The intracellular redox potential, which is determined by the level of oxidants and reductants, has been shown to play an important role in the regulation of cell growth. The principal intracellular reductant is NADPH, which is mainly produced by the pentose phosphate pathway through the actions of glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway, and by 6-phosphogluconate dehydrogenase. Previous research has suggested that an increase in G6PD activity is important for cell growth. In this article, we suggest that G6PD activity plays a critical role in cell growth by providing NADPH for redox regulation. The results show the following: 1) inhibition of G6PD activity abrogated growth factor stimulation of [3H]thymidine incorporation in all cell lines tested; 2) overexpression of G6PD stimulated cell growth, as measured by an increase in [3H]thymidine incorporations as compared with cells transfected with vector alone; 3) inhibition of G6PD caused cells to be more susceptible to the growth inhibitory effects of H2O2; 4) inhibition of G6PD led to a 30–40% decrease in the NADPH/NADP ratio; and 5) inhibition of G6PD inhibited cell anchorage and significantly decreased the growth-related stimulation of tyrosine phosphorylation.

Intracellular redox regulation is important for the regulation of cell growth (1–3). A critical modulator of the redox potential is NADPH, the principal intracellular reductant. Glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of the pentose phosphate pathway (PPP, Fig. 1), determines the production of NADPH by controlling the metabolism of glucose via the PPP (4–6).

Previous research has shown an association between the stimulation of cell growth and increased activity of the PPP that occurs over hours to days. For example, 1) kidney hypertrophy due to a variety of growth stimuli (e.g., unilateral nephrectomy or diabetes mellitus) is associated with an increased activity of the PPP due to increased G6PD activity (7, 8), 2) epidermal growth factor and insulin stimulated cell growth and increased G6PD activity in rat liver cells in culture (9), 3) growth hormone stimulated cell growth and increased G6PD activity in rat liver cells in culture (10), and 4) a wide variety of cancers and cultured tumor cells exhibit large increases in G6PD activity (11, 12). These findings suggest that G6PD activity is important for cell growth.

Research from our laboratory and others have shown that, in addition to the long-term stimulation of G6PD by growth factors, there is a stimulation of G6PD activity that occurs within seconds to minutes following exposure to growth factors (13–17). Specifically, our laboratory has shown that following stimulation of rat renal cells to grow using epidermal growth factor (EGF), an increase in the activity of G6PD was observed within seconds, maximal at 1 min, and back to baseline level in 60 min (14). In a search for the mechanism of this rapid activation of G6PD, we discovered that G6PD, an enzyme thought to exist unbound in the cytoplasm, is probably bound to an intracellular structure and translocates following growth factor stimulation (13, 14). Using a permeabilized cell system, we demonstrated that EGF and platelet-derived growth factor (PDGF) stimulate the release of G6PD from permeabilized cells (13, 14). We have further shown that the PDGF stimulation of G6PD translocation is dependent on tyrosine phosphorylation of the PDGF receptor (13) and is likely mediated by the signal transduction proteins phosphatidylinositol 3-kinase and phospholipase C-γ (13).

The previous work implies that there may be a mechanistic relationship between cell growth and G6PD activity. This report is designed to more directly assess the importance of G6PD activity on cell growth. The effects of both increases in G6PD activity via overexpression of G6PD and decreases in G6PD activity via the use of a G6PD inhibitor were used to address the importance and possible roles that G6PD may play in cell growth. The data suggest that proper G6PD activity is important for regulation of intracellular redox level during cell growth. The data further suggest that G6PD activity is important for proper cell anchorage and growth factor-stimulated tyrosine phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—[3H]Thymidine (85 Ci/mmol) was purchased from ICN. Cell culture medium, growth factors, and sera were obtained from Life Technologies, Inc. The TRMP cells (a dog kidney cell line) transfected with wild type PDGF receptors were generously provided by Dr. Jonathan Cooper (University of Washington, Seattle, WA). COS-7 cells were generously provided by Dr. Alex Toker (Harvard Medical School, Boston, MA). All other cells were from ATCC. Expression vector pcDNA3 was purchased from Invitrogen. Rabbit anti-rat antibody to G6PD was generously provided by Dr. Rolf Kletzien (Upjohn). Flag reagents and antibody were purchased from Eastman Kodak Co. Anti-phosphotyrosine monoclonal antibody was obtained from Upstate Bio.
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Measurement of Enzyme Activities—Enzyme activity assays were performed as described previously (13). Briefly, the conversion of NADP⁺ to NADPH catalyzed by the two dehydrogenase enzymes in the PPP was measured by the increase of absorbance at 341 nm due to the conversion of NADP⁺ to NADPH by either G6PD or by the second enzyme 6-phosphogluconate dehydrogenase (PGD). G6PD catalyzes the conversion of glucose 6-phosphate to 6-phosphogluconolactone, which is rapidly hydrolyzed to 6-phosphogluconate (the substrate for PGD).

To obtain accurate enzyme activities for G6PD and PGD activity alone and total dehydrogenase activity (G6PD + PGD) were measured separately. G6PD activity was calculated by subtracting the activity of PGD from total enzyme activity. To obtain the total dehydrogenase activity, substrates for both dehydrogenase enzymes were added to a cuvette. In another cuvette, substrates for the second enzyme, PGD, were added to obtain the activity of this enzyme. Substrate concentrations were (mM): glucose 6-phosphate (0.2), 6-phosphogluconate (0.2), and NADP⁺ (0.1). Samples were added to a cuvette containing buffer (50 mM Tris, 1 mM MgCl₂, pH 8.1). The increase of absorbance was determined in a Hitachi U-2000 dual beam spectrophotometer. Data are expressed in arbitrary absorption units.

Western Blotting—This was performed as described previously (13). Primary antibodies are described in the figure legends.

Reverse-phase high performance liquid chromatography (HPLC) was used with a 5-μm C-18 (25 cm) analytical column (Rennin) on a Hewlett-Packard 1050 HPLC. The mobile phase consisted of two eluents: 0.1 M KH₃PO₄ solution, pH 6.8 (Buffer A), and CH₃OH (Buffer B). The chromatographic conditions were the following: 2 min at 100% Buffer A, 20 min up to 25% Buffer B. The gradient was then returned to Buffer A in 2 min. The flow rate was 1 ml/min. Retention time and peak areas were simultaneously monitored at 254, 260, and 280 nm with a UV detector. Integration of peak areas at 254 nm was obtained for the quantitation of NADPH and NADP. Nucleotide standards included both α and β forms of NADP and NADPH. Quantification of reduced and oxidized nucleotides were calculated as the sum of α and β forms.

Tyrosine Phosphorylation—Confluent and quiescent cultures of Swiss 3T3 fibroblasts were incubated in DMEM in the absence or presence of G6PD inhibitor DHEA for 30 min at 37 °C. Cells were given 5 ng/ml PDGF for 10 min. Cells were then lysed using Nonidet P-40 lysis buffer containing 100 μM Na₃VO₄ and the cell lysates were boiled with SDS-PAGE sample buffer and fractionated on SDS-PAGE. The proteins were then transferred onto membranes and blotted with anti-Tyr(P) monoclonal antibody.

Immunoﬂuorescence Microscopy—Confluent and quiescent Swiss 3T3 cells were incubated for 30 min at 37 °C with either 10% calf serum or 10 ng/ml PDGF in DMEM in the absence or presence of G6PD inhibitor DHEA. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Incubation with monoclonal antibodies was carried out for 60 min using anti-Tyr(P) in 5% dry milk. After several washes with PBS, cells on coverslips were incubated with fluorescein isothiocyanate-conjugated goat anti-rat IgG for another 30 min. After final wash, the coverslips were mounted with mowiol containing p-phenylenediamine (1 mg/ml), an antifade agent.

Cell Adhesion Assay—For the assessment of cell detachment, Swiss 3T3 fibroblasts were cultured on 48-well plates for 24 h, rinsed twice with PBS, and incubated with DMEM in the absence and presence of G6PD inhibitor DHEA, 100 μM H₂O₂ or menadione. Cell detachment was observed under light microscope.

Statistical Analysis—For the statistical analysis, Student’s t test was used.

RESULTS

G6PD Activity Is Higher in Proliferating Cells—It has been reported that G6PD activity increases in growing cells (4, 7, 8). To confirm this, quiescent Balb/c 3T3 ﬁbroblasts were compared with serum-starved cells, and to actively growing cells. Adding 10% calf serum for 1 h stimulated G6PD activity up to 60% as compared with serum-starved cells. G6PD activity in actively growing cells was 3–4-fold higher as compared with serum-starved cells (Fig. 2A). This result suggests that both the stimulation of growth and being in the cell cycle increase...
the activity of G6PD. To determine whether the increase in G6PD activity was due to an increased expression of G6PD protein, Western blot analysis was done. Fig. 2B shows that the expression of G6PD protein in the proliferating cells during the time course of the experiment was similar to slightly increased over that seen in quiescent cells, suggesting that the higher G6PD activity in proliferating cells was likely due to both stimulation of preexisting G6PD enzyme as well as to modest increases in G6PD expression.

If G6PD activity is important for cell growth, we hypothesized that changes in G6PD activity should affect cell growth. The following experiments were designed to test this hypothesis.

**Dehydroepiandrosterone Inhibits the Enzymatic Activity of G6PD in Vivo**—Dehydroepiandrosterone (DHEA) is an endogenous steroid (21, 22). Although DHEA has multiple actions, it is known to be an inhibitor of G6PD (23). It has also been reported that DHEA can inhibit cell proliferation (21, 22, 24). However, other than a report in osteoblasts and osteosarcoma cells using insulin-like growth factor I (22), no one has determined the effect of DHEA on peptide growth factor stimulated cell growth. Initially, it was determined if DHEA inhibited G6PD activity in cultured cells. After incubating cells with or without DHEA for 24 h, the enzyme activity was assayed from the cell lysate. Incubation with DHEA decreased the activity of G6PD in all cell lines tested. The average inhibition by 100 μM DHEA ranged between 40 and 70% depending on the cell line. Representative results are shown in Fig. 3A from Balb/c 3T3 fibroblasts. DHEA inhibited the activity of G6PD in a dose-dependent manner with EC₅₀ of 4 μM. The data also show that DHEA did not inhibit the enzymatic activity of PGD, the second enzyme in the PPP. Fig. 3B shows that DHEA inhibited growth factor-stimulated [³H]thymidine incorporations in a dose-dependent manner. Note that the dose-dependent inhibition of G6PD activity by DHEA (Fig. 3A) was closely correlated with the DHEA-induced suppression of growth factor-stimulated [³H]thymidine incorporations (Fig. 3B). Fig. 3C shows that the decreased G6PD activity by DHEA was not caused by decreased expression level of G6PD protein, as indicated by an immunoblot using a specific antibody to G6PD.

**The Inhibitor of G6PD, DHEA, Abrogates the Stimulation of [³H]Thymidine Incorporation by Peptide Growth Factors and Serum**—To test the inhibitory effect of DHEA on cell proliferation, we examined [³H]thymidine incorporations following growth stimulation of serum-starved cells in a number of cell lines (Table I). PDGF, EGF, and serum increased [³H]thymidine incorporation by 1.3–7.6-fold increase over control (Table I). (Control level of [³H]thymidine incorporation is the level of [³H]thymidine incorporation in the quiescent cells in the absence of growth factor and in the absence of DHEA.) DHEA suppressed the growth factor stimulation of [³H]thymidine in-

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**Fig. 2. G6PD activity is stimulated by serum.** A, three experimental conditions were evaluated in Balb/c 3T3 fibroblasts. Cells were: 1) serum-starved for 48 h, 2) serum-starved for 48 h and then stimulated with 10% calf serum for 1 h, and 3) actively grown in medium containing 10% calf serum. Enzyme activity was measured from the lysate of the same number of cells. The y axis is given in arbitrary absorption units. The data are presented as average ± standard error from four experiments. Each experiment was done in triplicate. B, Western blot analysis of the same cell lysate using a specific antibody to G6PD to show the level of G6PD expression in both quiescent (lane 1) and proliferating (lane 2) Balb/c 3T3 fibroblasts. (*, p < 0.05; **, p < 0.01.)

**Fig. 3. DHEA inhibits the enzymatic activity of G6PD and inhibits the growth factor-stimulated incorporation of [³H]thymidine in a dose-dependent manner.** A, dose-response curve of the inhibition of G6PD activity by DHEA. Lysates from Balb/c 3T3 fibroblasts were assayed for enzyme activity in the presence of various concentrations of DHEA. The activity is expressed as the increase of light absorbance at 341 nm due to the conversion of NADP⁺ to NADPH. The data are presented as average ± standard error from three separate experiments, each run in at least triplicate. B, serum-starved Balb/c 3T3 fibroblasts were treated with various concentrations of DHEA in the presence of 5 ng/ml PDGF for 12 h. After the addition of 0.2 μCi of [³H]thymidine, cells were incubated for another 12 h. At the end of the experiment, the incorporation of [³H]thymidine was determined by scintillation counting of the trichloracetic acid-insoluble fraction. The data are presented as average ± standard error from four experiments, each run in triplicate. C, Western analysis using a specific polyclonal antibody to G6PD. Cell lysates were from Balb/c 3T3 fibroblasts. Lane 1, control cells not exposed to either DHEA or PDGF; lane 2, cells exposed to 100 μM DHEA only; lane 3, cells exposed to 5 ng/ml PDGF only; lane 4, cells exposed to 100 μM DHEA and 5 ng/ml PDGF.

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corporation in all cell lines tested. Specifically, DHEA abolished the PDGF-stimulated \(^{3}H\)thymidine incorporation in Swiss 3T3 and Balb/c 3T3 fibroblasts. DHEA inhibited EGF-stimulated \(^{3}H\)thymidine incorporation in TRMP cells (a dog kidney epithelial cell line that expresses EGF but not PDGF receptors (13, 25)). DHEA also abrogated EGF-stimulated \(^{3}H\)thymidine incorporation in PC12 cells. In addition, DHEA suppressed serum-stimulated \(^{3}H\)thymidine incorporation in PC12 cells and in Rin5mAF, an insulin-secreting cell line.

As noted above, this inhibition of \(^{3}H\)thymidine incorporations by DHEA was dose-dependent. A representative dose-response curve is shown in Fig. 3B (EC\(_{50}\) = 2 \(\mu M\)).

**COS-7 Cells Overexpressing G6PD Have Higher \(^{3}H\)Thymidine Incorporation as Compared with COS-7 Cells Expressing Endogenous Levels of G6PD—**If G6PD activity is important for cell growth, cells overexpressing G6PD should have a higher level of \(^{3}H\)thymidine incorporation as compared with cells expressing endogenous levels of G6PD. COS-7 cells were transiently transfected with either vector alone or with a construct of G6PD tagged with an epitope. Upon indirect immunofluorescence on intact cells and Western blotting, it was determined that approximately 30–50% of the cells expressed FLAG-G6PD. After 3 days of transfection, the cells expressed 2–3-fold higher G6PD levels as detected by Western blot analysis using both a monoclonal antibody against the Flag epitope and a polyclonal antibody against G6PD (Fig. 4A). This level of Flag-tagged G6PD expression gave rise to an almost 3-fold higher enzyme activity (Fig. 4B). No significant increase in the activity of PDG, the next enzyme in the PPP, was observed in COS-7 cells overexpressing G6PD.

Fig. 4C (compare the \(-cG6PD\) column with the \(+cG6PD\) column that was not exposed to DHEA) shows that the COS-7 cells overexpressing G6PD had a significantly higher serum-stimulated \(^{3}H\)thymidine incorporation as compared with cells transfected with vector alone, indicating that overexpression of G6PD can increase cell proliferation.

**DHEA Suppresses the Higher \(^{3}H\)Thymidine Incorporation Displayed in COS-7 Cells Overexpressing G6PD—**If the elevated \(^{3}H\)thymidine incorporations observed in the cells overexpressing G6PD are a result of the increased G6PD activity, then the G6PD inhibitor DHEA should suppress this elevation in \(^{3}H\)thymidine incorporations. Fig. 4C shows that the elevated \(^{3}H\)thymidine incorporation observed in COS-7 cells overexpressing G6PD was suppressed by 100 \(\mu M\) DHEA to almost the same level as detected in COS-7 cells transfected with vector alone. This level of inhibition of \(^{3}H\)thymidine incorporation by DHEA is much less than that seen in cells expressing only endogenous levels of G6PD (Fig. 3B). Since the COS-7 cells that overexpress G6PD (Fig. 4C) are actually a mixed population containing both cells that overexpress G6PD (30–50% of the total) and cells that express endogenous levels of G6PD, the level of inhibition of \(^{3}H\)thymidine incorporation caused by 100 \(\mu M\) DHEA is the sum total of the affect of DHEA on both overexpressing cells and cells expressing wild type levels of G6PD. It thus seems likely that overexpressing cells require a significantly higher concentration of DHEA to inhibit \(^{3}H\)thymidine incorporations as compared with cells expressing wild type levels of G6PD. Of note, DHEA inhibited the \(^{3}H\)thymidine incorporation of cells transfected with vector alone are inhibited by DHEA in a manner similar to untransfected cells (data not shown). Taken together, these results strongly suggest that the activity of G6PD plays an important role in cell growth.

The next series of experiments were designed to determine what is the critical product of G6PD activation that is required for cell proliferation. There are two principal end products of G6PD activation, NADPH, and via the PPP, ribose-5-phosphate. Deficiency of either separately or both together could account for the DHEA-suppressed cell growth. Similarly, increases in both or either alone could be required for enhancement of cell growth.

Ribose 5-Phosphate (R5-P) **Does Not Rescue Cells from DHEA-suppressed Growth—**R5-P does not readily cross cell membranes. Thus, to provide R5-P, Balb/c 3T3 cells were incubated with the combination of four ribonucleosides, or four deoxyribonucleosides. The nucleosides are metabolized to ribose-5-phosphate. This method has been routinely used to increase intracellular R5-P (22, 24). In this experiment, cell growth was measured by determining total protein content since the measurement of DNA synthesis by specific incorporation of \(^{3}H\)thymidine or bromodeoxyuridine could be interfered with by the high concentration of nonlabeled thymidine. Fig. 5 shows that neither ribonucleosides nor deoxyribonucleosides rescued cells from DHEA-suppressed growth.

**NADPH Is the Likely Critical PPP Product Required for Cell Growth—Activation of G6PD and PDG lead to the production of NADPH. Thus, inhibition of G6PD by DHEA should lower intracellular NADPH levels and render cells more susceptible to oxidative stress. To assess whether NADPH is the critical PPP product required for cell growth, we took the following two approaches.**

First, we evaluated the intracellular levels of NADPH and
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Increased activity of G6PD is associated with increased $[^{3}H]$thymidine incorporation. After 3 days of transfection, the same number of COS-7 cells were lysed for both Western analysis and enzyme activity. A Western blot analysis of G6PD-expression in COS-7 cells transfected with either vector alone or with Flag-tagged G6PD (see “Experimental Procedures”). In both blots (a and b), lane 1 is from lysates of cells transfected with Flag-tagged G6PD, and lane 2 is from lysates of cells transfected with vector alone. Blot a was blotted with a monoclonal antibody against Flag epitope. In blot b, the same blot used in a was stripped off and rebotted with a specific polyclonal antibody against G6PD. B, enzyme activity was measured as described under “Experimental Procedures.” The $x$ axis symbols are $-cG6PD$, which represents cells transfected with vector alone, and $+cG6PD$, which represents cells transfected with vector containing G6PD cDNA. The $y$ axis is given as arbitrary absorption units. The data are presented as average ± standard error from three experiments, each run in at least triplicate. (***, $p < 0.001$.) C, COS-7 cells overexpressing G6PD have a higher $[^{3}H]$thymidine incorporation, and DHEA inhibits the increased $[^{3}H]$thymidine incorporation seen in COS-7 cells overexpressing G6PD. Three days after COS-7 cells were transfected with either vector alone or with Flag-tagged G6PD, cells were treated with or without various concentrations of DHEA in the presence of $[^{3}H]$thymidine for 24 h. The data are presented as the percentage of increase in $[^{3}H]$thymidine incorporation over control COS-7 cells, $-cG6PD$ represents cells transfected with vector alone, and $+cG6PD$ represents cells transfected with vector containing G6PD cDNA. Data shown are the average ± standard error from five experiments, each run in at least triplicate. (*, $p < 0.05$; ***, $p < 0.001$ compared with control.)

NADP by HPLC analysis. Fig. 6 shows results from cells that were exposed to vehicle, 200 μM H$_2$O$_2$, or 100 μM DHEA for 1 h in the presence or absence of serum. The figure shows the effects of the treatments on the NADPH/NADP ratio. The NADPH/NADP ratio in the treated cells were compared with the NADPH/NADP ratio in control cells and expressed as a percentage of the control. Consistent with our data showing that G6PD activity is decreased by serum withdrawal, the data show that the NADPH/NADP ratio was decreased by serum withdrawal. In the absence of G6PD inhibitors, the NADPH/NADP ratio was not affected by a modest concentration of H$_2$O$_2$. This would be expected for cells that can properly defend against oxidative stress. Importantly, the data clearly show that in the presence and absence of serum, DHEA caused a 30–40% decrease in the NADPH/NADP ratio. This decrease in the NADPH/NADP ratio may be detrimental to cell growth.

Next, we designed an experiment to evaluate whether the inhibition of G6PD and the subsequent decrease in the NADPH/NADP ratio would affect cell growth under oxidative stress conditions. As previously noted, studies have shown that cells with low levels of G6PD activity have increased sensitivity to oxidative stress as compared with cells with normal levels of G6PD activity (5, 26). It has also been shown that low concentrations of H$_2$O$_2$ can stimulate cell growth (1, 3), whereas high concentrations can inhibit cell growth (1, 3). Although H$_2$O$_2$ inhibition of cell growth is probably multifactorial (27, 28), it is likely that the inhibition of cell growth is due, at least in part, to the elevation of intracellular oxidants. Thus, we hypothesized that COS-7 cells overexpressing G6PD should have an increased capacity for NADPH production and thus better defend against H$_2$O$_2$-inhibition of cell growth as compared with control cells expressing endogenous G6PD only. This was tested, and the results are shown in Fig. 7. COS-7 cells transfected with vector alone or transfected with cG6PD were grown in the presence or absence of serum. The figure shows the NADPH/NADP ratio in the treated cells were compared with that from control cells, and results expressed as a percent of the control. Data are means ± S.E. from five separate experiments.

Fig. 5. R5-P does not reverse DHEA-inhibited cell growth. Serum-starved Balb/c 3T3 cells were treated with or without 5 ng/ml PDGF along with the combination of four ribonucleosides (200 μM) or four deoxyribonucleosides (200 μM) in the absence or presence of 100 μM DHEA for 2 days. Cell growth was assessed by total protein content using Lowry assay after cells were lysed. The data are presented as average ± standard error from four experiments, each run in triplicate. (*, $p < 0.05$; ***, $p < 0.01$.)

Fig. 6. The G6PD inhibitor DHEA decreases the NADPH/NADP ratio. Cells were treated for 1 h with DMEM alone, 200 μM H$_2$O$_2$, or 100 μM DHEA in the absence or presence of 10% calf serum. NADPH and NADP were then assayed by reverse-phase HPLC as described under “Experimental Procedures.” The ratio of NADPH/NADP analyzed from control cells (cells in the presence of serum) in each experiment was assigned a value of 100%. The ratios of NADPH/NADP analyzed from all other groups were compared with that from control cells, and results expressed as a percent of the control. Data are means ± S.E. from four experiments.
in the presence of serum and 0, 30, and 60 μM H$_2$O$_2$. Control was the level of [H]$^3$H]thymidine incorporations in COS-7 cells transfected with vector alone that were grown in the presence of serum without H$_2$O$_2$. Fig. 7A shows that 60 μM H$_2$O$_2$ inhibited [H]$^3$H]thymidine incorporation to 40% of control levels in COS-7 cells transfected with vector alone. COS-7 cells overexpressing G6PD were exposed to both 60 μM H$_2$O$_2$ and menadione (a strong oxidant), or H$_2$O$_2$. For these studies, the Swiss 3T3 fibroblast cell line was evaluated.

Fig. 8 shows the results from cells that were evaluated 48 h after plating. To determine whether oxidant stress leads to cell detachment, the cells were exposed to two oxidants: menadione or H$_2$O$_2$. The results show that both oxidants caused a highly significant decrease in cell detachment. Next, we tested whether the G6PD inhibitor DHEA led to increased cell detachment. Similar to the oxidants, DHEA inhibited cell attachment. These results suggest that G6PD activity may be required to provide NADPH to maintain cell adherence.

**Inhibition of G6PD Activity by DHEA Decreases Tyrosine Phosphorylation**—Growth factor stimulation of tyrosine phosphorylation has also been shown to be important for cell growth (33). Thus, we explored whether the inhibition of G6PD activity by DHEA caused any changes in tyrosine phosphorylation. Fig. 9A shows a Western blot using a monoclonal antibody to phosphotyrosine in control cells. Fig. 9B and C shows cellular immunofluorescence using the antibody to phosphotyrosine in control and DHEA-treated cells. Pretreatment of cells with 100 μM DHEA led to a highly significant, dose-dependent inhibition of tyrosine phosphorylation both in the basal and the PDGF-stimulated conditions. This suggests that inhibition of G6PD may inhibit cell proliferation, at least in part, by inhibiting tyrosine phosphorylation.

**DISCUSSION**

**Published Research Shows a Strong Correlation between Stimulation of G6PD Activity and Stimulation of Cell Proliferation**—Previous research has strongly suggested that G6PD likely plays an important role in cell growth as many different models of cell growth are associated with increases in G6PD activity. In 1964, Epel (34) showed that fertilization of sea urchin eggs (a model of cell growth) resulted in the rapid
production of NADPH due to activation of G6PD. In 1968, Farquhar and Coe (7) studied dehydrogenase activity in a renal model of cell growth, unilateral nephrectomy. The increase in kidney size closely correlated with an increase in dehydrogenase activity. Similar results were obtained by Sochor et al. (8) in another model of renal cell growth, streptozotocin-induced diabetes in rats. In primary cultures of liver cells, the activity of G6PD was directly correlated with the rate of growth (9). EGF and insulin alone stimulated growth of the liver cells, and the addition of EGF and insulin together was a more potent stimulus to growth as compared with either growth factor alone. In all cases, the rate of increase in cell growth closely correlated to the rate of increase of G6PD activity. Moreover, Weber (12) has shown that a wide variety of tumor cells express large increases in G6PD activity, ranging from 3 to 20 times control (non-transformed) levels. Taken together, these results suggest that G6PD activity is associated with cell growth.

**Increased G6PD Activity by Overexpression Leads to Increased Cell Growth**—Our results confirm and extend previous observations by showing that activation of G6PD by growth factors occurs in many different cell types (see Table I) and by showing that an increased G6PD activity potentiates cell growth (Fig. 4). The increased \[^{3}H\]thymidine incorporations seen in the cells overexpressing G6PD were not due to transfection alone as the cells transfected with vector alone (expressing endogenous G6PD only) showed significantly lower incorporation of \[^{3}H\]thymidine. In addition to showing that increased G6PD activity can enhance cell growth, our data show that decreased G6PD activity is associated with decreased cell growth. DHEA, a known inhibitor of G6PD (35–38), abolished the increased \[^{3}H\]thymidine incorporations stimulated by serum or peptide growth factors in all cell lines tested (Table I). Moreover, there was a close correlation between the dose-dependent inhibition of G6PD activity by DHEA (Fig. 3A) and the dose-dependent inhibition of \[^{3}H\]thymidine incorporations by DHEA (Fig. 3B), again suggesting that G6PD activity and cell growth are closely related. Recently, Farquharson and colleagues (22) have published similar results using insulin-like growth factor 1 as a growth factor for cell lines derived from bone tissue. DHEA inhibited the insulin-like growth factor 1-stimulated proliferation of MG-63 cells, an osteosarcoma cell line, and rat osteoblasts. It is also possible that DHEA not only inhibited cell proliferation but led to cell death. To evaluate this, a dose-response curve evaluating DHEA and cell death was done. The data show that DHEA does cause cell death but at concentrations that are much higher than reported in the present article (data not shown). An extensive study of G6PD and cell death will be reported elsewhere.

DHEA is an uncompetitive inhibitor of G6PD (23). The molecular details of this inhibition have recently been shown to be due to the binding of DHEA to the ternary enzyme-coenzyme-substrate complex(es) (23). The exact mechanism by which DHEA inhibits cell growth has been controversial, as DHEA could exert its growth inhibitory effect through steroid receptor actions (39) as well as through the inhibition of G6PD. It is certainly possible that DHEA exerts its anti-proliferative effect by a mechanism that is independent of G6PD inhibition. However, considering the close correlation between G6PD activity and cell growth in either growth stimulatory conditions (i.e., cells exposed to growth factors or cells overexpressing G6PD) and in the growth inhibitory conditions, it is highly likely that the growth inhibitory effects of DHEA are mediated to a significant degree by the inhibition of G6PD.

**NADPH, Not R5-P, Is the Important Product of G6PD Activation Required for Cell Growth**—The consequences of inhibition of G6PD are decreased NADPH and via the PPP decreased production R5-P. If the supplement of nucleosides could reverse DHEA-abrogated cell proliferation, the lack of R5-P would be the sole critical product. Dworkin et al. (24) have reported that deoxy- or ribonucleosides partially reversed DHEA-induced growth inhibition of non-starved Hela TCRC-2 cells. Farquharson et al. (22) have also found that deoxynucleosides partially reversed DHEA-suppressed growth stimulation by insulin growth factor in MG-63 human osteosarcoma cells. In contrast, nucleosides did not reverse DHEA-suppressed growth of MCF-7 cells, a breast cancer cell line (39). Our data indicate that DHEA abolished PDGF-stimulated cell growth regardless of the presence of nucleosides (Fig. 5). These results indicate that DHEA-suppressed proliferation of Balb/c 3T3 cells was not due to the lack of R5-P for DNA synthesis. It is not clear whether the discrepancies among these studies are due to tissue specificity or experimental method. Biologically, since R5-P can be also provided by the conversion of fructose 6-phosphate and glyceraldehyde 3-phosphate via the catalysis of transaldolase and transketolase, the role of G6PD in providing R5-P is thought to be dispensable. However, considering the close correlation between G6PD activity and cell growth in either growth stimulatory conditions (i.e., cells exposed to growth factors or cells overexpressing G6PD) and in the growth inhibitory conditions, it is highly likely that the growth inhibitory effects of DHEA are mediated to a significant degree by the inhibition of G6PD.

**G6PD Activity Affects Cell Growth**

**Fig. 9.** The inhibition of G6PD by DHEA decreased PDGF-stimulated tyrosine phosphorylation. A, Western blot analysis of cell lysate using monoclonal antibody for phosphotyrosine showing that PDGF-stimulated tyrosine phosphorylation was suppressed by G6PD inhibitor DHEA in a dose-dependent manner. B and C, confluent and quiescent Swiss 3T3 fibroblasts were preincubated in DMEM in the absence (B) or presence of 100 μM DHEA (C) for 30 min at 37 °C. After being treated with 5 ng/ml PDGF for 10 min, cells were fixed, permeabilized, and incubated with monoclonal antibody for phosphotyrosine as described under “Experimental Procedures.”

NADPH, Not R5-P, Is the Important Product of G6PD Activation Required for Cell Growth—The consequences of inhibition of G6PD are decreased NADPH and via the PPP decreased production R5-P. If the supplement of nucleosides could reverse DHEA-abrogated cell proliferation, the lack of R5-P would be the sole critical product. Dworkin et al. (24) have reported that deoxy- or ribonucleosides partially reversed DHEA-induced growth inhibition of non-starved Hela TCRC-2 cells. Farquharson et al. (22) have also found that deoxynucleosides partially reversed DHEA-suppressed growth stimulation by insulin growth factor in MG-63 human osteosarcoma cells. In contrast, nucleosides did not reverse DHEA-suppressed growth of MCF-7 cells, a breast cancer cell line (39). Our data indicate that DHEA abolished PDGF-stimulated cell growth regardless of the presence of nucleosides (Fig. 5). These results indicate that DHEA-suppressed proliferation of Balb/c 3T3 cells was not due to the lack of R5-P for DNA synthesis. It is not clear whether the discrepancies among these studies are due to tissue specificity or experimental method. Biologically, since R5-P can be also provided by the conversion of fructose 6-phosphate and glyceraldehyde 3-phosphate via the catalysis of transaldolase and transketolase, the role of G6PD in providing R5-P is thought to be dispensable.

NADPH, the principal cellular reducing equivalent, is required by many antioxidant defense systems (26). Our data and
those of others strongly suggest that G6PD production of NADPH is critical for cell growth.

First, G6PD is the principal source of NADPH (26). The only other significant sources of NADPH are: (a) NADP-linked malic enzyme, which oxidatively decarboxylates malate to pyruvate; and (b) the mitochondrial enzyme, NADP-linked isocitrate dehydrogenase. Although NADPH production by the these two enzymes does contribute to the redox level of the cell, there is compelling evidence that G6PD is the principal source of NADPH utilized in redox regulation. Under oxidative stress conditions, many studies have shown that G6PD and the pentose phosphate pathway are routinely elevated (40–42). Although there is one study of lens epithelia that suggests that oxidative stress can increase the activities of all three NADPH producing enzymes (43), there are very few other studies that show any changes in malic enzyme and NADP-linked isocitrate dehydrogenase activities in response to oxidative stress. More importantly, work by Pandolfi et al. (5) using G6PD-deficient cell lines shows that the other two enzymes do not adequately replace the lack of NADPH production by G6PD, i.e. the G6PD-deficient cells had decreased growth rates, cloning efficiencies, and were highly sensitive to oxidative stress as compared with cells expressing endogenous levels of G6PD. The authors concluded that G6PD was critical for NADPH production and was the principal source of NADPH. Taken together, these results show that G6PD is the principal source of NADPH.

Second, control of redox has been suggested to be important for cell growth. Traditionally, redox control has been considered to be required mostly to maintain optimal redox levels for cell survival. However, recent evidence suggests that the oxidants themselves may be signals for growth. For example, it has been reported that the concentration of intracellular H2O2 rises after cells were exposed to PDGF. Prevention of the PDGF-induced increase in H2O2 by antioxidants (catalase or n-acetylcysteine) blocked the mitogenic effects of PDGF (2). Thus, H2O2 may act as a signaling molecule for cell growth. Presumably, if the intracellular H2O2 does rise upon growth stimulation, appropriate amounts of NADPH must be generated promptly to maintain an optimal redox status for cell growth. To determine whether the lack of NADPH is crucial for the inhibition of cell growth by DHEA, we evaluated the effects of DHEA on both the NADPH/NADP ratio and on cell growth at a biological redox threshold. This was achieved by the application of oxidative stress to COS-7 cells expressing different levels of G6PD. As seen in Fig. 6, DHEA decreased the NADPH/NADP ratio. Additionally, COS-7 cells overexpressing G6PD exhibited a higher growth rate in the presence of H2O2 as compared with the COS-7 cells transfected with vector alone, suggesting that a larger capacity of NADPH production sustained cell growth under oxidative stress conditions. This higher resistance to H2O2 rendered by the overexpression of G6PD was suppressed by DHEA. Taken together, these results suggest that the lack of NADPH is the significant consequence of G6PD inhibition by DHEA. As noted above, the metabolic role of G6PD has been recently studied by Pandolfi and colleagues (5) using targeted gene disruption. They found that G6PD-deficient cells were exquisitely sensitive to oxidative stress. Our results and those from Pandolfi et al. strongly suggest that NADPH, not N5-P, is the important product of G6PD that is important for cell growth regulation.

G6PD Activation Is Likely Required for Optimal Cell Anchorage—The results presented in this article along with other published data suggest that the activation of G6PD is to provide NADPH for the control of intracellular redox levels. To determine specific role(s) for G6PD activation during cell growth, intracellular events that have been shown to be affected by the redox potential of cells were evaluated. Growth-related events that have been shown to be associated, at least in part, with the redox level include cell anchorage (29, 44), cytoskeletal reorganization (31, 32), and phosphorylation (45). For this report, we focused on a role for G6PD in cell anchorage and tyrosine phosphorylation.

The attachment of cells to culture plates has been shown to be affected by oxidative stress. Zhang et al. (31) showed that H2O2 caused the detachment of neuronal PC-12 cells. Gailit and colleagues (43) showed similar results using a renal epithelial tubular cell line. These results suggest that increased oxidative stress can cause cell detachment. Our results using menadione and H2O2 confirm these findings (Fig. 8). The data shown in Fig. 8 show that DHEA also inhibited cell attachment. Thus, it is likely that G6PD activity is important in cell attachment after plating by maintaining an optimal redox potential.

The specific signals that regulate G6PD activation have not been fully determined. Clues to specific regulatory signals can be gleaned from the reported associations of G6PD activation with cell growth, cytoskeletal reorganization, and tyrosine phosphorylation. For example, other researchers have shown that the signal transduction protein, phosphatidylinositol 3-kinase (PI 3-kinase) is associated with cytoskeletal changes such as actin filament reorganization (42). In addition, the small GTP-binding proteins Rac and Rho have been shown to mediate cytoskeletal changes such as membrane ruffling (46) and stress fiber formation (47). Moreover, researchers have suggested that Rac and possibly Rho are downstream of PI 3-kinase (48). Our previous work has shown that the peptide growth factors, EGF and PDGF, stimulate release of G6PD from permeabilized cells likely via PI 3-kinase and by phospholipase C-γ (13). It is thus intriguing to speculate that the activation of G6PD associated with growth is mediated by a PI 3-kinase and Rac or Rho to provide the optimal intracellular redox potential for cell attachment and other growth-related events.

Inhibition of G6PD by DHEA Decreased Tyrosine Phosphorylation—Finally, increased tyrosine phosphorylation has been shown to be an important intracellular growth event (33). Inhibition of tyrosine phosphorylation inhibits cell growth in many cell growth systems (33). The level of tyrosine phosphorylation in cells is produced by a balance of tyrosine kinase and phosphotyrosine phosphatase activity. The DHEA-treated cells showed a significant decrease in the intracellular level of phosphotyrosines either due to decreased tyrosine kinase activity or increased phosphotyrosine phosphatase activity. The mechanism for this decreased phosphorylation is not clear. Although it may be due to the steroid actions of DHEA, a review of the literature concerning the effects of related steroids on phosphorylation reveals that androgens and estrogens promote tyrosine phosphorylation rather than inhibit tyrosine phosphorylation (41, 49). Thus it seems more likely that this decrease in tyrosine phosphorylation is due to decreases in NADPH or another as yet to be determined mechanism. Recently, Mazurek et al. (45) reported that extracellular AMP inhibits cell growth and inhibits tyrosine phosphorylation in breast cancer cell lines. The authors strongly suggest that the decreased phosphorylation is due to decreased NADH and NAD levels. The decrease in NADH levels is much greater as compared with decreases in the NAD levels favoring increases in oxidant levels in the cell. The authors believe that similar changes in NADPH/NADP system may cause the same decrease in tyrosine phosphorylation.3 Clearly, more research

3 S. Mazurek and E. Eigenbrodt, personal communication.
will be required to determine the exact role for G6PD activity in the regulation of tyrosine phosphorylation.

In conclusion, our results show the following. First, G6PD activity correlates with cell proliferation. Second, cells overexpressing G6PD proliferate at a higher rate as compared with cells expressing only endogenous levels of G6PD. Third, inhibition of G6PD activity by DHEA inhibited cell growth. Fourth, NADPH is the likely important product of G6PD that is required by growing cells. Fifth, G6PD activation possibly plays a role in cell anchorage and the growth factor stimulation of tyrosine phosphorylation.

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