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Growth hormone and adipose differentiation: Growth hormone-induced antimitogenic state in 3T3-F442A preadipose cells

(terminal differentiation/hormonal pathway)

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ABSTRACT **An additional activity for pituitary growth hormone is described—i.e., the *in vitro* induction of an antimitogenic state in murine 3T3-F442A preadipocyte fibroblasts. We previously developed a serum-free, hormonally defined medium permissive for the adipose differentiation of 3T3-F442A cells. When 3T3-F442A fibroblasts were maintained in serum-free medium without insulin but with growth hormone (2 nM), typical adipose differentiation did not occur. However, we found that growth hormone induced a state of cellular refractoriness to the mitogenic stimulus of fetal bovine serum as assayed by *de novo* DNA synthesis. The mitogen refractory condition (i.e., the antimitogenic state) was time-dependent (half maximal at ≈2.5 days) and growth hormone concentration-dependent (half maximal and maximal at ≈0.05 and 2.0 nM, respectively). The antimitogenic state was specifically induced by growth hormone and was not mediated by insulin-like growth factor I or prolactin. The growth hormone-induced antimitogenic state was completely reversible. The antimitogenic state was not induced by growth hormone in 3T3-C2 cells, a sister clone of 3T3 cells that exhibits essentially no adipose conversion. The kinetics for growth hormone-dependent commitment to adipose differentiation and induction of the antimitogenic state were similar. We suggest a relationship of growth hormone-induced antimitogenic state and the growth hormone-induced adipose differentiation of 3T3-F442A cells.**

Murine 3T3-F442A preadipocytes are immortal embryonic fibroblastic cells of mesenchymal origin (1). By tissue culture criteria, these cells are not transformed since the cells are highly contact inhibited during growth, require high concentrations of animal serum to grow, and are unable to grow in suspension culture (1). Growing cells exhibit no differentiated phenotypes. When 3T3-F442A cells achieve quiescence, serum factors are able to induce terminal adipose differentiation (2–4). The physiological relevance of this phenomenon is emphasized by the finding that subcutaneous injection of 3T3-F442A fibroblasts into nude mice yields fully developed fat pad tissue at the sites of injection (5).

Green and coworkers (6, 7) identified pituitary growth hormone (GH) as one of the adipogenic factors of serum. Adipose differentiation is under multihormone control as demonstrated by a number of groups (4, 7–10). We developed a serum-free, hormonally defined medium that is permissive for terminal adipose differentiation of 3T3-F442A fibroblasts (4). We found that both GH and insulin were required for adipose differentiation of 3T3-F442A cells (4). We concluded that the insulin requirement was mediated by the insulin receptor since insulin-like growth factor I (IGF-I) could not substitute for insulin even at micromolar concentrations (4).

GH did not induce any of the well-documented adipose phenotypes [e.g., high specific activity for the lipogenic marker enzyme glycerol-3-phosphate dehydrogenase (GPDH), development of fat inclusions, lipogenic and lipolytic hormonal responsiveness, and loss of proliferative capacity (1–13)]. However, GH did induce a distinct phenotype we designated the GH-primed phase of the adipose differentiation program (8, 11). Primed cells have entered a GH-induced, insulin-sensitive, and differentiation-permissive condition (8, 11). This is a regulated event in which quiescent cells become susceptible to GH. It is our hypothesis that the primed state is a differentiation-permissive quiescent state that has unique characteristics and determinants. When compared to quiescent cells that are not exposed to GH, primed cells exhibit a number of distinct features, including altered macromolecular synthesis, morphology, and mitogen responsiveness (8, 12). The primed state of 3T3-F442A preadipocytes is reversible in that all cells resume fibroblastic appearance and behavior when diluted to subconfluent density in a growth-permissive medium; i.e., they return to the typical growing fibroblastic cell state (4, 8, 11).

3T3-F442A preadipocytes that have been GH-primed in our serum-free medium are in a permissive condition for expression of the adipose program (4, 8). Insulin is required for cells to enter the expression phase of adipose differentiation (4, 8). Zezulak and Green (10) demonstrated that one of the functions of GH in 3T3-F442A cell differentiation was to endow newly formed adipocytes with IGF-I responsiveness not seen in undifferentiated cultures at confluence. IGF-I then induces a self-limited growth and division (two to four divisions) of fat cells [i.e., clonal expansion (13)]. We found that 3T3-F442A adipocytes generated in our serum-free medium did not clonally expand. This indicated that a serum factor(s), in addition to GH and IGF-I, was required for expansion (4, 11).

Our previous observation that GH-primed quiescent 3T3-F442A preadipocytes exhibited diminished DNA synthesis in response to human platelet-derived growth factor (PDGF) and insulin (8) led us to further characterize this phenomenon. Cells exposed to GH for 3 days exhibited nearly 50% decreased DNA synthesis in response to insulin and PDGF in serum-free medium. However, the importance of this phenomenon was unclear since PDGF and insulin are weakly mitogenic for growth-arrested 3T3-F442A cells and do not have the ability to support cell growth and division (R.E.C., unpublished observations; ref. 8). In the present study, we found that 3T3-F442A cells in serum-free medium developed a GH-dependent refractoriness to the potent growth signals of fetal bovine serum (FBS) (i.e., an antimitogenic state). The GH-induced antimitogenic state was characterized and its significance for the role of GH in

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Abbreviations: GH, growth hormone; Met-hGH, recombinant human growth hormone; PDGF, human platelet-derived growth factor; IGF-I, human insulin-like growth factor I; FBS, fetal bovine serum; GPDH, glycerol-3-phosphate dehydrogenase.

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differentiation is discussed within the context of a cell cycle model for adipose differentiation.

MATERIALS AND METHODS

The composition of serum-free medium and the sources of the components were previously described (4). Recombinant human growth hormone (Met-hGH) was a gift from Genentech and Eli Lilly, bovine prolactin was from the National Pituitary Program (lot AFP-6300), human recombinant IGF-I was a gift from Daniel Burleigh, Jr., of International Minerals and Chemicals (Northbrook, IL), bovine GH was purified in our laboratory (14), culture-grade PDGF was from Collaborative Research, [^{3}H]thymidine (specific activity = 20 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear, calf serum was from GIBCO, and FBS was from HyClone.

3T3-F442A and 3T3-C2 cells were generously provided by Howard Green of Harvard University. Growth, maintenance, and counting of cells were as described (4, 8, 11). For experiments, cells were grown in surface culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) calf serum. Subconfluent monolayers were detached by trypsin, washed by centrifugation with Hanks' balanced salt solution (HBSS), and plated in 6- or 24-well dishes (GIBCO) in DMEM/10% calf serum at a density of 8 $\times$ 10^3 cells/cm^2. After 4 hr of incubation at 37°C in a humidified incubator gassed with 5% CO_2/95% air, the medium was aspirated; the monolayers were washed with HBSS; and serum-free medium (4), without insulin (or IGF-I), and the indicated concentrations of Met-hGH (or other hormones being tested) were added.

Colony-forming efficiency was determined as described (4). Experiments were run in triplicate for each time point, and each point was plated out in at least duplicate. The plating efficiency was generally 50% and 25% for cells in DMEM/10% calf serum and serum-free medium (without GH or insulin), respectively.

Differentiation was quantified by determining the specific activity of the adipose marker enzyme GPDH as described (4).

Cellular commitment times for adipose differentiation in response to GH were determined in serum-free medium. Cells were grown to near confluence in the nonadipogenic medium of DMEM/5% cat serum. Monolayers were then washed extensively with HBSS followed by the addition of serum-free medium as described (4). The zero time of the experiment was at the addition of serum-free medium. At this time the serum-free medium contained Met-hGH (2 nM) and insulin (1.6 μM), a condition that induces adipose differentiation (4). After the indicated times (see Fig. 1*C*), the medium was aspirated, and the monolayers were washed extensively with HBSS. After washing, the residual levels of Met-hGH were determined by radioimmunoassay and were $\leq 1.0 \times 10^{-13}$ M, which is two or three orders of magnitude lower than the EC_{50} for Met-hGH-induced adipose differentiation of 3T3-F442A preadipocytes (4, 6). After washing, the cells were fed fresh serum-free medium without GH and incubated for 20 days. Then the extent of differentiation was determined by assaying GPDH-specific activities. The positive control for the commitment experiment was cells maintained in the original serum-free medium with Met-hGH (2 nM) and insulin (1.6 μM) for the entire 20 days (i.e., full differentiation as a function of time of GH exposure). Each point is the average of triplicate determinations.

DNA synthesis (7) was assayed 18–24 hr after the addition of FBS or PDGF plus insulin to cultures by aspirating the medium and replacing it with identical medium containing 1–10 μCi of [^{3}H]thymidine per ml. After a 1-hr pulse, the isotope was removed, the monolayers were washed with HBSS, and DNA was precipitated with cold 10% (wt/vol) trichloroacetic acid. Precipitates were deposited on glass fiber filters and washed extensively with ice-cold 10% trichloroacetic acid. After the filters were dried and solubilized with scintillation cocktail, radioactivity was determined by liquid scintillation counting. For experiments conducted in 24-well dishes, data are presented as cpm per well since cell numbers did not change detectably in serum-free medium in the presence or absence of GH during the course of the experiments. Some experiments were repeated in 6-well dishes. Prior to trichloroacetic acid precipitation, cells were detached by trypsinization; one sample was used for protein determination by the Bradford method (15), as described (4), and a second sample was used to determine DNA synthesis. Data were expressed as cpm per μg of cell protein. Similar results were obtained whether data were expressed as cpm per well or cpm per μg of cell protein.

RESULTS

In serum-free medium, 3T3-F442A fibroblasts do not divide, and DNA synthesis progressively diminishes to zero by 4 days of incubation (Fig. 1*A*). This experiment demonstrates that cell number is not changing in serum-free medium without insulin or GH and the cells are becoming quiescent as evidenced by the loss of DNA synthesis (Fig. 1*A*). When Met-hGH (2 nM) was included in the serum-free medium, the cell number did not change. When the medium was supplemented with serum (5% FBS), cell number increased and was accompanied by DNA synthesis (Fig. 1*A*). The monolayer in serum appeared 100% confluent by day 4, which accounts for the decrease of DNA synthesis at this time.

For measurement of DNA synthesis, 3T3-F442A cells were plated at a subconfluent density in DMEM/10% calf serum. After 4 hr of incubation to allow cell attachment, the monolayers were washed. Serum-free medium, without insulin and with or without Met-hGH (2 nM), was added, and this was considered the zero time (Fig. 1*B*). 3T3-F442A fibroblasts were exposed to serum (5% FBS) for 20 hr prior to the time points indicated in Fig. 1*B*. The ability of 3T3-F442A cells incubated with Met-hGH to mount a mitogenic response to serum diminished with time of Met-hGH exposure (Fig. 1*B*). The data are presented relative to control cultures in serum-free medium without Met-hGH. The ability of these control cultures to mitogenically respond to serum did not change appreciably over the time course of the experiment (data not shown). Met-hGH (2 nM) pretreatment of 3T3-F442A cells resulted in a progressive decrease in the ability of cells to synthesize DNA in response to serum, with a maximal inhibition of 80% achieved by day 5 and a half-maximal inhibition achieved after 2.5 days of Met-hGH exposure (Fig. 1*B*). Thus, in serum-free medium, Met-hGH induced cells to enter an antimitogenic state, defined as a reduced ability to respond to the mitogenic action of FBS as compared to matched control cells not exposed to GH.

Met-hGH (2 nM) treatment of 3T3-C2 cells in serum-free medium for up to 5 days did not inhibit the ability of cells to synthesize DNA in response to serum (5% FBS) added 20 hr prior to the assay (Fig. 1*B*). This result suggests that the GH induction of the antimitogenic state may be related to the differentiating activity of GH for 3T3-F442A cells.

The connection between the GH-induced antimitogenic state and GH-induced commitment to differentiate for 3T3-F442A cells is further supported by the kinetics of the two processes (Fig. 1*C*). The cells required 2 days of exposure to Met-hGH in order to subsequently achieve 50% of the maximal differentiation obtained with 20 days of continuous exposure to both Met-hGH and insulin (Fig. 1*C*). Thus the kinetics for the Met-hGH-induced commitment to differentiate and the induction of the antimitogenic state are similar (Fig. 1*C*).

Met-hGH-mediated induction of the antimitogenic state was entirely reversible as determined by colony-forming

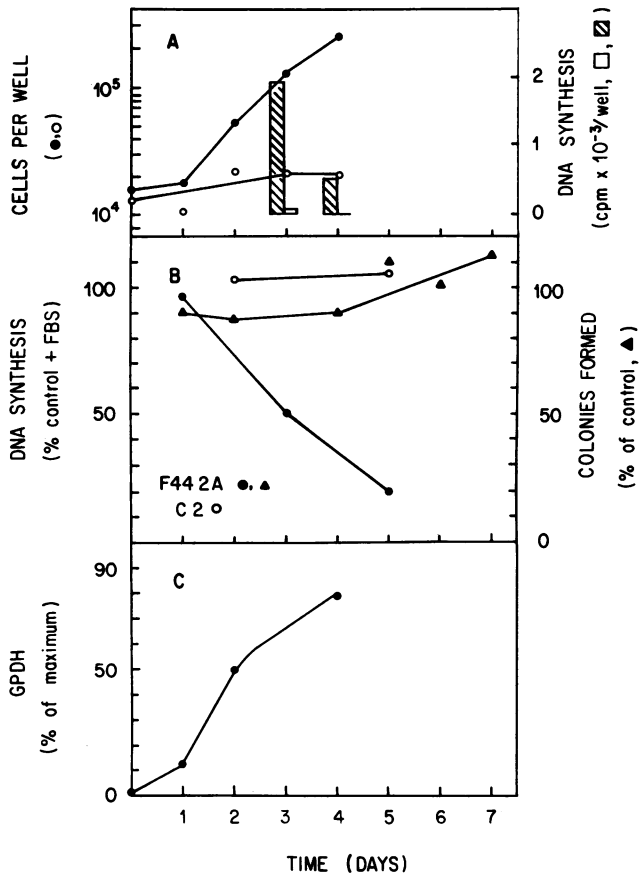

FIG. 1. Growth, DNA synthesis, and differentiation of 3T3-F442A preadipocytes. (*A*) Cells were plated at the indicated densities in serum-free medium (○, □) or serum-free medium supplemented with 5% (vol/vol) FBS (●, ■). Cell number and DNA synthesis were determined at the indicated times. (*B*) 3T3-F442A (●) and 3T3-C2 (○) cells were plated in serum-free medium as described above. Half the cultures received Met-hGH (2 nM) and the other half did not (controls). At 20 hr prior to the indicated times, 5% FBS was added to all cultures, at 1 hr prior to the indicated times cultures were pulsed with [^{3}H]thymidine, and DNA synthesis was determined. Data are expressed as the percentage of the activity seen in GH-treated cultures relative to control cultures. In a parallel experiment with 3T3-F442A cells (▲), the cells were treated with and without 2 nM Met-hGH, and at the indicated times the colony-forming efficiency of the cultures was determined. Data are expressed as the results of the GH-treated cultures relative to control cultures not treated with GH. (*C*) Commitment experiments were performed on 3T3-F442A cells. Cells were exposed to 2 nM Met-hGH in serum-free medium with insulin for the incubated times. Monolayers were washed extensively and incubated in GH-free, serum-free medium with insulin for 20 days at which time specific activity for GPDH was determined. Data are expressed relative to control cells maintained continuously in the presence of 2 nM Met-hGH and insulin.

efficiency of Met-hGH-treated cells (Fig. 1*B*). 3T3-F442A cells were maintained in serum-free medium without insulin in the presence or absence of Met-hGH (2 nM). At the indicated times (Fig. 1*B*), cells were replated at clonal density (100 cells per 100-mm plate) in DMEM/10% calf serum (i.e., a growth-permissive medium). After 2 weeks of incubation the plates were fixed and stained, and macroscopic colonies (64 or more cells per colony) were counted. The data are expressed as the percentage of colonies formed by matched cultures treated identically but without Met-hGH treatment (Fig. 1*B*). Under conditions that elicit the Met-hGH-mediated antimitogenic state for 3T3-F442A cells, there was no decrease in the ability of Met-hGH-treated cells to replate and initiate growth to macroscopic colonies (Fig. 1*B*).

3T3-F442A preadipocytes developed the mitogen refractory condition in a dose-responsive manner, with half-maximal and maximal activity seen at ≈0.05 and 2.0 nM Met-hGH, respectively (Fig. 2). Bovine GH (10 nM) exhibited substantial antimitogenic activity for 3T3-F442A preadipocytes, whereas glucagon (20 nM), bovine prolactin (2 nM), and IGF-I (17 nM) had no antimitogenic activity (data not shown). It is not surprising that IGF-I was not antimitogenic for 3T3-F442A fibroblasts, because we previously demonstrated that IGF-I (17 nM) was mitogenic for 3T3-F442A cells (12), indicating that the antimitogenic action of GH was not mediated by an IGF-I-dependent mechanism.

We found expression of the GH-induced antimitogenic state to be inversely related to the concentration of FBS employed as the mitogenic stimulus (Fig. 3). After 5 days of treatment with Met-hGH (2 nM), at 10% and 20% FBS, DNA synthesis was reduced 50% and 44%, respectively. At 0.2% and 5% FBS, DNA synthesis was inhibited 95% and 75% compared with control cells not exposed to Met-hGH, respectively (Fig. 3). The mitogenic signals of serum could partially offset the antimitogenic state if present in sufficiently high concentrations. Thus a cell's behavior with respect to mitogenesis is affected by the quality and quantity of extracellular signals impinging upon it.

Under the assay conditions employed for DNA synthesis (i.e., determination 20 hr after the addition of the mitogenic signal), the effect of GH may be to lengthen the prereplicative lag rather than actually preventing the proliferative response (16, 17). For the embryonic nontransformed murine cell line C3H/10T½ (clone 8), the prereplicative time increased from 12 to ≈21 hr, with extended quiescence prior to replication (16). It was found that when quiescent monolayers of the murine embryonic cell line AKR-2B were exposed to transforming growth factor type β the mitogenic responsiveness of cells was inhibited when assayed 24 hr after mitogenic stimulation (17). However, when cells were assayed for DNA synthesis 48 hr after mitogenic stimulation, transforming growth factor type β did not inhibit DNA synthesis and was in fact mitogenic itself (17). The apparent antimitogenic activity of transforming growth factor type β for AKR-2B cells was actually induction of a prereplicative lag such that induced DNA synthesis by quiescent cells was readily detected 48 hr after mitogenic stimulation but not at 24 hr (17). We therefore performed an experiment to determine if the reduced DNA synthesis observed in 3T3-F442A cells treated with GH resulted from such a prereplicative lag (17). 3T3-

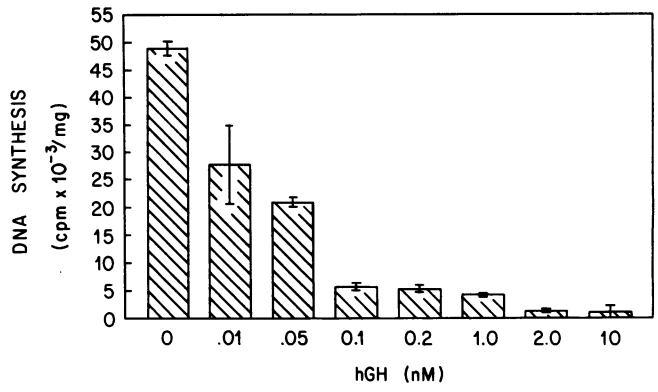

FIG. 2. GH concentration dependence for the antimitogenic state. 3T3-F442A cells were incubated in serum-free medium for 5 days with the indicated concentrations of hGH. Twenty hours prior to harvesting cells, 4% FBS was added, 1 hr prior to harvest [^{3}H]thymidine was added, and DNA synthesis was assayed. Data are expressed relative to control cultures that received no hormonal treatment but were stimulated with FBS. The values are the means ± SD.

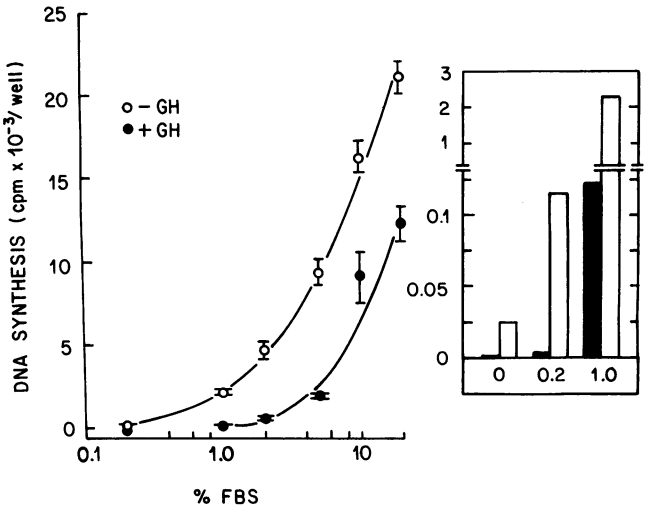

FIG. 3. GH-induced antimitogenic state and serum concentration. 3T3-F442A cells were maintained in serum-free medium with or without 2 nM Met-hGH for 5 days. Twenty hours prior to harvesting cells, the indicated concentrations of FBS were added to cultures. Cells were labeled with [^{3}H]thymidine, and DNA synthesis was determined. The values are the means ± SD. The bar graph is an enlargement of the lower serum concentrations.

F442A fibroblasts were treated for 5 days with Met-hGH (2 nM) in serum-free medium. Cells then received serum (5% FBS), and DNA synthesis was assayed from 16 to 51 hr after the addition of serum by the standard assay procedure (Fig. 4). The inhibition of DNA synthesis was constant for the duration of the experiment, suggesting that Met-hGH was not acting to increase the prereplicative lag time prior to the onset of DNA synthesis (Fig. 4). When Met-hGH was present after the addition of FBS, the antimitogenic effect was reversed between 3 and 4 days after FBS addition, whereas when Met-hGH was removed immediately prior to FBS addition, the antimitogenic state dissipated after 24 hr (data not shown). Thus the presence of Met-hGH was not required during FBS stimulation to maintain the antimitogenic state for 1 day, but the presence of the hormone was required during the stimulation phase to maintain the antimitogenic state for several days.

In our previous report, we found that 3 days of Met-hGH treatment of 3T3-F442A fibroblasts in serum-free medium resulted in ≈50% inhibition of the DNA synthetic response of

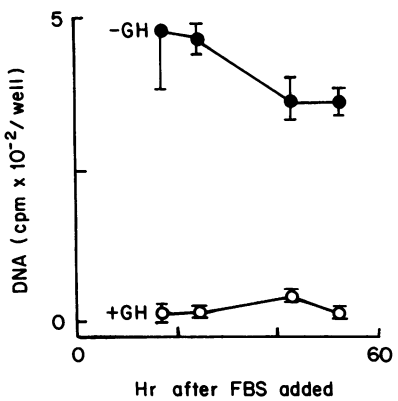

FIG. 4. GH-induced antimitogenic state: prereplicative time. 3T3-F442A cells were maintained in serum-free medium with (○) or without (●) 2 nM Met-hGH for 5 days. At time zero, all cultures received 5% FBS. At 1 hr prior to the indicated times, cells were labeled with [^{3}H]thymidine, and at the indicated times DNA synthesis was determined. The values are the means ± SD.

cells to PDGF (2.5 units/ml) and 1.6 μM insulin (8). When the experiment was repeated, except that incubation with Met-hGH was for 5 days, 95–100% inhibition of DNA synthesis resulted (R.E.C., unpublished data).

DISCUSSION

GH treatment of quiescent 3T3-F442A preadipocytes rendered cells refractory to serum-induced DNA synthesis (i.e., an antimitogenic state). It is important to note that our definition of antimitogenic is that resting cells develop refractoriness to mitogenic signals. We consider that the GH-induced antimitogenic state and GH-induced adipose differentiation of 3T3-F442A fibroblasts are related based on several experiments. First, GH does not induce the antimitogenic state for 3T3-C2 cells. This is a sister clone of the 3T3-F442A cells that differentiates at such a low frequency that it essentially does not exhibit detectable adipose conversion (2–7, 8, 10–13). Second, both phenotypes exhibit similar kinetics of onset (i.e., the antimitogenic state and commitment to differentiate) (Fig. 1 *B* and *C*). Finally, both processes exhibit similar Met-hGH concentration dependencies (Fig. 2; ref. 6). Additionally, other peptide hormones, most notably prolactin and IGF-I do not induce either process (Fig. 2; refs. 6 and 8). This means that neither response is lactogenic or mediated by IGF-I (Fig. 2; refs. 8 and 18).

Green and coworkers described a dual effector model for GH action as a differentiation agent (10, 19). Subsequently, a similar hypothesis was put forward to explain the somatogenic activity of GH in bone growth (19). Both arguments postulate a dual effector mechanism for GH's action based upon the differentiating activity of the hormone (19, 20). The dual role is that GH first induces differentiation of precursor cells that are insensitive to the mitogenic actions of IGF-I in the undifferentiated resting state (10, 19). As a consequence of differentiation, the young adipocytes acquire sensitivity to the mitogenic (somatogenic) properties of IGF-I (19, 20). It is also of interest that GH, but not IGF-I, inhibited growth of epiphysial primary chondrocytes of the intermediate zone *in vitro* (21). Although an antimitogenic activity for GH appears to be anomalous, our interpretation is that the antimitogenic state is related to the differentiation program. Thus, our observation and interpretation of the GH-induced antimitogenic state in 3T3-F442A preadipocytes is entirely consistent with the dual effector theory of GH action.

A relationship of GH-induced adipose differentiation and the GH-induced antimitogenic state is consistent with our previous proposal of a multihormone, multistep, sequential pathway for the adipose differentiation of 3T3-F442A fibroblasts (8, 11). We consider existing data to be consistent with a sequential pathway with different steps under the regulation of different hormones. However, we do not preclude the existence of parallel steps that may be under multihormone control.

We have previously described the GH-primed 3T3-F442A preadipocyte as representing a phase of the adipose differentiation program (8, 11). The description of a reversible antimitogenic state (Fig. 1*B*) for primed cells permits refinement of our previously described cell cycle hormonal pathway analysis of adipose differentiation (8).

It has previously been shown that competence factors for 3T3 cell mitogenesis (e.g., PDGF and fibroblast growth factor) can block adipose differentiation of 3T3-F442A cells (22). We found that PDGF could block the GH- and insulin-induced adipose conversion of 3T3-F442A cells in serum-free medium (R.E.C., unpublished data). Based upon this study and the results of previous studies (8, 21), we suggest that an action of GH in differentiation is to uncouple or block the action of competence factors for mitogenesis (i.e., PDGF). The role of PDGF antagonism might be related to the cell

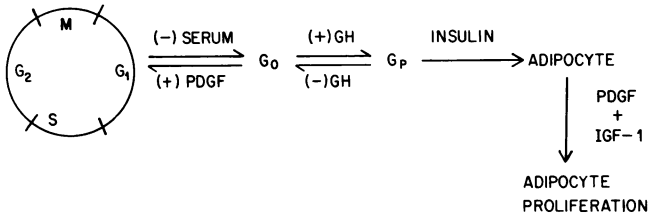

FIG. 5. Postulated hormonal pathway of adipose differentiation of 3T3-F442A preadipocytes. Initially, cells exit the cell cycle due to serum deprivation in our system. Cells at G_0 are then substrates for GH, which reversibly induces the differentiation of the primed state G_p. Reversal requires removal of GH and addition of PDGF, serum, or other competence factors. Subsequently insulin, in an irreversible step, promotes fat accumulation. Clonal expansion of the differentiated cells (i.e., adipocyte proliferation) is limited to two to four cell divisions and is under the hormonal control of PDGF and IGF-I.

cycle stage of the preadipocyte. A prerequisite of differentiation is that cells be quiescent [i.e., at the G_0 stage of the cell cycle (Fig. 5)]. It is well documented that PDGF drives quiescent 3T3 cells in G_0 back into the cell cycle (23, 24) [i.e., a nonpermissive condition for differentiation (22)]. A role of GH to antagonize competence factors for cell growth and induce the primed state implies an active, hormonally regulated process. Although primed cells are quiescent, this does not imply the simple absence of growth-related activities. Our demonstration of distinct properties for primed cells (8, 12), including the antimitogenic state, argues this to be an active regulated event. Consistent with this notion is the report by Schneider *et al.* (25) in which a unique set of genes of NIH 3T3 cells were induced by quiescence and repressed by growth signals.

In our model (Fig. 5) GH acts on G_0 cells to produce primed cells (G_p). G_p cells exhibit a number of distinct phenotypes (8, 12), including the antimitogenic state. This step is reversible (Fig. 1*B*) by removal of GH and placing cells in a growth-permissive environment. We suggested that G_p cells are in a differentiation-permissive condition; i.e., they become sensitive to the differentiating activity of insulin (4). Insulin then induces expression of the adipose phenotypes [e.g., fat accumulation (4)]. We have represented this as an irreversible step (Fig. 5). This is based upon our observation that 3T3-F442A adipocyte cultures replated and gave rise to macroscopic colonies with reduced efficiency relative to matched control cells that were not differentiated (4). The reduction of plating efficiency was roughly equivalent to the extent of adipose differentiation (4). The original description of 3T3 cell differentiation reported that adipocytes were unable to replate and reinitiate growth (2). The irreversibility is an operational definition based upon the inability of 3T3-F442A fat cells to grow upon extended incubation after replating in growth medium. There have been reports that primary fat cells are able to grow and assume fibroblast-like morphology in culture (26, 27). However, these primary fat cells were from newborn rats and were grown in batch culture initiated at 10^5 cells per flask (27). Our results are not necessarily inconsistent with these findings (27), because the growth measured might have resulted from cells in the population that had not terminally differentiated.

Finally, in our model the newly generated fat cells undergo clonal expansion under the influence of IGF-I (10) and serum factors (11). In preliminary experiments we found that when 3T3-F442A cells were differentiated in serum-free medium with Met-hGH and insulin the addition of PDGF and IGF-I a week after initiation of differentiation, a time at which cells were already adipocytes, resulted in clonal expansion of the adipocytes (R.E.C., unpublished data). We have thus represented the final state of adipose differentiation as being under the control of PDGF and IGF-I (Fig. 5). This scheme suggests the process of clonal expansion (i.e., the self-limiting growth of differentiated cells) uses the same hormonal signals as fibroblast growth. Clonal expansion requires the presence of both competence factors and progression factors, PDGF and IGF-I, respectively (23, 24).

Our model is similar to that previously proposed by Scott and coworkers (28–31) for the adipose differentiation of BALB/c 3T3 T fibroblasts; however, several differences in the details of the two cell and culture systems employed make direct comparisons difficult. Most importantly, G_p is a condition achieved by a defined set of hormonal conditions, with GH being the principal effector, whereas GH was reported not to be an effector of BALB/c 3T3 T adipose differentiation, unlike our Swiss 3T3-F442A cells (31).

We have demonstrated that GH induces an antimitogenic state in 3T3-F442A fibroblasts by an IGF-I-independent mechanism. The antimitogenic state induced by GH (i.e., the transient antagonism of growth signals) may be important to the role of GH as an adipose differentiation agent.

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1. Todaro, G. J. & Green, H. (1963) *J. Cell Biol.* **17**, 299–311.
2. Green, H. & Kehinde, O. (1974) *Cell* **1**, 113–116.
3. Kuri-Harcuch, W. & Green, H. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6107–6109.
4. Guller, S., Corin, R. E., Mynarcik, D. C., London, B. M. & Sonenberg, M. (1988) *Endocrinology* **122**, 2084–2089.
5. Green, H. & Kehinde, O. (1979) *J. Cell. Physiol.* **101**, 169–172.
6. Morikawa, M., Nixon, T. & Green, H. (1982) *Cell* **29**, 783–789.
7. Nixon, T. & Green, H. (1984) *Endocrinology* **114**, 527–532.
8. Guller, S., Sonenberg, M., Wu, K.-Y., Szabo, P. & Corin, R. E. (1989) *Endocrinology* **125**, 2360–2367.
9. Smith, P. J., Wise, L. S., Berkowitz, R., Wan, C. & Rubin, C. S. (1988) *J. Biol. Chem.* **263**, 9402–9408.
10. Zezulak, K. M. & Green, H. (1986) *Science* **233**, 551–553.
11. Guller, S., Sonenberg, M. & Corin, R. E. (1989) *Endocrinology* **124**, 325–331.
12. Guller, S., Corin, R. E., Wu, K.-Y. & Sonenberg, M. (1989) *Biochem. Biophys. Res. Commun.* **163**, 895–901.
13. Pairault, J. & Green, H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5138–5142.
14. Free, C. & Sonenberg, M. (1966) *J. Biol. Chem.* **241**, 5076–5082.
15. Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem.* **79**, 544–560.
16. Miska, M. & Bosman, H. B. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1140–1144.
17. Shipley, G. D., Tucker, R. F. & Moses, H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4147–4151.
18. Morikawa, M., Green, H. & Lewis, U. J. (1984) *Mol. Cell. Biol.* **4**, 228–231.
19. Green, H., Morikawa, M. & Nixon, T. (1985) *Differentiation* **29**, 1995–1998.
20. Nilsson, A., Isgaard, J., Lindahl, A., Dahlstrom, A., Skottner, A. & Isaksson, O. P. G. (1986) *Science* **233**, 571–574.
21. Lindahl, A., Nilsson, A. & Isakasson, O. P. G. (1987) *J. Endocrinol.* **115**, 263–271.
22. Hayashi, I., Nixon, T., Morikawa, M. & Green, H. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3969–3972.
23. Harrington, M. A. & Pledger, W. J. (1987) *Methods Enzymol.* **147**, 400–407.
24. Stiles, C. D. (1985) *Cancer Res.* **45**, 5215–5218.
25. Schneider, C., King, R. M. & Philipson, L. (1988) *Cell* **54**, 787–793.
26. Sugihara, H., Yonemitsu, N., Miyabara, S. & Yun, K. (1989) *Differentiation* **31**, 42–49.
27. Sugihara, H., Yonemitsu, N., Miyabara, S. & Toda, S. (1987) *J. Lipid Res.* **28**, 1038–1045.
28. Willie, J. J., Jr., & Scott, R. E. (1982) *J. Cell. Physiol.* **112**, 115–122.
29. Wier, M. L. & Scott, R. E. (1985) *Cancer Res.* **45**, 3339–3345.
30. Sparks, R. L., Estervig, D. N. & Scott, R. E. (1988) in *Growth Factors, Tumor Promoters, and Cancer Genes*, eds. Colburn, N. H., Moses, M. L. & Stanbridge, E. J. (Liss, New York), pp. 171–182.
31. Sparks, R. L. & Scott, R. E. (1986) *Exp. Cell Res.* **165**, 345–352.