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Appearance of a cytosolic protein that stimulates glyceraldehyde-3-phosphate dehydrogenase activity during initiation of renal epithelial cell growth

(BSC-1 cells/K-depleted rats/mouse fibroblasts/glycolysis)

H. N. AITHAL, MARGARET M. WALSH-REITZ, AND F. GARY TOBACK

Department of Medicine, The University of Chicago, Chicago, Illinois 60637

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ABSTRACT **Rats fed a K-deficient diet exhibit accelerated kidney growth and enhanced activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PD; D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase, EC 1.2.1.12). Cultures of renal epithelial cells (BSC-1 line) served as a model for this phenomenon because a reduction in the medium K concentration from 5.4 to 3.2 mM resulted in augmented growth and increased G3PD activity. Mixing the soluble supernatant fraction from cells grown in low-K medium (3.2 mM) with that from cells grown in control medium indicated that the cytosol of low-K cells contained a positive modifier of G3PD activity. Appearance of modifier activity that ocurred within 1 hr after exposure of cells to low-K medium was blocked by cycloheximide but not by actinomycin D. Modifier activity was also observed in mouse fibroblasts stimulated by low-K medium and in proliferating renal medullary tissue from rats fed a K-deficient diet. A single protein that contained G3PD-stimulating activity was isolated from the soluble supernatant of BSC-1 cells exposed to low-K medium. This protein was not detectable in control cells. The material yielded a single band on NaDodSO$_4$/polyacrylamide gel electrophoresis with an apparent M_r of 62,000. These results suggest that a new protein with the capacity to stimulate G3PD activity appears in the cytosol during the initiation of cell growth.**

Little is known about the mechanisms by which a mitogenic signal is transduced into the intracellular biochemical events that lead to initiation of DNA synthesis and cell growth. The Warburg effect is a familiar example in which malignant growth is linked in an unknown way to an alteration in cell metabolism—i.e., activation of aerobic glycolysis (1–3). In kidney tissue from rats fed a K-deficient diet, both accelerated cell growth and enhanced glycolysis are also observed, and both can be reversed by the return of K to the diet (4–6). Although there is generalized renal growth, proliferation is most striking in epithelial cells of the collecting tubules in the inner stripe of the red medulla (5, 6). The physiological purpose of the adenomatous hyperplasia in this nephron segment is not known but could represent an attempt by the organ to increase tubular absorptive surface area to limit further loss of K in the urine.

We recently observed that renal growth induced in rats fed a K-deficient diet can be simulated in tissue culture by exposing renal epithelial cells of the BSC-1 line to medium with a decreased K concentration (7). This experimental manipulation induced accelerated growth of BSC-1 cells and increased uptake of glucose analogs (7).

We have now observed that rapid activation of a key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (G3PD; D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase, EC 1.2.1.12), occurs in these growing cells. Study of the mechanism of G3PD activation revealed that a new protein with the capacity to stimulate the enzyme appeared in the cytoplasm after exposure of cells to the mitogenic signal.

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MATERIALS AND METHODS

Renal Tissue from K-Depleted Rats. Male Sprague–Dawley rats (Sprague–Dawley Farms, Madison, WI) weighing 200–250 g each were fed a K-deficient diet for up to 8 days (5). Slices from the inner stripe of renal red medulla were cut with a Stadie–Riggs microtome (8). A 10% (wt/vol) tissue homogenate was prepared in 0.25 M sucrose containing 10 mM Tris·HCl (pH 7.5). Cell debris, nuclei, and mitochondria were sedimented by centrifugation at 9,000 × *g* for 10 min at 0–4°C and discarded. The remaining supernatant was centrifuged at 100,000 × *g* for 60 min at 0–2°C to remove microsomes and was then used as an enzyme source for determination of G3PD activity.

The effect of low-K diet on renal DNA synthesis was assessed in medullary slices. Each slice (22–43 mg) was incubated in 3 ml of Krebs–Ringer bicarbonate medium containing [*methyl*-^{3}H]thymidine (6.7 Ci/mmol, 2.7 μCi per flask, New England Nuclear; 1 Ci = 3.7 × 10^{10} becquerels) for 1 hr as described (9). Tissue DNA was estimated by the method of Giles and Meyers (10).

Cell Cultures. Epithelial cells from the African green monkey kidney line (BSC-1) (11, 12), a gift from R. W. Holley (Salk Institute, La Jolla, CA), and a strain of mouse fibroblasts, line clone 1 D (13), a gift from G. S. Getz (University of Chicago), were used for study. BSC-1 cells were grown in Dulbecco–Vogt modified Eagle medium (containing 25 mM glucose) with 1.6 μM biotin and 1% calf serum as described (12). High-density cultures were prepared by plating 2 × 10^6 cells in 100-mm plastic dishes (Falcon), and the medium was changed twice per week until there were 6–7 × 10^6 cells per dish. Three days after the last medium change, the cells were washed with modified Eagle medium, and fresh medium containing 16 μM biotin and 0.01% serum was added. The cultures became quiescent and were used for study 3 days later. Low K cells and control cells were obtained by aspirating the spent medium and then adding fresh medium containing K at 3.2 mM (low-K) or 5.4 mM (control) and 0.5% serum; the cells were used for study 1 hr later unless otherwise indicated.

Mouse fibroblasts were treated as above except that 9 × 10^6 cells were plated and 30 × 10^6 cells per dish were obtained in high-density quiescent cultures.

Preparation of Soluble Supernatant Fraction. Cells were scraped into a tube, suspended in 0.25 M sucrose containing 10

Abbreviations: G3PD, glyceraldehyde-3-phosphate dehydrogenase; P$_i$/NaCl, phosphate-buffered saline.

mM Tris·HCl (pH 7.5), and collected by centrifugation. The cells were resuspended in dilute phosphate-buffered saline (P_i/NaCl) (1:5) and homogenized. Sufficient 0.45 M sucrose was added to bring the sucrose concentration in the homogenate to 0.25 M. The soluble supernatant fraction of the homogenate was prepared as described above for rat renal tissue.

Enzyme Assays. The activities of G3PD and 6-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) in the soluble supernatant were determined by standard procedures (14, 15). For the determination of kinetic constants for G3PD activity the two invariant substrates were held at the concentrations specified by Wang and Alaupovic (14). Protein was estimated by the method of Lowry *et al.* (16). The results were expressed as μmol of NAD^+ reduced per min per mg of protein.

Soluble supernatant fractions from control and low-K cells were mixed in different proportions as specified. Each fraction was added to 0.5 ml of 0.1 M Tris·HCl (pH 8.6) and preincubated for 2 min at room temperature before addition of the reactants required to perform the enzyme assay (final volume, 1 ml). The predicted value for enzyme activity was calculated from each of the known activities in the mixture.

Purification of Positive Modifier. BSC-1 cells (control or low-K) from 30 dishes were scraped into a tube and suspended in 0.25 M sucrose/10 mM Tris·HCl, pH 7.5, and the soluble supernatant fraction was prepared as described above. This material was applied to a column of blue Sepharose CL-6B (Pharmacia) (1 × 30 cm) which had been equilibrated with P_i/NaCl (pH 6.8) and was eluted with 30 ml of P_i/NaCl. The capacity of material in each 0.4-ml fraction to stimulate G3PD activity in soluble supernatant prepared from control cells was assayed as described above. The presence of G3PD-stimulating activity in a fraction indicated that it contained the positive modifier. The fractions that comprised the void volume were free of G3PD activity but did contain the positive modifier and were subjected to further purification. Protein eluted from the column was monitored spectrophotometrically by measuring absorbance at 280 nm. G3PD activity bound to blue Sepharose CL-6B was eluted from the column with 10 mM Tris·HCl, pH 8.6/10 mM NAD^+ (17). Recovery of enzyme activity (45%) was similar in low-K and control cells and to that reported (17).

Fractions containing modifier activity were dialyzed against 1 mM phosphate buffer (pH 6.8), lyophilized, and then dissolved in 0.25 ml of P_i/NaCl. This material was applied to a column of Sephadex G-150 (medium; Pharmacia) (1 × 50 cm) that had been equilibrated with P_i/NaCl (pH 6.8). The column was eluted with P_i/NaCl, and fractions containing modifier activity were pooled, dialyzed, and lyophilized. The powder obtained was dissolved in 0.2 ml of 10 mM Tris·HCl (pH 7.8) and applied to a column of DEAE-Sephacel (Pharmacia) (0.7 × 20 cm) equilibrated with 10 mM Tris·HCl (pH 7.8) and eluted with a linear 0–0.5 M NaCl gradient (30 ml) in this buffer. The fractions exhibiting modifier activity were subjected to dialysis and lyophilization as described above, and the material obtained was dissolved in 0.2 ml of 10 mM phosphate buffer (pH 6.8) and applied to a column of Cellex-CM (Bio-Rad) (0.7 × 10 cm) equilibrated with 10 mM phosphate buffer (pH 6.8). Elution was with 10 mM phosphate buffer (pH 6.8) with a linear 0–0.5 M NaCl gradient (25 ml). Modifier fractions were treated as above, dissolved in 0.2 ml of P_i/NaCl (pH 6.8) and applied to a column of Sephadex G-150 (1 × 50 cm) equilibrated with P_i/NaCl. Elution of the column with P_i/NaCl resulted in a single protein peak which contained modifier activity. $NaDodSO_4$/polyacrylamide disc gel electrophoresis of this material (10 μg) was carried out in a 5% polyacrylamide gel (18, 19). The gel was stained with Coomassie blue and destained by the method of Weber and Osborn (19).

Data were compared by Student's *t* test; *P* values < 0.05 were accepted as significant.

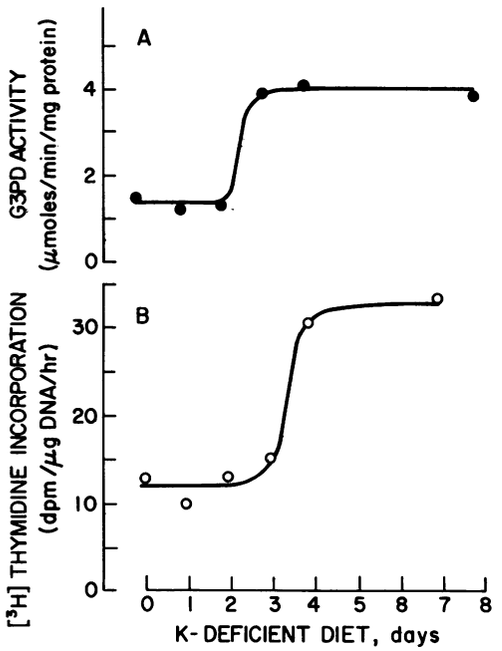

FIG. 1. G3PD activity and initiation of DNA synthesis in kidney tissue from K-depleted rats. Enzyme activity in the soluble supernatant fraction (*A*) and [*methyl*-^{3}H]thymidine incorporation into DNA (*B*) in slices from the inner stripe of kidney red medulla were measured in animals fed a K-deficient diet. Each value is the mean for three to six rats (SEM, <5%).

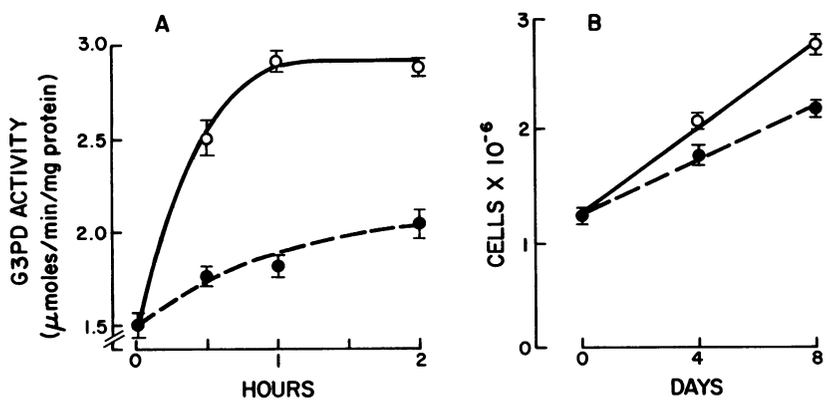

FIG. 2. Effect of low-K medium on G3PD activity and growth of BSC-1 cells. (*A*) G3PD activity in the soluble supernatant fraction of high-density quiescent cultures was measured at different times after exposure of the cells to medium with K at 3.2 mM (low-K) (○) or 5.4 mM (control) (●) and 0.5% serum. Each value is the mean ± SEM for three or four experiments performed in duplicate. (*B*) Cells were plated in medium with 1% serum and grown to confluence in 60-mm dishes as shown on day 0 on the graph. Sets of plates were then changed to medium with K at 3.2 mM (○) or 5.4 mM (●) and 0.5% serum; the medium was changed twice per week. For counting, cells were washed, detached with crystalline trypsin, dispersed, and counted in a hemocytometer (12). Values are means ± SEM of at least six dishes of cells.

RESULTS

Growth and G3PD Activity in Renal Cells. G3PD activity in the soluble supernatant fraction was increased in renal tissue obtained from the inner stripe of red medulla of rats fed a K-deficient diet for 3 days (Fig. 1*A*). This increment in enzyme activity preceded the increase in [*methyl*-^{3}H]thymidine incorporation into DNA, which occurred on day 4 in slices obtained from this renal zone (Fig. 1*B*).

High-density quiescent cultures of renal epithelial cells from the BSC-1 line were exposed to low-K medium to simulate K-depletion *in vivo*. This maneuver stimulated G3PD activity in the soluble supernatant fraction by 40% after 30 min of exposure to low-K medium and by 60% after 2 hr ($P < 0.001$) (Fig. 2*A*). Treatment of BSC-1 cells with two known growth-promoting stimuli, calf serum (10%) or mouse epidermal growth factor (50 ng/ml), for 1 hr had different effects on G3PD activity: serum augmented activity to the same extent as did exposure to low-K medium, whereas the growth factor had no effect. The activity of 6-phosphofructokinase was not altered in cells exposed to low-K medium for 1 hr. Growth of BSC-1 cells was 30% greater in low-K medium than in control medium after 8 days ($P < 0.02$) (Fig. 2*B*).

G3PD-Stimulating Activity in the Soluble Supernatant Fraction of BSC-1 Cells Exposed to Low-K Medium. When supernatant from cells exposed to low-K medium was mixed with supernatant from control cells, G3PD activity was greater than predicted (Table 1). A small amount of supernatant protein (0.6 μg) from low-K cells stimulated G3PD activity in control supernatant (4 μg) by the maximal amount (60%) (Fig. 3). These mixing experiments indicated that a positive modifier of G3PD activity was present in the supernatant of cells exposed to low-K medium.

The supernatant from low-K cells retained its capacity to stimulate G3PD activity (in the mixing experiment) after dialysis at 4°C overnight. However, boiling abolished this stimulatory effect, which suggested that the positive modifier could be a thermolabile protein. Inclusion of cycloheximide in the medium during exposure of BSC-1 cells to low-K medium eliminated the stimulatory activity, whereas actinomycin D did not (Fig. 4). Thus, appearance of the positive modifier was apparently dependent on the synthesis of one or more proteins and was independent of DNA-dependent RNA synthesis.

Table 1. G3PD-stimulating activity in BSC-1 cells exposed to low-K medium

Soluble supernatant from	G3PD activity, μmol/min/mg protein
Quiescent cells	1.24 ± 0.05
Control cells	1.37 ± 0.03
Low-K cells	2.31 ± 0.05*
Control cells + low-K cells (mixture):	
4.0 μg + 0.6 μg	2.50 ± 0.09* (1.50 ± 0.06)†
2.2 μg + 3.0 μg	2.58 ± 0.11* (1.91 ± 0.07)†

High-density quiescent cultures of BSC-1 cells were exposed to low-K medium for 1 hr. The mixture of supernatant fractions from control cells and low-K cells contained the amounts of protein shown. Each value represents the mean ± SEM of at least three experiments performed in triplicate. The difference in enzyme activity between control cells and quiescent cells is not significant.

* G3PD activity in supernatants from low-K cells was greater than that in control cells ($P < 0.001$).

† Values in parentheses are those predicted from the observed specific activity of each supernatant added to the mixture. Observed values for G3PD activity in the mixture of supernatants were greater than predicted ($P < 0.001$).

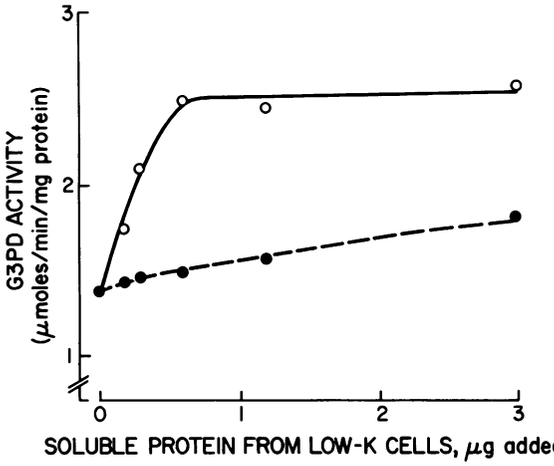

FIG. 3. Stimulation of G3PD activity in the soluble supernatant fraction of control high-density BSC-1 cells by addition of supernatant from cells exposed to low-K medium for 1 hr. Values for enzyme activation obtained by mixing (○) were greater than predicted (●). Each point is the mean of two determinations with variance <10%.

The effect of the positive modifier from the supernatant of low-K cells on G3PD activity from control cells was assessed by measuring the K_m and V_{max} of each substrate in the reaction: glyceraldehyde 3-phosphate, NAD^+, and arsenate in place of phosphate. Arsenate was used because G3PD does not distinguish between it and phosphate, and the instantaneous arsenolysis of 1-arseno-3-phosphoglycerate that follows its formation

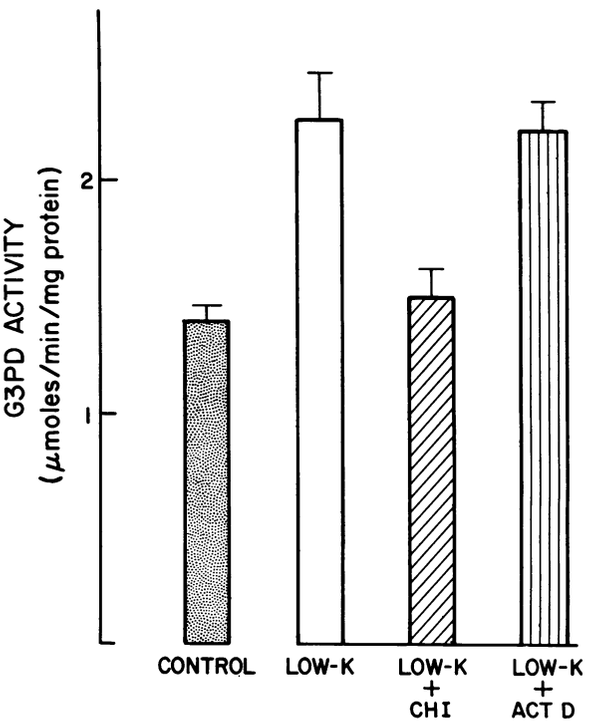

FIG. 4. Effect of cycloheximide and actinomycin D on G3PD activity in BSC-1 cells exposed to low-K medium. High-density quiescent cells were exposed to medium containing K at 3.2 mM (low-K) or 5.4 mM (control) and 0.5% serum for 1 hr. Cycloheximide (100 μg/ml) (CHI) or actinomycin D (25 μg/ml) (ACT D) was added to low-K cells at 0 time. Cycloheximide and actinomycin D inhibited [^{14}C]leucine and [^{3}H]uridine incorporation, respectively, into acid-insoluble material by 95% under these conditions. Each value represents the mean ± SEM for three experiments performed in duplicate. The increment in G3PD activity observed in low-K cells compared to control ($P < 0.005$) was abolished in the presence of cycloheximide.

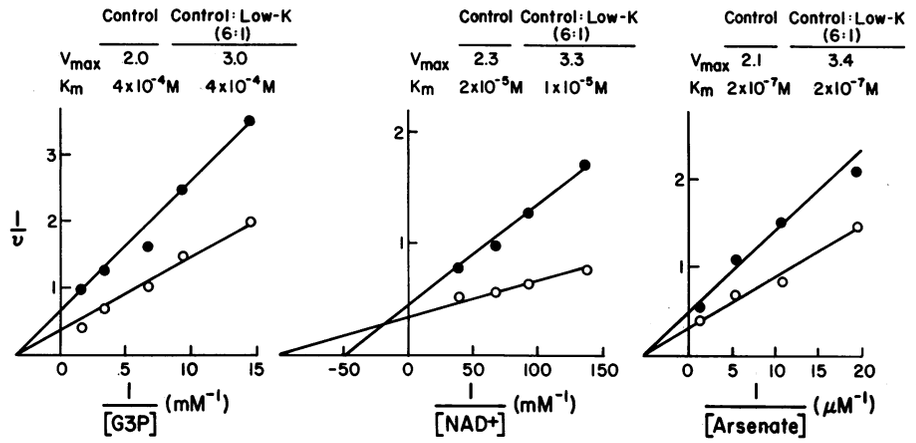

FIG. 5. Stimulation of G3PD activity in the soluble supernatant of control high-density BSC-1 cells by addition of supernatant from cells exposed to low-K medium for 1 hr. The mixture of supernatant fractions from control cells and low-K cells contained protein in the ratio 5 μg:0.8 μg. G3PD activity in the mixture (○) is compared to activity in control supernatant (●) for each of the three substrates in the reaction. Each value is the mean of two experiments performed in duplicate with variance <10%. G3P, glyceraldehyde 3-phosphate.

drives the reaction to completion. The V_{max} for each substrate was increased by about 50% by the addition of 1 vol of low-K supernatant to 6 vol of control supernatant (Fig. 5). The apparent K_m for NAD^+ was halved by low-K supernatant whereas the apparent K_m for each of the two other substrates did not change. A similar decrease in apparent K_m was observed when supernatant from low-K cells alone was compared directly to that of control cells, which indicated that the effects observed in the mixing experiments were maximal. These results show that the positive modifier from low-K cells alters the kinetic properties of the enzyme directly or indirectly.

Exposure of mouse fibroblasts to low-K medium stimulated G3PD activity by 75% (Table 2) and enhanced cell growth (data not shown). G3PD-stimulating activity was detected in the supernatant fraction of epithelial and fibroblastic cells exposed to low-K medium and of kidney medullary tissue from K-depleted rats by the results of the mixing experiments (Table 2).

Isolation of a Positive Modifier of G3PD Activity from Soluble Supernatant Fraction of BSC-1 Cells Exposed to Low-K Medium. Modifier activity was separated from G3PD activity by passing the supernatant fraction from low-K cells through a column of blue Sepharose CL-6B. The void volume contained modifier activity but was free of G3PD activity. In contrast, neither modifier nor G3PD activity was present in the void volume obtained from the soluble supernatant of control cells. Enzyme activity eluted from the column with NAD^+ was similar in low-K and control preparations (3.2 and 3.4 μmol/min per mg of protein, respectively). Addition of void volume fractions containing modifier to the eluted enzyme fraction from low-K or control preparations stimulated G3PD activity to the same extent. Thus, a positive modifier was responsible for the augmented G3PD activity observed in low-K cells (Fig. 2*A*).

The isolation procedure was begun with 12.5 mg of soluble supernatant protein from low-K cells. Passage through a blue Sepharose CL-6B column yielded 5.2 mg of protein in the void volume, and subsequent purification on a Sephadex G-150 column gave 1.8 mg of protein in a broad peak. This material was purified further on columns of DEAE-Sephacel, Cellex-CM, and Sephadex G-150 which resulted in a single peak that contained 30 μg of protein (Fig. 6). A 50% stimulation of G3PD activity in control supernatant (7.5 μg of protein) was achieved by adding progressively smaller amounts of protein in fractions from low-K cells obtained during the purification procedure: 1.6 μg of soluble supernatant protein from low-K cells, 1.1 μg of protein from the void volume of the blue Sepharose column, and 0.2 μg of protein from the peak isolated from the final Sephadex G-150 column. The last material exhibited a single band on $NaDodSO_4$/polyacrylamide disc gel electrophoresis when stained with Coomassie blue and had an apparent molecular weight of 62,000 (Fig. 6 *Inset*).

DISCUSSION

The results show that the onset of renal epithelial and fibroblastic cell growth induced by low-K medium was associated with activation of a key glycolytic enzyme, G3PD. This increase in enzyme activity in renal epithelial cell cultures was apparently mediated by a new cytosolic protein with an apparent molecular weight of 62,000.

It is not clear from the present experiments whether the pos-

Table 2. G3PD activity in kidney epithelial cells and mouse fibroblasts exposed to low-K medium and in kidney tissue from K-depleted rats

Enzyme source	Activity, μmol/min/mg protein: Control	Activity, μmol/min/mg protein: Low-K	Stimulation by low-K, % above control	Mixture, % above predicted
Monkey kidney epithelial cells (BSC-1 line)	1.4 ± 0.03	2.3 ± 0.05*	64	66
Rat kidney tissue (inner red medulla)	2.5 ± 0.06	3.5 ± 0.04*	40	54
Mouse fibroblasts (line clone 1 D)	1.2 ± 0.02	2.1 ± 0.04*	75	33

High-density quiescent cultures of BSC-1 cells and fibroblasts from line clone 1 D were exposed to low-K medium for 1 hr. Rats were fed the K-deficient diet for 4 days before kidney tissue was obtained. G3PD activity was assayed in the soluble supernatant fraction prepared from each cell type. The mixture of supernatant fractions from control cells and low-K cells contained protein in the ratio of 6:1. Values are means ± SEM for at least three determinations.

* G3PD activity in low-K cells or tissue from K-deficient rats was greater than its respective control ($P < 0.001$).

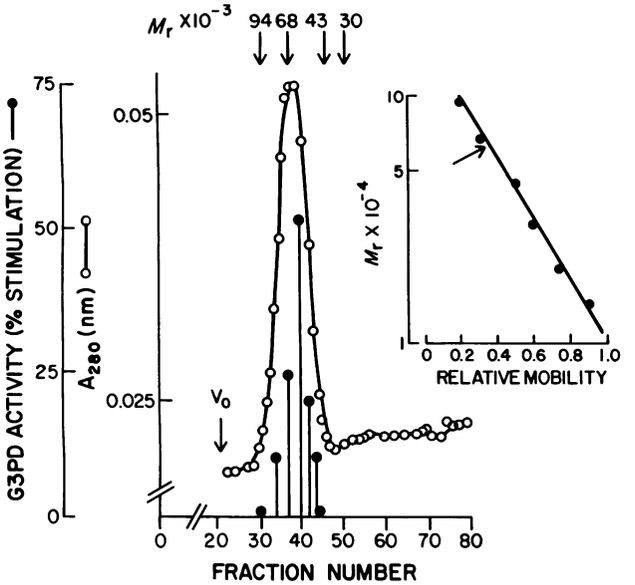

FIG. 6. Characterization of a protein that stimulates G3PD activity. The protein was isolated from the soluble supernatant fraction of high-density quiescent BSC-1 cells exposed to low-K medium for 1 hr and was assayed for G3PD-stimulating activity in supernatant from control cells. The stimulator of G3PD activity was purified by chromatography on blue Sepharose CL-6B, Sephadex G-150, ion-exchange columns, and then Sephadex G-150. The profiles of G3PD-stimulating activity and protein (A_{280}) eluted from the final Sephadex G-150 column are shown. The vertical arrows indicate the position of protein samples of known molecular weight (from left to right: phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase). (*Inset*) Apparent molecular weight of the protein peak obtained from the Sephadex G-150 column displayed after NaDodSO$_4$/polyacrylamide disc gel electrophoresis. The arrow indicates that the apparent molecular weight of the protein was 62,000. The standards used were (from top to bottom): phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lactalbumin. V_0, void volume.

itive modifier activates G3PD directly or exerts its effect through one or more intermediate molecules. Intrinsic enzyme activity in low-K and control cells appeared to be similar after removal of modifier activity from enzyme activity in low-K cells by affinity chromatography. Thus, enzyme activity was not permanently altered by exposure to the low-K growth stimulus but was only increased by the presence of the modifier. Furthermore, the modifier stimulated G3PD activity derived from low-K and control cells equally well. The modifier did not appear to increase G3PD activity by activation of previously inactive molecules in the soluble supernatant because: (*i*) enzyme activity eluted from the blue Sepharose column was similar in low-K and control cells, and (*ii*) the apparent K_m for one of the substrates (NAD^+) was altered (Fig. 5).

The relationship between the onset of cell growth and activation of G3PD is not known. Experiments in K-depleted rats indicate that the Na content of growing renal tissue is increased (20). This finding and observations in other cell types studied in culture indicate that enhanced Na influx (21–23) and stimulation of glycolysis (24, 25) are early events during mitogenesis. We propose that augmented Na influx during the onset of low-K induced growth could activate Na,K-ATPase and thereby increase hydrolysis of ATP. The P_i released from ATP could serve as a substrate for G3PD and facilitate glycolysis and regeneration of ATP (14, 26). ADP released by ATP hydrolysis could be reutilized for ATP synthesis in the phosphoglycerate kinase and pyruvate kinase reactions. Stimulation of G3PD activity by the appearance of its positive modifier would be expected to enhance glycolytic ATP production for use by Na,K-ATPase and other energy-requiring reactions during cell proliferation. This proposal suggests that one step in the mechanism by which an extracellular mitogenic signal is transduced into an intracellular event is the generation of a cytosolic protein that activates a glycolytic enzyme in the growing cell. Further studies will be required to determine if the appearance of this protein is necessary for specific growth-promoting stimuli to accomplish the initiation of DNA synthesis (27, 28).

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