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Effect of Activating and Inactivating Mutations of G_s- and G_{i2}-Alpha Protein Subunits on Growth and Differentiation of 3T3-L1 Preadipocytes

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Abstract Previous investigations have demonstrated that both G_s- and the G_i-family of GTP-binding proteins are implicated in differentiation of the 3T3-L1 preadipocyte. In order to further analyze the role of $G_s\alpha$ vs. $G_{i2}\alpha$, which are both involved in adenylate cyclase modulation, we transfected undifferentiated 3T3-L1 cells with two sets of G-protein cDNA: the pZEM vector with either wild type, the activating (i.e., GTP-ase inhibiting) R201C-$G_s\alpha$ or the inactivating G226A(H21a)-$G_s\alpha$ point mutations, or the pZIPNeoSV(X) retroviral vector constructs containing the $G_{i2}\alpha$ wild type or the missense mutations R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A(H21a)-$G_{i2}\alpha$.

The activating [R201C]$G_s\alpha$-mutant did not significantly affect the differentiation process, i.e., increase in the steady-state levels of G-protein subunits, gross appearance, or insulin-elicited deoxy-glucose uptake into 3T3-L1 adipocytes, despite a marked initial increase in hormone-elicited adenylate cyclase activity. The [H21a]$G_s\alpha$-mutant, on the other hand, enhanced the degree of differentiation slightly, as evidenced by an augmented production of lipid vesicles and insulin-stimulated deoxy-glucose uptake. However, an expected increase in mRNA for hormone-sensitive lipase was not seen. Secondly, it appeared that both activating [R179E]$G_{i2}\alpha$ or [Q205L]$G_{i2}\alpha$ mutants reduced cell doubling time in non-confluent 3T3-L1 cell cultures, while [H21a]$G_{i2}\alpha$ slowed proliferation rate. Furthermore, it seemed that cell proliferation, as evidenced by thymidine incorporation, ceased at a much earlier stage prior to cell confluency when cultures were transfected with the [R179E]$G_{i2}\alpha$ or [Q205L]$G_{i2}\alpha$ mutants. Upon differentiation with insulin, dexamethasone, and iBuMeXan, the following cell characteristics emerged: the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants consistently enhanced adenylate cyclase activation and cAMP accumulation stimulated by isoproterenol and corticotropin over controls. Deoxy-glucose uptake was also super-activated by the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants. Finally, steady-state levels of hormone sensitive lipase mRNA were dramatically increased by [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ over differentiated controls. The inactivating [H21a]$G_{i2}\alpha$-mutant obliterated all signs of preadipocyte differentiation.

It is concluded that G_{i2} plays a positive and much more important role than G_s in 3T3-L1 preadipocyte differentiation. Cyclic AMP appears to play no role in this process. J. Cell. Biochem. 64:242–257.

Key words: GTP-binding proteins; site-directed mutagenesis; stable transfection; adenylate cyclase; glucose uptake; hormone sensitive lipase; cell growth; cell differentiation

Abbreviations used: AC, adenylate cyclase, EC 4.6.1.1; DMEM, Dulbecco's minimum essential medium; FCS, foetal calf serum; FGF, fibroblast growth factor; $G_{i1,2,3}\alpha$, alpha subunit of the inhibitory G proteins $G_{i1,2,3}$; G_p, alpha subunit of the phospholipase C stimulatory G protein; GPDH, glycerophosphate dehydrogenase; G-protein, GTP-binding protein; $G_s\alpha$, alpha subunit of the stimulatory G protein G_s; HSL, hormone sensitive lipase; iBuMeXan, isobutylmethyl xanthine; IGF, insulin-like growth factor; PLC, phospholipase C, EC 3.1.4.3; [R179E]$G_{i2}\alpha$-mutant, cells expressing a mutated $G_{i2}\alpha$; R179E-$G_{i2}\alpha$, $G_{i2}\alpha$ point (missense) mutation; SSPE, NaCl/sodium phosphate/EDTA; TGF_β, transforming growth factor β.

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Previous studies have demonstrated that both $G_s\alpha$- and $G_{i2}\alpha$-activating (with low intrinsic GTP-ase activity) mutants enhance cell proliferation [1–4]. Swiss 3T3 cells transfected with the Q227L-$G_s\alpha$ mutation in a pMV-7 vector enhanced cell proliferation in serum-starved cells in the presence of insulin [1]. Hermouet et al. 1991 [2] showed that NIH 3T3 cells with a pZipNeoSV(X) vector construct containing the Q205L-$G_{i2}\alpha$ point mutation displayed a considerably reduced doubling time. G-proteins have also been implicated in preadipocyte differentiation [5–8]. 3T3-L1 fibroblasts altered phenotype when incubated with insulin, dexametha-

sone, and the phosphodiesterase inhibitor iBuMeXan for 3 to 7 days with ensuing enhancement of $G_s\alpha$, $G_i\alpha$, and $G\beta$ protein levels [6]; augmented hormone-sensitive adenylate cyclase activities [9]; increased transcription of hormone sensitive lipase [10]; elevation in fatty acid synthase [11], ATP-citrate lyase, acetyl CoA carboxylase, and pyruvate decarboxylase [12] enzyme activities; as well as increased conversion of radiolabelled precursors into lipids [13]. Despite an inconsistent alteration in $G_s\alpha$ of 3T3-L1 cells upon differentiation [6,14], it is conceivable that G-proteins involved in adenylate cyclase modulation [15] may play a role in preadipocyte growth and differentiation.

G-proteins are heterotrimeric proteins which serve as intermediaries between activated membrane receptors and their effector enzymes and/or ion channels [16]. The α-subunit binds GTP and diplays intrinsic GTPase activity which modulates the activity of a given effector molecule. The domains involved in GTP-binding are highly conserved among the diverse members of the G-protein family. The G-3 region implicated in the GTP-induced conformational change in the α-subunit, displays the consensus sequence DVGGQR in most α-subunits. Consequently, mutations that alter GTP-hydrolysis may modulate the rate of hormonal signal transduction. The R201-$G_s\alpha$ position serves as substrate for cholera toxin catalyzed ADP-ribosylation rendering the α-subunit low in GTPase activity [16]. Naturally occurring missense mutations of R201-$G_s\alpha$ to C/H201-$G_s\alpha$ in growth hormone secreting pituitary adenomas are responsible for chronically elevated cAMP levels [17]. A single base mutation of Q61 in the G-3 region of p21ras leads to constitutive activation through a decrease in GTPase activity [15]. Mutation of the corresponding amino acid Q227 in $G_s\alpha$ also renders cAMP formation constitutively enhanced [18]. Modulation of the adjacent position G226 to A226 blocks adenylate cyclase activation, because the α-subunit is unable to assume the active GTP-bound configuration [19]. The mutated protein is, however, capable of normal receptor coupling [16]. Such a mutant would serve as a dominant inhibitor. The multiple pertussis toxin sensitive G-proteins are encoded by distinct genes [20]; however, their specific functions are still incompletely mapped.

In order to elucidate the influence of $G_s\alpha$- and $G_i\alpha$-proteins on cell proliferation and differentiation, we constructed the point mutations R201C-$G_s\alpha$ and G226A(H21a)-$G_s\alpha$ and homologous missense mutations in the $G_{i2}\alpha$ gene. Given the conservation of the GTP-binding site, the latter mutations should serve as either constitutively active (R179E, G205L) and inactive (G204A(H21a)) forms of $G_{i2}\alpha$. Such mutants would be expected to either stimulate or inhibit $G_{i2}\alpha$-mediated signalling pathways. All mutants were stably transfected into 3T3-L1 fibroblasts (preadipocytes) and tested for their ability to affect cell growth and differentiation as assessed by thymidine incorporation, modulation of G-protein levels, adenylate cyclase activation, deoxy-glucose uptake, and transcription of hormone-sensitive lipase.

MATERIALS AND METHODS

Site Directed $G_s\alpha$ and $G_{i2}\alpha$ Mutations and Construction of Expression Vectors

The insert of plasmid DJG18, encoding a rat α_s and α_{i2} cDNAs (provided by R. Reed, Johns Hopkins University, Baltimore, MD) was subcloned into M13mp19 and site-directed mutagenesis was performed by a modification of the method of Nakamaye and Eckstein, 1986 [21]. The $G_s\alpha$ cDNAs were inserted into the pGEM-vector containing a metallothionein promoter according to standard procedures [2]. Ten micromoles/l of $CdCl_2$ used to enhance $G_s\alpha$ expression did not affect the differentiation of control and vector-transfected cells. The mutagenic oligonucleotides used to create the most important $G_{i2}\alpha$ mutants had the following sequences: R179Eα_{i2}; 5′-GCCTGTGGTCTTCACTTCGGT-CCGCAGCACATC-3′ and G204Aα_{i2}; 5′-CCGCT-CAGATCGCTGGGCGCCCACATCAAACAT-3′. Mutations were confirmed by dideoxy sequencing. Wild-type and mutant α_{i2} cDNAs were ligated into the EcoRI site of a shuttle vector pSP72NotI and then into the NotI site of a modified retroviral vector, pZIPNeoSV(X) [22]. In the latter constructs, the transcription of α_{i2} cDNAs and the G418-resistance gene is directed by the Moloney murine leukemia virus long terminal repeat.

Transfection and Clone Selection

Cell lines were transfected with 1 µg of linearized DNA per 10 cm plate by calcium phosphate precipitation. Twenty-four hours after transfection, cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing G418 (0.60

mg/ml) for at least 3 weeks. G418-resistant clones were selected by serial dilution. Ten clones were selected for each construct, and the studies described here were performed with the clones (three) demonstrating the highest level of α_{i2} expression.

Cell Cultures and Differentiation Procedures

Cell culture reagents and Geneticin (G418) were purchased from Biofluids (Rockville, MD) and GIBCO (Middlesex, England). 3T3-L1 cells were grown in monolayer cultures in DMEM with high glucose (25 mM) and supplemented with 10% foetal calf serum (FCS) and standard penicillin-streptomycine and fungizone concentrations. Medium was changed three times a week. Consistent and reliable differentiation of cells was accomplished in the presence of insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM) (abbreviated ins/dex/iBuMeXan) for 8 consecutive days after cell confluency. To achieve consistent and reproducible differentiation, it proved necessary to expose the 3T3-L1 cells to ins/dex/iBuMeXan for at least 5 days. During the last 4 days of differentiation, cell medium was changed every 24 h. To date, differentiation of controls, vector- and Gα-transfected cells has been predictable and consistently reproducible provided that the cell density in the stock culture flask never exceeded 20% confluency prior to each passage.

Preparation of Subcellular Particulate Fractions

The medium was removed and the cells scraped in ice cold 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and pelleted (700*g*, 10 min, 4°C). The cell pellet was washed once with the same buffer, resuspended in 20 volumes of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and eventually homogenized on ice using a rotating knife for 10 s. The homogenate was subsequently filtered through nylon mesh and centrifuged at 27,000*g* for 30 min at 4°C. Finally, the pellet was resuspended in 10 volumes of Tris-EDTA buffer containing 0.1% bovine serum albumin, using the rotating knife for 5 s.

Western Blotting and Immunostaining

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [23]. Cell membrane samples were prepared for electrophoresis by dilution in sample buffer and heating to 100°C for 5 min. Proteins were electrophoretically transferred from gel to PVDF Immobilon-P filters (Millipore, Bedford, MA) with constant current (100 mA) for about 12 h in a Bio-Rad (Richmond, CA) Transblot™ apparatus. After transfer, the filters were incubated for 2 h with phosphate-buffered saline (PBS with 25 mM phosphate, pH 7.3) including 5% fat free dry milk powder. Subsequent incubation lasted for 6–24 h at room temperature in PBS containing 1% dry milk powder and anti G-protein antisera. After being washed with PBS containing 0.1% Tween 20, the filters were incubated with [^{125}I]-protein A (150,000 cpm/ml, Amersham, Arlington Heights, IL) in PBS containing 1% dry milk powder for 1 h at room temperature. The filters were then washed extensively with phosphate buffered saline containing 0.1% Tween 20 and dried prior to autoradiography.

G-Protein α-Subunit Antibodies

Rabbit antisera against synthetic decapeptides corresponding to the predicted C-terminal amino acid sequences of different G protein α-subunits were a generous gift from Dr. Allen M. Spiegel (NIH, NIDDK, Bethesda, MD). Antisera were used at final dilutions of 1/200 to 1/400.

Gross Appearance of Cell Cultures

Cells were observed under microscope at ×150 magnification during the exponential phase of growth, at confluency and after 8 days of differentiation. Photographs in penetrating light were taken of all cell strains subsequent to 8 days of differentiation.

Adenylate Cyclase (AC) Assay

AC activity was measured in 20 µl aliquots of crude subcellular fractions containing 20–50 µg of protein per assay tube [24]. The total incubation volume was 50 µl and consisted of 1 mM ATP (including 1.8×10^6 cpm of [α-^{32}P]-ATP; Amersham), 10 µM GTP, 2.8 mM $MgCl_2$, 1.4 mM EDTA, 1 mM cAMP (containing approximately 2.5×10^3 cpm of [8-^{3}H]-cAMP; Amersham), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl, pH 7.4, in the absence or presence of corticotropin (1 µM) or isoproterenol (1 µM). Incubations were carried out at 35°C for 20 min. Reactions were stopped with 0.1 ml of a solution comprising 10 mM cAMP, 40 mM ATP,

and 1% SDS. The [^{32}P]-cAMP formed and the [^{3}H]-cAMP added to monitor recovery (65–80%), were isolated using Dowex and aluminium oxide chromatography.

Estimation of cAMP Accumulation

3T3-L1 cells were plated at a cell density of 50,000 cells in 1 ml wells and grown to confluency or differentiated for 8 days in DMEM with FCS as indicated in 24 well plates. The medium was replaced with DMEM (1 ml per well) containing 10 mM Na-Hepes (pH 7.5), 1 mM iBuMeXan, and hormones as indicated. The plates were incubated for 20 min and then placed on ice. The incubation medium was replaced with 1 ml of 0.1 M HCl/0.1 mM $CaCl_2$ and the plates stored at −20°C. After thawing, the samples were acetylated by adding 20 µl of acetic anhydride/triethylamine (1:2.5 v/v). Cyclic AMP was measured by RIA, and DNA content in each well was determined to normalize for differences in cell number. DNA was determined using diphenylamin/acetaldehyde according to a standard colourimetric procedure [25]. Optical density was read at 580 nm after 36 h of incubation.

2-Deoxy Glucose Uptake

Each well of six-well plates was seeded with 50,000 transfected cells in 1 ml DMEM and grown to confluency or differentiated as indicated. During a 10 min period of incubation in the differentiation medium (containing ins/dex/iBuMeXan and 25 mM glucose), the incorporation of [^{3}H]-2-deoxy glucose (400 nCi/well) was measured in 0.5 ml 1N NaOH lysate of repeatedly washed (3 times in cold PBS) cells neutralized with 0.5 M HCl.

^{3}H-Thymidine Incorporation

Cells were plated at 1,000 cells per ml per well and fed 0.1 µCi of [^{3}H]-thymidine for 1 h six times during exponential growth in 6.5% horse and 3.5% FCS. Subsequent to each incubation period, the cells were washed (3 times) with ice-cold PBS and solubilized with 0.1% SDS. Material precipitated by 20% TCA was dissolved in 1N NaOH and neutralized with 1N HCl.

Hormone Sensitive Lipase (HSL) mRNA Estimation (Northern Blots)

Rat full-length HSL cDNA [10] contained within Bluescript was kindly furnished by Dr. M.C. Schotz (Wadsworth Medical Center, CA) and cut with BamHI and HindIII. Ten nanograms each of the 2.1 and 1.1 kb fragments were used in a standard random priming probe synthesis. RNA filters were hybridized over night at 42°C. Washing (three times) was accomplished by using 6 × SSPE (ambient temperature), 2 × SSPE (45°C), and 1 × SSPE (52°C) for 10 min in the presence of 0.2% SDS. Autoradiograms were scanned in an automatic XRS OmniMedia Scanner.

Statistical Evaluations

Levels of G-proteins were assessed by autoradiography and considered significantly different when deviating more than 35%. This cut point represents an average 2 × SD of 6 replicate scannings of Northern and Western blots. Functional assays were analyzed by non-parametrical analysis of variance (Kruskall-Wallis test), and binary comparisons accomplished by the Wilcoxon rank test at the level of $P = 2\alpha = 0.05$.

RESULTS

G-Protein Expression in Transfected Cells

Expression of $G_s\alpha$ and $G\alpha_{i2}$. 3T3-L1 clones resistant to G418 were obtained by transfecting cells with vectors alone and various $G_s\alpha$ and $G_{i2}\alpha$ cDNA constructs. After serial dilution cloning, 10 clones containing each construct were screened for G-protein subunit protein expression by immunoblotting with RM and AS7 affinity purified antisera [2] specific for $G_s\alpha$ and $G_{i1/i2}\alpha$, respectively. 3T3-L1 cells do not express $G_{i1}\alpha$ mRNA (J.O. Gordeladze, unpublished observations), hence immunoblots are construed to reflect the $G_{i2}\alpha$ protein only. For further analyses, we selected three clones which constitutively expressed the highest levels of $G_s\alpha$ or $G_{i2}\alpha$. Cells were differentiated as indicated, and 20 µg of crude membrane protein was separated by gel electrophoresis, blotted, and immunocomplexed as described in Materials and Methods.

The cells were also checked for the presence of correct mutations by reverse transcriptase (RT) polymerase chain reaction (PCR), and restriction enzyme analysis using a unique restriction enzyme for each missense mutation introduced. All clones used contained the correct construct. Both immunoblots and RT-PCR analyses demonstrated that transfected cells

lost some of their $G_s\alpha$ expression after 3 months in continuous (stock) culture. Hence, stock cell clones used were always grown and frozen in large quantities and never kept for more than 2 months in culture before discarded (data not shown).

In figures where data for control cells are not shown, results were not discernible from observations obtained with vector-transfected cells. For the convenience of the reader, the various G-protein mutations and their effects on GTP-ase activity and ensuing impact on effector systems are summarized in Table I.

Effect of $G_s\alpha$ on 3T3-L1 Cells

G-protein levels in $G_s\alpha$-transfected cells before and after differentiation. Vector (pZEM)-transfected cells (grown in the presence of 10 μM $CdCl_2$ to maximally activate the metallothionein promoter) showed identical intrinsic $G_{i2}\alpha$ levels, while the wild type $G_s\alpha$ ([Wtype]$G_s\alpha$) and the mutants [R201C]$G_s\alpha$ and [G226A]$G_s\alpha$ showed an approximate 4–5-fold higher amount of immunoreactive $G_s\alpha$ (Fig. 1, top). Subsequent to incubation (differentiation) with insulin (10 μg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM), the total amounts of either $G_{i2}\alpha$ or $G_s\alpha$ attained approximately the same levels, irrespective of construct.

Growth in monolayer culture; gross appearance. Subsequent to differentiation of 3T3-L1 cells transfected with control DNA, pZEM-vector, Wtype-$G_s\alpha$, or the mutations R201C-$G_s\alpha$ and G226A-$G_s\alpha$, standard microscopic pictures with ×150 magnification were taken. It appeared that pZEM-transfected cells were confluent (i.e., rounded up), and some 85–95% of the cells contained lipid droplets (Fig. 2). The cells containing the Wtype-$G_s\alpha$ and R201C-$G_s\alpha$ mutations were undistinguishable from vector-transfected cells in terms of shape, size, or lipid accumulation (data not shown). However, the clones expressing the H21a-$G_s\alpha$ mutant seemed to differentiate better, since most cells were generally larger and contained more lipid (Fig. 2).

TABLE I. Designation, Effect on Intrinsic GTP-ase Activity, and Impact on Effector Activities Exerted by the Different Missense $G_s\alpha$ and $G_{i2}\alpha$ Mutations Transfected Into 3T3-L1 Cells

Mutation	Impact on GTP-ase	Impact on Effector
R201C-$G_s\alpha$		
R179E-$G_{i2}\alpha$	−	+
Q205L-$G_{i2}\alpha$		
G226A(H21a)-$G_s\alpha$	Chronic	−
G204A(H21a)-$G_{i2}\alpha$	α^{GDP}-state	

Adenylate cyclase (AC) activation and cAMP accumulation. AC activation was accomplished in cell membranes incubated without or with corticotropin (1 μM) or isoproterenol (1 μM) for 20 min at 35°C in the presence of 1 mM [α-^{32}P]-ATP and 0.4 mM free Mg^{2+}. Cyclic AMP accumulation was performed on whole cells in the presence or absence of the same hormones for 20 min, and RIA analyses completed on cell lysates. DNA was determined on remaining cell debris. It appeared that net AC activation by corticotropin and isoproterenol were doubled in pZEM-transfected cells after differentiation. The introduction of the H21a-$G_s\alpha$ mutation almost completely blocked the enhancement of hormone-elicited AC activation before differentiation, while after differentiation it was slightly increased over vector-transfected cells. Wtype-$G_s\alpha$ and R201C-$G_s\alpha$ transfected cells apparently enhanced both pre- and post-differentiated hormone-elicited membrane AC activation to the same extent (i.e., by some 2- and 3-fold, respectively) (Fig. 3A). Adenosine-mediated reduction in corticotropin-enhanced AC activation did not differ between 3T3-L1 clones without or with the various $G_s\alpha$ constructs (data not shown).

Translated into cAMP accumulation, control and pZEM-transfected cells became 2–3 times more sensitive to corticotropin and isoproterenol after differentiation. Interestingly, hormone-stimulated cAMP accumulation in H21a-$G_s\alpha$ transfected cells were lower than in pZEM-transfected cells before differentiation, while approximately 5-fold higher in differentiated cells. Both the Wtype-$G_s\alpha$ and R201C-$G_s\alpha$ constructs enhanced preadipocyte cAMP accumulation by hormones approximately 2-fold, while only 2- to 3-fold subsequent to differentiation (Fig. 3B).

Deoxy-glucose uptake. [^{3}H]-deoxy-glucose uptake during 10 min at confluence and 8 days subsequent to differentiation was measured in the presence of insulin-containing differentiation medium and depicted as dpm/million cells per min. Essentially the same trend as for cAMP accumulation emerged (Fig. 4). Deoxy-glucose uptake into pZEM-transfected cells was increased by a factor of 4 subsequent to differentiation. By introducing the H21a-$G_s\alpha$ construct,

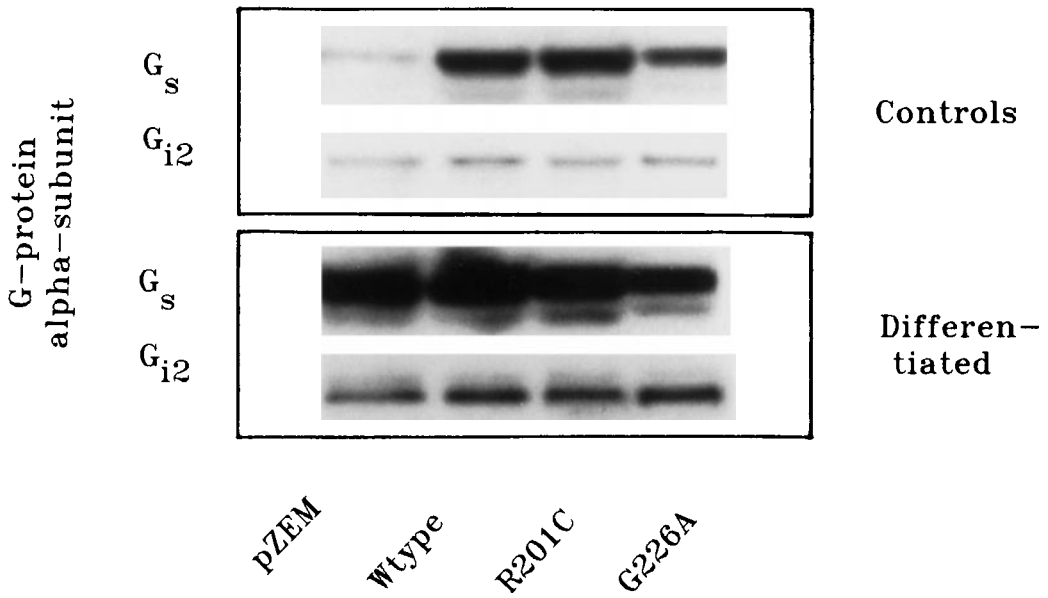

Fig. 1. Expression of $G_s\alpha$ and $G_{i2}\alpha$ subunits in 3T3-L1 cells transfected with vector (pZEM), wild type (Wtype) $G_s\alpha$, and the mutations R201C-$G_s\alpha$ and G226A(H21a)-$G_s\alpha$ before and after differentiation in DMEM containing 10% FCS fortified with insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM) for 8 consequtive days. Crude membrane proteins (20 µg/lane) were resolved on 8–16% SDS Tris-glycine gels and transferred to nitrocellulose filters (according to standard Novex procedure). Blots were incubated with the AS7 ($\alpha_{i1/2}$) and RM (α_s) antibodies [50]. Detection of α-subunit levels was accomplished by autoradiography of antigen-antibody complexed with [^{125}I]-protein A.

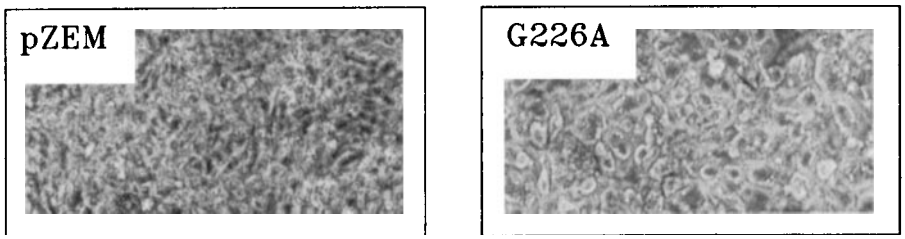

Fig. 2. Gross appearance of 3T3-L1 cells differentiated with insulin (10 µg/ml), dexamethasone (10 nM) and iBuMeXan (0.5 mM) for 8 consecutive days in DMEM with 10% FCS. The pictures represent ×150 magnifications of cells transfected with vector (pZEM) and the mutant [G226A(H21a)]$G_s\alpha$, respectively.

both pre- and post-differentiated [^{3}H]-deoxy-glucose uptake was upregulated some 1.7–1.8-fold compared to pZEM-transfected clones. Cells containing the Wtype-$G_s\alpha$ or R201C-$G_s\alpha$ constructs were equal to pZEM-transfected clones in terms of [^{3}H]-deoxy-glucose uptake both in the pre- and post-adipocyte stage (Fig. 4).

Effect of $G_{i2}\alpha$ on 3T3-L1 Cells

G-protein levels in $G_{i2}\alpha$-transfected cells before and after differentiation. Cell clones containing the $G_{i2}\alpha$ wild type (Wtype-$G_{i2}\alpha$) and the mutants [R179E]$G_{i2}\alpha$, [Q205L]$G_{i2}\alpha$, and [G204A]$G_{i2}\alpha$ showed an approximate 5–7-fold higher amount of immunoreactive $G_{i2}\alpha$ as compared to the vector (pZipNeoSX(V))-transfected cells prior to differentiation (Fig. 5, top). Immunoblotting conducted with the RM antibody on the same membrane fractions, showed little if any variation in $G_s\alpha$. After differentiation for 8 days, both G-protein subunit increased, except for $G_s\alpha$ in H21a-$G_{i2}\alpha$ containing cells (Fig. 5, bottom).

Effects of $G_{i2}\alpha$ on 3T3-L1 cell proliferation. Growth rate was studied in the presence of 1, 5, and 10% FCS in DMEM in the exponential phase of proliferation. Controls and cells containing vector (pZipNeoSX(V)), Wype-$G_{i2}\alpha$, and the mutations R179E-$G_{i2}\alpha$, Q205L-G_{i2}, and G204A-$G_{i2}\alpha$ were seeded at a density of 1,000 cells/well. At indicated time intervals, they were trypsinated and counted in a haemocytometer. Doubling time was based on regression analyses of 8–12 time intervals and computed as $1/(b.\ln 2)$ from the equation $y = ae^b$. As can be

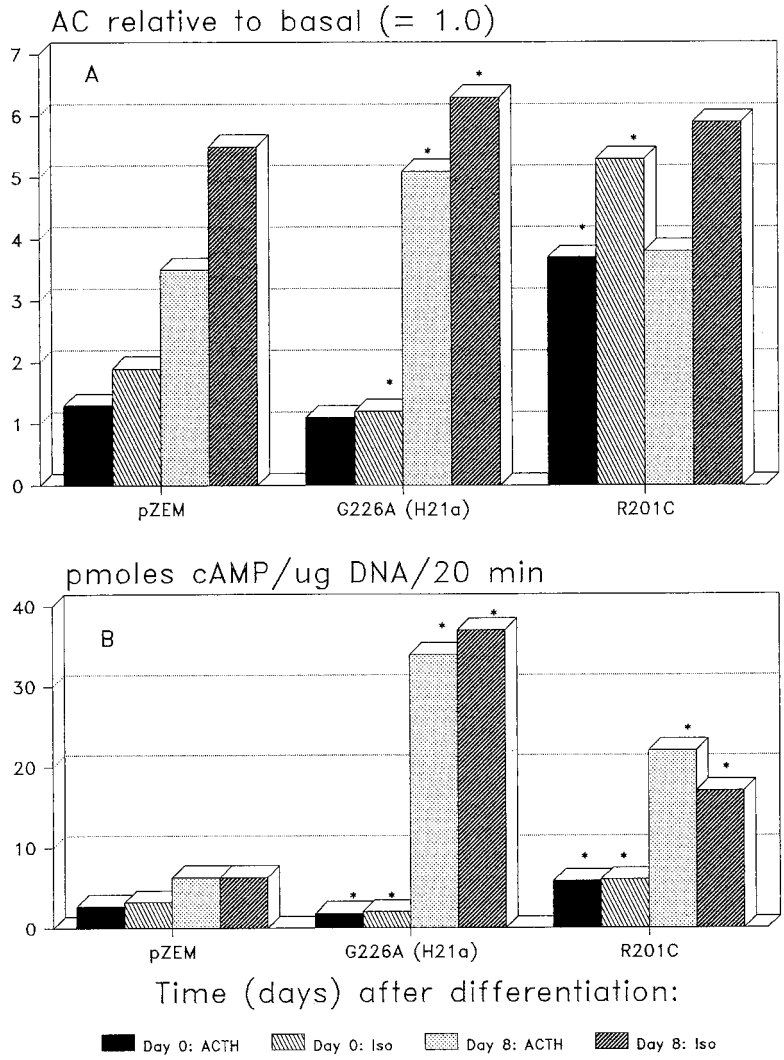

Fig. 3. Adenylate cyclase activation **(A)** and cAMP accumulation **(B)** in 3T3-L1 cells at confluence (t_0) and 8 days (t_8) of differentiation with insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM). Cells were transfected with either vector (pZEM) or the G226A(H21a)-$G_s\alpha$ or R201C-$G_s\alpha$ constructs, and crude cell membranes or whole cells subjected to incubations in the absence or presence of corticotropin (1 µM) or isoproterenol = Iso (1 µM). Adenylate cyclase activation was assayed in the presence of 0.4 mM free Mg^{2+}, 1 mM ATP including $1.8 \cdot 10^6$ cpm of [α-^{32}]P-ATP, 0.1 mM Ca^{2+}, 1 mM cAMP comprising 2,500 cpm of [8-^{3}]H-cAMP, a regenerating system (myokinase, creatine kinase and creatine phosphate) for ATP and 25 mM Tris-HCl, pH 7.4. Incubations were carried out for 20 min at 35°C, and [^{32}P]-cAMP formed was separated on ion exchange and alumina columns. Adenylate cyclase activation is depicted as values relative to basal (=1.0). Cyclic AMP accumulation was accomplished with the same hormones in serum free DMEM containing 0.5 mM iBuMeXan for 20 min at 37°C. Cyclic AMP was measured in a HCl-Ca^{2+} lysate by a conventional RIA method and expressed as pmol cAMP/µg DNA per 20 min of incubation. All figures represent mean of quadruplicate determinations. *$P < 0.05$ (Wilcoxon rank test, vector- and $G_s\alpha$-transfected cells vs. controls). Coefficients of variation (CVs) for the adenylate cyclase and cAMP were 3.4 and 7.8%, respectively.

seen from Figure 6, the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants displayed a doubling time of some 2–3 h less than controls and vector-transfected cells in the presence of 1% FCS. The H21a-$G_{i2}\alpha$ mutation yielded a doubling time of 17.4 h, some 3 h higher than for controls. In 5% FCS, the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants multiplied at the same rate as control cells, while the H21a-$G_{i2}\alpha$ induced doubling time was still elevated by 3 h. Differences were not seen between any cell clones in the presence of 10% FCS (data not shown).

Thymidine incorporation in the presence of 1, 5, and 10% FCS was measured as 10,000 cpm of [^{3}H]-thymidine in TCA-precipitable material per 60 min at various time intervals. FCS (10%)

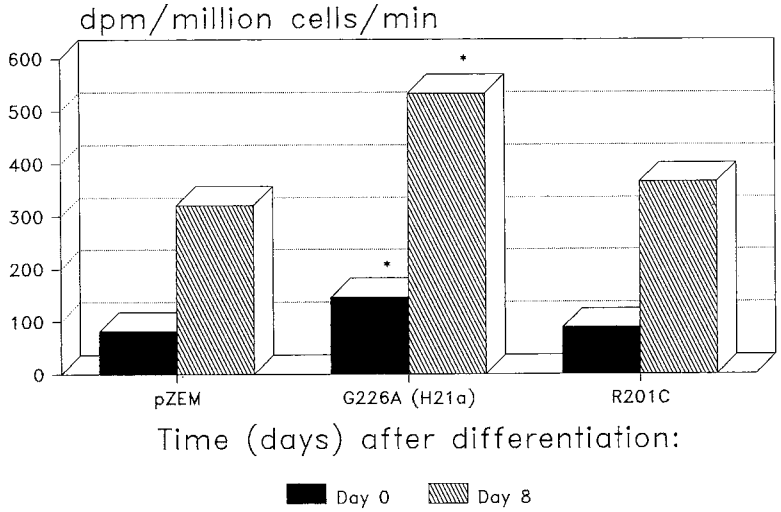

Fig. 4. Insulin-elicited deoxy-glucose uptake in confluent undifferentiated and differentiated 3T3-L1 cells. Phenotypic differentiation of cells previously transfected with either vector (pZEM) or the G226A(H21a)-$G_s\alpha$ and R201C-$G_s\alpha$ constructs, was completed in the presence of insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM). [^{3}H]-deoxy-glucose contents were measured in the NaOH-lysate of cells exposed to differentiation medium subsequent to a 10 min incubation period, and given as dpm/million cells per min. All results are shown as means of quadruplicate determinations. *$P < 0.05$ (Wilcoxon rank test, vector- and $G_s\alpha$-transfected cells vs. controls). Coefficient of variation (CV) for the assay was 6.8%.

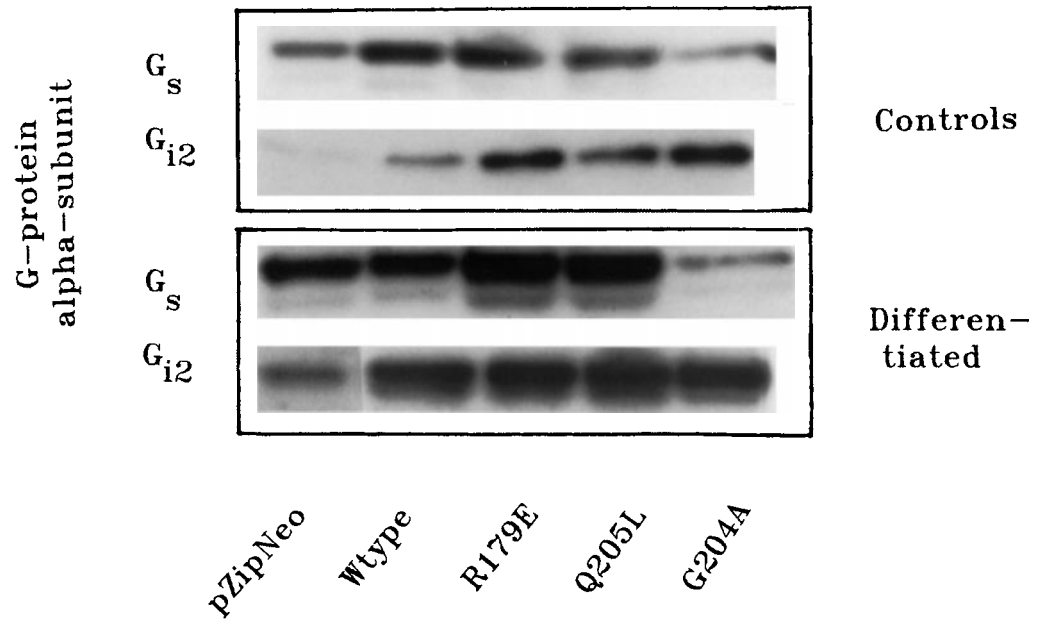

Fig. 5. Expression of $G_s\alpha$ and $G_{i2}\alpha$ subunits in 3T3-L1 cells transfected with control (placental) DNA, vector (pZipNeoSX(V)), wild type $G_{i2}\alpha$ (Wtype-$G_{i2}\alpha$), and the mutations R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A(H21a)-$G_{i2}\alpha$, before and after differentiation in DMEM containing 10% FCS plus insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM) for 8 consecutive days. Crude membrane protein fractions (20 µg/lane) were resolved on SDS Tris-glycine gels, trans-blotted, and the α-subunits visualized with antisera [50] and [^{125}I]-protein A as described in the legend to Figure 1.

gave essentially no differences (data not shown) between cell clones, while 1% FCS (data not shown) supplementation resulted in a profile very much like the one with 5% FCS shown in Figure 7. Cells transfected with the various $G_{i2}\alpha$-constructs outlined above were grown in late exponential phase and showed striking differences. Control cells, vector-transfected and Wtype-$G_{i2}\alpha$-transfected clones displayed similar and initially constant rates of de novo DNA

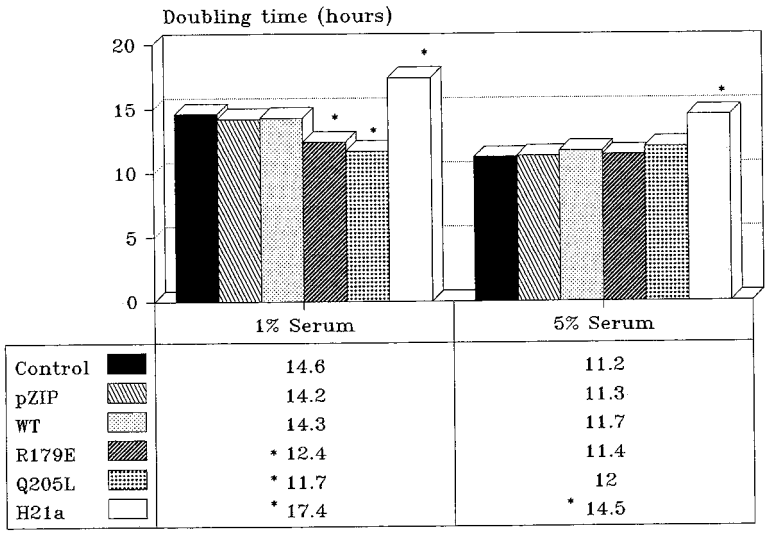

Fig. 6. Growth Rate of 3T3-L1 Fibroblasts Expressing Wild Type (Wtype = WT) $G_{i2}\alpha$ and the Mutated $G_{i2}\alpha$ Constructs R179E, Q205L, and G204A (H21a). The doubling times were estimated by cell counting during the exponential phase of growth in 10 cm Petri dishes 4 days prior to confluence in DMEM containing 1 and 5% FCS, respectively. *$P < 0.05$ (Wilcoxon rank test, vector- and $G_{i2}\alpha$-transfected cells vs. controls).

synthesis plateauing at some 40 h of incubation. The H21a-$G_{i2}\alpha$ clone exhibited a constant but slower incorporation rate which was linear for at least 60 h. However, the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants plateaued and decreased their incorporation rate at an early stage, indicating a complete halt in cell proliferation.

Growth in monolayer culture; gross appearance. Subsequent to differentiation of 3T3-L1 cells transfected with control DNA, vector (pZipNeoSX(V)) or the mutations R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A-$G_{i2}\alpha$, pictures at $\times$150 magnification were taken (Fig. 8). It appeared that Ctrl (A) and pZipNeo (B) cells were confluent and spherical, of which some 85–95% contained lipid droplets. The cells expressing the [R179E]$G_{i2}\alpha$ (D) and [Q205L]$G_{i2}\alpha$ (E) missense mutations appeared as megalocytes with huge lipid droplets; however, they grew to a much lesser density. Interestingly, the H21a-$G_{i2}\alpha$ (F) expressing clones were unable to stay in culture as monolayers. They became partly detached, grew as multilayers to a very high density, and retained their typical shape as preadipocytes with protrusions and long cellular processes. Detached cells were still viable as evidenced by a standard dye exclusion test (data not shown).

Adenylate cyclase (AC) activation and cAMP accumulation.

Measurements of AC activities and cAMP accumulation were accomplished as described earlier. Net AC activation by adrenocorticotrophic hormone (corticotropin) and isoproterenol were doubled in controls and vector (pZipNeoSX(V))-transfected cells after differentiation. Transfection with the Wtype-$G_{i2}\alpha$, R179E-$G_{i2}\alpha$, and Q205L-$G_{i2}\alpha$ constructs apparently enhanced both pre- and post-differentiated cell corticotropin- and isoproterenol-stimulated AC activities by some 3- and 3–4-fold, respectively. The introduction of the H21a-$G_{i2}\alpha$ mutation completely blocked the enhancement of hormone-elicited AC activation (Fig. 9A). As a test for the inherent properties of the various $G_{i2}\alpha$ constructs, adenosine-mediated reduction in corticotropin-enhanced AC was measured. Percentage reduction in the various 3T3-L1 clones ranged R179E-$G_{i2}\alpha$ = Q205L-$G_{i2}\alpha$ > Wtype-$G_{i2}\alpha \gg$ controls = pZipNeoSX(V); H21a-$G_{i2}\alpha \sim$ 0 (data not shown).

Corresponding cAMP accumulation showed that controls and vector-transfected cells became 2–3 times more sensitive to corticotropin and isoproterenol after differentiation. As for the AC activation, Wtype-$G_{i2}\alpha$ and especially the R179E-$G_{i2}\alpha$ and Q205L-$G_{i2}\alpha$ constructs enhanced preadipocyte cAMP accumulation by hormones. Furthermore, the Wtype-$G_{i2}\alpha$ and the R179E-$G_{i2}\alpha$ and Q205L-$G_{i2}\alpha$ mutations augmented hormone-stimulated cAMP accumulation in differentiated cells by some 8–10-fold over controls and vector-transfected cells. Interestingly, cAMP accumulation in H21a-$G_{i2}\alpha$

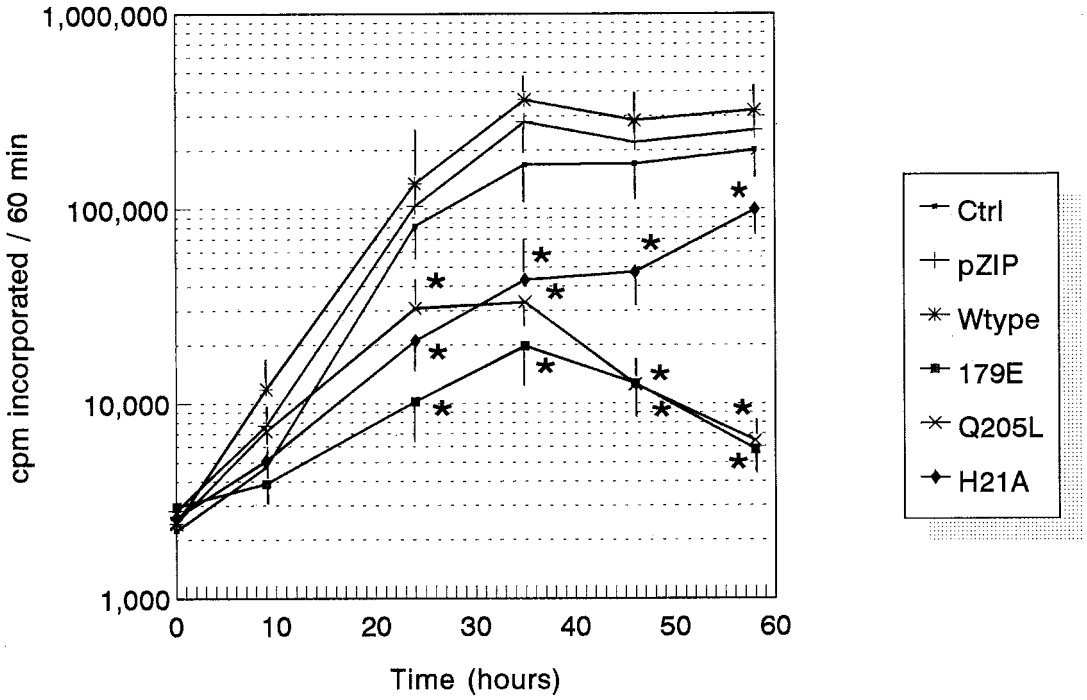

Fig. 7. Rate of DNA synthesis in 3T3-L1 preadipocytes transfected with control (placental) DNA, vector (pZipNeoSX(V)), wild type $G_{i2}\alpha$ (Wtype$G_{i2}\alpha$), and the mutated R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A(H21a)-$G_{i2}\alpha$ constructs. Cells were plated at 1,000 cells/ml/well in 24-well plates and incubated in DMEM with 5% FCS. [^{3}H]-thymidine was incorporated as described in Materials and Methods at the time intervals indicated and depicted as cpm incorporated per 60 min. Values represent mean ± SEM of 4 replicates for controls (Ctrl), vector (pZipNeoSX(V)), transfected cells, or cells containing Wtype-$G_{i2}\alpha$, R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A(H21a)-$G_{i2}\alpha$ constructs, respectively. *$P < 0.05$ (Wilcoxon rank test, vector- and $G_{i2}\alpha$-transfected cells vs. controls).

transfected cells was in fact lower than in controls (and vector-transfected cells) (Fig. 9B).

Deoxy-glucose uptake. As before, the [^{3}H]-deoxy-glucose uptake was measured during 10 min at confluence and 8 days subsequent to differentiation. Essentially the same trend as for cAMP accumulation emerged (Fig. 10). Deoxy-glucose uptake into controls and pZipNeoSX(V)-transfected cells were increased by a factor of 4 subsequent to differentiation. By introducing Wtype-$G_{i2}\alpha$ and the R179E-$G_{i2}\alpha$ and Q205L-$G_{i2}\alpha$ constructs, both pre- and post-differentiated deoxy-glucose uptake were upregulated some 2-fold compared to controls (and vector-transfected cells). Surprisingly, clones containing the H21a-$G_{i2}\alpha$ construct were inferior to endogenous $G_{i2}\alpha$ (in control and vector-transfected cells) in terms of deoxy-glucose uptake both in the pre- and pan-adipocyte stage.

Hormone sensitive lipase. Figure 11 shows Northern blots of mRNA for hormone sensitive lipase in pre- and post-differentiated 3T3-L1 cells. Post-differentiated cells containing Wtype-$G_{i2}\alpha$ and the R179E-$G_{i2}\alpha$ and Q205L-$G_{i2}\alpha$ constructs displayed a tremendous (>50-fold) increase in amount of message for this enzyme. However, post-differentiated vector-transfected cells exhibited a non-significant alteration compared to corresponding pre-differentiated cells. Interestingly, the introduction of H21a-$G_{i2}\alpha$ completely blocked the transcription of hormone sensitive lipase despite incubation in differentiation medium.

DISCUSSION

Concerted G-Protein Gene Regulation

The 3T3-L1 cell lines stably transfected with wild type (Wtype) $G_s\alpha$ or $G_{i2}\alpha$-cDNA, or the R201C-$G_s\alpha$/R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G226A(H21a)-$G_s\alpha$/G204A(H21a)-$G_{i2}\alpha$ constructs consistently overexpressed $G_s\alpha$ or $G_{i2}\alpha$ proteins by a factor of 5–6 or 4–5, respectively. Irrespective of being activating or inactivating, these constructs did not induce compensatory alterations of intrinsic $G_s\alpha$ or $G_{i2}\alpha$. There are conflicting findings in the literature as to whether compensatory G-protein regulation ensue [26–28]. Hermouet et al. have demonstrated that $G_s\alpha$ was marginally and inconsistently reduced

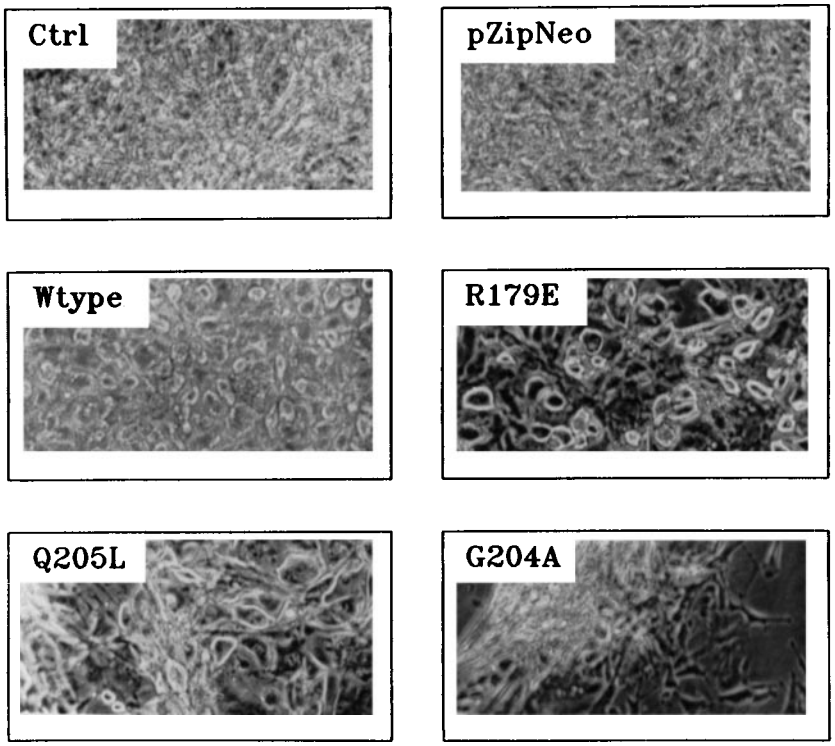

Fig. 8. Gross appearance of 3T3-L1 cells differentiated with insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM) for 8 consequtive days in DMEM containing 10% FCS. The pictures represent ×150 magnifications of cells transfected with placental DNA (Ctrl), vector (pZipNeoSX(V)), or wild type $G_{i2}\alpha$ (Wtype-$G_{i2}\alpha$), and the mutations R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A(H21a)-$G_{i2}\alpha$, respectively.

upon transfection of NIH 3T3 cells with various $G_{i2}\alpha$ constructs [26]. Since overexpression of $G_s\alpha$ or $G_{i2}\alpha$ does not lead to complementory G-protein gene regulation in 3T3-L1 cells, the present cell line constitutes a suitable model to study the impact of each of these G-proteins on cell growth and differentiation.

Cell Proliferation

Interestingly, none of the $G_s\alpha$-constructs affected 3T3-L1 fibroblasts proliferation rate. However, cells transfected with the activating [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants displayed lowered doubling times in the presence of 5% (v/v) serum. This reduction in doubling times was comparable to those seen by Hermoutet et al. [2] with the same vector constructs transfected into NIH 3T3 cells. The GTP-binding protein $G_{i2}\alpha$ has been shown to couple to receptors with 7 transmembrane regions like those binding somatostatin in GH pituitary adenoma cells [23] and catecholamines in platelets [29], and might be responsible for the reduction of adenosine-elicited adenylate cyclase in 3T3-L1 cells containing either the [Wtype]$G_{i2}\alpha$ or the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants (J.O. Gordeladze, unpublished observations). However, these constructs, if exerting an impact on cAMP accumulation at all, actually evoked an increase in corticotropin- and isoproterenol-elicited cAMP production. If one assumes that hormones which inhibit adenylate cyclase do so by releasing $G\beta\gamma$ to complex with $G_s\alpha$ and block its effect, the overexpression of $G_{i2}\alpha$ may in fact complex with intrinsic $G\beta\gamma$ to yield a marginally enhanced basal cAMP accumulation.

Cyclic AMP has been found to enhance cell proliferation in cell systems like the thyroid FRTL cell line [30], osteoblasts [31], and Swiss 3T3 cells [32]. The effect of activating $G_{i2}\alpha$ mutants may thus be explained by the altered cAMP, if it were not for the fact that the activating or inactivating [R201C]$G_s\alpha$ or [G226A]$G_s\alpha$ mutants did not at all affect growth rate. However, the effect of the various $G_{i2}\alpha$-constructs was only evident when the growth medium was depleted of serum containing mitogenic activity, which can be attributed to a variety of of growth factors. The majority of these factors

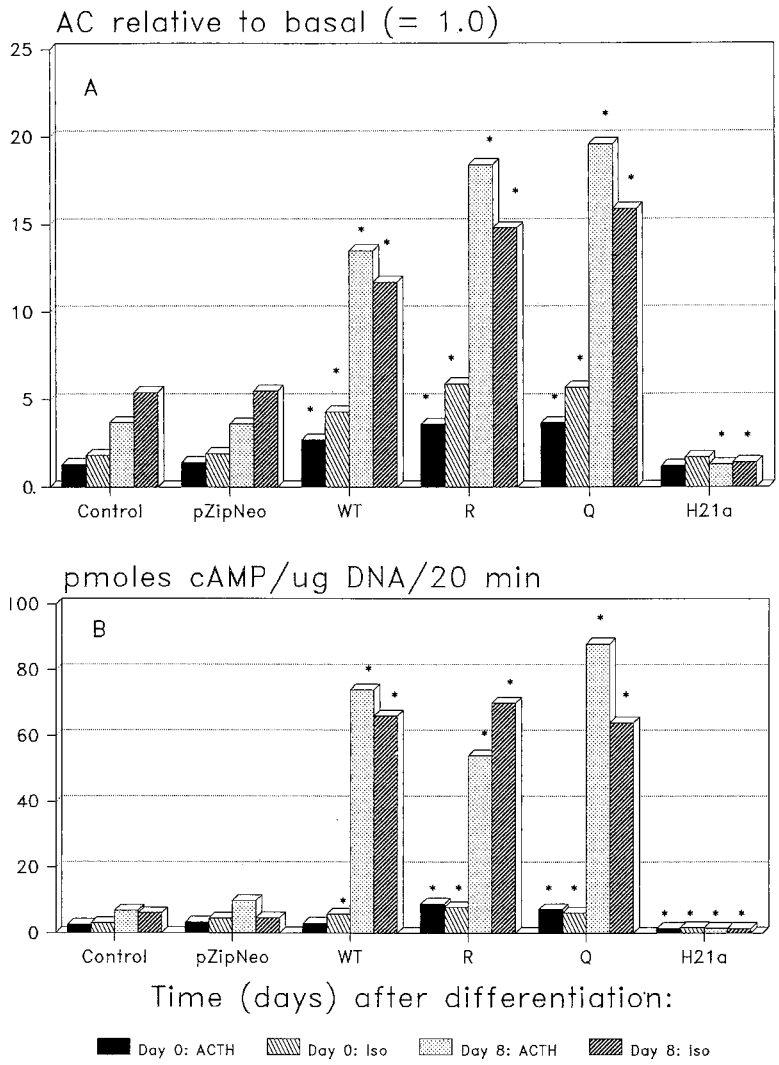

Fig. 9. Adenylate cyclase activation **(A)** and cAMP accumulation **(B)** in 3T3-L1 cells at confluence (t_0) and 8 days (t_8) of differentiation with insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM). Cells were transfected with either placental DNA (controls), vector (pZipNeoSX(V)), or wild type $G_{i2}\alpha$ (Wtype-$G_{i2}\alpha$), and the mutations R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, and G204A(H21A)-$G_{i2}\alpha$. Then cell membranes or intact cells were subjected to incubations in the absence or presence of corticotropin (1 µM) or isoproterenol = Iso (1 µM). Adenylate cyclase activation and cAMP accumulation were assayed and expressed as outlined in the legend to Figure 3. All figures represent mean of quadruplicate determinations. $*P < 0.05$ (Wilocoxon rank test, vector- and $G_{i2}\alpha$-transfected cells vs. controls). Coefficients of variation (CVs) for the adenylate cyclase and cAMP were 3.8 and 7.1%, respectively.

transduce their signals either through protein tyrosine kinase associated receptors (i.e., EGF, insulin) or through G-protein coupled systems (i.e., bombesin, thrombin, bradykinin, vasopressin) [16]. Since the latter hormones mainly work through phosphatidyl inositol biphosphate (PIP_2) hydrolysis stimulated by G_p, which is different from $G_{i2}\alpha$, the latter G-protein might convey the signals of some growth factors. It has been shown that the growth factors $TGF\beta_1$ and FGF somehow work through pertussis toxin (pertussis toxin)-sensitive G-proteins [33,34], and that the insulin-like growth factor II (IGF-II) receptor in fact couples directly to $G_{i2}\alpha$ [35]. LaMorte et al. [36] showed that microinjections of a $G_{i2}\alpha$ antibody (AS) into Balb/c 3T3 fibroblasts resulted in abolition of IGF-II-induced DNA synthesis and cell proliferation. Hence, it is conceivable that growth factor stimulation may be mediated through $G_{i2}\alpha$. In fact, $G_{i2}\alpha$ (*Gip2*) mutants like [R179E]$G_{i2}\alpha$ (i.e., [R179C]$G_{i2}\alpha$ and [R179H]$G_{i2}\alpha$) have been found in tumour cells of both the adrenals and ovaries where IGFs play a major role in cell regulation [16,37].

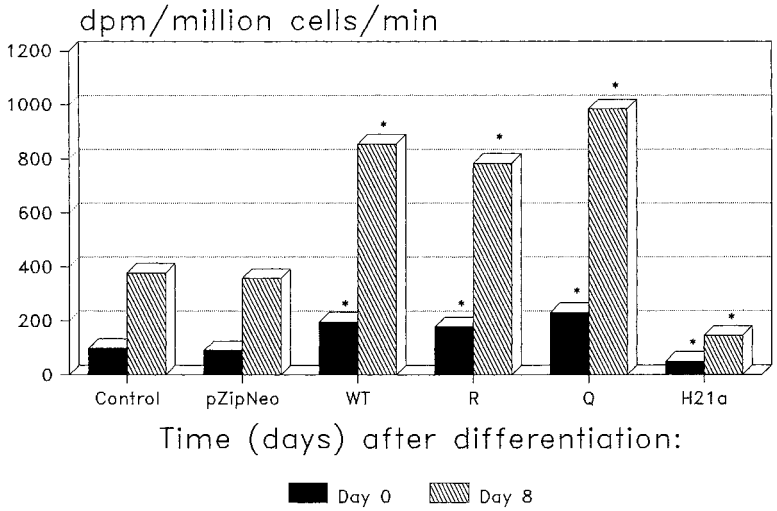

Fig. 10. Insulin-elicited deoxy-glucose uptake in confluent undifferentiated and differentiated 3T3-L1 cells. Phenotypic differentiation of cells transfected with either placental DNA (controls), vector (pZipNeoSX(V)), and wild type $G_{i2}\alpha$ (Wtype-$G_{i2}\alpha$), or the mutations R179E-$G_{i2}\alpha$ (R), Q205L-$G_{i2}\alpha$ (Q), and G204A(H21a)-$G_{i2}\alpha$, was completed in the presence of insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM). [^{3}H]-deoxy-glucose contents were measured as described in the legend to Figure 4, and given as dpm per million cells per min. All results are shown as means of quadruplicate determinations. *$P < 0.05$ (Wilcoxon rank test, vector- and $G_{i2}\alpha$-transfected cells vs. controls). Coefficient of variation (CV) for the assay was 7.8%.

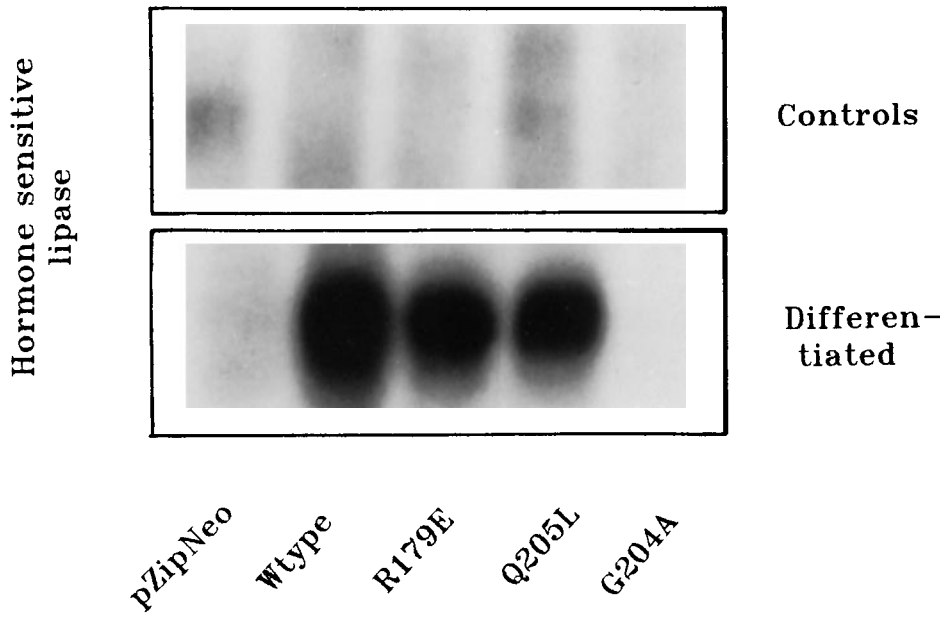

Fig. 11. Northern blot of hormone sensitive lipase in confluent control and differentiated 3T3-L1 cells transfected with vector (pZipNeoSX(V)) or wild type $G_{i2}\alpha$ (Wtype-$G_{i2}\alpha$), and the mutations R179E-$G_{i2}\alpha$, Q205L-$G_{i2}\alpha$, or G204A(H21a)-$G_{i2}\alpha$. Differentiation was accomplished after 8 consecutive days of incubation with insulin (10 µg/ml), dexamethasone (10 nM), and iBuMeXan (0.5 mM). Total RNA was isolated by the RNAzol™ method (Cinna-Biotecx Lab. Int., Inc., Friendswood, TX), and 20 µg separated on denaturing 1% agarose gels, capillary blotted onto nitrocellulose filters, and hybridized (by random priming) with the [^{32}P]-labelled 1 and 2 kB cDNA (BamHI and HindIII) fragments. Satisfactory autoradiography was obtained after 3 days.

Introducing the [G204A(H21a)]$G_{i2}\alpha$ mutant, which may interact with receptors, but does not assume the GTP-bound state, markedly enhanced cell doubling time was also seen with NIH 3T3 cells [2]. Since this mutant barely had an effect on intracellular cAMP accumulation, it is conceivable that excessive H21a-α_{i2} protein binds to and displaces intrinsic wild type α_{i2} from their receptors, thus blocking the proliferative effect of serum growth factors. Previously,

a role in cell proliferation had been evoked for the pertussis toxin-sensitive G-proteins [38]. $G_o\alpha$ was shown to reduce cell division in Y1 adrenal cells [39]. $G_{i1}\alpha$ is apparently not expressed in 3T3-L1 cells, and $G_{i3}\alpha$ does not seem to affect cell proliferation in NIH 3T3 cells [40]. Hence, $G_{i2}\alpha$ may be the sole pertussis toxin-sensitive G-protein involved in cell proliferation, at least in 3T3-L1 preadipocytes.

Most studies of the effect of $G_{i2}\alpha$ have been performed on cells which do not change phenotype. However, the 3T3-L1 fibroblasts assume the adipocyte phenotype when incubated with insulin, dexamethasone, and iBuMeXan for 3 to 7 days [11] with ensuing enhancement of $G_s\alpha$, $G_i\alpha$, and $G\beta$ protein levels [6]. It has been shown that lymphoma Nb2-Sp cells express their highest levels of $G_{i2}\alpha$ mRNA when reaching confluence and cease to proliferate [41]. This has been interpreted as coinciding with lowered cAMP, which is necessary for proliferation of these cells [42]. In the absence of differentiation medium, the rate of thymidine incorporation (which may be construed as proliferation rate of non-confluent euploid cells) into 3T3-L1 cell, DNA was dramatically reduced and even ceased in cells transfected with the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants prior to reaching confluence. This may be interpreted as a toxic reaction to the overexpression of activating G-protein mutations rather than representing a genuine halt in cell proliferation. However, the parallel enhancement of differentiation markers strongly indicates that $G_{i2}\alpha$ plays a constitutive role in the differentiation process of 3T3-L1 fibroblasts into adipocytes.

Cell Differentiation

Acquisition of adipocyte characteristics entails rounded shape with accumulation of lipid droplets [11], enhanced incorporation of acetate into triglycerides [13], and increased lipid metabolizing enzymes like fatty acid synthase, ATP-citrate lyase, acetyl CoA carboxylase, pyruvate dehydrogenase, glycerophosphate dehydrogenase (GPDH), and hormone sensitive lipase (HSL) [11,12,43]. Furthermore, insulin receptor number and adenylate cyclase susceptibility to corticotropin and β-adrenergic agonists are increased [44,45]. Finally, this transition is associated with an increase in $G_s\alpha$, $G_i\alpha$ (as estimated by pertussis toxin-catalyzed ADP-ribosylation) and $G\beta$ yielding an enhanced $G\alpha/G\beta$ ratio [6]. Recently, it was demonstrated that antisense oligonucleotides towards $G_s\alpha$ enhanced differentiation in 3T3-L1 cells, while forskolin and cAMP analogues failed to induce differentiation [46]. These data indicate that G_s in fact inhibit adipocyte differentiation through a mechanism involving an effector system different than the adenylate cyclase. The present work, which demonstrates that only the cell clones containing the inactivating [G226A]$G_s\alpha$ mutant enhanced differentiation despite lowered initial hormone-elicited adenylate cyclase activation, corroborates these findings.

It was demonstrated that pre-exposure of 3T3-L1 cells with pertussis toxin prior to cell differentiation with insulin, dexamethasone, and iBuMeXan partly reduced insulin-elicited glucose uptake, GPDH activity, and isoproterenol-stimulated cAMP accumulation [47]. This directly implicates one of the $G_i\alpha$ proteins in the differentiation process without involving cAMP as the second messenger. Moxham et al. 1993 [48] induced a $G_{i2}\alpha$-specific antisense RNA in vivo in mice and demonstrated that adipocytes in the transgenes exhibited elevated basal cAMP levels, and that body fat was markedly reduced. In line with these experiments, we were able to show that differentiation of 3T3-L1 cells overexpressing wild type $G_{i2}\alpha$ ([Wtype]$G_{i2}\alpha$), and especially the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants, developed the adipocyte phenotype to a larger extent than controls and vector (pZipNeoSV(X)) transfected cells. Individual cells became enlarged and apparently stored larger amounts of lipids per cell. Furthermore, adenylate cyclase activation and cAMP accumulation elicited by either corticotropin or isoproterenol known to enhance HSL activity, as well as insulin-elicited glucose uptake, were markedly increased over differentiated controls and vector transfected cells. As expected, both controls and vector transfected cells displayed increased levels of $G_s\alpha$ and $G\beta$ upon differentiation, however, these levels were not further increased by either [Wtype]$G_{i2}\alpha$ or the [R179E]$G_{i2}\alpha$ and [Q205L]$G_{i2}\alpha$ mutants. Accordingly, the augmented hormone-elicited adenylate cyclase activation and cAMP accumulation are due to de novo large expression of corticotropin- and isoproterenol-receptors. The reason why cells transfected with the [R201C]$G_s\alpha$ mutant demonstrated lower hormone-stimulated adenylate cyclase activity than the G226A-$G_s\alpha$ transfected clones, may reside in the fact that differentiation dramatically induces the endogenous level of $G_s\alpha$, or also the steady-state amounts of G_s-sensitive adenylate cyclase isoenzymes.

Finally, steady-state levels of hormone sensitive lipase (HSL) mRNA were dramatically increased. HSL is one of several gene products, like adipsin (28K) and aP2 = FSE2 [43] which are regarded as specific adipocyte markers. Consequently, the super-normal levels of HSL mRNA seen in cells containing the Wtype-$G_{i2}\alpha$, R179E-$G_{i2}\alpha$, and Q205L-$G_{i2}\alpha$ constructs definitely underscore the impact of $G_{i2}\alpha$ in 3T3-L1 differentiation. However, if $G_{i2}\alpha$ is not the sole regulator of variables pertaining to acquisition of the adipocyte phenotype, it appears to be a prerequisite for the process. The H21a-$G_{i2}\alpha$ construct blocked, and in fact, reduced both cell shape alteration, hormone-sensitive adenylate cyclase activity, and cAMP accumulation, as well as insulin-elicited glucose uptake below levels of undifferentiated controls and vector-transfected 3T3-L1 cells before and after attempts to differentiate with hormones and iBuMeXan. Furthermore, cells treated with differentiation medium started to grow in multilayers, detached from the dish surface.

The effector regulated by $G_{i2}\alpha$ in these cells is apparently not adenylate cyclase nor phospholipase C (J.O. Gordeladze, unpublished observations). One possible mechanism is the stimulation of mitogen-activated protein kinase (MAPK) [49]. We are currently investigating this hypothesis in our laboratory.

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