Changes in Actin Content During Induced Myeloid Maturation of Human Promyelocytes

By William H. Meyer and Thomas H. Howard

Actin is an important cytoskeletal protein; new actin synthesis occurs during differentiation of many motile cells. To better understand the process of myeloid maturation, the change in actin content during induced maturation of HL-60 human promyelocytic leukemia cells was studied. HL-60 cells induced toward myeloid maturation by a 5-day exposure to dimethylformamide showed an 86% increase in a 43,000 mol wt protein comigrating with rabbit muscle actin on dodecyl sulfate polyacrylamide gels. To further demonstrate that this was an increase in actin content, the total actin content of lysed HL-60 cells was measured by the ability of actin to inhibit DNAase I. Using this assay, actin content of HL-60 cells increased 96% during induced differentiation. The amount of incorporation of $^4$H-leucine into actin doubled after a 5-day exposure to dimethylformamide, suggesting the increase in actin was due primarily to new synthesis. Total new protein synthesis increased 2–7-fold during differentiation. Additional analysis of polyacrylamide gels showed increased quantities and new synthesis of a high molecular weight protein comigrating with rabbit muscle myosin. This study shows that actin content increases during myeloid maturation. It also demonstrates that the HL-60 cell line is a useful model to study both functional and biochemical events during human myeloid differentiation.

ACTIN IS A ubiquitous protein in eukaryotic cells and has been implicated in most well documented cases of whole cell motility. Actin synthesis occurs during differentiation and maturation of many motile cells. In early differentiating Dictyostelium discoideum, the synthesis of actin rises from 8% to about 25% of total protein synthesis. Muscle-specific actin (α) and myosin synthesis increase at the time of myoblast fusion, an early phase of myoblast differentiation in quail skeletal myoblasts. A mouse myeloid leukemia cell line (M1) also shows an increase in actin content and actin synthesis during induced differentiation. Thus, actin synthesis may be a critical factor in motile cell maturation. To better appreciate the role of actin during human myeloid differentiation, we examined changes in actin content and actin synthesis during induced differentiation of HL-60 cells.

The HL-60 cell line, a human promyelocytic cell line that can be induced toward myeloid maturation by a variety of agents, provides an excellent model to study biochemical changes during myeloid maturation. In its uninduced form, the HL-60 cell is relatively nonmotile. As the cell is induced to differentiate, it morphologically and functionally resembles more mature myeloid forms, becomes motile, and responds to chemotactic peptides. Changes occur in surface glycoproteins and lactate dehydrogenase isoenzyme patterns. This article reports that the actin content of HL-60 cells increases twofold during induced myeloid maturation and that this increase is due primarily to new actin synthesis. In addition, analysis of HL-60 whole cell proteins by dodecyl sulfate polyacrylamide gel electrophoresis demonstrates changes in a number of other proteins, including a significant increase in a 205,000 mol wt protein comigrating with rabbit muscle myosin.

MATERIALS AND METHODS

Cells

HL-60 cells (a gift from Dr. Curt Civin, Johns Hopkins Hospital) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (GIBCO) at 37°C in 5% CO₂ humidified environment. Myeloid maturation was induced by exposing cells at 2–4 x 10⁶ cells/ml to 0.1 M N,N-dimethylformamide (Sigma, St. Louis, MO). Maturation was assessed by light microscopic morphology and nitroblue tetrazolium (NBT) reduction.

Polyacrylamide Gels

Discontinuous sodium dodecyl sulfate polyacrylamide gels (SDSPAGE) (3% stacking, 7% resolving) were run using the method of Laemmli. The buffer system was 0.057 M Tris, 0.192 M glycine, 0.05% SDS, pH 8.3. The cells were disrupted in 0.05 M Tris-HCl, 10% glycerol, 1% SDS, 2% β-mercaptoethanol, 0.001% bromophenol blue (pH 6.8) (sample buffer), and boiled for 5 min. Equal amounts of protein, usually 20 μg, were loaded in the wells. The following proteins were used for molecular weight markers: carbonic anhydrase 29,000, rabbit muscle actin 43,000, bovine serum albumin 66,000, phosphorilase-B 97,400, β-galactosidase 116,000, and rabbit muscle myosin 205,000 (Sigma). Gels were stained with Coomassie brilliant blue. To determine the percent of stainable protein per band, wet gels were scanned at 500 nm on a Schoeffel 3000 D scanning spectrodensitometer. The areas under peaks were measured using a Graf-Pen sonic digitizer programmed to record areas. For experiments designed to measure new actin synthesis and for fluorograms, 100,000 cpm/well were loaded on the gel.

Protease Inhibition

Proteases have been reported to decrease quantities of actin, myosin, and actin-binding protein during solubilization of human...
polymorphonuclear leukocytes. Consequently, cells were pretreated for 5 min at 4°C with 0.002M diisopropylfluorophosphate and then washed twice in ice-cold 0.15M NaCl prior to disruption or lysis.

**Protein Determinations**

Protein concentrations were assayed by the Lowry method as modified by Hartree. Bovine serum albumin was used for standard curves.

**Leucine Incorporation**

Cells were washed twice and then resuspended at 10³ cells/ml in Hanks' buffered salt solution. One milliliter of cells was exposed to 10 μl ³H-leucine (50 Ci/mole, ICN, Irvine, CA) for 30 min at 37°C. One hundred microliters of labeled cells was applied to Whatman filter disks; leucine incorporation was measured by the method of Mans and Novelli. In some experiments, following the leucine incorporation, the cells were washed thoroughly, treated with diisopropylfluorophosphate, and solubilized in sample buffer. Following separation of these labeled proteins by SDS-PAGE, the incorporation of leucine into actin was determined by identifying the band comigrating with rabbit muscle actin, excising this band with a razor blade, extracting the radiolabeled protein with a 9:1 Protocol:water (New England Nuclear, Boston, MA) mixture, and counting the extracted label in Omnifluor (New England Nuclear) dissolved in toluene.

**Fluorography**

The ³H-leucine in polyacrylamide gels was detected by the method of Bonner and Laskey.

**DNAase I Assay**

The DNAase assay, as described by Blikstad et al., was used with some modifications to measure the total actin content of cells. One and one-half milliliters of DNA substrate [40 μg/ml calf thymus DNA (Sigma) dissolved in 0.1M Tris-HCl, 4 mM MgSO₄, 1.8 mM CaCl₂, pH 7.5] was reacted with 10 μl of DNAase I (0.1 mg/ml) for baseline determinations. Standard curves of DNAase I inhibitory activity were constructed using rabbit muscle actin prepared from acetone powder by the method of Spudich and Watt. The incorporation of leucine into actin was determined by identifying the band comigrating with rabbit muscle actin, excising this band with a razor blade, extracting the radiolabeled protein with a 9:1 Protocol:water (New England Nuclear, Boston, MA) mixture, and counting the extracted label in Omnifluor (New England Nuclear) dissolved in toluene.

**RESULTS**

**Actin in HL-60 Cells**

In the uninduced state, about 90% of HL-60 cells were promyelocytes. Wright's-stained cytopsin preparations showed cells with a large nuclear-cytoplasmic ratio and prominent nucleoli. The cytoplasm stained deeply basophilic with prominent azurophilic granules. After a 5-day exposure to dimethylformamide (induced HL-60), the cells became smaller. Typically, 85% of cells were viable by trypan blue staining. The cytoplasm was pink to slightly bluish, and no prominent granules were seen. The nucleus became more dense with a significant proportion of the cells showing multiple lobes. Table 1 indicates the degree of maturation attained after a 5-day exposure to dimethylformamide. The ability of these cells to reduce nitroblue tetrazolium (NBT) to insoluble blue-black formazan was used as a functional assay of myeloid maturation. As Table 1 indicates, the vast majority of the induced HL-60 cells reduced NBT after a 5-day exposure to dimethylformamide. A marked increase in the ability of HL-60 cells to reduce NBT was noted between 48 and 72 hr of exposure to dimethylformamide. At 48 hr, 42% of cells showed NBT reduction; by 72 hr, 75% of cells reduced NBT.

**Polyacrylamide Gels**

There are a number of differences in the electrophoretic patterns of the uninduced versus the induced HL-60 cells. The differences noted by visual inspection of these Coomassie-blue-stained gels are indicated by the letters a–o in Fig. 1. Although most of the changes represent an increase in the amount of protein after differentiation, some decreases were observed (band d—mol wt about 96k; band h—mol wt about 55k; band m—mol wt about 29.5k; band o—mol wt about 17k).

The band comigrating with rabbit muscle actin (band k) showed a significant increase from uninduced HL-60 cells to HL-60 cells exposed to dimethylformamide for 5 days. Densitometer scans of gels confirmed the increase in the 43,000 mol wt band comigrating with rabbit muscle actin from 4.11% ± 0.91% (uninduced HL-60 cells) to 7.64% ± 0.56% (induced HL-60 cells) of total stainable protein. Although this band almost certainly represents HL-60 cell actin, it is possible that other proteins besides actin contribute to this band. However, it is unlikely that proteolytic
fragments accounted for this change, because no higher molecular weight band decreased in intensity to the degree that band k increased, and because all cells were pretreated with diisopropylfluorophosphate.

Of additional interest was the dramatic increase in band a, a 205,000 mol wt protein comigrating with rabbit muscle myosin. On gels of uninduced HL-60 cells, this protein was virtually undetectable or very faintly visible (0.01\% ± 0.02\% stainable protein). However, following a 5-day exposure to dimethylformamide, this protein increased to 0.28\% ± 0.22\% of all stainable protein. Using a molecular weight of 205,000 for band a and 43,000 for band k (actin), the molar ratio of bands k:a is 130 in the induced cells.

The HL-60 cells induced to mature with dimethylformamide are smaller than uninduced HL-60 cells and contain less protein per cell. Using hemocytometer cell counts, we estimated that uninduced HL-60 cells contained about 90 pg protein per cell. Dimethylformamide-induced HL-60 cells contained about one-half this amount of protein per cell. Gels of uninduced and induced HL-60 cells, equalized for cell number rather than cell protein, showed similar intensity of the actin band; however, in this instance, the lanes of uninduced HL-60 cells contained greater overall staining, as would be expected. Scans of these polyacrylamide gels continued to show a relative increase of band k, the

43,000 mol wt protein that comigrates with rabbit muscle actin.

**DNAase I Assay**

The DNAase I assay for actin\(^5\) was used to verify that the increase in band k on polyacrylamide gels represented an increase in HL-60 cell actin. A typical standard curve for actin inhibition of DNAase I is shown in Fig. 2. This standard curve indicates that purified actin had 105,660 U/mg of inhibitory activity.

---

**Fig. 1.** SDS-polyacrylamide gel separation of HL-60 whole cell proteins after disruption in 10\% glycerol, 1\% SDS, 2\% \(\beta\)-mercaptoethanol. Gels were stained with Coomassie blue and destained in methanol-acetic acid. Lane 1: molecular weight markers: rabbit muscle myosin 205k, \(\beta\)-galactosidase 116k, phosphorylase 97.4k, bovine serum albumin 66k, rabbit muscle actin 43k, carbonic anhydrase 29k. Lanes 2,3: uninduced HL-60 cells. Lanes 4,5: HL-60 cells exposed to dimethylformamide for 5 days; 20µg protein was placed on each well. Letters indicate bands that change in staining intensity.

---

**Fig. 2.** One microgram DNAase is reacted with 1.5 ml DNA substrate (40 µg/ml) at 30\( ^\circ\)C, with increasing amounts of actin. Each point is the mean of quadruplicate determinations. Change in absorbance per minute is recorded at 260 nm. The line is extrapolated to the x-axis, equaling that amount of actin that 100\% inhibits 1 µg DNAase. x-intercept = 0.946 equivalent to 105,660 U of inhibitory activity/mg protein.
ty, a value similar to previously published levels.\textsuperscript{15} To measure actin in cell lysates, inhibitory activity was read directly from a standard curve constructed with 10 \( \mu l \) of the guanidine HCl solution added.\textsuperscript{16}

Using the DNAase assay, HL-60 cells induced to mature with dimethylformamide contained about twice as much actin as the uninduced HL-60 cells. As Table 2 indicates, uninduced HL-60 cells contained 5.3 \( \mu g \) actin/100 \( \mu g \) cell protein. After induction of maturation with dimethylformamide, these cells contained 10.3 \( \mu g \) actin/100 \( \mu g \) cell protein. This twofold change in actin content was consistently reproducible, as noted in Table 2.

**New Protein Synthesis**

\( ^3H \)-leucine pulse labeling of HL-60 cells was used to determine the rate of new protein synthesis. In some experiments, labeled cells were pipetted onto filter disks, and the trichloroacetic acid precipitable radioactivity was determined. In other experiments, labeled cells were solubilized in sample buffer in preparation for gel electrophoresis; a volume of these solubilized labeled cells was counted. Both methods for determination of new protein synthesis gave similar results.

Incorporation of \( ^3H \)-leucine during HL-60 cell differentiation was from two to over seven times greater than control (uninduced HL-60 cell) levels. There was considerable variability from one experimental trial to the next; however, all trials showed similar patterns of \( ^3H \)-leucine incorporation. Figure 3 shows a single representative experiment. There was increased incorporation of leucine occurring within the first 24 hr of exposure to dimethylformamide (DMF). A second peak of radiolabel incorporation occurred after 4 days. Uninduced HL-60 cells showed no change in incorporation of leucine as cell density increased.

Labeled solubilized cell proteins were separated by SDS-PAGE and the radioactivity in the actin band (the 43,000 mol wt protein comigrating with rabbit

![Fig. 3. Uptake of \(^3H\)-leucine by HL-60 cells exposed to dimethylformamide for various times. After indicated exposure to dimethylformamide (DMF), cells were exposed to \(^3H\)-leucine for 30 min, washed, and solubilized in sample buffer at 400 \( \mu g \) ml protein. The radiolabel in this solubilized protein was counted after addition of Protosol. Brackets indicate 1 SD.](image)

| Table 2. Actin Content of Induced and Uninduced HL-60 Lysates Using the DNAase I Assay |
|-------------------------------|-------------------|-----------------|-----------------|-----------------|
| Cells                         | Total Cell Protein (\( \mu g/100 \mu l \) Lysate) | Total Cell Actin (\( \mu g/100 \mu l \) Lysate) | \( \mu g \) Actin per 100 \( \mu g \) Protein | Mean |
| Uninduced HL-60               | 4.3              | 6.6             | 5.3             |
| Induced HL-60                 | 10.3             | 10.3            | 10.3            |

Induced HL-60 cells are cells exposed to 0.12 \( M \) dimethylformamide for 5 days. Cellular actin in lysates is measured by adding 10 \( \mu l \) 1.5 \( M \) GuHCl solution to 10 \( \mu l \) of cell lysate and using solution to inhibit 1 \( \mu g \) of DNAase I. The rate of reaction is read directly from standard curves of actin inhibition of DNAase I, with 10 \( \mu l \) GuHCl.
muscle actin) was determined. Figure 4 shows the results of a representative trial for leucine incorporation into the actin band. In the experiment shown, 1.73% ± 0.16% of the total cpm placed on the gel is found in the actin band of uninduced HL-60 cells. After a 5-day exposure to DMF, 3.22% ± 0.05% of the total cpm is found in the actin band. Although some variability is noted in the shape of this curve, particularly at the points for 48- and 72-hr exposure to DMF, multiple experiments (n = 5) have consistently shown a doubling of the percent cpm in the actin band in day 5 induced HL-60 cells relative to uninduced HL-60 cells, indicating a twofold increase in the rate of actin synthesis.

Fluorograms of these gels confirm the above data. The intensity of the band comigrating with rabbit muscle actin (43 k) increased following exposure of HL-60 cells to dimethylformamide, as seen in Fig. 5. This figure also shows increasing radiolabel present in the band comigrating with rabbit muscle myosin (205 k) following exposure to DMF. Increasing amounts of radiolabel are also present in a number of other unidentified high molecular weight proteins.

DISCUSSION

The HL-60 cell line has been used extensively as a model for human myeloid maturation. Morphologically, this cell can be induced to differentiate from a promyelocyte toward mature myeloid forms, including bands and segmented cells. During this differentiation, the cells acquire many functional characteristics of mature myeloid forms, including bacterial ingestion and killing, increased generation of superoxide radicals, increased reduction of nitroblue tetrazolium, and increased chemotaxis. Increases in motility (including chemotaxis) of HL-60 cells may be due to multiple factors, including changes in actin quantity and composition, changes in actin-associated proteins, changes in membrane glycoproteins, and changes in chemotactic peptide receptors. Although multiple changes probably occur, increases in actin content during maturation and differentiation have been a prominent characteristic of motile cell maturation of other cell types.

These data show an increase in actin content during myeloid maturation of HL-60 cells. After 5 days of exposure to dimethylformamide, HL-60 cells morphologically and functionally mature to later myeloid forms, as has been previously reported. At the end of this 5-day exposure, induced HL-60 cells contain...
about twice as much actin per milligram cell protein as do uninduced HL-60 cells using two distinctly different assay techniques. The actin content of induced HL-60 cells, using other inducers of myeloid maturation, has not been investigated.

Scanning polyacrylamide gels is an accurate method for quantification of cellular actin, if care is taken to prevent proteolysis. Likewise, if the DNAase I assay is performed under carefully controlled conditions, it provides a useful method for the measurement of total cellular actin. That these two methods give similar estimates of total cellular actin [5.25 μg% (DNAase I assay) versus 4.11% (gels)] in uninduced HL-60 cells and a similar magnitude of increase in cell actin in induced HL-60 cells (96% increase in actin, DNAase I assay, versus 86% increase in actin, gels) shows that actin content per milligram cell protein increases during myeloid maturation of HL-60 cells.

The increase in total cellular actin in HL-60 cells during myeloid maturation, expressed per milligram total cellular protein, raised a number of important questions. Of particular interest was whether the increase in actin was due to new actin synthesis. The data presented show that HL-60 cells synthesize new protein during myeloid differentiation. Although this work tends to confirm Fontana et al.'s observations, the time course of labeled leucine incorporation and the absolute rate of leucine incorporation are dissimilar. Our data show that increased total protein synthesis occurs within the first 24 hr of exposure to DMF (earlier than Fontana et al. noted), and a second peak of protein synthesis occurs after a 4-day exposure to DMF, not previously seen. The significance of this pattern of protein synthesis is unknown. In addition, it is unclear why we are seeing a somewhat different pattern of leucine incorporation than has been reported previously.

In addition, the rate of leucine incorporation into actin, relative to leucine incorporation into total protein, doubles in day-5 induced HL-60 cells. This doubling of the relative rate of leucine incorporation into actin may primarily account for the twofold increase in total actin content in differentiating HL-60 cells. The time course of leucine incorporation into actin is of interest. Fontana et al. showed in differentiating HL-60 cells that the development of adherent cells with oriented shape, leading front migration, and 1H-f-met-leu-phe binding all were at about control levels following a 2-day exposure to dimethylformamide, but then showed marked increases at 4- and 6-day exposures, correlating well with our observations of leucine incorporation into actin. This is of interest because actin may play a crucial role in some of these motility-related events.

Finally, analysis of polyacrylamide gels has demonstrated a large number of other changes in protein composition of induced HL-60 cells. Of particular interest is the marked increase in the 205,000 mol wt protein (band a) comigrating with rabbit muscle myosin. Fluorograms suggest significant increases in the rate of leucine incorporation into this high molecular weight protein. To date, we have not identified this protein; however, two findings suggest that this protein may be HL-60 cell myosin. First, this protein comigrates with rabbit muscle myosin and has the same approximate molecular weight as myosin from macrophages, fibroblasts, platelets, and granulocytes. Second, the molar ratio of actin to this protein is about 130, well within the range of molar ratios of actin to myosin in other myeloid cells.

The HL-60 promyelocytic leukemia cell line has proved to be an excellent model for the study of myeloid differentiation. This study suggests that the HL-60 cell model will be useful in relating functional and biochemical events during human myeloid maturation.

ACKNOWLEDGMENT

The author is grateful to Dr. Robert Bloodgood for assistance with fluorography and discussions, to Shirley Hensel for technical assistance, and to Alice Buchanan for preparation of the manuscript.

REFERENCES

9. Pantazis P, Lazarou SA, Papadopoulos NM: Isoenzymes of
Changes in actin content during induced myeloid maturation of human promyelocytes

WH Meyer and TH Howard