Definition of the Activities and Properties of c-myc Required to Inhibit Cell Differentiation

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Abstract

Previous studies have shown that high levels of c-myc inhibit cell differentiation. The goal of this study was to define the activities and properties of c-myc that are necessary and/or sufficient for this effect. A series of mutant human c-myc genes were stably transfected into the 3T3-L1 preadipocyte cell line and assayed for their capacity to block differentiation into adipocytes. Results of the differentiation tests were then correlated with other known activities and properties of the mutants. Our studies show that the ability of c-myc to inhibit 3T3-L1 cell differentiation requires its transforming activity, and the ability of c-myc to bind sequence-nonspecific DNA and to form oligomers is not sufficient for this effect. Thus, the ability of c-myc to inhibit cell differentiation may be central to its role as a transforming oncogene.

Introduction

The product of the c-myc protooncogene has a pivotal role in the control of cell proliferation and differentiation. Deregulated expression of c-myc inhibits differentiation of many cell types (1–4) and promotes cell transformation both in vitro and in vivo (5–8). The effect of c-myc on terminal differentiation is not due to significant alterations in normal growth control mechanisms but instead is due to an inability of cells to commit to a pathway leading to the terminally differentiated state (2–4). For example, 3T3-L1 cells expressing a recombinant c-myc gene arrest growth in G0/G1 at confluence and complete one round of DNA replication in response to differentiation inducers similar to normal cells. However, in contrast to normal cells, cells expressing high levels of c-myc can reenter the cell cycle following treatment with differentiation inducers and fail to differentiate (3). Similar findings were made with mouse erythroleukemia cells (1, 4). These results argue that c-myc inhibits cell differentiation by preventing irreversible withdrawal from the cell cycle, a requisite for terminal differentiation (3). Taken together, these studies support the view that cell proliferation and terminal differentiation represent alternative and mutually exclusive pathways for cells and indicate a role for c-myc in the decision-making process.

Like the product of other oncogenes, c-myc possesses several distinct activities and properties that are encoded by separate domains (9). These activities include the ability to transform cultured cells (5, 6, 9), to bind sequence-nonspecific DNA (10, 11), and to form oligomers in vitro (12). If the ability of c-myc to inhibit cell differentiation is pertinent to its role as an oncogene, then the transforming activity of c-myc should be required for this effect. Thus, in this study we defined the activities and properties of c-myc that are required for its ability to inhibit cell differentiation. This was accomplished by testing a series of mutant c-myc genes for their capacity to inhibit differentiation of 3T3-L1 preadipocytes (13, 14) and then correlating these results with other activities and properties of the mutants.

Results

One wild-type and 16 mutant c-myc genes were utilized in this study and are shown schematically in Fig. 1. These mutants were chosen because, together, they systematically mutate the entire c-myc coding sequence, and their activities and properties have been previously characterized. The transforming activity of nearly all of the mutants has been measured using two different assays (9). The REC assay measures the ability of c-myc to cooperate with an activated c-Ha-ras to transform primary rat embryo cells, and the Rat-1a cell assay measures the ability of c-myc alone to transform an established cell line. Human c-myc (439 amino acids) was divided into four regions on the basis of mutational inactivation of its transforming activity (Fig. 1). In addition, many of these mutants have been examined for their ability to bind sequence-nonspecific DNA and to form oligomers in vitro (11, 12). Stable 3T3-L1 cell lines expressing each c-myc gene were established by cotransfection with the dominant selectable marker gene, pSV2neo (15). As a control, 3T3-L1 cells were also cotransfected with pUC-8 and pSV2neo. Following selection in G418, pooled transfectants representing approximately 200–400 individual colonies were obtained for each c-myc construct.

Expression of the human c-myc RNA was quantified in each cell line by S1 nuclease analysis using a probe that hybridizes to sequences contained in exon 1. This probe was selected since mRNA from all of the mutant c-myc genes protects the same length fragment, which facili-

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4 The abbreviation used is: REC, rat embryo cell.
Inhibition of Cell Differentiation by c-myc

<table>
<thead>
<tr>
<th>Transforming activity</th>
<th>% Inhibition of G-3-P DH</th>
<th>Comments</th>
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<tbody>
<tr>
<td>pM21 (wild-type)</td>
<td>+ +</td>
<td>100</td>
</tr>
<tr>
<td>In6</td>
<td>+ +</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>In40</td>
<td>+ +</td>
<td>102 ± 6</td>
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<tr>
<td>In55</td>
<td>+ +</td>
<td>87 ± 6</td>
</tr>
<tr>
<td>D7-91</td>
<td>+ +</td>
<td>&lt;10</td>
</tr>
<tr>
<td>In105</td>
<td>+ +</td>
<td>&lt;10</td>
</tr>
<tr>
<td>D145-262</td>
<td>+ +</td>
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<tr>
<td>In262m</td>
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<tr>
<td>D371-412</td>
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</tr>
<tr>
<td>D414-433</td>
<td>+</td>
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Fig. 1. Summary of the properties and activities of mutant c-myc genes. Top, the 439-amino acid human c-myc protein with portions encoded by exons 2 and 3 indicated above the box. The protein can be divided into four regions (I, II, III, and IV shown below the box), based on how mutations in these regions affect transforming activity (9). The amino acids that divide these regions are indicated in italics. Shaded box A, amino acids (265-317) responsible for c-myc sequence-non-specific DNA-binding activity (11); shaded box B, amino acids (371-439) necessary for oligomerization in vitro (12). Boxes below, the various mutant c-myc genes used in this study, with the position of insertion mutations (In) indicated by a line and the extent of deletion mutations (D) indicated by a hatched box. The amino acid positions affected by the insertions and deletions are indicated by the numbers following In and D, respectively. The activity of the mutants in the REC cotransformation assay with EJras or in the Rat-1a cell transformation assay is indicated (+, 26-100%; +, 3-25%; +, 0-2% of wild-type c-myc activity (9)). NT, mutants not tested in a particular assay. Inhibition of induction of 3T3-L1 cell glycerophosphate dehydrogenase (G-3-P DH) activity by the various mutants is given as a percentage of the inhibitory activity of the wild-type human c-myc gene (pM21, 100%); and mutants having less than 10% wild-type activity are grouped together (<10%). The results represent the average (± range) of two separate experiments in which two different plasmid DNA preparations were used in the transfections. The specific activity of glycerophosphate dehydrogenase in cells transfected with pM21 was 6.5% (46.2 ± 10.6 milliunits/mg protein) of that in cells transfected with pUC-8 (708 ± 31 milliunits/mg protein). To facilitate the comparison among results of the transformation assays and inhibition of induction of glycerophosphate dehydrogenase activity, mutants scored as + in the transformation assays but having 10% or less of wild-type activity are indicated with an asterisk. The ability of several key mutants to bind sequence-non-specific DNA or to form oligomers (tetramers) in vitro is indicated under Comments.

tates the comparison of c-myc RNA levels among cell lines. As shown in Fig. 2, each pooled cell line expressed the human c-myc mRNA at readily detectable and comparable levels. In contrast, RNA from 3T3-L1 cells transfected with pUC-8 did not protect the human c-myc probe. We attempted to measure the amount of c-myc in cell lines by Western blot analysis using three different c-myc-specific antisera but found that the level was below detection. Other studies have also found that c-myc is difficult to detect, even in cells where a pronounced phenotypic effect is observed (3, 9). However, a previous study using the same mutant c-myc genes showed that the steady-state concentration of mutant c-myc protein correlates well with mRNA levels in COS-7 cells (9). Thus, the ability of these mutant c-myc genes to inhibit 3T3-L1 cell differentiation reflects the specific activity and not the concentration of its gene product.

All pooled cell lines were growth arrested at confluence and tested for their ability to differentiate into adipocytes following exposure to agents that promote terminal differentiation (3, 16). The degree of differentiation was monitored by staining cells for lipid with Oil-Red-O and by quantifying the activity of glycerophosphate dehydrogenase, an adipocyte-specific marker. Similar to previous studies which utilized a recombinant mouse c-myc gene (3), high expression of the human c-myc RNA in 3T3-L1 cells completely blocked the conversion of cells into adipocytes (Fig. 3c). Less than 5% of cells expressing the wild-type c-myc RNA accumulated cytosolic triglyceride under conditions which induced significant differentiation of normal 3T3-L1 cells (Fig. 3a). Mutants that contained small in-frame insertions in region
I (amino acids 1–105) were as active as the wild-type gene in their ability to prevent differentiation (Fig. 3e). In contrast, mutant D7–91, which deletes most of region I, did not inhibit 3T3-L1 cell differentiation (Fig. 3f). Similar results were obtained for mutants involving regions II (amino acids 106–143) and III (amino acids 144–319): in-frame insertion mutations had little effect on the ability of c-myc to inhibit 3T3-L1 cell differentiation, but deletions in these regions completely abolished this activity (Fig. 3, g–k). Thus, the presence of these regions is important for the ability of c-myc to prevent terminal differentiation, although small alterations may be tolerated. Mutations involving region IV (amino acids 320–439) gave different results; both insertions and deletions completely abolished the ability of c-myc to inhibit 3T3-L1 cell differentiation. For example, cells expressing mutant m370m differentiated into adipocytes to the same extent as normal 3T3-L1 cells (l). Similar results were obtained with D371–412 and D414–433, which delete different portions of region IV (Fig. 3, m and n). Thus, region IV of c-myc is more sensitive to insertional mutational inactivation than regions I, II, and III.

These conclusions were substantiated by examining the ability of each mutant to prevent induction of glycerophosphate dehydrogenase (sn-glycerol-3-phosphate: NAD+ 2-oxidoreductase, EC 1.1.1.8). The specific activity of glycerophosphate dehydrogenase increases 300-fold upon differentiation of 3T3-L1 cells, and the level of this enzyme correlates well with the degree of differentiation (17, 18). The results of these studies are summarized in Fig. 1 and are compared to other known activities and properties of the mutants. The ability of each mutant to inhibit the induction of glycerophosphate dehydrogenase is expressed relative to that of wild-type human c-myc (pM21; 100% inhibition); and those having less than 10% of wild-type activity, which was considered negligible, are grouped together (<10%). The results of these studies agree well with the lipid-staining assays in that small insertions in regions I, II, and III had little or no effect on the ability of c-myc to inhibit induction of glycerophosphate dehydrogenase; however, large deletions in these regions abolished this effect of c-myc. Similarly, this assay also showed that region IV is more sensitive to insertional mutational inactivation than regions I, II, and III.

Discussion

These results allow several conclusions concerning the activities and properties of c-myc that are necessary and/or sufficient for its ability to inhibit cell differentiation. Mutants of c-myc that are transformation defective, as measured by the REC or Rat-1a cell transformation assay, are unable to inhibit 3T3-L1 cell differentiation, and mutants having greater than 25% of wild-type transforming activity do inhibit differentiation. These findings argue that the transforming activity is required for this effect of c-myc. Beyond this, the results of this study agree more closely with those of the Rat-1a cell transformation assay. Region III (amino acids 144–319) which is totally dispensable for cooperation with an activated c-Ha-ras in the transformation of rat embryo cells, is required for inhibition of 3T3-L1 cell differentiation and for efficient transformation of Rat-1a cells (see D145–262, D265–317; Fig. 1). Thus, it follows that if the Rat-1a cell and REC transformation assays are measuring different interactions of c-myc in the cell, interactions responsible for transformation of Rat-1a cells are similar to those responsible for inhibition of 3T3-L1 cell differentiation. Rat-1a cells overexpressing human c-myc acquire altered growth properties as assayed by their ability to form foci in monolayer cultures, to exhibit anchorage-independent growth, and to form tumors in athymic mice (9). In contrast, 3T3-L1 cells expressing high levels of normal human c-myc do not display these properties. Although similar cellular interactions are required to see phenotypic changes in 3T3-L1 cells and Rat-1a cells, the manifestations of c-myc overexpression differ in different

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1. S. O. Freytag, unpublished experiments.
Enforced expression of c-myc prevents 3T3-L1 cell differentiation without affecting early steps in the differentiation pathway (3). Similar to normal cells, 3T3-L1 cells expressing high levels of c-myc arrest growth in G0/G1 at confluence, traverse the cell cycle once, and reenter G0/G1 upon treatment with differentiation inducers. However, unlike normal cells, cells expressing high levels of c-myc are capable of reentering the cell cycle following treatment with differentiation inducers. Such cells do not irreversibly withdraw from the cell cycle and thus fail to terminally differentiate.

Materials and Methods

Cell Culture. Methods for growing, differentiating, and staining 3T3-L1 cells were as previously described (3).

DNA Transfections. The c-myc plasmids used in these studies have been described in detail elsewhere (9). All transfections were performed using plasmid DNA purified on CsCl-ethidium bromide density gradients (21). Subconfluent monolayers of 3T3-L1 cells (1 × 10⁶ cells/100-mm dish) were cotransfected with 50 µg of each c-myc plasmid plus 5 µg of pSV2neo (15) using the CaPO₄ precipitation method (22). Five hours after addition of the DNA precipitate, cell monolayers were shocked with 15% (v/v) glycerol (prepared in Dulbecco’s modified Eagle’s medium) for 1 min, washed with 10 ml of Dulbecco’s modified Eagle’s medium, and refed with fresh growth media. The next day, cells were detached by trypsinization, replated into three 100-mm dishes, and subjected to selection in growth medium containing 1.0 mg/ml G418 (40% active; Gibco Laboratories, Grand Island, NY). Two weeks later, the number of colonies was recorded, and cells were pooled by trypsinization.

Analysis of RNA. Procedures for the isolation of total cellular RNA and for S1 nuclease assays were as previously described (3). Fifty µg of total cellular RNA were used in each S1 nuclease assay. DNA probes were 5’-end labeled with [γ-³²P]ATP and T4 polynucleotide kinase using standard procedures (21).

Enzyme Assays. Assays for glycerophosphate dehydrogenase (sn-glycerol-3-phosphate:NAD⁺-2-oxidoreductase. EC 1.1.1.8) were performed as follows. Cell monolayers were washed twice with Dulbecco’s phosphate-buffered saline (pH 7.4) and harvested in 1 ml of ice-cold Dulbecco’s phosphate-buffered saline (pH 7.4)
by scraping. Cells were collected by centrifugation (8000 \( \times g \) for 1 min), resuspended in 0.25 ml of ice-cold 10 mm Tris-HCl-150 mm NaCl (pH 7.6), and lysed by sonication on ice. A clarified cell extract was prepared by centrifugation at 8000 \( \times g \) for 10 min at 4\(^\circ\)C. Glycerophosphate dehydrogenase activity was measured in clarified cell extracts using conditions previously described (23). All assays were performed in duplicate and were linear with time and protein. One unit of specific activity (S.A.) equals the conversion of 1 \( \mu \)mol of substrate into product/min/mg of protein at 25\(^\circ\)C. The percentage of inhibition of glycerophosphate dehydrogenase activity by the mutant c-myc genes was calculated as follows:

\[
1 - \frac{(S.A. \text{ of mutant cell line} - S.A. \text{ of pM21 cell line})}{(S.A. \text{ of pUC-8 cell line} - S.A. \text{ of pM21 cell line})} \times 100
\]

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References