Temporal Mapping of the Differentiation Pathway of the Murine Erythroleukemia Cell

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ABSTRACT

These studies are concerned with "mapping" the temporal order of the precommitment events in the differentiation pathway of the Friend erythroleukemia cell. We have used a single-block procedure in which a differentiation-specific inhibitor of a temperature-sensitive (ts) differentiation-defective mutation was used to block the differentiation program. Later, the block was removed, differentiation was allowed to proceed, and the time required to reach a reference marker was monitored. These studies have indicated that the mutations tsC2GPl and tsBSA, the poly(ADP-ribose) polymerase inhibitor 3-aminobenzamide, and the glucocorticoid hormone dexamethasone blocked functions which are required just prior to commitment. We have also used a double-block procedure involving two consecutive restrictive conditions, which suggests that the 3-aminobenzamide- and tsC2GPl-blocked functions constitute a part of a sequentially ordered pathway leading to terminal differentiation. The convergence of the 3-aminobenzamide, dexamethasone, and ts mutant blocks just prior to commitment suggests that the blocked functions may be part of a major control mechanism for commitment.

In these studies, we have introduced a cytochalasin B-based assay to monitor commitment. The use of cytochalasin B permits a direct assay for commitment and obviates the need for colony-forming assays using semisolid medium, which have inherent problems such as efficiency of plating.

INTRODUCTION

The FEL cell is a retrovirus-transformed cell blocked in the pathway of terminal differentiation. These transformed cells may be induced to differentiate following treatment with a variety of chemical or physical agents (1). The cellular responses to these inducing agents include changes in the transcription of the c-fos, c-myb, and c-myc protooncogenes, the synthesis of histone H1, spectrin, and surface antigens, alterations in calcium flux and cAMP metabolism, and other changes (for reviews, see Refs. 2-4). During the early phase of differentiation, the removal of the inducing agent results in the arrest of the differentiation program and a reversion to the uninduced state. At a later stage, the induced cell becomes irreversibly destined to terminal differentiation. Here, we will refer to the point in time when the inducer is no longer required for the expression of the differentiated state as the "commitment point." This distinction is made since commitment has been considered a process which is acquired gradually in a series of steps (5), whereas the transition to an irreversibly committed state appears to be a sharply defined time point, as shown in these studies. The postcommitment events include the formation of hemoglobin, cessation of DNA replication, and cell death (2-4).

We have charted a part of the differentiation pathway of the FEL cell, using ts differentiation-defective mutants and differentiation-specific inhibitors which characterize the differentiation program. The ts mutants grow normally at both restrictive (39°C) and permissive (34°C) temperatures but are ts for DMSO-induced differentiation. The poly(ADP-ribose)polymerase inhibitor 3AB and the glucocorticoid hormone DEX were both previously shown to inhibit FEL cell differentiation, with minimal effects on cell growth (5-9). Our studies with the ts mutants and inhibitors have indicated that there is a convergence of critical events (functions) at the commitment point and that the 3AB- and tsC2GPl-blocked functions appear to be arranged in a linear pathway leading to hemoglobin accumulation.

MATERIALS AND METHODS

Chemicals. DMSO was obtained from Fisher Scientific (NJ). 3AB was purchased from Pfaltz and Bauer (Waterbury, CT), which was dissolved in 95% ethanol as a 1 mM stock solution and kept at 4°C, and benzidine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Cytochalasin B, which was obtained from Aldrich Chemical Co. (Milwaukee, WI), was dissolved in 95% ethanol as a stock solution (0.2 mg/ml) and stored at -20°C. Iscove's modified Dulbecco's medium (Iscove's medium), penicillin, and streptomycin were obtained from Gibco (Burlington, Ontario, Canada). Fetal bovine serum from Bocknek was purchased from BDH (Toronto, Ontario, Canada). Bacto-agar was obtained from Difco Laboratories (Detroit, MI).

Cell Culture. FEL cells were cultured in Iscove's medium containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). The ts differentiation-defective mutants were isolated following overnight treatment of FEL cells (745A) with N-methyl-N'-nitro-N-nitrosoguanidine (0.1 μg/ml; Sigma Chemical Co.) and growth at 34°C. These cells were subsequently treated with repeated (12 or more) cycles of exposure to inducing agent at 39°C for 30 h, followed by growth at 34°C for 72 h in the absence of inducing agent. The inducing agents used for tsC2GPl and tsBSA were 1.5% DMSO and 5 mM hypoxanthine, respectively. Clones were isolated at 34°C using the soft agar method (9) and tested for the formation of benzidine-positive cells at 39°C and 34°C. The ts clones were reisolated using the same method. The ts mutants used in these experiments have slightly decreased growth rates, compared to the parental 745A strain, at both 34°C and 39°C. The ts phenotype refers only to the differentiation properties. When these mutants were cloned in soft agar in the presence of 1.5% DMSO at 39°C, they formed large colonies, indicating that they were blocked prior to the nonproliferative stage of differentiation (10). Mutant and "wild-type" cell lines were maintained at 34°C and 37°C, respectively, in a humidified atmosphere with 5% CO2. The wild-type strain reported in all of the experiments was clone K derived from strain 745A. Clone K was selected on the basis of its high induction (>95%) with 1.5% DMSO and its relative stability on extended incubation with DMSO.

Induction of Differentiation. The general protocol for induction involved early logarithmic phase FEL cells, which were suspended in Iscove's medium at a concentration of 1×106/ml and induced with 1.5% DMSO under various conditions. In experiments involving...
changes in the medium (such as changes from a DMSO-containing to
DMSO-free medium), the cells were centrifuged, washed with Iscove’s
medium, and suspended at a concentration of 5 × 10⁷/ml for the second
treatment. The presence of benzidine-positive cells was assayed at times
specified with each experiment.

In experiments involved in testing the effect of cytochalasin B on
differentiation, cells (1 × 10⁷/ml) were incubated with 1.5% DMSO
and varying concentrations of cytochalasin B for different intervals of
time and were assayed for the formation of benzidine-positive cells, cell
numbers, and cytotoxicity. Cytochalasin B was also tested for its effect
on maturation of benzidine-positive cells by first incubation of cells
with DMSO for 56 h, followed by a second incubation in DMSO-free
medium containing cytochalasin B (0.75 μg/ml), for various intervals
of time, and assays for benzidine-positive cells, cell numbers, and
cytotoxicity. In the single-block experiments, the cells (1 × 10⁷/ml)
were initially grown in the presence of 1.5% DMSO and 3AB (10 mM)
or 1.5% DMSO and DEX (1.0 μM) for 56 h. The second-stage incu-
bation involved treatment of cells (5 × 10⁷/ml) with DMSO alone for
various intervals of time, followed by incubation in DMSO-free medium
in the presence of cytochalasin B for maturation. The presence of
benzidine-positive cells was assayed at 104 h (total incubation period).

In the single-block experiment for ts mutant cells, the initial incubation
was performed in the presence or absence of 1.5% DMSO at 39°C for
56 h. The second incubation was carried out at 34°C and otherwise
treated similarly to the single-block experiments involving 3AB and
DEX. The double-block experiments involved basically two separate
experiments. In the first experiment, mutant tsC2GPl was initially
incubated in the presence of 1.5% DMSO at 39°C for 55 h. The cells
were subsequently incubated in the presence of DMSO, 3AB (10 mM),
and cytochalasin B (0.75 μg/ml) at 34°C for 55 h and assayed for the
presence of benzidine-positive cells. The second experiment in the
double-block procedure involved a reverse order for the blocks. The
initial incubation was at 34°C with 1.5% DMSO and 3AB (10 mM) for
67 h, followed by a second incubation with DMSO and cytochalasin B
(0.75 μg/ml) at 39°C for 40 h. The rationale for the choice of incubation
times is described in “Results.”

Benzidine Staining for Hemoglobin. The cells were stained for he-
moglobin using the benzidine staining procedure, as described by Go-
palakrishnan and French Anderson (11), usually 96 h after start of the
experiment.

Viability Tests. The cells were centrifuged, stained with trypan blue
(0.04% in phosphate-buffered saline), and counted microscopically (12).

Soft Agar Assay. This procedure was similar to one previously
described (9), except that a double agar layer was used. The lower layer
contained Iscove’s medium with 10% FBS and 0.3% agar, and the
upper layer contained Iscove’s medium with 20% FBS and 0.3% agar;
cells were added after the lower layer had solidified. The colonies were
stained with benzidine after 4 days of culture.

RESULTS

Commitment Assays. Commitment has been assayed by plat-
ing induced cells in soft agar, methylcellulose, or plasma clots,
in the absence of inducer (8–10, 13, 14). These methods are
informative, in that they permit the scrutiny of the resulting
colonies, but they require a high efficiency of plating and are
time consuming. Cytochalasin B, which was previously shown
to block cytokinesis with minimal effects on differentiation of
FEL cells (15, 16), was used here as an alternative method to
assay for committed cells.

The following experiments were performed to test the effect
of cytochalasin B on differentiation, cell division, and viability.

When FEL cells were induced with 1.5% DMSO in the presence
of varying concentrations (0.75, 1.0, and 1.25 μg/ml) of cyto-
chalasin B, there was little or no effect on differentiation, as
measured at 48 and 60 h after DMSO addition (Fig. 1A). There
were <0.2% benzidine-positive cells at 0, 24, and 36 h. Under
these experimental conditions, cell numbers remained relatively
constant (Fig. 1B), and cytotoxicity, as measured by trypan
blue staining, indicated >90% viability at 48 and 60 h (data not
shown). Since cytotoxicity increased with longer exposure time
and higher cytochalasin B concentrations, usually the exposure
time was 48 h or less and the cytochalasin B concentration was
0.75 μg/ml. The cytochalasin B concentrations used did not
induce differentiation or enucleation (data not shown).

In the cytochalasin B-based commitment assay, typically cells
were induced with DMSO for varying periods, centrifuged,
washed with Iscove’s medium, incubated in the presence of
cytochalasin B (0.75 μg/ml) and in the absence of DMSO, and
subsequently assayed for benzidine-positive cells. In order to
determine the time required for committed cells to express the
differentiated phenotype (maturation), FEL cells were induced
with DMSO for 56 h, incubated in DMSO-free medium in the
presence or absence of cytochalasin B for varying periods of
time, and assayed for benzidine-positive cells. At 56 h, there
were approximately 9% benzidine-positive cells. The benzidine-
positive cells increased to 50–60% within 12 h (68-h total
incubation time) and remained at this level for 48 h (104-h total
incubation time) (Fig. 2A). Also, the cell numbers were approxi-
ately constant over this period of exposure to cytochalasin B
(Fig. 2B). In contrast, when cytochalasin B was left out, the
percentage of benzidine-positive cells increased to 50–60%
following 12 h of incubation in the absence of DMSO, but after
20 h the percentage of benzidine-positive cells decreased be-

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**Fig. 1.** Effect of cytochalasin B on cell di-
vision and differentiation of DMSO-induced
cells. FEL cells (1 × 10⁷/ml) were treated
simultaneously with 1.5% DMSO and differ-
ent concentrations of cytochalasin B and were
assayed for benzidine-positive cells (A) and cell
density (B) at subsequent time points. The
percentage of benzidine-positive cells at 0 time
was <0.1%. ▲, no cytochalasin B; △, 0.75; ☐,
1.0; and □, 1.25 μg/ml cytochalasin B. Each
time point represents the mean of three ex-
periments. In this and all subsequent experiments,
error bars represent the standard deviation.
cause of the continued replication of uninduced cells and the limited replication of committed cells. Cytotoxicity tests indicated ~98% viable cells in the presence of cytochalasin B and ~99% viable cells in the absence of cytochalasin B after 48 h of exposure. Since the total number of benzidine-positive cells in the absence of cytochalasin B was 2.2 times the number of committed cells in the presence of cytochalasin B, it appears that each committed cell yielded, on average, 2.2 benzidine-positive cells. These results indicate that the addition of cytochalasin B to the medium provides a means of estimating the number of committed cells without complications introduced by cell replication. The estimate of benzidine-positive cells could be made 12–48 h after the addition of cytochalasin B, since the number and percentage of benzidine-positive cells remain approximately constant over this period.

Another means of assessing the cytochalasin B-based assay is to compare this assay with the previously used soft agar assay (9). During the early period following the induction with DMSO, the soft agar assay shows significantly increased numbers of "benzidine-positive" colonies, compared to the benzidine-positive cells in the cytochalasin B-based assay (Fig. 3). We noted that, at 28 h (following DMSO addition), almost 90% of the "benzidine-positive" colonies were composed of both benzidine-positive and -negative cells ("mixed") colonies. Both "mixed" and completely benzidine-positive ("pure") colonies were counted as benzidine-positive. Generally these mixed colonies were larger than the pure benzidine-positive colonies. However, the proportion of mixed to pure colonies decreased by 46 h, and commitment measured by the soft agar method was similar to the cytochalasin B-based assay. At subsequent times, both assay methods gave similar results and it should be noted that in the following experiments only these later times are involved. The predominance of mixed colonies during the early period following the addition of the inducing agent has been previously noted (17), but it is not known whether commitment in these mixed colonies occurred at the time of transfer to the semisolid medium or at a later time. The kinetics for the formation of committed cells, as measured by the cytochalasin B assay, preceded the appearance of benzidine-positive cells by approximately 12–15 h. These data confirm the finding (Fig. 2) which showed that maturation of the benzidine-positive cells required 12 h in the presence of cytochalasin B. The time required to reach ~50% commitment by the cytochalasin B assay was approximately 56–62 h at 37°C.

Single-block Experiments to Determine the Temporal Order of Events (Functions). In the single-block method, the cells were induced under restrictive conditions and released from the block, and the time required to reach a specific reference marker in the differentiation pathway was determined. The time at which a blocked event (or function) is normally being consummated is indicated by the time required to reach the reference marker (commitment point) after the removal of the block. FEL cells were induced with 1.5% DMSO at 37°C and at the indicated times were removed and plated in the absence of DMSO in soft agar (D) or in medium containing cytochalasin B (O). The cells were stained with benzidine and counted after 4 days in soft agar. In the cytochalasin B assay, the cells were stained after a total incubation period of 96 h. The appearance of benzidine-positive cells in a culture continuously treated with DMSO and assayed at various time points is indicated (C).

Fig. 3. Comparison of the soft agar and cytochalasin B assays for commitment. Cells (1 x 10^6/ml) were induced with 1.5% DMSO at 37°C and at the indicated times were removed and plated in the absence of DMSO in soft agar (D) or in medium containing cytochalasin B (O). The cells were stained with benzidine and counted after 4 days in soft agar. In the cytochalasin B assay, the cells were stained after a total incubation period of 96 h. The appearance of benzidine-positive cells in a culture continuously treated with DMSO and assayed at various time points is indicated (C).
ment, and the kinetics for the appearance of committed cells after the removal of the 3AB or DEX blocks were almost identical. As a control, FEL cells were incubated in 3AB- or DEX-containing medium (without DMSO) for 56 h and subsequently incubated in the presence of DMSO, as in the other examples. These experiments show that 3AB or DEX treatment itself does not change the cellular response to DMSO and that 3AB or DEX together with DMSO permitted the early steps of the DMSO-induced differentiation program. Similar experiments were performed with two mutants, tsC2GP1 and tsBSA. Here, the general experimental protocol was similar to that described above, except that the initial incubation in the presence or absence (control) of DMSO was carried out for 56 h at 39°C (without inhibitor). The subsequent steps were similar to the previous experiment, except that the incubations were carried out at 34°C. We noted that by 2.5–5 h after the down-shift from the restrictive (39°C) temperature to the permissive (34°C) temperature, there was an increased percentage of committed cells (Fig. 5). The control shows that, in the absence of DMSO for 56 h and otherwise treated as above. For the control, between 0 and 12 h after removal of inhibitor, the percentage of benzidine-positive cells was <0.1%.

The sequential order of the functions deduced by comparing the commitment point or hemoglobin formation as a reference marker. In the first experiment, tsC2GP1 cells were induced with DMSO in the presence of 3AB at 34°C and subsequently transferred to DMSO-containing medium and incubated at 39°C. In the second experiment, the order of the blocks was reversed. The cells were initially induced at 39°C and were subsequently transferred to a medium containing DMSO and 3AB and incubated at 34°C. In both experiments, cytochalasin B was included in the second incubation to prevent cell multiplication (see “Materials and Methods” for details). The rationale for the choice of incubation times was based on the following experiments. The initial incubation times were chosen so that the 39°C and 34°C treatments were physiologically equivalent. We noted that, when wild-type cells were induced with DMSO, the time required for ~50% of the cells to be committed was 67 and 55 h at 34°C and 39°C, respectively (data not shown). The second incubation times were selected by monitoring the time required for the numbers and percentages of benzidine-positive cells to stabilize. When the first incubation was extended so that a greater number of cells would be committed, we found that the cells showed a reduced capacity to differentiate. When the second incubation period was prolonged, the cells were prone to lysis (data not shown). While the choice of these incubation times may appear arbitrary in part, the experiment has a sufficient number of controls which indicate the cellular capacity to respond during each incubation period (Table 1). When the order of the blocks was 3AB followed by the restrictive temperature (39°C), differentiation was blocked (Table 1, A1), but where the order was reversed, with the restrictive temperature first followed by 3AB, differentiation occurred (Table 1, B1). These results suggest that the 3AB-blocked function precedes the tsC2GP1-blocked function. The sequential order of the functions deduced by comparing the reciprocal arrangement of blocks (Table 1, A1 and B1) is corroborated by the additional data provided in the A1–7 and B1–4 series. Control A3 indicates the reversibility of the 3AB block, A4 indicates the effectiveness of two consecutive 3AB treatments, and A1 and A2 indicate that the block is maintained.
when the shift is made from 3A8 to 39°C, suggesting that the tsC2GP1-blocked function follows the 3AB-blocked function or that 3AB and the tsC2GP1 mutation block the same function. Control A5 indicates that the differentiation occurs at 34°C during the first treatment. A6 shows the effect of the second incubation at 39°C with DMSO, and A7 (compared with B4) illustrates the ts phenotype. In the B1–4 series, a comparison of B1 and B2 with B4 indicates the 3AB block had little effect when 3AB was included in the second incubation, suggesting that the 3AB-blocked function was operative during the initial 39°C incubation and that 3AB and tsC2GP1 block separate functions. This confirms the conclusion derived in the A1–7 series that the 3AB block precedes the tsC2GP1 block. Controls B3 and B4 indicate the effectiveness of the ts blocks.

We found that the double-block procedure was difficult to apply with the tsB5A mutant, because of the elevated cytotoxicity resulting from the simultaneous exposure to 1.5% DMSO and 10 mM 3AB. We have noted that some of the other ts isolates also show altered cytotoxicity to DMSO and 3AB.

**DISCUSSION**

We have initiated a study of the differentiation pathway of the FEL cell, using ts differentiation-defective mutants and inhibitors. Previous studies have indicated that the developmental pathway is temporally ordered (18) but does not necessarily involve an obligatory sequential series of events (15, 19). These earlier studies involved products associated with differentiation, such as histone H1 (10), hemoglobin, or terminal cell division. In the studies reported here, the functions assayed are those required for differentiation.

We have used single-block and double-block procedures to determine the temporal order of events in the differentiation pathway. In the single-block method, the cells were induced under restrictive conditions and released from the block, and the time interval between the removal of the block and a reference marker was determined. We found that the “timing” of the 3AB- and DEX-blocked functions was indistinguishable and occurred just prior to the commitment point. In previous studies, it was shown that commitment occurred shortly after the removal of 3AB (9) or DEX (20) from hexamethylenbisacetamide-induced FEL cells. The ordering of events using the single-block procedure is applicable only in cases where the blocks are readily reversible, such as in the case of 3AB and DEX. This method is not strictly applicable to the ts mutants where the mode of reversion may be complex and allele-specific. Nevertheless, these studies have indicated that the tsC2GP1 and tsB5A mutant blocks are also temporally located just prior to commitment. One interpretation of these results could be that these blocked functions appear to be timed just prior to the commitment point because they represent independent or random functions which are required to complete the commitment program. However, the double-block experiments suggest that the 3AB- and tsC2GP1-blocked functions are not random functions but are components of a linear pathway in which an event (or function) depends on a prior event. Therefore, it appears that these functions may be a part of a pathway which is closely linked temporally to the commitment point and a major control point in the differentiation program.

The double-block procedure is designed to order the sequence of events (or functions) in the pathway. The double-block procedure involves two experiments. In the first experiment, the ts mutant is induced with DMSO at the restrictive temperature and subsequently incubated at a permissive temperature with 3AB and DMSO. If there is a linear pathway in which the ts function precedes the 3AB-blocked function, then differentiation would be inhibited, but if the 3AB-blocked function precedes the ts function, then differentiation should occur. In the second experiment, the blocks are applied in reverse order, and the conclusions of the first experiment can be confirmed. The double-block procedure is basically similar to a method used to order gene-controlled steps and demonstrate dependent pathways in the yeast cell cycle (21, 22). We have included several additional controls so that each experiment (Table 1, A and B) is internally controlled and can be evaluated independently. Our interpretation of the double-block experiment is a linear pathway with an invariant order: 3AB-blocked function → tsC2GP1-blocked function - commitment → hemoglobin (benzidine-positive cells). The tsC2GP1 function and commitment are bracketed since these experiments do not distinguish between these functions.

The studies presented here have shown that both 3AB and DEX appear to inhibit a step(s) that is critical for commitment. Other investigators have noted that inhibitors of poly(ADP-ribose) polymerase and DEX inhibit ADP-ribosylation (23) and that both inhibitors similarly affect the expression of mouse mammary tumor virus expression (24). A common action of the glucocorticoid hormone and poly(ADP-ribose) polymerase inhibitor might be rationalized if the protein target of the hormone itself were ADP-ribosylated or if the hormone interacted with an ADP-ribosylated protein. Attempts to distinguish the 3AB- and DEX-blocked functions using the double-block procedure have been unsuccessful.

In summary, these studies, involving ts mutants and inhibitors of the differentiation pathway of the FEL cell, have indicated that some of the events preceding the commitment point are temporally arranged in a linear pathway with an invariant order. The convergence of ts mutant and inhibitor-blocked functions just prior to the time of commitment suggests that the blocked functions may be part of a major regulatory control mechanism for commitment.
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