharide (LPS) of Gram-negative bacillus, etc. (11, 22, 23, 37, 38, 60), the Ml cells differentiate either to neutrophil granulocytes or to macrophages and gain motility and phagocytic activity accompanied by the induction of other differentiated functions and the loss of mitotic activity.

The D^- line is a subline of the Ml line (26). Not even a high concentration of the D factor induces differentiation in this subline.

Eagle's minimum essential medium (MEM) (Nissui Seiyaku Co., Tokyo, Japan), with a double concentration of amino acids and vitamins, was used with 8% heat-inactivated horse serum.

Cells harvested from ~20 Falcon dishes first were cultured in a 3-liter flask, then in a 10-liter flask with constant agitation by a magnetic stirrer. The cell density was maintained at 0.7–1.0 × 10^6/ml by a daily supply of fresh medium.

One experiment usually needed two bottles for the cultures of $>10^{10}$ cells. To induce differentiation, CM and LPS were added to the medium for the final 3 d of culture at concentrations kept constant at 2–3% and 0.5 μg/ml, respectively. This D factor–treated culture finally yielded 1.6–2.1 × 10^{10} cells with 74–85% viability and ~75% phagocytic activity. Under the same culture conditions, 2.5–2.9 × 10^{10} D^- cells were harvested with 87–96% viability.

CM

Primary or secondary rat embryo cells were seeded at 1 × 10^7 cells/10 ml/10-cm Petri dish. The culture fluid was harvested after 3–4 d, then centrifuged at 2,500 rpm for 15 min, after which it was kept at −20°C until used. The activity for the induction of differentiation in Ml cells was >10 times that of CM from mouse embryo cells.

Test for Phagocytosis

After incubation with CM or LPS for 3 d, the cells were transferred to serum-free medium containing polystyrene latex particles (1 drop/20 ml of medium, average diameter 1 μm; Dow Chemical Co., Indianapolis, IN) and incubated for another 4 h. The cells were washed thoroughly, and the number of cells phagocytizing the particles was counted under a microscope.

Purification of Actin

The procedure of Gordon et al. (14) was used for treated or untreated Ml and D^- cells, with minor modifications.

After two washings in phosphate-buffered saline, the cells were resuspended in 2 vol of cold G-buffer (0.1 mM $CaCl_2$, 0.5 mM ATP, 0.75 mM 2-mercaptoethanol, 3 mM imidazole-HCl, pH 7.5) and homogenized by ~20 strokes in a Potter-Elvehjem homogenizer (Kontes Co., Vineland, NJ), then centrifuged at 100,000 *g* for 60 min at 4°C. The supernatant was harvested carefully without disturbing the floating lipid and microsome fractions, then these latter fractions were centrifuged at 150,000 *g* for 45 min and their supernatant was combined with the first supernatant. The combined supernatants were loaded on a 2.6 × 30 cm DEAE-Sephadex A-50 column equilibrated with D-buffer (the same as G-buffer except for 10 mM imidazole-HCl) containing 0.1 M KCl and were eluted with 1,000 ml of a 0.1–0.5 M linear KCl gradient. Actin-containing fractions were detected by the DNase I–inhibition assay (32) and were concentrated fivefold by ultrafiltration through a UM-10 membrane (Amicon Corp., Lexington, MA). This concentration is important for recovery of the polymerized actin in a high yield because the actin will be left in the unpolymerized state if the concentration is lower than the critical value. These concentrated samples were combined with $MgCl_2$ and phenylmethylsulfonyl fluoride (PMSF) to give the concentrations of 2 and 0.5 mM, respectively, after which they were left for 6 h at room temperature, then centrifuged at 100,000 *g* for 180 min at 25°C. The pellet containing polymerized actin was suspended in 10 ml of G-buffer, after which the suspension was dialyzed against 50 vol of the same buffer with 0.02% NaN_3 for 3 d, then centrifuged at 100,000 *g* for 150 min at 4°C. The supernatant was loaded on a 1.6 × 90 cm Sephadex G-150 column, which had been equilibrated with G-buffer, and was eluted with the same buffer. After fractions that showed DNase I–inhibiting activity had been concentrated with a UM-10 filter, they were dialyzed against G-buffer for 3 d, then centrifuged at 100,000 *g* for 150 min at 4°C. The supernatant was used as purified G-actin.

Preparation of Rabbit Skeletal Muscle Actin and HMM

Actin was purified from the acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (55). HMM was prepared by trypsinization of skeletal muscle myosin according to the method of Yagi et al. (61).

Biochemical Measurements

For the DNase I–inhibition assay, Lindberg's method was used (32).

To check equilibrium viscosity, samples to be polymerized were incubated overnight at 25°C or for 2 d at 5°C. For the tests of polymerization kinetics, the incubation time was shown in each experiment. Viscosity was measured with a Cannon-Manning semimicro viscometer, size 75 (Poulten, Selfe & Lee, Ltd., Essex, England).

To check the HMM-ATPase reaction, the assay mixture contained 2 mM ATP, 25 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$ for Mg^{2+}-ATPase or 0.5 M KCl plus 2 mM EDTA for K^+-EDTA-ATPase, HMM, and the actin samples. The reaction was started by adding the ATP solution and was stopped by adding an equivalent volume of 20% TCA, after which the mixture was centrifuged immediately to measure the free phosphate in the supernatant by the Fiske-Subbarow's method (12).

SDS PAGE was carried out on a 10% polyacrylamide gel slab according to Laemmli's method (29). After being stained by Coomassie Brilliant Blue R, the gel slab was scanned by a computerized gel scan spectrophotometer UWA 401 (Union Giken Co., Hirakata, Japan).

For isoelectric focusing gel electrophoresis, purified actins were applied to gels (13 cm × 2 mm inside diameter) and isoelectrically focused by the technique of O'Farrell (47) with 5% pharmalyte (pH 4–6.5).

For two-dimensional analysis of tryptic peptide, iodination of the actin band in SDS PAGE and electrophoresis followed by thin-layer chromatography were carried out by the methods of Bray and Brownlee (3) and Sargent and Vadlamudi (53), respectively, as described in a previous paper (44).

Protein concentration was measured by the method of Lowry et al. (34).

RESULTS

Purification of M1-cell Actin

As stated in Materials and Methods, a 16-liter suspension culture gave ~2.2–2.7 × 10^{10} cells of untreated and 1.6–2.1 × 10^{10} cells of CM- and LPS-treated cells. Since the cell volume increased during differentiation, the packed volumes of both materials were almost the same. The phagocytosis rate for the untreated Ml cells was always <5%, whereas that of the CM-treated cells was 74.6 ± 11.3% (mean ± standard deviation, in five independent experiments).

As the first step in the purification of actin, the cell extract was chromatographed on DEAE-Sephadex A-50 with a KCl linear gradient (Fig. 2 *a*). Untreated and CM-treated cell extracts showed similar elution patterns as seen by the adsorption at 290 nm, except for a marked increase in the pass-through fraction of the treated cell extracts. This was also the case for Mml subline extracts, a macrophage line which spontaneously differentiated from the Ml cells. The D^- subline, which is insensitive to CM, showed no increase in pass-through fraction even after treatment with CM (data not shown).

The DNase I–inhibition assay detected actin-containing fractions with a peak at 55 m℧ conductivity that corresponds to 0.39 M KCl. As shown in Fig. 2 *b*, actin was concentrated around this fraction. Fractions eluted with 0.36–0.42 M KCl were pooled, then concentrated by UM 10 ultrafiltration and purified further: one cycle of polymerization/depolymerization, in a 32–40% yield, followed by chromatography on a Sephadex G-150 column, which yielded 10% of the total actin with 92–99% purity.

There were no differences in the recovery and purity of actins from treated and untreated Ml cells. Recovery was influenced greatly by the step of polymerization/depolymerization, especially depolymerization. In a successful case, ~60% of the actin present in the DEAE fractions was recovered with 92% purity, and 26.4% of the total actin in the starting cell extract was recovered with 99.3% purity in the final step, Sephadex G-150.

Fig. 3 shows the Coomassie-stained gel slabs after SDS PAGE in each purification step.

Characterization of the Actin

DNASE I–INHIBITORY ACTIVITY: DNase I inhibiting rates were plotted as a function of dose of actin purified from skeletal muscle, untreated and CM-treated Ml cells (Fig. 4). There was little difference in the inhibitory activity of these three actin species although skeletal muscle actin gave a slightly higher value than the others. On the basis of this dose-response curve, we calculated the actin contents in the cell extracts: 48

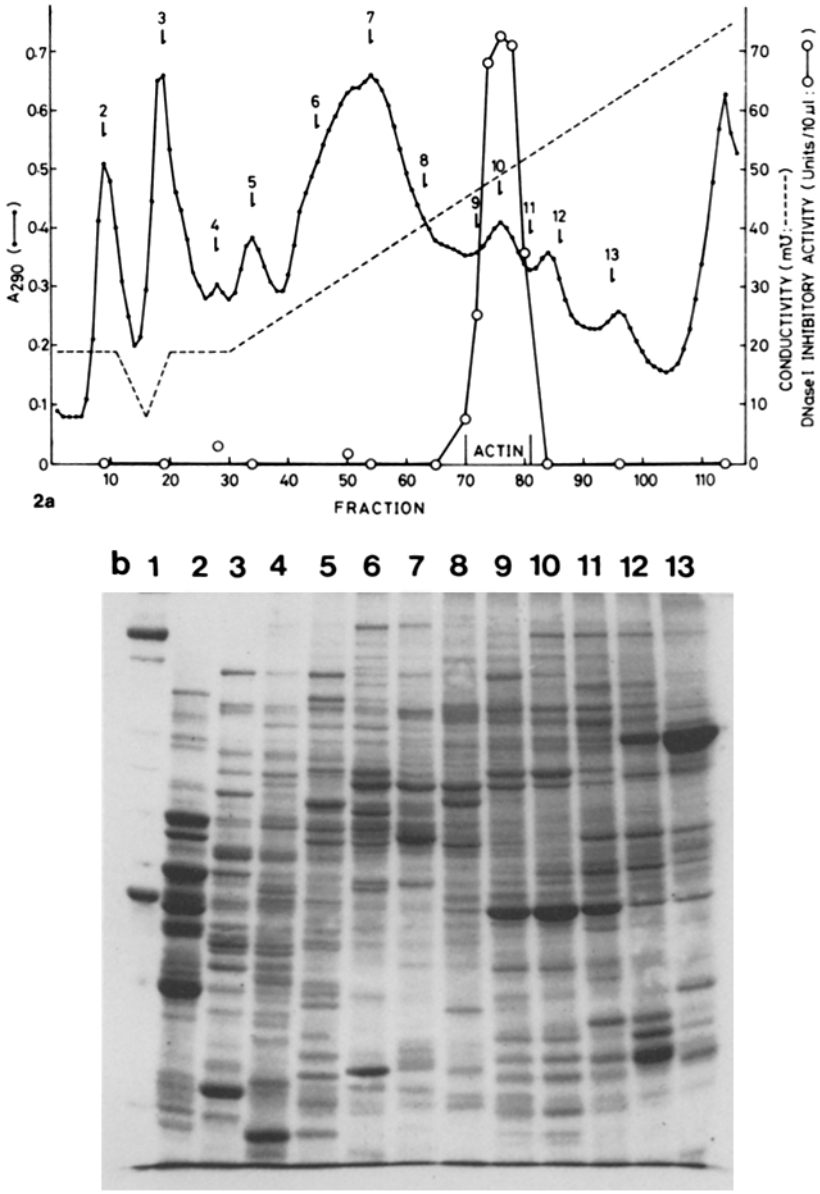

FIGURE 2 Fractionation of the M1-cell extract on DEAE-Sephadex A-50. (*a*) The low-ionic-strength Ml-cell extract (40–60 ml, 15–20 mg of protein/ml) was applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm) previously equilibrated with 0.1 M KCl in D-buffer, followed the procedure of Gordon et al. (14). To protect the actin from direct exposure to the 0.1 M KCl, 20–30 ml of G-buffer was added to the column immediately before and after application of the sample. After elution with 100 ml of 0.1 M KCl in D-buffer, the column again was eluted with 1 liter of a linear gradient of 0.1–0.5 M KCl in D-buffer. The absorbance at 290 nm and the conductivity were measured at room temperature. A protein of each fraction (5–20 µl) was assayed for DNase I–inhibitory activity as described in Materials and Methods. The activity unit was defined according to Blikstad et al. (1). (*b*) SDS PAGE of each fraction. A 1-mm-thick gel slab was stained with Coomassie Brilliant Blue, then destained and dried on filter paper. (*1*) Myosin and actin purified from rabbit skeletal muscle. *2–13* correspond to fractions indicated in the elution pattern of chromatography.

µg/mg protein for control Ml and 61 µg/mg protein for CM-treated Ml cells, as reported previously (44).

HMM K^+-EDTA-ATPASE-INHIBITORY ACTIVITY: It is known that actins from both skeletal-muscle and nonmuscle cells are capable of inhibiting skeletal muscle HMM K^+-EDTA-ATPase activity. As shown in Fig. 5, Ml-cell actins, regardless of whether untreated or CM-treated, inhibited this enzyme activity more effectively than did skeletal-muscle actin.

ACTIVATION OF HMM MG^{2+}-ATPASE: Activation of HMM Mg^{2+}-ATPase by actin is an important process from which the muscle gains energy for contraction. It is reasonable to assume that the same process occurs in nonmuscle cells carrying out their locomotive and phagocytic functions.

HMM Mg^{2+}-ATPase activity increased linearly as increasing concentrations of actin were added. From these values we calculated the specific activities of the actins (Table I). The specific activity of the control Ml-cell actin was about one third that of skeletal-muscle actin. These values are close to those given for skeletal-muscle and *Acanthamoeba* actins by Gordon et al. (14). In contrast, the actin from CM-treated Ml cells showed a specific activity almost twofold that of untreated Ml actin. Each value fluctuated, depending on the different lots of HMM as well as the actin preparations, but the relation was reproducible.

To determine how the specific activity is elevated during cell differentiation, we studied the kinetics of HMM Mg^{2+}-ATPase activation in detail. As reported by Eisenberg and Kielley (10) and by Gordon et al. (13), the relationship between the activation of skeletal-muscle HMM Mg^{2+}-ATPase and the dose of muscle as well as nonmuscle actins is described by a double reciprocal plot, from which the V_{max} (the extrapolated ATPase activity at an infinite dose of actin) and K_{app} (the actin dose required for half-maximal activation) are given. This plot was applied to actins from untreated and CM-treated Ml cells. As shown in Fig. 6, the two actins activated the Mg^{2+}-ATPase to the same V_{max} as that of skeletal-muscle actin. However, the K_{app} of untreated Ml-cell actin was threefold that of skeletal-muscle actin; three times as much Ml-cell actin as muscle actin was required to reach half-maximal activation. This figure also shows that the K_{app} of differentiated Ml-cell actin is about half that of untreated-cell actin.

STORAGE STABILITY OF ACTIN: How long isolated ac-

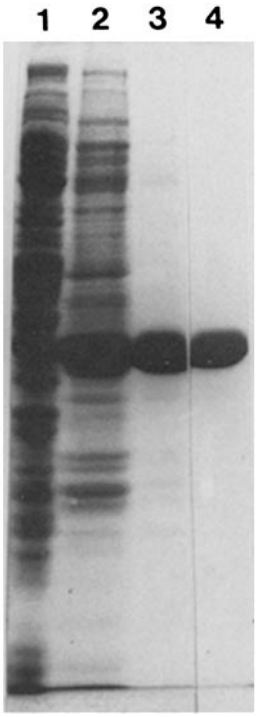

FIGURE 3 SDS PAGE of M1-cell actin at different stages of purification. (*1*) Crude extract, (*2*) DEAE fraction, (*3*) polymerized/depolymerized supernatant, and (*4*) Sephadex fraction.

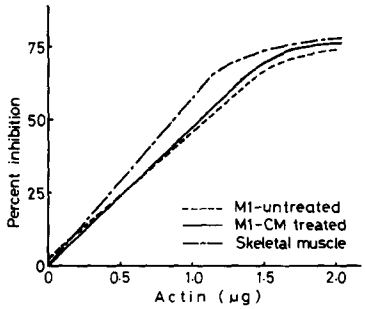

FIGURE 4 Inhibition of DNAase I activity by actins purified from untreated and CM-treated M1 cells, and from rabbit skeletal muscle. The DNAase I-inhibition assay was carried out as described by Lindberg (32). The hydrolysis rate of the substrate DNA solution was calculated from the linear relationship between the length of incubation and the increase in absorbance at 260 nm. The actin solution was added to the reaction mixture in a range of 5–40 μl, which was considered negligible in comparison with the volume of the substrate (3 ml).

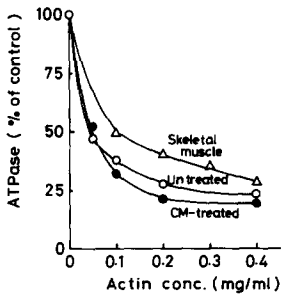

FIGURE 5 Inhibition of the K^+-EDTA-ATPase of HMM by the M1-cell and skeletal-muscle actins. The ATPase assay was carried out as described in Materials and Methods. The M1-cell and skeletal-muscle actins were used after being polymerized in 0.1 M KCl in G-buffer at 25°C. Control values of ATPase activity without actin varied between 102 and 106 μM P_i/μM HMM · min at 30°C in the three experiments.

tins in storage maintain their ability to polymerize needed to be determined. In Fig. 7, the time courses for the polymerization of actins in the presence of either 2 mM $MgCl_2$ or 0.1 M KCl are compared for fresh and ~2-d-stocked actin samples in G-buffer in a refrigerator (5°–8°C). In both salt solutions, the final specific viscosity of skeletal-muscle actin did not differ before or after storage. In contrast, polymerization of M1-cell actin in the presence of $MgCl_2$ was not influenced by storage at all, but in the presence of KCl both the polymerization rate

TABLE I

Specific Activity of Actins for the Activation of HMM Mg^{2+}-ATPase

	Mg-ATPase*
Untreated M1	13.3
CM-treated M1	24.3
Skeletal muscle	33.3

ATPase was assayed as described in Materials and Methods by incubating the reaction mixture which contained 0.4 μM rabbit skeletal-muscle HMM and various concentrations of actin (0.2–1.2 μM) at 30°C for 30 min. Because the activation of HMM Mg^{2+}-ATPase increased linearly with increasing concentration of actin within this range of actin concentration, its slope was used to calculate the specific activities of the actins.

* μM P_i/μM HMM head·s·mg actin.

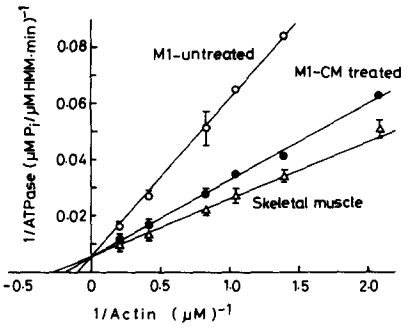

FIGURE 6 Activation of the Mg^{2+}-ATPase of HMM by M1-cell and skeletal-muscle actins. Double reciprocal plots are shown for the activation of the Mg^{2+}-ATPase as a function of the actin concentration. Actin was polymerized in 5 mM $MgCl_2$ in G-buffer at 25°C for 60 min, then added to the reaction mixture. The Mg^{2+}-ATPase activity of HMM at 30°C in the absence of actin was 10.3 μM P_i/μM HMM · min.

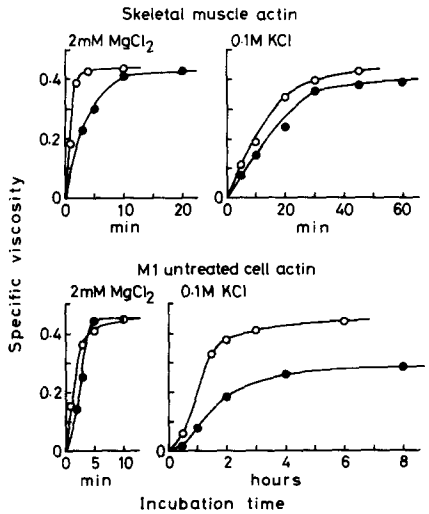

FIGURE 7 Storage stability of the M1-cell and skeletal-muscle actins. Untreated M1-cell and rabbit skeletal-muscle actins were tested for polymerization at 25°C, after being stocked for 2 (○) and 20 (●) d for M1-cell or 23 (●) d for muscle-actin in a refrigerator (5–8°C). The purified actins were kept as solutions in G-buffer. The aged actin was centrifuged and its protein concentration again was measured before the polymerization test, although neither a precipitate nor any decrease in the protein concentration of the supernatant was observed.

and the final viscosity markedly decreased. Because of this, we used Ml-actin samples within 3 d of isolation.

POLYMERIZATION OF ACTIN: The G-F transformation is one of the basic properties of actin molecules. Muscle and nonmuscle actins differ as to the critical concentration below which they do not polymerize, even when other conditions are satisfactory (13). With salt concentrations of 2 mM $MgCl_2$ or 0.1 M KCl at varying incubation temperature, we plotted the specific viscosities as a function of actin concentrations.

As shown in Fig. 8, the specific viscosities of all of the actin samples were linear increases. The reduced viscosity that represents the slope of increase in a given actin sample, except for the D^--cell actin, was almost the same under all the conditions tested. This differs from the results of Korn's group (13, 14) which reported a decreased slope at 5°C, as compared with 25°C, in the presence of $MgCl_2$. Only the actin from the D^- subline showed a slight decrease in its reduced viscosity at 5°C in the presence of either $MgCl_2$ or KCl. The reduced viscosity of CM-treated-cell actin was slight but reproducibly lower than that of untreated-Ml-cell actin, indicative of the presence of shorter filaments (4).

The critical concentration of Ml-cell actin in the presence of $MgCl_2$ increased a little at 5°C, although the critical concentration of muscle actin did not. A more dramatic difference was found at 5°C in the presence of KCl; the critical concentration was 0.10 mg/ml for muscle actin, 0.66 mg/ml for untreated Ml-cell actin, and 0.50 mg/ml for CM-treated Ml-cell actin. The value of 0.98 mg/ml for the D^--subline actin appears to be the highest value ever reported (13). This extraordinarily high value raises the question as to whether it is due to the denaturing of actin molecules, but that is unlikely because a sample of this actin showed a high viscosity when it was transferred at 25°C and incubated for another day (data not shown).

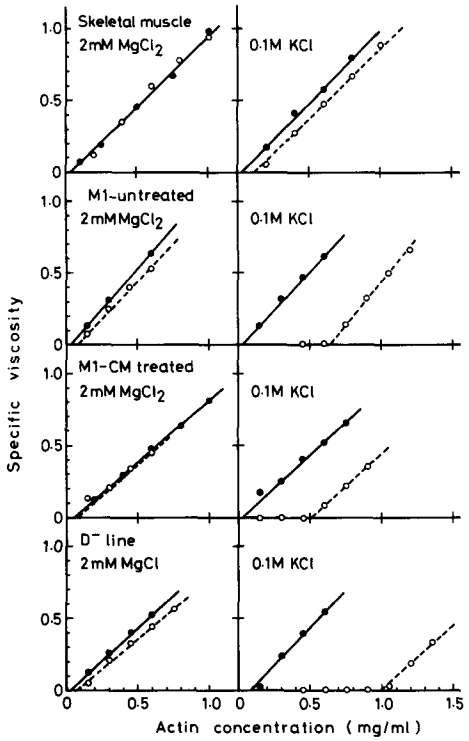

FIGURE 8 Polymerization of the M1-cell and rabbit skeletal-muscle actins. The equilibrium specific viscosity at 25°C (●) and 5°C (○) was plotted, after incubation for 1 d at 25°C or for 2 d at 5°C, as a function of the concentrations of the actins purified from skeletal-muscle, untreated M1, CM-treated M1, and D^- subline cells. All samples contained 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.75 mM β-mercaptoethanol, 3 mM imidazole-HCl pH 7.5, 0.02% NaN_3, and either 2 mM $MgCl_2$ or 0.1 M KCl. Viscosity was measured in a size 75 Cannon-Manning semimicro viscometer. The flow time for the buffer was 127 s at 25°C and 225 s at 5°C.

TIME COURSE OF ACTIN POLYMERIZATION: The marked differences in the critical concentration led us to study the kinetics of actin polymerization. In the presence of $MgCl_2$, actins have completed polymerization within 5–10 min, and there was no clear difference detected in the different actin species (Fig. 9). However, a marked difference was revealed for polymerization in the presence of KCl. The length of time required to reach the plateau of polymerization was 30–45 min for skeletal muscle, 60–90 min for CM-treated Ml, and 120–180 min for untreated Ml cells. The D^--subline-cell actin showed the same pattern as the control Ml-cell actin.

To disclose which stage of actin polymerization, nucleation or elongation, is responsible for the delay in polymerization, we inoculated F-actin fragments from rabbit skeletal muscle into a sample of D^--cell actin. Whereas the specific viscosity of the D^--cell actin without such nuclei was only 0.057 after 30-min incubation, inoculation with only F-actin fragments at 0.03 mg/ml brought about a dramatic increase (0.472) in the specific viscosity within 30 min (Fig. 10). This means that, in the presence of F-actin nuclei, even D^--actin completed polymerization within 30 min. Thus, the apparent delay in the time course for actin polymerization is probably due to the delay in nuclei formation (15).

Differences in the critical concentration for polymerization in KCl at 5°C, differences in the time course of polymerization in KCl at 25°C, and differences in the K_{app} in the HMM Mg^{2+}-ATPase activation described for the four actin species are compatible. Induction of locomotive and phagocytic activities in the Ml cells appears to be intimately related to those changes in the properties of the actin molecule, although interference by minor contaminants can not be excluded, as pointed out in the report by MacLean-Fletcher and Pollard (36).

COMPOSITION OF ISOACTINS AND PEPTIDE MAPPING: To disclose whether or not the functional differences we observed are due to different actins, we attempted to show some chemical differences in the actins. Fig. 11 shows the pattern of isoactins analyzed by an isoelectric focusing. While skeletal-muscle actin produced a single α-band, actins from control Ml, CM-treated Ml, and D^- subline cells proved to consist of a mixture of more alkaline β- and γ-species. Interestingly, the ratios of β- to γ-actins were remarkably different among these three states of cells (Table II). The rather high β/γ ratio in the Ml-cell actin was decreased by CM-treatment, becoming closer to the value reported for other nonmuscle cells (13). On the other hand, the β/γ ratio in the D^- subline was extraordinarily high, as was observed in Fig. 11 where the γ-actin was only a trace.

A two-dimensional analysis of [^{125}I]-labeled tryptic peptides of actins revealed the presence of two different spots between skeletal muscle and Ml-series cells (Fig. 12): spot *A1* found in skeletal muscle is absent in Ml cells, and the location of spot *A2* appears different. As for the difference among the Ml-series cells, the following points can be indicated: spot *B1* is almost undetectable in control and D^- cells but evidently is present in CM-treated cells; furthermore, spot *B2*, which appears to be absent in D^- cells but present in control Ml cells, is more markedly labeled in CM-treated Ml actin.

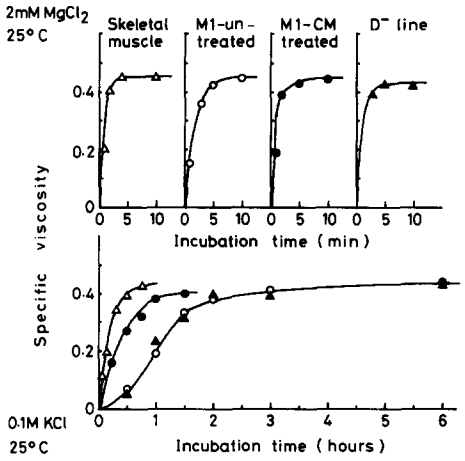

FIGURE 9 Kinetics of actin polymerization. The specific viscosity at 25°C was plotted as a function of the length of incubation for four kinds of purified actin. All samples contained 0.5 mg/ml of actin, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.75 mM β-mercaptoethanol, 3 mM imidazole-HCl pH 7.5, 0.02% NaN_3, and either 2 mM $MgCl_2$ or 0.1 M KCl.

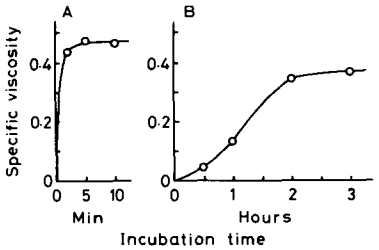

FIGURE 10 The effect of F-actin fragment on the polymerization of D^- cell actin. Purified rabbit skeletal-muscle actin (1 mg/ml) was polymerized for 1 h at 25°C in the presence of 0.1 M KCl. Just before use, it was sonicated for 15 s and added to the actin solution of D^- cells to be tested. Reaction solution consisted of 0.5 mg/ml D^--cell actin, 0.1 M KCl, and 0.03 mg/ml skeletal-muscle F-actin fragments in G-buffer. Other conditions were the same as described in the legend for Fig. 9. F-actin fragments were added in *A*, and not in *B*.

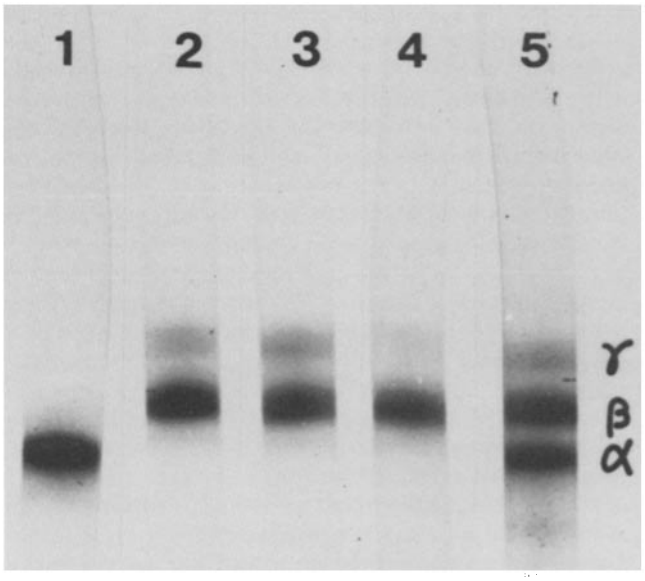

FIGURE 11 Isoelectric focusing gels of muscle and M1-cell actins. Purified actins were applied to gels (13 cm × 2 mm inside diameter) and isoelectrically focused by the O'Farrell's technique (47) with 5% pharmalyte (pH 4–6.5). The gels are (*1*) rabbit skeletal-muscle actin, (*2*) untreated M1-cell actin, (*3*) CM-treated M1-cell actin, (*4*) D^- subline-cell actin, and (*5*) a mixture of rabbit skeletal-muscle and untreated M1-cell actins.

TABLE II

The Ratio of β- to γ-Actin Isomers

	Exp. 1	Exp. 2
Untreated M1	4.1 ± 0.5	5.6 ± 1.2
CM-treated M1	2.6 ± 0.6	3.0 ± 0.1
D^- subline	7.4 ± 1.2	7.8 ± 1.2

This table shows the results of two experiments in which the actin samples were independently isolated. The isoelectric focusing gels stained with Coomassie Blue were densitometrically scanned, and the areas occupied by each isoactin were cut and weighed. Values represent mean ± standard error using five gels for each actin sample.

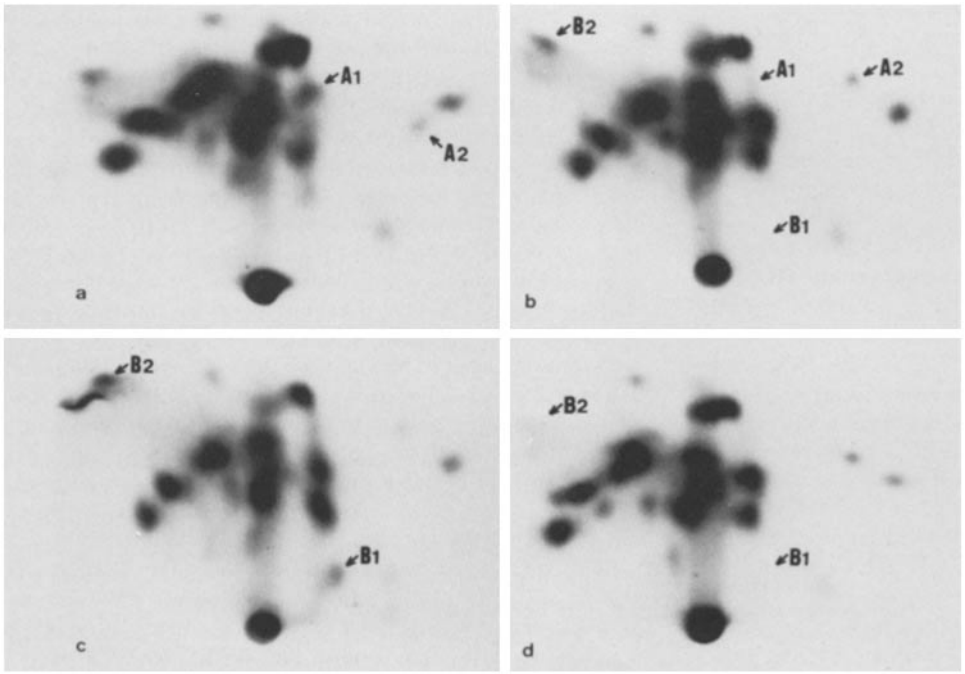

FIGURE 12 Autoradiogram of ^{125}I-labeled tryptic peptides. Electrophoresis was carried out from right to left, and chromatography from bottom to top. The apparently different peptide spots between skeletal muscle (*a*) and M1 cells (*b*) are marked by *A1* and *A2*. *B1* and *B2* show the differences of peptide spots among untreated M1 (*b*), CM-treated M1 (*c*), and D^- subline (*d*) actins.

DISCUSSION

In the purification of actin from rabbit alveolar macrophages, as their starting material, Hartwig and Stossel (17) used a gelled extract that contained most of the cellular actin and was sedimentable by centrifugation at 12,000 *g* for 10 min. This procedure, however, could not be used for Ml cell extract because their experimental conditions, incubation with 75 mM KCl and 2 mM $MgCl_2$ at room temperature for 1.5 h, did not cause this cell extract to gel. The failure to gel is explained by the very small content of a high molecular weight gelling factor, an actin-binding protein, in this cell line (data not shown).

Incubation of the crude Ml-cell extract with KCl or $MgCl_2$ followed by centrifugation at 100,000 *g* for 3 h, as compared with the CM-treated Ml and Mml line (macrophage line) extracts, did not concentrate actin in the pellet. This agrees with a previous report in which the ability to sediment [^{35}S]-methionine-labeled actin was compared before and after CM treatment (44). This suggests two possibilities: some defects in the actin molecule so that there can be no polymerization, or the presence of an inhibitor that prevents actin from polymerizing. The actin fraction isolated by DEAE-Sephadex A-50 chromatography was precipitable upon the addition of $MgCl_2$. This suggests the presence of an inhibitor for actin polymerization in the crude cell extract. This also is supported by the normal polymerization pattern of purified actin at 25°C (Figs. 8 and 9). Actually, we have identified a polymerization inhibitor in the extract of untreated Ml and D^- subline cells. This inhibitor, named API, was eluted just in advance of the actin fraction in DEAE-Sephadex chromatography. The API purified by further steps proved to be a 71,000-dalton protein (45).

The actin preparations used in the present experiments were >93% pure. The impurity was due to macroscopically undetectable components and a minor band at 36,000 daltons. This band, which was sometimes absent in the polymerization/depolymerization step and detectable only at the final step, may represent a partly hydrolyzed actin molecule. In some experiments, we extracted from the untreated Ml cells an actin sample with 99% purity that still showed a low polymerization ability. However, as suggested by MacLean-Fletcher and Pollard (36), a possibility that minor contaminants that were undetectable in our SDS PAGE interfered with the polymerization process was not completely excluded.

An excellent method for estimating the intracellular F-actin ratio was devised by Blikstad et al. (1) based on the fact that only G-actin inhibits DNase activity. In this and our previous reports (44), a standard curve of DNase I inhibition was produced by increasing the concentration of G-actin. Using this standard curve, we determined the total actin content in the crude cell extracts in the presence of guanidine-HCl. This value was the same as that determined by densitometry of the SDS PAGE of the [^{35}S]methionine-labeled cellular protein.

The activation of skeletal-muscle HMM Mg^{2+}-ATPase by actin, one of the most important properties of actin to produce energy for cellular functions, was more effective with muscle actin than with Ml-cell actin. This made us wonder whether there is a degeneration of actin during the purification process, which is unlikely because the inhibition of HMM K^+-EDTA-ATPase, another characteristic property of actin, was higher with Ml actin than with muscle actin, although we do not know why.

According to the model of Eisenberg et al. (10) in which the rate-limiting step of ATP hydrolysis occurs before the interaction between HMM and actin, the V_{max} for the actin-HMM-Mg^{2+}-ATPase activation should be constant whatever the source of actin. This was proved right in our present experiments. On the other hand, the specific activity for Mg^{2+}-ATPase activation by Ml-cell actin was about one third that for muscle actin, and CM treatment of the Ml cells increased the specific activity about twofold. These findings are consistent with the changes in K_{app}: that of untreated Ml-cell actin was threefold that of muscle actin, and that of the CM-treated Ml-cell actin was one half that of untreated Ml-cell actin.

In the experiments for HMM Mg^{2+}-ATPase activation, actin was used in the assay after being polymerized in $MgCl_2$. This may be why we found that our value for the K_{app} of skeletal-muscle actin differed from that found by others who used G-actin. In fact, when we used unpolymerized actin, the K_{app} of skeletal-muscle actin was 9.1 μM, almost the same as that reported by Gordon et al. (14). Our experimental conditions exclude the possibility that the different rate and extent of actin polymerization between the control and CM-treated Ml cells indirectly influences the activation of HMM Mg^{2+}-ATPase.

The possibility that rapid depolymerization of Ml F-actin in the assay solution might bring about low activation of Mg^{2+}-ATPase is excluded because of the linearity of the double reciprocal plot (Fig. 6). If the F-actin of the Ml line were depolymerized more rapidly than that of CM-treated cells under the conditions of acto-HMM, the concentration of F-actin would be less than that assumed for low actin concentrations where depolymerization would be favored. Thus, the double reciprocal plot would produce a concave line instead of the straight line.

HMM Mg^{2+}-ATPase activation by Ml-cell actin was extraordinarily low, and treatment with CM which induced differentiation in this cell line produced the specific activity and K_{app} of the normal values for nonmuscle cells reported by Gordon et al. (13, 14). Since Mg^{2+}-ATPase activation must play a central role in energy production for cell motility, the induction of locomotive and phagocytic activities in the Ml cell line probably is correlated with increased activation.

Differentiation of the Ml-cell line also brought about two differences in the polymerization of actin at 25° and 5°C in the presence of 0.1 M KCl. First, the abnormally high critical concentration for polymerization of the Ml-cell actin at 5°C decreased after differentiation to a value close to that of other nonmuscle actins. Second, there were differences in the time courses of polymerization at 25°C in the presence of 0.1 M KCl. When complete polymerization is defined as the point above which viscosity does not increase even after further incubation, the time required to reach complete polymerization of Ml actin is about fourfold that for muscle actin, but it was shortened to twofold after CM treatment. Thus, we concluded that the polymerizability of actin is as follows: skeletal muscle > CM-treated Ml > untreated Ml > D^- subline. This agrees with the results of our previous report (44) in which unpurified actins were used. These differences in polymerizability, however, were undetectable in the presence of $MgCl_2$. The conditions unfavorable for the polymerization of cellular actin, the absence of $MgCl_2$ and a low temperature, may magnify the masked minor differences in actin molecules before and after differentiation.

Whether or not these functional differences of actin are due to any chemical changes in actin molecules was the next problem. Peptide mapping and isoelectric focusing revealed the presence of different peptides and the different β/γ isoactin

ratio before and after the differentiation. It is interesting to note that the density of spot *B2* in the peptide map followed the order, CM-treated Ml > control Ml > D^- subline, which corresponds to the induction of differentiation: differentiated cells, cells that can be induced to differentiate, and uninducible cells.

Sakiyama et al. (52) reported the deletion of γ-actin in mouse fibroblast L cells. According to Leavitt et al. (31), normal T cells synthesize β-actin as the predominant form, but leukemic T cells synthesize almost equal amounts of β- and γ-actin. In contrast, the leukemic Ml cell line synthesizes predominantly β-actin, and the proportion of β- to γ-actin decreased as the differentiation to macrophages was induced. The synthesis of two actin-isomers in a single cell line is regulated depending on the states of cells, leukemic (neither motile nor phagocytic) or nonleukemic (motile and phagocytic). This presents a very attractive problem of the regulation of gene expression coupled to the activation of cytoskeletal structures for cell locomotion.

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