CHANGES IN CONTRACTILE PROTEINS DURING DIFFERENTIATION OF MYELOID LEUKEMIA CELLS

I. Polymerization of Actin

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ABSTRACT

Quantitative and qualitative changes in cellular actin were followed during differentiation of a myeloid leukemia cell line, namely Ml, which was inducible with conditioned medium (CM). During 3 d of incubation with CM, when the Ml cells differentiated to macrophages and lost their mitotic activity, the actin content, F-actin ratio in total actin, and the actin synthesis showed an increase. A greater difference before and after differentiation was found in the ability of G-actin to polymerize. Actin harvested from CM-treated cells showed a greater ability to polymerize, depending on the increased concentration of $MgCl_2$ and/or KCl and proteins, as compared with the actin from untreated Ml cells. Actin harvested from the Mml cell line, a macrophage line, had a particularly high polymerizability with or without CM treatment. In contrast, the actin from the D^- subline, which is insensitive to CM, showed almost no polymerization.

In recent years, the molecular mechanism of cell motility and phagocytosis has been studied with special attention given to the possible involvement of actomyosin in these cellular functions (7, 15, 23, 36, 38). In nonmuscle cells, actin is not always in a filamentous or organized state as seen in muscle cells, but there is a time-to-time conversion between monomeric and filamentous states under a certain control mechanism, depending on the demand from cellular functions; resting, dividing, moving, and phagocytizing. Those nonmuscle cells contain a higher concentration of actins than the critical concentration required for polymerization, but most appear to be prevented from polymerization (4, 42). Profilin, one of the inhibiting factors of actin polymerization, has been isolated from calf spleen (6) and human platelets (31). On the other hand, Bray and Thomas (4), Abramowitz et al. (1), and Gallagher et al. (10) reported that there are two kinds of actin in nonmuscle cells, one can be readily polymerized and the other cannot. For a study of the role of actin in cellular functions, therefore, not only the actin content but also the state of actin has to be taken into consideration.

Most studies have dealt with actively moving cells (8, 11, 37) or with mature phagocytes (13, 14, 41). The process of transition from the immature or a nonmotile state has apparently been documented only by Tilney who used Echinoderm sperm (42, 43).

We have established a myeloid leukemia cell line (Ml) which can be induced to differentiate to macrophages by a variety of differentiation-stimulating (D) factors (17, 19, 20, 29, 33). Before contact with the D factors, Ml cells are morphologically myeloblasts and are neither motile nor

phagocytic. In this cell line, D factors induce locomotory and phagocytic activities (18, 34), the Fc receptor for immunoglobulin (27), elevation of lysosomal enzyme activities (22, 24, 32), the helper function for antibody production (44), and change in cell morphology (19) accompanied by loss of leukemogenicity (20). It has also been demonstrated that cytochalasin B inhibits the manifestation of motility and phagocytosis even in the presence of D factors, and that removal of the drug restores both functions only if the cells have been treated with D factors beforehand (21). If the actomyosin system regulates the locomotory and phagocytic activities in this cell line, then changes should be found in the contractile proteins before and after treatment with D factors.

In the present study, we used the original Ml cell line, the D^- subline resistant to the D factor, and the Mml line which spontaneously differentiated from the Ml line to macrophages. We reported the qualitative and quantitative changes in cellular actin.

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 cultured in our laboratory since 1969 (19). The cells of this line are morphologically myeloblasts and have neither motility nor phagocytic activity. When incubated with conditioned medium, however, the cells differentiate into macrophages and gain motility and phagocytic activity as well as the characteristics of mature macrophages (18–20).

The Mml line was derived from the Ml line and has been maintained in vitro as a macrophage cell line (30). This line shows very active phagocytosis without incubation with conditioned medium.

The D^- line is also a subline of the Ml line (21). With this subline, not even a high concentration of conditioned medium (CM) induces the differentiation seen in the original Ml line cells.

Eagle's minimum essential medium (Nissui Seiyaku Co.) with a double concentration of amino acids and vitamins (EM) was used with 10% heat-inactivated horse serum.

Conditioned Medium (CM)

Secondary mouse embryo cells were seeded at 10^6 cells/6 cm petri dish. The culture fluid was harvested after 5 d and centrifuged at 2,500 rpm for 15 min, after which it was kept at −20°C until use.

Test for Phagocytosis

After incubation with CM for 3 d, the cells were centrifuged and resuspended in serum-free EM containing polystyrene latex particles (1 drop/20 ml of EM, average diameter 1 μm, Dow Chemical Co.) and incubated for another 4 h. The cells were then collected and washed thoroughly. The number of viable cells that phagocytized the particles was counted under a microscope in order to calculate the rate of phagocytosis (Fig. 1 *a* and *b*). Cell viability was checked with 0.25% trypan blue solution.

Test for Cell Motility

Ml cells or the sublines were seeded in agar as reported previously (19, 20). 1.5 ml of CM was poured over 10 day-old colonies, and the colony morphology was checked after 4 d. When motility had been induced, cells began to migrate out of the colony into the agar as shown in Fig. 1 *c* and *d*.

Preparation of Rabbit Skeletal Muscle Actin

Actin was purified from the acetone powder of rabbit skeletal muscle by the method of Spudich and Watt (40). This sample showed a single band on SDS-polyacrylamide gel electrophoresis (Fig. 5).

SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel (10%) electrophoresis in sodium dodecyl sulfate (SDS) was carried out on 5-mm diameter cylindrical gels. Laemmli's method (25) was followed except that *N,N'*-dialyltartardiamide was used instead of methylene-bis-acrylamide to make the sliced gel more soluble in the 2% periodate solution (pH 7.0). Skeletal muscle actin was added to the sample at 2 μg/gel tube for co-electrophoresis. After staining the gel with Coomassie Brilliant Blue R then destaining it in acetic acid-methanol, the actin band was sliced and made soluble by Anker's method (2). It was then used in the test for radioactivity with Bray's scintillation fluid in a Nuclear Chicago counter.

To check the radioactivity of the total protein of the gel, part of the sample was combined with TCA at a final concentration of 10%. The TCA-insoluble radioactivity trapped on a Millipore filter was counted in toluene scintillation fluid.

Two-Dimensional Analysis of Tryptic Peptides

Iodination of the actin band in gel electrophoresis was carried out by Bray and Brownlee's method (3). Briefly, the stained actin band was cut from the gel, then chopped into pieces, and eluted with phosphate buffer containing 0.1% SDS. The protein was precipitated with 0.2 M KCl, and was destained by washing it in acetone after which it was dried in a vacuum. The dry samples were suspended in phosphate buffer containing 0.1% SDS and iodinated with [^{125}I]sodium iodide and chloramine T solution. After oxidation with performic acid, the samples were incubated with TPCK-trypsin for 16 h. Residual radioactive contaminants were removed by Sephadex G25 gel filtration.

Electrophoresis and thin-layer chromatography were carried out according to the method of Sargent and Vadlamudi (39). Two samples of the tryptic digest of the muscle and the Ml cell actin bands were applied to a single thin-layer of silica gel (Chromatogram sheet Type 6061, 20 × 20 cm, Eastman Kodak Co.) so that the origins were in symmetrical positions against the midline of the sheet. The layer was sprayed with a solution of pyridine:acetic acid:water (25:1:225 by volume) and electrophoresed under 300 V for 2 h. After drying, the layer was cut along the midline. Ascending chromatography was carried out at right angles to the electrophoresis with a solvent of butanol:acetic acid:water (3:1:1 by volume) for 2.5 h. These thin layers were air-dried, then autoradiographed.

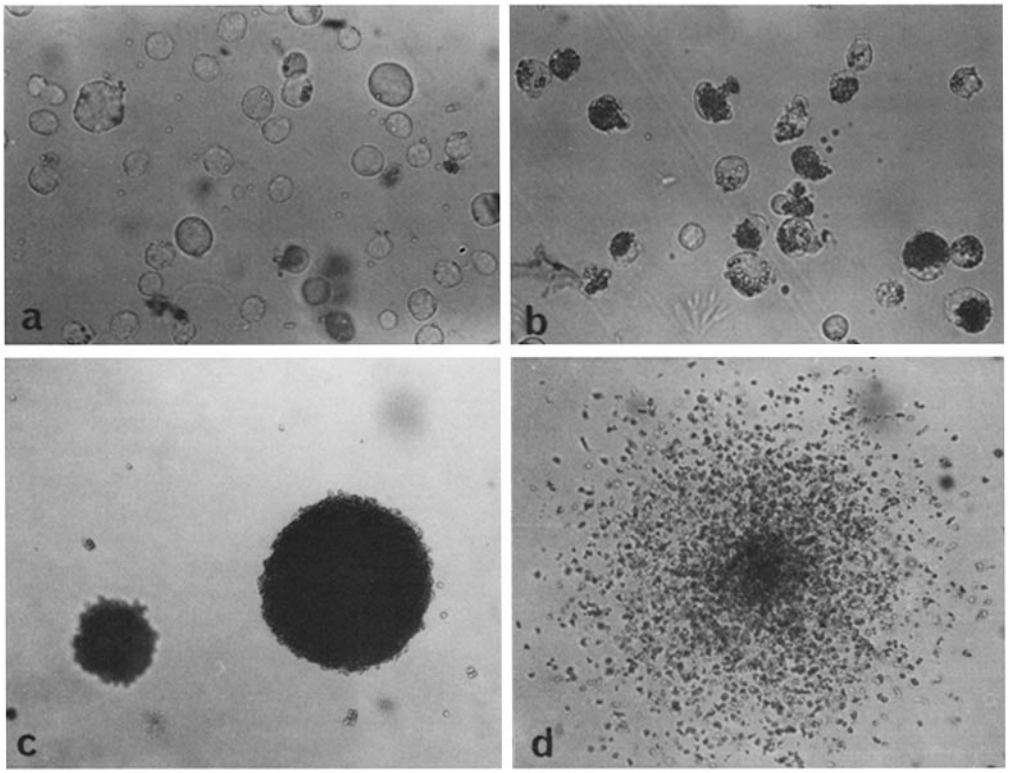

FIGURE 1 Tests for phagocytosis (*a* and *b*) and cell motility (*c* and *d*). The Ml control cells are shown in *a* and *c*. Cells differentiated by incubation with CM for 2 d are shown in *b* and *d*. (*a* and *b*) × 400. (*c* and *d*) × 50.

DNase I Inhibition Test for the Determination of Actin Content

The Ml cell actin was purified by the method of Gordon et al. (11) to give a single band in SDS-polyacrylamide gel electrophoresis (manuscript submitted for publication). Using this actin sample, the inhibition rate of DNase I activity was plotted against increasing actin concentration according to Lindberg's method (26), and the curve was used as a standard dose-response curve for the assay of actin content (Fig. 2). There was no difference in the inhibiting activity of actins from control Ml and that from CM-treated cells (data not shown).

After washing the collected cells three times in cold PBS, the cell suspension was divided into two groups, one for the determination of protein concentration, and another for actin content. The latter was lysed in 5 mM potassium phosphate buffer (pH 7.6), 150 mM NaCl, 2 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM dithiothreitol (DTT), 0.5% Triton X-100, and 0.01 mM phenyl methyl sulfonyl fluoride (lysis buffer). A part of the extract was added to an equal volume of guanidine-HCl buffer (guHCl; 1.5 M guanidine-HCl, 1 M sodium acetate, 1 mM $CaCl_2$, 1 mM ATP, 20 mM Tris-HCl pH 7.5) to depolymerize F actin; thereafter the actin content was calculated from the DNase I inhibition assay (5, 26) using the standard dose-response curve and the amount of total protein added to the assay system.

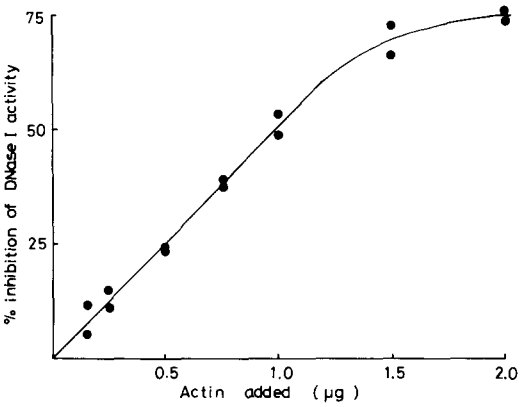

FIGURE 2 Standard dose-response curve of DNase I inhibitory activity of actin. Actin was purified by the method of Gordon et al. (11) from Ml line cells. 15 μl of DNase I enzyme solution (50 μg/ml) was mixed with various amounts of actin, followed by the addition of the substrate solution according to the procedure of Lindberg (26).

F-Actin Ratio

The F-actin ratio was determined in the following two ways. (*a*) By DNase inhibition test. This assay was based on the method of Blikstad et al. (5). The cells were harvested by centrifugation, washed three times with cold PBS, and lysed in the lysis buffer. The amount of G-actin was measured by the DNase I inhibition assay, and then a portion of the lysate was added with an equal volume of guHCl buffer for the determination of total actin. F-actin ratio was calculated from the total actin content from which was subtracted the G-actin content. (*b*) By centrifugation. Cells were incubated with [^{35}S]methionine at 0.5 μCi/ml for 3 d. After washing the labeled cells in cold PBS, the packed cells were resuspended in 0.05 M Tris-HCl buffer, pH 7.0. This cell suspension was homogenized for 3 min in a Potter-Elvehjem homogenizer with a mechanically driven Teflon pestle at 25°C, then centrifuged at 100,000 *g* for 2.5 h at 25°C. The supernate, which was assumed to contain monomeric actin, was used for SDS-polyacrylamide gel electrophoresis, without further treatment. The pellet, which was assumed to contain F-actin, was resuspended in 1 ml of depolymerizing buffer (0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 2 mM Tris-HCl buffer pH 8.0) (40), then sonicated at four cycles of 15 s each after which the mixture was used for SDS-polyacrylamide gel electrophoresis. Radioactivities incorporated into the actin band were examined in the supernate and the sediment (9).

Polymerizability of G-Actin

The [^{35}S]methionine-labeled cells (1 μCi/ml for 12 h) were washed three times in PBS, then resuspended in 2 vol of sucrose buffer (0.34 M sucrose, 10 mM DTT, 0.5 mM ATP, 1 mM EDTA, 20 mM Tris-maleate buffer, pH 7.0) (13). This cell suspension was homogenized for 6 min in an ice-chilled, Potter-Elvehjem homogenizer with a mechanically driven Teflon pestle at 0°C, centrifuged at 100,000 *g* for 1 h at 4°C, and the supernate was used as the crude extract. This extract was incubated at 30°C for 1.5 h under varied concentrations of $MgCl_2$, KCl, and protein, then centrifuged at 100,000 *g* for 2.5 h or 1 h. The precipitate was dissolved in depolymerizing buffer, and the radioactivities in total protein and in actin band were counted as mentioned above. To calculate the ratio of polymerized actin to total actin (Fig. 8*b*), we determined the actin contents not only for precipitate but also for supernate after the polymerization.

The protein concentration in crude extract was measured by the method of Lowry et al. (28). We diluted the cell homogenate with about 100 times the volume of distilled water before the determination. At this dilution, the interfering effects of DTT and EDTA on Lowry's assay were negligible.

Reagents

The reagents used were: ATP (Sigma Chemical Co., St. Louis, Mo.), dithiothreitol (Wako Junyaku Co.), 2-mercaptoethanol (Wako Junyaku Co.), *N-N'*-dialyl-tartardiamide (Eastman Kodak Co., Rochester, N. Y.), 1-[^{35}S]methionine (The Radiochemical Centre, Amersham Corp., Arlington Heights, Ill.; sp act 840 Ci/mmol), [^{125}I]sodium iodide (New England Nuclear, Boston, Mass.; 17 Ci/mg, NEZ-033H), TPCK-trypsin (Worthington Biochemical Corp., Freehold, N. J.).

RESULTS

Quantitative Change in Actin

To compare the rate of actin synthesis before and after the differentiation, we labeled the cells with [^{35}S]methionine in the final 12 h of the 3 d culture with or without 50% CM, when the CM-treated cells had ceased to proliferate (Fig. 3). The cell homogenates were used for SDS-polyacrylamide gel electrophoresis after which the radioactivity incorporated into the band corresponding to the purified rabbit skeletal muscle actin was determined. As shown in Table I, there was a slight increase in the treated cells, but no change in D$^-$ subline which did not differentiate but continued to proliferate despite the presence of CM. The increase in radioactive actin in the CM-

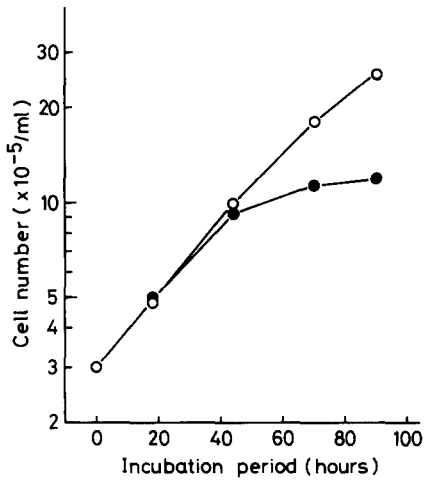

FIGURE 3 Growth curve of Ml cells with or without CM. Ml cells were seeded at 3 × 10^5/ml with (●) or without (○) 25% CM.

TABLE I

Rate of Actin Synthesis

Cell line	Treatment	Phagocytosis	$\frac{\text{Actin}}{\text{Total protein}} \times 100$
		%	
Ml	None	0.5	4.4
Ml	CM 25%	59.7	5.7
D$^-$	None	0	4.4
D$^-$	CM 25%	0	4.3
Mml	None	92.2	8.5

Cultures were labeled with [^{35}S]methionine for the final 12 h in the 3-d incubation with or without CM. Cells were harvested, then homogenized, and used for SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. Rate of actin synthesis was calculated by dividing the disintegrations per minute in the actin band by the disintegrations per minute in the total protein.

exposed cells could represent rapid turnover of actin rather than increase in actin content.

In the next experiment, the cell extracts were applied to the DNase I inhibition test so as to compare the actin contents in steadily proliferating cells and in those undergoing differentiation. As shown in Table II, there was also an increase in the actin content of CM-treated cells. Although the increase in actin/total protein ratio was not so much, the actin content in single cells increased more than two-fold after the treatment with CM, since the cellular protein content also increased.

The content and the synthesis rate of actin in Mml cells which are actively locomotory and phagocytic without exposure to CM, were higher than those of actin in CM-treated Ml cells.

TABLE II

Actin Content

Cell line	Treatment	Phagocytosis	Protein content	Actin / Total protein × 100	Actin content
		%	*mg/10⁸ cells*		*mg/10⁸ cells*
Ml	None	1.5	17.3	4.8	0.83
Ml	CM 50%	53.0	32.5	6.1	1.98
D⁻	None	0	10.5	4.9	0.51
D⁻	CM 50%	0	11.2	4.5	0.50
Mml	None	92.0	32.8	7.8	2.56

Cells were incubated with or without CM for 3 d. After testing for phagocytosis, the actin content in the cell homogenates was assayed by the DNase I inhibition test as described in Materials and Methods. This table represents an average of four experiments.

To identify the nature of the "actin" band from the cell extract and to check for possible contaminants comigrating in the actin band, we radioiodinated the isolated band and then prepared a tryptic peptide map using the two-dimensional method described in Materials and Methods. As the control, the actin purified from rabbit skeletal muscle was processed in the same way. As shown in Fig. 4, radioactive peptides obtained from the Ml cell extract corresponded in shape and mobility to those of rabbit skeletal muscle actin, except for one or two spots. Thus, the band of the Ml cell extract was shown to contain pure cellular actin.

Ratio of F-Actin

Unlike muscle tissue, nonmuscle cells must change the rigidity of their cell peripheries, depending on phagocytosis and cellular movement. This process is probably under the control of contractile proteins (41). Even if the total amount of actin is little increased after treatment with CM, the polymerized actin would occupy a greater part of the total actin in treated cells compared with its content in untreated cells.

As described in Materials and Methods, F-actin content was determined in two ways. First, it was calculated from the difference between G-actin content and total actin content, determined by the DNase I inhibition test before and after the addi-

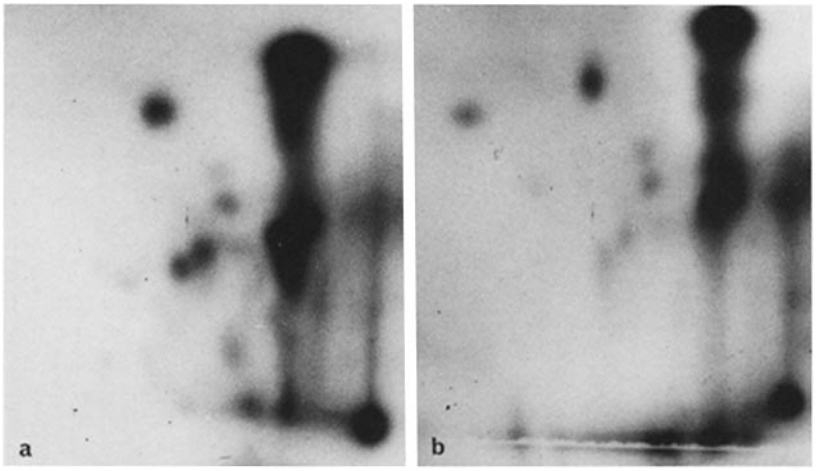

FIGURE 4 Autoradiogram of [^{125}I]-labeled tryptic peptides from (*a*) rabbit skeletal muscle actin and (*b*) presumably actin in the Ml cell extract. Electrophoresis was from right to left, and chromatography from bottom to top.

tion of guHCl, respectively. Second, it was calculated from the actin contents in the supernate and the sediment after centrifugation, both of which were determined by radioactivity to be incorporated into the actin band in the SDS-polyacrylamide gel electrophoresis. While there was no change in D^- subline treated with or without CM, a slight increase was always detected in CM-treated Ml cells (Table III). It should be noted that the results obtained by these two different assays agreed.

Polymerizability of G-Actin

Although there were some increases in the content and synthesis rate of cellular actin and in the ratio of polymerized actin in the CM-treated cells, these increases may not be sufficient to account for the dramatic changes in locomotory and phagocytic activities induced by CM (Fig. 1). This raises the question of whether there is a qualitative change in the characteristics of the actin recovered from CM-treated cells. Thus, we compared the polymerization of G-actin in treated and untreated Ml cells.

Effects of Salt Concentration

Treated or untreated cells were homogenized in 0.34 M sucrose buffer, then centrifuged at 100,000 *g* for 1 h. The supernate was used as the cell extract. The protein concentration of the extract was fixed at 4 mg/ml for both cell extracts to maintain the same actin concentration in both extracts, because the actin contents in these supernates were almost the same for treated and untreated cells: 7.5% of the total protein for untreated, and 7.7% for treated cells. These extracts were incubated at 30°C for 1.5 h with various concentrations of salts as described below, then were centrifuged at 100,000 *g* for 2.5 h, after which the actin content in the precipitates was determined (see Fig. 5).

TABLE III
Ratio of F-Actin

Cell line	Treatment	Percentage F-actin in total actin: By DNase I inhibition	Percentage F-actin in total actin: By centrifugation
Ml	None	48.3	48.3
Ml	CM 25%	62.7	62.3
D^-	None	47.2	41.8
D^-	CM 25%	40.6	48.6

For the DNase I inhibition test, the F-actin was calculated from a difference between G-actin content and total actin content before and after the incubation with guanidine HCl. For the centrifugation test, the cultures were labeled with [^{35}S]methionine for 72 h. After centrifugation at 100,000 *g* for 2.5 h, the supernate and precipitate dissolved in the depolymerizing buffer were electrophoresed as described in Materials and Methods.

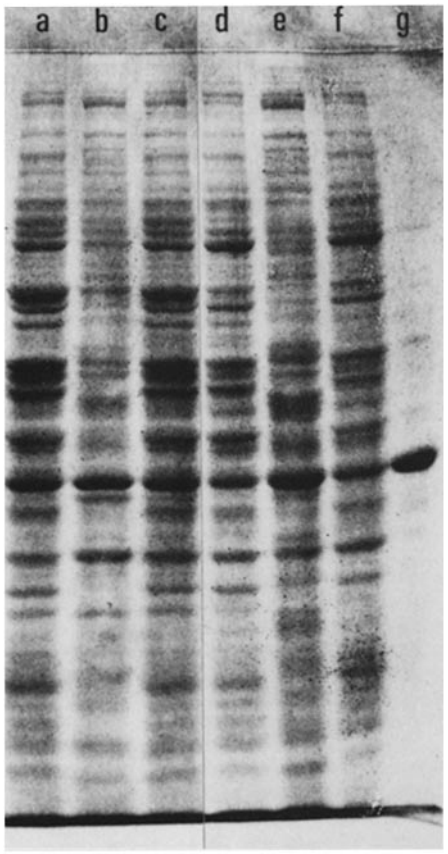

FIGURE 5 Electrophoresis of cell extracts on 10% SDS polyacrylamide slab gels stained with Coomassie Brilliant Blue. The cell homogenate was centrifuged at 100,000 *g* for 1 h at 4°C, and the supernate was used as crude extract. After the protein concentration was adjusted to 4 mg/ml, the extract was incubated with 2 mM $MgCl_2$ at 30°C for 1.5 h, then centrifuged at 100,000 *g* for 2.5 h at 25°C. The precipitate was dissolved in depolymerizing buffer. (*a–c*) Ml control cells. (*d–f*) Ml cells treated with CM. (*a* and *d*) Crude extract. (*b* and *e*) Precipitate. (*c* and *f*) Supernate. (*g*) Rabbit skeletal muscle actin.

EFFECT OF $MgCl_2$: An increasing concentration of $MgCl_2$ produced a linear increase in the polymerized actin in CM-treated cells but not in untreated cells (Fig. 6 *a*). The cell extract from the

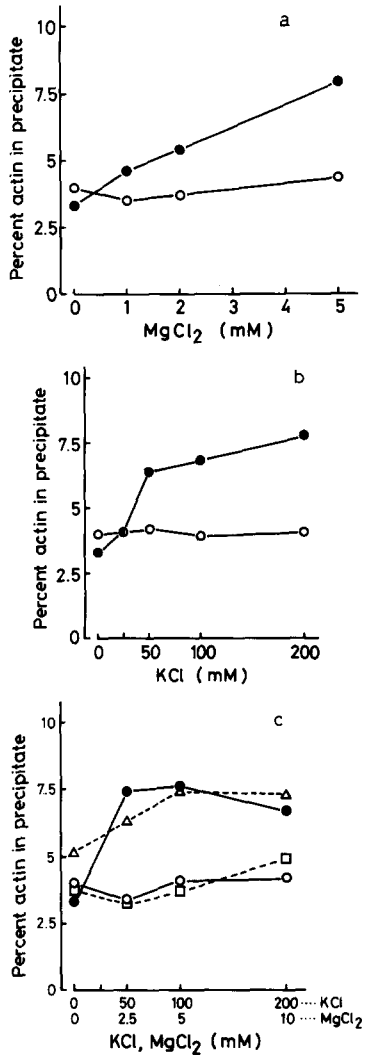

FIGURE 6 Effects of the salt concentration on actin polymerization. In *a*, *b*, and *c*, the protein concentration of the crude cell extract was adjusted to 4 mg/ml with 0.34 M sucrose buffer and combined with salt solution to give the final concentration indicated in the figure. The mixtures were incubated at 30°C for 1.5 h, then centrifuged at 100,000 *g* for 2.5 h at 25°C. The pellet was dissolved in depolymerizing buffer, ultrasonicated, and the mixture was used for electrophoresis. Ordinate: counts in actin band/total counts in the pellets applied to gel electrophoresis. Abscissa: final concentration of salts. CM-treated Ml cells, ●. Untreated Ml cells, ○. Untreated D^- subline, □. Untreated Mml subline, △.

D^- line did not show such an enhancement even after the CM treatment (Fig. 7 *a*).

EFFECT OF KCl: With CM-treated cells, polymerization of actin was enhanced rapidly by an increased concentration of KCl up to 50 mM yet was enhanced slowly at higher concentrations. This enhancement was not observed either in untreated Ml cells (Fig. 6 *b*) or in treated D^- subline cells (Fig. 7 *b*).

COMBINED EFFECT OF $MgCl_2$ AND KCl. As shown in Fig. 6 *c*, 100 mM KCl plus 5 mM $MgCl_2$ had the highest polymerization effect on G-actin from CM-treated Ml cells as well as untreated Mml cells, but this combination had no effect on actin from untreated Ml and D^- subline cells.

EFFECTS OF PROTEIN CONCENTRATION

It is well known that there is a critical concentration of actin below which the actin solution is unable to polymerize even though other conditions are optimal, and if the actin concentration is above the critical point, the polymerization rate will parallel the concentration of actin (11, 35).

To check for possible differences in the critical concentration required for polymerization, we tested untreated and treated Ml cell extracts for actin polymerization in the same way as described above and under the most favorable salt conditions, 100 mM KCl plus 5 mM $MgCl_2$. In this experiment, not only the actin content in the pre-

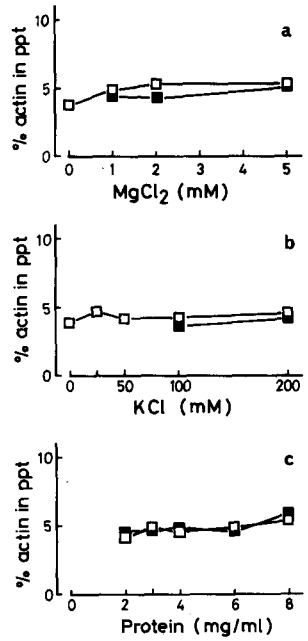

FIGURE 7 Effects of the concentration of $MgCl_2$ (*a*), KCl (*b*), and protein (*c*) on polymerization of the actin harvested from the D^- subline. CM-treated D^-, ■. Untreated D^-, □.

cipitates (Fig. 8 *a*) but also the ratio of precipitated actin to total actin (Fig. 8 *b*) were calculated as described in Materials and Methods. In the untreated cell extract, polymerization was slightly enhanced only when the protein concentration was more than 3 mg/ml. Since the actin content in the cell extract used for polymerization tests was 7.5%, the apparent critical concentration of actin might be calculated to be 0.23 mg/ml. However, in the CM-treated cell extract which contained 7.7% of actin, polymerization began from the starting protein concentration of 1 mg/ml and increased rapidly with an increasing concentration of protein. So, the critical concentration of actin

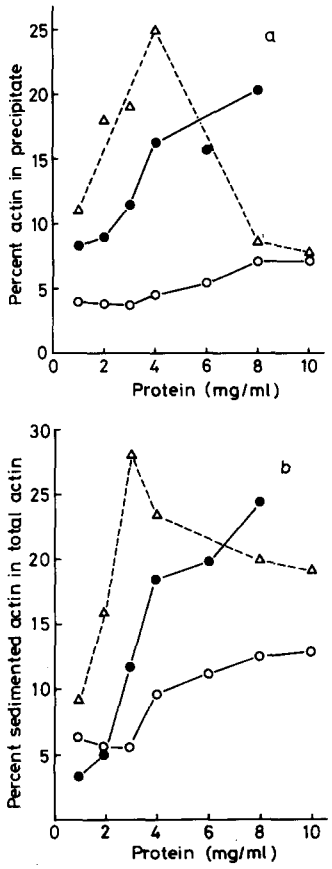

FIGURE 8 Effects of protein concentration on actin polymerization. The final concentrations of KCl and $MgCl_2$ were 100 mM and 5 mM, respectively. Ordinate: (*a*) counts in actin band/total counts in the pellets applied to gel, (*b*) counts in sedimented actin/counts in total actin. Abscissa: final concentration of protein in cell extract used for polymerization tests. CM-treated Ml cells, ●. Untreated Ml cells, ○. Untreated Mml subline, △.

from CM-treated cells might be calculated to be 0.08 mg/ml. This means that the critical concentration of actin required for polymerization is one-third that of original Ml cells by treatment with CM.

The extract from the Mml line showed a peculiar curve (Fig. 8 *a* and *b*). Polymerization was always sensitively enhanced when the concentration of proteins was increased up to 4 mg/ml, but the higher concentration brought about a marked decrease in polymerized actin. This could be reproduced several times.

The results for the D^- subline are shown in Fig. 7 *c*. Polymerization of actin in this subline did not depend on the concentration of protein, regardless of the absence or presence of CM.

In the experiments shown in Figs. 7 *c* and 8 *a* and *b*, polymerized actin was determined by centrifuging the cell extract at 100,000 *g* for 1 h after it had been incubated at 30°C for 1.5 h. Replications in which the extract was centrifuged for 2.5 h gave the same results.

DISCUSSION

Untreated Ml cells proliferate more rapidly than do CM-treated cells, and mitosis and cytokinesis are known to require the involvement of actomyosin filaments. Nevertheless, the actin content in CM-treated cells showed an increase as compared with untreated Ml cells. If radioactivity were added during the final 12 h of the 3 d incubation period when CM-treated cells had lost their mitotic activity in compensation for acquiring motility but untreated cells were still undergoing steady growth, then the synthesis of actin in the CM-treated cells (5.7%) was higher than in the control (4.4%). Although we did not find the great increase reported by Hoffman-Liebermann and Sachs (16), this difference was statistically significant ($P < 0.05$) in experiments repeated five times. It indicates that actin synthesis for cell motility was enhanced as the result of differentiation, although the difference in synthesis was partly masked by the fact that actin was also necessary for cell division.

It is worthwhile to mention that the actin content of differentiated cells is probably underestimated in the present report, because the CM-exposed population always contained undifferentiated cells which escaped the effect of CM treatment.

The Mml line became confluent 3 d after

seeding, and the growth rate slowed down. This was accompanied by a decrease in the production of total cellular protein which resulted in a marked increase in the rate of actin synthesis, the actin probably being utilized for motility and phagocytosis.

The actin recovered in the soluble fraction from CM-treated Ml cells could polymerize with increasing concentrations of $MgCl_2$ and/or KCl, but the actin from untreated Ml cells could not. This inability to polymerize the G-actin in Ml cells probably explains why the Ml line always had a low yield of actin (data not shown) in the purification method by Spudich and Watt (40), in which repeated polymerization-depolymerization is the basic procedure.

The G-actin from Mml cells that do not require contact with CM to maintain motility and phagocytic activity responds sensitively to an increased concentration of salts without CM treatment. In contrast, the actin from the D^- subline could not polymerize even after treatment with CM. These findings suggest that the ability to polymerize actin found in the CM-treated cells is a specific phenomenon related to the induction of motility and phagocytosis in this cell line, and not an unspecific agglutination or binding to membranous material of actin caused by the CM treatment.

The polymerization of actin is also dependent on the concentration of this protein component. Below a critical concentration, the actin cannot polymerize even when the other conditions are satisfactory. According to Gordon et al. (11), who tested changes in the specific viscosity of actin samples from skeletal muscle and *Acanthamoeba*, the critical concentration of actin was 0.07 mg/ml for both muscle and *Acanthamoeba* in the presence of 2 mM $MgCl_2$, but 0.03 mg/ml for muscle and 0.09 mg/ml for *Acanthamoeba* in the presence of 0.1 M KCl at 25°C. They also compared the changes in specific viscosity of actins from skeletal muscle, *Acanthamoeba*, and nonmuscle cells including human platelets, embryonic chick brain, and rat liver, and reached the conclusion that the characteristics of actins from nonmuscle cells were much the same and resembled those of actin from *Acanthamoeba* more than those of actin from skeletal muscle (12). For the cell extract of CM-treated Ml cells, the threshold of the protein concentration for actin polymerization was 1 mg/ml. Since the actin content in this cell extract was 7.7%, the critical contentration of actin was calculated to be 0.08 mg/ml. The critical concentration of actin from untreated Ml cells was calculated to be 0.23 mg/ml. This means that the critical concentration of actin for polymerization was one-third that of original Ml cells, by treatment with CM.

From the results shown in Fig. 7 *c*, we conclude that the actin from D^- cells may not be able to polymerize or that the critical concentration required for polymerization may be extraordinarily high, over 0.44 mg/ml (6 mg/ml × 7.4%). Treatment with CM had no effect on the actin from D^- cells. Increased concentrations of KCl, $MgCl_2$, and protein could not induce polymerization of this actin. This loss of the ability to polymerize is probably related to the insensitivity of this subline to CM.

These findings, however, do not exclude the presence of a regulatory factor that inhibits or promotes the polymerization of cellular actin. In fact, the peculiar behavior of the Mml cell sap shown in Fig. 8 strongly suggests this possibility. Our next step is to check a possible difference in the physicochemical nature of actins before and after the treatment with CM, and to identify a regulatory factor which may interfere with the polymerization of actin.

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