ABSTRACT

Transformation is associated with profound structural and quantitative changes in the cytoskeleton. Herein we report studies using F-actin, a major cytoskeletal protein, as a quantitative marker for transformation cells, focusing on separating the effects of the cell cycle, cell differentiation, and transformation. The model system for these studies consisted of three lymphoblastic cell lines, one untransformed line (RPMI) and two transformed lines, one (HL-60) of which can be induced to differentiate and the other (Daudi) which cannot. The relation of F-actin levels to cell cycle was studied by flow cytometry with the use of fluorescein-phallolidin to label F-actin and propidium iodide to label DNA. F-Actin levels in transformed Daudi and HL-60 lines were only two-thirds that of the untransformed RPMI cells. Histograms of the distribution of F-actin showed that the transformed lines consisted of two cell populations, one having an F-actin content near that of untransformed cells and the other having much less. Cell cycle analysis showed that F-actin in untransformed cells increased 10-15% as cells entered the S compartment, remaining approximately constant through G2 and M phases of the cell cycle, but in transformed cells the major increase in F-actin occurred during G2 + M phase. Double-label studies with rhodamine-phalloidin for F-actin and Ki-67 monoclonal antibody for dividing cells (cells at late G2, S, G1, and M) measured with quantitative fluorescence image analysis showed that the mean F-actin content of dividing cells was twice that of nondividing cells. These results suggested that most of the cell division-related F-actin increase occurred during late G1 phase in untransformed cells. Differentiation of HL-60 cells with dimethyl sulfoxide or retinoic acid normalized the F-actin content of the nondividing cell population, but dimethyl sulfoxide and retinoic acid produced no detectable change in F-actin in the undifferentiable Daudi cells. A tumor promoter (12-0-tetradecanoylphorbol-13-acetate) inhibits differentiation of hematopoietic cells, resulted in a 32% decrease in the mean F-actin content of RPMI cells due to the appearance of a new subpopulation of low F-actin content. The 12-O-tetradecanoylphorbol-13-acetate-induced changes reversed slowly after removal of 12-O-tetradecanoylphorbol-13-acetate but more rapidly in the presence of retinoic acid. These results indicate that F-actin can serve as a marker for cellular transformation and provides a tool for studying the mechanisms of cellular differentiation that may lead to a better understanding of the oncogenic process.

INTRODUCTION

Actin, one of the major components of the cytoskeleton, is a ubiquitous structural protein in eukaryotic cells. It is important in a variety of cell functions, including regulation of cell shape, motility, secretion, intracellular transport, endocytosis and exocytosis, and cell division (1, 2). Actin exists in the cytoplasm as either G-actin monomers, or as reversibly assembled polymeric F-actin filaments. Fluorescence microscopy, with fluorescein-labeled phalloidin, a highly specific probe for F-actin (3), has established that transformed cells, most commonly fibroblasts, showed profoundly altered patterns of actin filaments (3–8), suggesting that quantitative measurements of F-actin levels might serve as a useful biological marker for transformation. Actin levels may reflect cancer-related changes at a molecular level through several mechanisms: a point mutation in an actin gene (9–11), altered regulation of F-actin assembly by pp60c-src interaction with phosphorylated vinculin (12–16), alterations in the actin polymerization process (17, 18), or alterations under the control of other oncogenes (19, 20). For example, microinjection of a protein with a molecular weight of 21,000, the gene product of c-Ha-ras oncogene, altered the amount and pattern of F-actin in NIH 3T3 cells in a dose-dependent way (19). In addition, alterations of the cytoskeleton induced by tumor promoters appear to facilitate the expression of DNA-damaging tumor-initiating events (7, 20–21), suggesting a relationship between cytoskeleton and cellular mechanisms of growth inhibition.

The studies reported here investigate the use of cellular F-actin levels as a quantitative marker for cellular transformation in relation to cell differentiation. While conventional biochemical studies of F-actin levels in cells yield a mean content per cell, no information concerning cell population is obtained, and in order to obtain such information, flow cytometry and QFIA3 cytology were used in this study. To assure accurate measurement of F-actin, optimal fixation conditions were identified for the simultaneous measurement of DNA and F-actin. The effects of cell cycle and cell division of F-actin levels were determined. A model of cellular differentiation, the HL-60 cell, which reversibly assumes a differentiated or undifferentiated phenotype (17, 22–26), was characterized concerning the relationship between F-actin and cellular differentiation. Finally, the effects of tumor promoters such as TPA on F-actin levels were investigated with the untransformed cell line. These studies were carried out in hematopoietic cells, which neither form stress fibers nor attach to a substrate, in order to eliminate differences in adhesion between transformed and untransformed cells as factor.

MATERIALS AND METHODS

Materials. Fluorescein-labeled phalloidin was obtained from Molecular Probes, Inc., Eugene, OR, and RNase, DMSO, RA, and TPA were obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions

2 The abbreviations used are: QFIA, quantitative fluorescence image analysis; TPA, 12-O-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide; RA, retinoic acid; PBS, phosphate-buffered saline; PF, paraformaldehyde; MCN, mean channel number; TAS, texture analyzing system; PI, proliferation index.

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2 To whom requests for reprints should be addressed, at Department of Urology, Oklahoma University Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190.
were prepared to contain 2.0 mg/ml propidium iodide in 1.12% (w/v) sodium citrate, RA freshly dissolved in absolute ethanol (23), and 5.0 mg/ml TPA in DMSO (20). The monoclonal mouse antibody to proliferation antigen Ki-67 was from DAKO Corp., Santa Barbara, CA, and biotinylated goat anti-mouse IgG and streptavidin-fluorescein were from Bethesda Research Laboratories, Gaithersburg, MD.

Cells and Cell Culture. Untransformed lymphoblasts (RPMI 7666, ATCC 114-CCL), transformed lymphoblasts (Daudi, ATCC 213-CCL), and promyelocytic leukemia cells (HL-60, ATCC 240) were all obtained from American Tissue Culture Collection, Rockville, MD. The RPMI and Daudi cells were maintained in RPMI 1640 medium at 37°C in a humidified atmosphere of 5% CO2 in air supplemented with 10% fetal bovine serum, while HL-60 cells were supplemented with 20% heat-inactivated fetal bovine serum. Stock cultures were maintained in exponential growth by subculturing every 4 to 6 days. The cells were grown at 1 to 2 × 10^6/ml in 25-cm² plastic culture flasks before addition of one of the agents. Cells were treated with DMSO or RA as described in Tables 4-6. Cells were counted with a hemocytometer, and viability was determined by trypan blue exclusion.

Fixation and Staining Samples for Flow Cytometry. Cultured cells were harvested, diluted to 10⁶ cells/ml, washed by two cycles of differential centrifugation at 500 × g, and the final pellet was resuspended in 0.5 ml PBS to which was added either 3.5 ml PBS, or 0.5 ml 4, 8, or 16% PF and 3 ml PBS to give final concentrations of 0, 0.5, 1, 2, and 4% PF. After 15 min, cells were washed as above and the cell membranes were permeabilized in 5 ml of either 25, 50, 75, or 95% ethanol for at least 2 h. Fixed cells were stable for at least 2 weeks at 4°C.

For double labeling of F-actin and DNA, a two-step incubation was used. Cells were pelleted at 500 × g for 5 min and resuspended in 0.5 ml PBS with the addition of 50 μl of fluorescein phalloidin. The cells were equilibrated for 30 min at room temperature in the dark. Cells, were pelleted and treated with 1 ml of 10 mg/ml RNase for 30 min, followed by 10 μl of propidium iodide stock for 30 min. Cells were kept on ice in the dark until analysis by flow cytometry.

Double Labeling of F-Actin and Ki-67 on Slides. Cells were harvested and fixed in 2% PF as described above. The cells were counted with a hemocytometer, and 250,000 cells were spun onto special slides (Code-On slides; Fisher Scientific) using a Cytospin (Shandon; Cytospin 2) at 500 rpm for 10 min. Cells on the slides were then fixed with acetone (cold, −20°C or room temperature) for 3 to 5 min. Drying was prevented. After washing with a special buffer (automation buffer, Fisher Scientific, Code-On series), slides were treated in a computer-controlled immunology slide stainer (BM30; Fisher Scientific), using the following sequence: incubation with Ki-67 at a 1/10 dilution for 30 min, three wash cycles, incubation with biotinylated goat anti-mouse IgG at a 1/20 dilution for 30 min, three wash cycles, incubation with fluorescein-streptavidin at a 1/100 dilution for 30 min, three wash cycles, and a final incubation with rhodamine-phalloidin at a dilution of 1/10, followed by a final three wash cycles.

Quantitation of F-Actin Fluorescence Intensity by QFIA. Cells were labeled for F-actin and Ki-67 protein as described above, mounted with nonfluorescent mounting medium (Aqua-Mount; Lerner Lab., Stamford, CT), and scanned with the TAS using excitation with the Leitz N2 filter block (Wild Leitz USA, Inc., Rockleigh, NJ). The excitation wavelength was isolated by using a high-performance interference short pass filter of 530–560 nm. The dichroic beam-splitting mirror was a reflection short pass filter of 580 nm, and the emission was isolated with a long pass 580 nm suppression filter. The TAS scans the slide in a raster pattern, locates fluorescent objects, applies image analysis algorithms to identify the cells, and measures F-actin fluorescence intensity. The scan was halted after detecting 40 objects that meet specific image criteria for cells. After completion of the scan, the TAS returned to each object and Ki-67 was visually scored positive or negative after switching the filters to an “12” emission filter block with a bandwidth of 450–490 nm, dichroic beam-splitting mirror to an RKP510, and the emission filter to an LP515. The F-actin intensities and Ki-67 status were determined for approximately 100 cells/slide. The intensities were normalized to that of a fluorescent phosphor particle (NBS 1022). This normalization process converts the intensities to phosphor particle units, an absolute, although arbitrary, unit of fluorescence.

Flow Cytometric Analysis. An Epics 541 with a single 5 W argon laser by Coulter Electronics Corp. of Hialeah, FL, was used to measure simultaneously DNA and F-actin. Optics were aligned by using DNA beads purchased from Coulter Immunology, Inc. The green signal was isolated with a 530 nm short pass filter, and the red signal with a 570 nm long interference filter. A 550 nm dichroic mirror was used to split the signal. Excitation was with the blue (488 nm) laser beam operating at 200 mW. The cells, double labeled with fluorescein-phalloidin and propidium iodide, were analyzed at a flow rate of 2000 to 4000 cells/s.

The output voltage pulse amplification was adjusted such that the mode of the G0 population of cells from an untransformed cell line fell into channel 40. Compensation of color cross-over was achieved by adjusting the red (propidium iodide) or green (fluorescein) compensation voltage to align the G0 population to channel 40. Immediately thereafter the other samples were processed.

The flow cytometry data were analyzed with the Easy 88 system and GATEWAY software. In order to diminish the influence of cell size and debris on F-actin quantification, a unique data analysis program was developed by setting a gate on the histogram of 90-degree light scattering (granularity) versus forward angle light scattering (cell size).

Another gate was set on the histogram of green (F-actin) versus red (DNA). All the subsequent analyses were based on that specific area. The MCN has been used to represent the F-actin level of a particular cellular population.

RESULTS

Determination of Optimal Fixation Condition. Fig. 1 illustrates the effect of different fixation conditions on the measurement of F-actin in RPMI cells. Similar data (not shown) were also obtained from transformed cells. Optimal values of F-actin (MCN) were achieved with at least 0.5% PF following fixation with 25–50% ethanol, or with 25% ethanol alone. Higher concentrations of ethanol led to a progressive loss in the F-actin-labeled phalloidin signal.

Fig. 2 illustrates the effect of ethanol and PF fixation on measurement of DNA content. PF fixation severely degraded DNA histograms, regardless of the ethanol concentration. The coefficient of variation of the G0 + G1 peak increased from 4.5–7% in the absence of PF to 17–28% with 4% PF, with a dramatic change in the histogram shape. Therefore we chose overnight fixation in 25% ethanol alone as the optimal fixation condition for both DNA and F-actin.

F-Actin in Untransformed and Transformed Cell Lines. Table 1 shows the mean actin content per cell in each of the three cell lines.
cell lines, the actin content of G2 + M cells was significantly higher (P < 0.05) than that of G0 + G1 or S phase cells. In both transformed cell lines (Daudi and HL-60) contained about one-third less F-actin than did the untransformed RPMI cells. Those differences are highly significant (P < 0.005).

The two transformed lines (Daudi and HL-60) contained about one-third less F-actin than did the untransformed RPMI cells. Those differences are highly significant (P < 0.005). Unlike the other two cell lines, histograms of F-actin content of HL-60 cells usually display two peaks. One peak is comprised of about 45 to 60% of the cells and peaks near channel 70, while the second peak, which consists of the remaining cells, peaks near channel 155.

F-Actin Changes during Cell Division. Dual-label flow cytometry for DNA and actin with windows set according to the DNA histogram, and data were reanalyzed by flow cytometry. The response of F-actin to RA is dose dependent between 0.01 and 1.0 μM. At 4 μM RA, the F-actin value decreased to 151 ± 3.8, possibly reflecting toxicity. As shown in Fig. 3 the percentage of cells in the low F-actin population decreased significantly from 36 ± 2.5 in control cells to 10 ± 4.3 in 1.25% DMSO-treated cells and 12 ± 2.4 in 1.0 μM RA-treated cells, values that are similar to those observed with RPMI cells.

Correlation of F-Actin with HL-60 Cell Differentiation. Table 4 shows the effect on mean F-actin content of a 6-day incubation of HL-60 cells with various concentrations of the differentiation-inducing agents DMSO and RA. The F-actin content increased from the characteristic control level of MCN = 118 ± 7.2 to 151 ± 1.3 in cells treated with 1.25% DMSO and to 166 ± 6.3 in cells treated with 1.0 μM RA (P < 0.02). These values are similar to the levels observed in untransformed RPMI cells. The response of F-actin to RA is dose dependent between 0.1 and 1.0 μM. At 4 μM RA, the F-actin value decreased to 151 ± 3.8, possibly reflecting toxicity. As shown in Fig. 3 the percentage of cells in the low F-actin population decreased significantly from 36 ± 2.5 in control cells to 10 ± 4.3 in 1.25% DMSO-treated cells and 12 ± 2.4 in 1.0 μM RA-treated cells, values that are similar to those observed with RPMI cells.

**Table 1** Mean F-actin content of untransformed and transformed cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>F-Actin (MCN)</th>
<th>P versus RPMI</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>162 ± 7.4</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Daudi</td>
<td>106 ± 2.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HL-60</td>
<td>117 ± 6.5</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

*Mean ± SD of five independent experiments.

**Table 2** Mean ± SD (4 independent experiments) F-actin content of cells in different compartments of the cell cycle

<table>
<thead>
<tr>
<th>Cell line</th>
<th>G0 + G1</th>
<th>S</th>
<th>G2 + M</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>150 ± 4.5</td>
<td>170 ± 3.7</td>
<td>173 ± 3.4 ±0.27</td>
</tr>
<tr>
<td>RPMI + 0.5 μg/ml TPA</td>
<td>113 ± 2.7</td>
<td>117 ± 1.7</td>
<td>138 ± 6.1*&lt;0.01 vs. G0 + G1</td>
</tr>
<tr>
<td>Daudi</td>
<td>98 ± 2.5</td>
<td>109 ± 4.0</td>
<td>120 ± 3.7°&lt;0.01 vs. G0 + G1</td>
</tr>
<tr>
<td>HL-60</td>
<td>110 ± 4.7</td>
<td>115 ± 3.2</td>
<td>126 ± 3.8°&lt;0.01 vs. G0 + G1</td>
</tr>
<tr>
<td>HL-60 + 1 μM RA</td>
<td>135 ± 3.7</td>
<td>150 ± 3.6</td>
<td>172 ± 2.6*&lt;0.01 vs. G0 + G1</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with G0 + G1 phase cells.

**Table 3** F-Actin levels (mean ± SD) measured by QFIA in dividing (KI-67 positive) and nondividing (KI-67 negative) cells measured by QFIA

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Whole population</th>
<th>KI-67 positive</th>
<th>KI-67 negative</th>
<th>% of positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>F-Actin*</td>
<td>0.80 ± 0.43</td>
<td>1.00 ± 0.58</td>
<td>0.52 ± 0.37*</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>155</td>
<td>141</td>
<td>130</td>
</tr>
<tr>
<td>Daudi</td>
<td>F-Actin*</td>
<td>0.27 ± 0.10*</td>
<td>0.48 ± 0.26*</td>
<td>0.29 ± 0.16*</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>66</td>
<td>41</td>
<td>68</td>
</tr>
</tbody>
</table>

*Mean ± SD of F-actin fluorescence intensity (phosphor particle units) measured by QFIA based on 3 independent experiments.

*P < 0.01 compared with KI-67 positive.

*N, number of cells measured.

Table 4 shows the effect on mean F-actin content of a 6-day incubation of HL-60 cells with various concentrations of the differentiation-inducing agents DMSO and RA. The F-actin content increased from the characteristic control level of MCN = 118 ± 7.2 to 151 ± 1.3 in cells treated with 1.25% DMSO and to 166 ± 6.3 in cells treated with 1.0 μM RA (P < 0.02). These values are similar to the levels observed in untransformed RPMI cells. The response of F-actin to RA is dose dependent between 0.1 and 1.0 μM. At 4 μM RA, the F-actin value decreased to 151 ± 3.8, possibly reflecting toxicity. As shown in Fig. 3 the percentage of cells in the low F-actin population decreased significantly from 36 ± 2.5 in control cells to 10 ± 4.3 in 1.25% DMSO-treated cells and 12 ± 2.4 in 1.0 μM RA-treated cells, values that are similar to those observed with RPMI cells.
The PI, the percentage of cells in S + G2 + M, independently confirmed the effect of those agents in inducing differentiation. The PI decreased from 36 ± 5.3 in control cells to 17 ± 1.1 in 1.25% DMSO-treated cells and to 19 ± 2.0 in 1.0 μM RA-treated cells (P < 0.05). Daudi cells, which cannot be induced to differentiate by either agent, showed no change in either actin content or PI after incubation with DMSO and RA (data not shown).

Effect of TPA on F-Actin Content of RPMI Cells and Coeffect of RA

As shown in Table 5, the F-actin decreased from 170 ± 2.7 in untreated cells to 116 ± 1.5 in cells treated with 0.1 and 0.5 μg/ml TPA, respectively (P < 0.05). Cells treated with 1.0 μg/ml TPA showed intermediate values. Toxicity at the higher TPA concentrations is suggested by a decrease in viability to 70 – 80% at 1.0 μg/ml TPA, as compared with >95% normally observed. As shown in Fig. 4, a population of cells with low actin content appeared following treatment with TPA. However, treatment with TPA did not alter the DNA histogram. The small amount of DMSO introduced with TPA was without effect, as shown by controls incubated with 20 μl DMSO. When cells that had been treated with 0.5 μg/ml TPA for 6 days were treated with 0, 0.1, or 1.0 μM RA for an additional 6 days, the F-actin content (MCN) increased from 130 ± 4.1 to 140 ± 4.0 in 0.1 μM RA and 147 ± 4.0 (P < 0.05) in 1.0 μM RA-treated cells (Table 6). Although the mean F-actin content was still lower than in the control, the increase in F-actin was manifested by a marked reduction of the low F-actin cell population (Fig. 4). Simply removing the cells from TPA led to an increase in F-actin to MCN of 130 ± 4.1 from 116 ± 1.5 (P < 0.05).

### Table 4 F-Actin levels in HL-60 cells as a function of cell differentiation induced by DMSO or RA

<table>
<thead>
<tr>
<th>F-Actin amount (MCN)</th>
<th>Low F-actin population (%)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>118 ± 7.2</td>
<td>36 ± 2.5</td>
</tr>
<tr>
<td>Ethanol (10 μl)</td>
<td>119 ± 8.5</td>
<td>38 ± 3.5</td>
</tr>
<tr>
<td>DMSO (% v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>133 ± 1.3</td>
<td>21 ± 1.0</td>
</tr>
<tr>
<td>1.25</td>
<td>151 ± 1.3</td>
<td>10 ± 43</td>
</tr>
<tr>
<td>RA (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>139 ± 9.5</td>
<td>19 ± 1.9</td>
</tr>
<tr>
<td>0.5</td>
<td>146 ± 7.4</td>
<td>15 ± 1.4</td>
</tr>
<tr>
<td>1.0</td>
<td>166 ± 6.3</td>
<td>12 ± 2.4</td>
</tr>
<tr>
<td>2.0</td>
<td>155 ± 2.3</td>
<td>12 ± 2.7</td>
</tr>
<tr>
<td>4.0</td>
<td>131 ± 3.8</td>
<td>15 ± 0.8</td>
</tr>
<tr>
<td>RPMI cells</td>
<td>162 ± 7.4</td>
<td>7 ± 2.1</td>
</tr>
</tbody>
</table>

* Mean ± SD of three independent experiments.  
* P < 0.05 compared with control.  
* P < 0.01 compared with control.

### Table 5 Effect of TPA on untransformed lymphoblast cells (RPMI)

<table>
<thead>
<tr>
<th>TPA (μg/ml)</th>
<th>F-Actin (MCN)</th>
<th>Low F-actin population (%)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>170 ± 2.7</td>
<td>7 ± 1.5</td>
<td>18 ± 2.2</td>
</tr>
<tr>
<td>0.1</td>
<td>140 ± 10.1</td>
<td>22 ± 6.4</td>
<td>25 ± 8.2</td>
</tr>
<tr>
<td>0.5</td>
<td>116 ± 1.5</td>
<td>43 ± 2.1</td>
<td>21 ± 0.2</td>
</tr>
<tr>
<td>1.0</td>
<td>132 ± 13.7</td>
<td>23 ± 2.3</td>
<td>21 ± 0.3</td>
</tr>
</tbody>
</table>

DMSO only (20 μl)  | 169 ± 1.6 | ND* | 26 ± 1.2 |

* Mean ± SD of three independent experiments.  
* P < 0.05 compared with no TPA-treated cells.  
* P < 0.01 compared with no TPA-treated cells.  
* ND, not done.

### Table 6 Effect of RA on TPA-treated RPMI cells

<table>
<thead>
<tr>
<th>RA (μM)</th>
<th>F-Actin (MCN)</th>
<th>PI (%)</th>
<th>F-Actin (MCN)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>172 ± 0.5</td>
<td>22 ± 1.8</td>
<td>130 ± 4.1</td>
<td>33 ± 3.5</td>
</tr>
<tr>
<td>0.1</td>
<td>ND*</td>
<td>ND</td>
<td>140 ± 4.0</td>
<td>28 ± 2.8</td>
</tr>
<tr>
<td>1.0</td>
<td>167 ± 1.4</td>
<td>22 ± 1.8</td>
<td>147 ± 4.0</td>
<td>27 ± 4.2</td>
</tr>
</tbody>
</table>

* Mean ± SD of three independent experiments.  
* ND, not done.

* P < 0.05 compared with no RA-treated cells.
DISCUSSION

Cancer is a disease in which cells subvert or escape from the normal controls of cellular growth and differentiation (21, 27, 28). Growth and differentiation are controlled, at least in part, by endocrine/paracrine mechanisms involving external signals, usually peptides, binding to cell surface receptors linked to proteins on the inner cell membrane that, in turn, generate internal second messenger substances. These second messengers induce changes in both the cytoplasm and the nucleus that lead to cell division. In addition, the level of differentiation and, hence, morphology, of a cell can also be altered in response to either external or internal signals resulting from carcinogenesis (28). Because each particular tissue or cell type has its own specific rules for regulation of growth and maintenance of differentiation, there are therefore many different mechanisms by which cells escape control. However, the biochemical consequences of the escape from control seem to have a common thread in affecting the cellular cytoskeletal elements (7, 21). This commonality suggests that changes in the cytoskeleton may well be sensitive markers for transformed phenotypes.

A number of investigators have used semiquantitative techniques to demonstrate that transformed cell lines and tumor cells contain less F-actin compared with normal cell lines and tissues (3–8, 29, 30). Most investigators, using nonquantitative immunohistochemistry and cytochemistry, have studied cytological structural changes rather than biochemical quantitative changes. In this study we have focused upon quantitative changes in actin levels throughout the cell cycle in hematopoietic cells and in relation to cell differentiation, using dual labeling and quantitative flow and image analysis techniques. Our models consisted of an untransformed cell line (RPMI) and two transformed cell lines, one of which (HL-60) can be induced to differentiate and the other (Daudi) which cannot.

Cell cycle-related changes in F-actin were studied by using both flow cytometry and QFIA (31, 32). The combination of these two techniques showed that the division-related F-actin increase occurs in the untransformed cells during the late G1 phase, but in the G2 + M phase in the transformed cells. As depicted in Fig. 5, flow cytometry divides the cell cycle into three compartments and does not distinguish G0 and early G1 from late G1, while KI-67 divides the cell cycle into two compartments and does distinguish late G1 from G0 and early G1 (33–35). KI-67-positive RPMI cells have twice the F-actin content of KI-67-negative cells (Table 3), while S and G2 + M cells show a much smaller relative increase (170 versus 150 in RPMI cells; Table 2). These finding demonstrate the major increase in F-actin content occurs in late G1 stage. Earlier reports of increased actin mRNA levels in late G1 cells in phytomagglutinin-stimulated lymphocytes (36) suggested that G-actin, the precursor of F-actin, was synthesized in late G1 phase.

![Diagram](image)

Fig. 5. Cell cycle analysis by flow cytometry and QFIA. Flow cytometry divides cell cycle into G0 + G1, S, and G2 + M compartments but does not differentiate G0 (G1, early) from G1 (G1, late). QFIA with KI-67 divides the cell cycle into G0 + G1, and G2 + M compartments.

The HL-60 human promyelocytic leukemia cell line can be induced to differentiate when treated with RA, dimethylformamide, or DMSO (17, 22–26) and has been used to study the relationship of cell differentiation to oncogene expression (22), glycosaminoglycan biosynthesis (23), and actin polymerization (17, 24–26). In our study, differentiation of HL-60 cells increased their F-actin content 41%, approximating the quantitative content characteristic of untransformed RPMI cells. The changes in F-actin did not represent a nonspecific response to RA or DMSO, since no changes in F-actin levels were seen in the Daudi cells, which do not differentiate in response to these agents. A previous study using biochemical analysis rather than cytometry showed an 86–96% increase in total actin following differentiation induced by dimethylformamide (17).

The phorbol ester, TPA, modulates cellular differentiation; in hematopoietic cells it inhibits differentiation (28). Several biochemical actions have been specifically identified. TPA activates protein kinase C which negatively regulates actin polymerization (14, 37). TPA also alters the morphological distribution of F-actin, causes the disappearance of gap junctions, and modifies the expression of several oncogenes (20, 27). Treatment of untransformed RPMI cells with TPA caused F-actin levels to decrease to a value characteristic of transformed cells. This effect reflected cytoplasmic alterations unrelated to proliferation since the DNA histogram, as measured by the proliferation index, was unchanged. Thus the changes in F-actin levels appears to be a specific marker for differentiated cells. Treatment of TPA-treated cells with RA causes the F-actin levels to increase, but not to the levels of cells not treated with TPA. The effect occurs spontaneously in the absence of RA at a lower rate.

By treating the untransformed cells with TPA and the HL-60 cells with RA and examining the relationship of the increase in F-actin with cell cycle, the difference in the timing (i.e., relation to cell cycle) of F-actin increases between untransformed and transformed cells could be understood. Differentiation of the HL-60 cells increased the amount of F-actin to levels seen in untransformed cells but did not alter the timing (Table 2), the major increase occurring in G2 + M regardless of whether the cells were differentiated or not. However, treating the RPMI cells with TPA not only decreased the amount of F-actin to levels characteristic of the transformed cells but altered the timing to that of the transformed cells as well. This suggests that the difference in timing may well relate to altered polymerization of G-actin. Protein kinase C activity is enhanced by phorbol esters (21, 27), and increased protein kinase C activity has been reported to decrease actin polymerization (14, 37). Because net synthesis of G-actin peaks during late G1 (36), but as shown here the increase in F-actin does not appear in transformed or TPA-treated cells until G2 + M, our data suggest the hypothesis that the increased protein kinase C activity of transformed or TPA-treated cells may inhibit actin polymerization until the G2 + M phase.

In summary, our data show that the F-actin content of transformed cells (Daudi and HL-60) is approximately two-thirds of that of similar but untransformed cells (RPMI), even considering that dividing cells contain nearly twice the F-actin content of resting cells, and transformed cells divide at a higher rate than do untransformed cells. The identification of cells with low F-actin content displaying the timing of F-actin appearance in the cell cycle characteristic of transformed cells may well prove useful in distinguishing cancer-related processes in cells from benign processes, because cancer-related processes are much more likely to reflect alterations in cellular differen-
tiation than are benign processes. Because of the ability of retinoids and DMSO to correct the cytoskeletal F-actin defect, quantitative determination of F-actin may serve as an intermediate end point marker for monitoring chemopreventive therapy.

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REFERENCES

Cellular F-Actin Levels as a Marker for Cellular Transformation: Relationship to Cell Division and Differentiation


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