Decreased Alkaline Phosphatase in Cells Transformed by Rous Sarcoma Virus

Artrice V. Bader, Joan Kondratick, and John P. Bader

ABSTRACT

Chick embryo cells transformed by either of two strains of Rous sarcoma virus (Bryan high titer or Schmidt-Ruppin) have low levels of alkaline phosphatase activity compared with nontransformed chick embryo cells. Essentially no differences in acid phosphatase activity were observed between these transformed and nontransformed cells. A virus mutant, RSV-BH-Ta, induces temperature-dependent transformation in infected cells. At 41°C, the transformation-nonpermissive temperature, alkaline phosphatase activities were similar to those of chick embryo cells. Shifting these cells to 37°C resulted in a change to transformed morphology and a progressive loss of enzyme activity, requiring 18 to 24 hr to reach the level of transformed cells. Rat embryo cells transformed by murine sarcoma virus also contained lower alkaline phosphatase levels than did nontransformed cells. These observations suggest that decreased alkaline phosphatase activities may be a general property of transformed cells.

INTRODUCTION

A number of metabolic changes occur after cells become transformed by tumorigenic viruses. Several of these changes have been found in RSV-transformed CE cells, including increased uptake of glucose and possibly of other exogenous metabolites (4, 8), increased hyaluronic acid synthesis (6, 91), increased protease synthesis (22), and decreased cyclic adenosine monophosphate levels (16). Increases in water content (4) and in uptake of sodium ions (unpublished observations) are characteristics of cells transformed by the Bryan strain of RSV (RSV-BH) and are accompanied by vacuolization of the cytoplasm (2, 3, 5). The association of water and ions with vacuoles has been observed in other organisms, e.g., the contractile vacuole of certain protozoa. Micropuncture analysis of amöebas indicated that the contractile vacuole was involved in osmoregulation (17), and cytochemical studies with electron microscopy revealed a concentration of alkaline phosphatase in the membrane of this vacuole (7). This association of water and ions in vacuoles containing alkaline phosphatase prompted us to investigate the activity of alkaline phosphatase in cells transformed by RSV-BH. Unexpectedly, these transformed cells were found to contain enzyme activities substantially lower than activities of nontransformed cells.

Vacuoles found in RSV-BH-transformed cells are similar to but morphologically distinguishable from most described lysosomes. Organelles suspected to be lysosomes have been shown to contain acid phosphatase activity (1, 12). For clarification of a possible relationship of vacuoles to lysosomes, an examination of acid phosphatase activity was made. No substantial differences in acid phosphatase activity were observed between vacuolated and nontransformed cells.

A mutant of RSV-BH, RSV-BH-Ta, induces temperature-dependent transformation in infected cells, but after a shift of temperature from 41 to 37°C all of the characteristics of transformed cells appear, including cytoplasmic vacuolization (2, 3, 5). Vacuolization in this system develops without a requirement for new RNA or protein synthesis, allowing the possibility of a controlled examination of the changes of alkaline phosphatase levels relative to vacuolization.

Another strain of RSV, the Schmidt-Ruppin strain (RSV-SR), transforms cells without inducing vacuolization (5). Possible changes in alkaline phosphatase levels in nonvacuolated transformed cells were examined in such RSV-SR-infected cells, and decreased enzyme levels were found to be associated with transformation rather than with vacuolization.

The generality of the decrease in alkaline phosphatase levels in transformed cells was further examined in rat embryo cells transformed with MSV (Moloney strain). Decreased levels of enzyme were found in these mammalian cells as well, suggesting that a low level of alkaline phosphatase is a general property of transformed cells.

MATERIALS AND METHODS

Virus. The Bryan "high titer" strain of RSV (RSV-BH), a transforming virus, is mixed with Rous-associated virus (RAV), a subgroup A nontransforming avian leukemia virus. The mutant RSV-BH-Ta, also called tdBEIBH, transforms infected cells at 37°C, but when placed at 41°C these infected cells revert to normal phenotype. RSV-BH-Ta stocks also contain RAV and RAVo, but the presence of these viruses has no effect on the transformation process. Another transforming virus, the Schmidt-Ruppin strain of RSV (RSV-SR), transforms CE cells to a phenotype morphologically different from RSV-BH or RSV-BH-Ta. The Moloney strain of MSV, a reproduction-defective virus, contained a murine leukemic virus (Rauscher strain) as a helper virus in mixed infections.

Cell and Media. CE cells were prepared from 10-day-old embryos and replated at 2- to 3-day intervals. Cultures...
were grown in Eagle's minimal essential medium supplemented with dextrose (2 g/liter final concentration), sodium pyruvate (5 mm), 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), 5% fetal bovine serum, penicillin (50 units/ml), streptomycin (50 μg/ml), tylosin (50 μg/ml), and gentamicin (20 μg/ml). Cultures were maintained in humidified CO₂ atmosphere incubators at 41, 39, or 37°, as dictated by individual experiments.

Cells were infected (2 to 10 focus-forming units/cell) as secondary cultures within 24 hr after transfer and redistributed at 2- to 3-day intervals thereafter. Transformation was evident in infected cells within 1 or 2 days after infection, and practically all cells in infected cultures were transformed within 6 days after infection. Infected cultures were used for experiments on the second day after replating before cells became confluent.

**Assay for Enzyme Activity.** Culture plates were washed 2 times with Tris-buffered 0.9% NaCl solution (pH 7.4) prior to adding 0.5 ml of 0.5% Triton X in 10 mm Tris buffer (pH 9.5) for 30 min at room temperature. Plates were gently rotated during this 30-min period. Sets of duplicate or triplicate cultures were examined for each test. Aliquots of 0.2 ml were removed from each of the plates after the 30-min incubation with detergent and frozen at -20° to be used for protein determinations (11). To the remaining 0.3 ml, 5 ml of p-nitrophenyl phosphate (pH 9.5) were added to each plate. The plates were gently rotated, and 1-ml aliquots were immediately removed from each plate and placed in 1 ml of 10% cold trichloroacetic acid to inhibit further reaction. The remaining extract-substrate mixture was incubated at room temperature and monitored visually for the release of p-nitrophenol, recognized by the development of a yellow color. At appropriate times, 90 or 120 min in most assays, a second 1-ml aliquot was mixed with 10% cold trichloroacetic acid. Samples were centrifuged, supernatants were made alkaline again with NaOH, and the yellow color was analyzed spectrophotometrically at 410 nm. Acid phosphatase was measured by essentially the same procedure, except that the reaction progressed at pH 5.0 in sodium acetate buffer.

**RESULTS**

**Cellular Alkaline Phosphatase Activity.** Alkaline phosphatase activity of CE cells and CE cells transformed by the vacuolating virus, RSV-BH, were compared (Table 1). The nontransformed CE cells contained more than 5 times as much activity as did the cells transformed by RSV-BH, as measured during a 90-min incubation. In the same experiment, cells infected with a nonvacuolating transforming virus, RSV-SR, contained even lower activities of alkaline phosphatase, suggesting that the decrease in enzyme activity was associated with transformation and was unrelated to vacuolization.

In similar cultures virtually no differences in acid phosphatase activities were found between transformed and nontransformed CE cells (Table 1).

The kinetics of p-nitrophenol release was examined in these transformed and nontransformed cells to ensure that rates of alkaline phosphatase activity were being determined (Chart 1). Release of p-nitrophenol occurred essentially linearly over the 2-hr time period in extracts of nontransformed CE cells. During the same time period, little activity was seen in extracts from cells transformed by either RSV-BH or RSV-SR.

The relative inactivity of RSV-transformed cells could be a result either of decreased enzyme synthesis or of the synthesis of an inhibitor of CE alkaline phosphatase. Mixing extracts of CE cells and cells transformed by RSV-BH resulted in essentially the same activity as was found in CE cells alone, and no indication of an alkaline phosphatase inhibitor was found.

The Temperature-dependent System. Cells infected with the virus mutant, RSV-BH-Ta, were phenotypically transformed at 37°, but at 41° they behave as nontransformed cells (3). When these RSV-BH-Ta-infected cells were grown at the 2 temperatures, alkaline phosphatase activities were similar to those described previously for transformed and nontransformed cells; enzyme activities were low in cells maintained at the transformation-permissive temperature (37°) and high in cells maintained at the transformation-nonpermissive temperature (41°), (Table 1; Chart 2). Acid phosphatase levels were similar at the 2 temperatures.
Mutant-infected cells were shifted from 41 to 37° for examination of the rate of decline of enzyme activity during the development of the transformed state (Chart 3). A gradual decrease in alkaline phosphatase activity was observed following the temperature shift, but 8 hr after the initial change more than 50% of the original activity still remained. Vacuolization of these cells occurred much more rapidly, as described previously (3), and cells were fully transformed morphologically within 8 hr after temperatures were shifted. When examination was performed at later times, 18 and 24 hr, enzyme levels were commensurate with the levels observed in the transformed cells described previously.

A possible involvement of macromolecular synthesis in the enzyme decrease was examined with actinomycin D and cycloheximide. At levels that effectively inhibited RNA synthesis (actinomycin D, 2 μg/ml) or protein synthesis (cycloheximide, 2 μg/ml), substantial decreases in alkaline phosphatase activities were found in cells incubated at 41°, as well as in cells shifted to 37°, and no conclusion about requirements for RNA or protein synthesis could be drawn. 1-β-D-Arabinofuranosylcytosine (10^-4 M), an inhibitor of DNA synthesis, had no effect on the decrease of alkaline phosphatase activity in cells shifted to 37° under conditions where activity was maintained at 41°.

Mutant-infected cells grown at 37° and containing low levels of alkaline phosphatase activity regained enzymatic activity within 24 hr after shifting to 41°. An exact time course of this reversal was not examined.

Effects of Cell Density. Cell density is known to affect the capacity for glucose uptake (18), the rate of glycolysis (20), and perhaps other metabolic aspects of CE cells (4). The effect of cell density on alkaline phosphatase levels was examined. Levels of enzyme were found to increase with increasing cell density in nontransformed CE cells (Chart 4); about a 4-fold difference in activity was observed between the highest and lowest cell densities tested. Nonetheless, even at the lowest cell densities examined, enzyme activities substantially exceeded those usually found in RSV-transformed cells. In several experiments with RSV-BH-transformed cells, no effect of cell density on alkaline phosphatase activity was found. The decreased cellular growth rate caused by high cell density possibly was responsible for increased enzyme activity. This was examined with the use of 1-β-D-arabinofuranosylcytosine. Inhibition of DNA synthesis and cellular division by 1-β-D-arabinofur-
an osylcytosine (10⁻⁴ M) had little effect on alkaline phosphatase activity over an 18-hr period.

Effects of Glucose Depletion. Cells transformed by RSV-BH have an increased capacity for glucose uptake (4, 8). An increased capacity for glucose uptake also can be induced in nontransformed CE cells by subjecting cells to glucose starvation for 8 hr (13). The possibility that increased uptake of glucose is directly related to lowered alkaline phosphatase levels was examined by measuring alkaline phosphatase activities in nontransformed CE cells depleted of glucose (Table 2). No changes in alkaline phosphatase levels were found, although the rate of glucose uptake, as measured with [³H]-2-deoxy-D-glucose, increased more than 2-fold as a result of glucose starvation.

Effects of Hyperosmolar Medium. An increase in osmolarity of the culture medium with NaCl was reported to increase the activity of alkaline phosphatase in a variety of cells containing initially high or low levels of activity (9, 15). The effects of NaCl concentrations between 100 ml and 200 mn on enzyme activities of CE and RSV-BH-transformed cells were examined. In several experiments, cells were grown for as long as 4 days with daily changes of medium containing the various NaCl concentrations, and alkaline phosphatase activities were determined. Differing NaCl concentrations induced no differences in alkaline phosphatase activity in CE cells or in RSV-BH-transformed cells. Also, no differences in growth of cells attributable to osmolarity of the medium were observed in either CE or transformed cells.

Effects of K⁺ and Ouabain. The possibility that a potassium-dependent ATPase of CE cells contributed to the phosphatase activity that degrades p-nitrophenyl phosphate was examined. The presence or absence of K⁺ in the incubation mixture had no effect on p-nitrophenol release. Also, ouabain, an inhibitor of potassium-dependent ATPase, as well as of Na⁺-K⁺-ATPase, had no effect on alkaline phosphatase activity of CE cells.

Enzyme Activities in Rat Cells. The possibility that decreased alkaline phosphatase activity was peculiar to transformed avian cells was examined by comparing rat embryo cells with these same cells transformed by MSV. Although activities were generally lower in rat embryo cells than in CE cells, essentially the same result was observed (Table 3); MSV-transformed cells contained lower amounts of enzyme activity than did their nontransformed counterparts. As anticipated from the results in CE cells, doubling the number of cells plated resulted in an increased specific activity of alkaline phosphatase in the rat embryo cells but had little effect on the activity of MSV-transformed cells.

Table 2

<table>
<thead>
<tr>
<th>Medium</th>
<th>Alkaline phosphatase activity (µoles/mg protein/hr)</th>
<th>[³H]Deoxyglucose uptake (dpm/mg protein/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ glucose</td>
<td>1.66</td>
<td>8,460</td>
</tr>
<tr>
<td>- glucose</td>
<td>1.76</td>
<td>22,500</td>
</tr>
</tbody>
</table>

DISCUSSION

The possibility that vacuolated transformed cells may contain increased amounts of alkaline phosphatase, suggested by the occurrence of this enzyme in protozoan vacuoles, was not borne out by experiments presented here. In contrast, lower alkaline phosphatase activities were found in RSV-BH-transformed cells than in nontransformed CE cells. The decreased alkaline phosphatase activities were found to be a general property of virus-transformed cells. CE cells transformed by another strain of RSV, RSV-SR, as well as rat embryo cells transformed by a murine tumor virus, MSV, contained lower alkaline phosphatase activities than did their nontransformed counterparts. In an earlier study, Sela and Sachs (19) reported decreased alkaline phosphatase activities in hamster cells transformed by chemical carcinogens or by the DNA tumor viruses, SV40 and polyoma virus. These observations suggest that low alkaline phosphatase levels are a characteristic property of transformed cells. However, the possibility that low levels of alkaline phosphatase are required for the maintenance of cancer remains to be determined.

The increase in alkaline phosphatase activity with increasing cell density and decreasing cellular growth rates superficially suggests that the enzyme is involved in regulation of cellular growth. A role for alkaline phosphatase in regulation of DNA synthesis was suggested by Melnykovych et al. (14), who found fluctuations in enzyme activity during various stages of the cell cycle. Nonetheless, no cause-effect relationship has been established. The mere inhibition of DNA synthesis is insufficient to induce alkaline phosphatase, as shown here by the failure of 1-β-D-arabinofuranosylcytosine to affect enzyme levels. It seems unlikely, therefore, that the induction of alkaline phosphatase by bromodeoxyuridine, as shown by Koyama and Ono (10), is due to the effect of bromodeoxyuridine on DNA synthesis.

Growth rates of nontransformed CE cells in sparse culture are similar to those of RSV-BH-transformed cells, but enzyme levels of the transformed cells were consistently lower than those of nontransformed cells. An opposite effect of transformation is observed in cellular capacity for glucose uptake. Transformed cells take up glucose at more than double the rate of nontransformed cells, even when growth rates are similar (4, 8). Also, in nontransformed cells the capacity for glucose uptake decreases with increasing cell density and lower growth rates (18), in contrast to the increased activity of alkaline phosphatase occurring under the same conditions. It is possible that
alkaline phosphatase levels have something to do with capacity for glucose uptake. For example, differential rates of hydrolysis of phosphorylated glucose, or glucose analogs, would allow variable escape of the test sugar from cells, affecting measurements of uptake. It seems more likely, however, that alkaline phosphatase and glucose uptake are inversely coordinate activities, affected in opposite ways by identical physiological conditions. Alkaline phosphatase activity and capacity for glucose uptake are not stringently coordinated, however. As shown here, induction of increased capacity of glucose uptake by glucose starvation failed to affect the activity of alkaline phosphatase.

The idea that vacuoles of RSV-BH-transformed cells are lysosomes, while not eliminated, receives no support from these experiments. Little, if any, difference in acid phosphatase activity was seen between RSV-BH-transformed, vacuolated cells and nontransformed, nonvacuolated CE cells. In other studies, mammalian cells that were induced from low to high alkaline phosphatase activity exhibited no changes in acid phosphatase activity (10). The relative unresponsiveness of acid phosphatase activities to varying physiological conditions suggests that this activity is a constitutive activity not under strict cellular regulation.

REFERENCES

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