Repression of Adipogenesis by Adenylyl Cyclase Stimulatory G-protein α Subunit Is Expressed within Region 146-220*

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The heterotrimeric G-protein α subunit stimulatory with respect to adenylyl cyclase (G\textsubscript{sa}) represses adipogenesis of 3T3-L1 mouse embryonic fibroblasts. Depression occurs in response to inducers, to oligodeoxynucleotides antisense to G\textsubscript{sa}, and to overexpression of heterotrimeric G-protein α subunit 2, inhibitory with respect to adenylyl cyclase (G\textsubscript{sib}). Constitutive expression of G\textsubscript{sa} blocks adipogenesis and was exploited as an assay, in which chimeras of G\textsubscript{sa} and G\textsubscript{sib} were expressed stably in 3T3-L1 cells to define the region controlling adipogenesis. N-terminal analysis revealed region 146-220 of G\textsubscript{sa} as a repressor of adipogenesis; substitution of G\textsubscript{sib} abolished the ability of the chimera to repress adipogenesis in response to inducers. Expression of a chimera in which the 146-235 region of G\textsubscript{sa} was embedded in G\textsubscript{sib} fully repressed adipogenesis in response to the inducers. C-terminal analysis revealed no loss of function for truncated G\textsubscript{sa}, lacking the terminal 38 residues. The repressor domain for adipogenesis maps to a region that includes switch domains I and II and is spatially distinct from the regions mapped for control of adenylyl cyclase.

Mouse embryo fibroblast 3T3-L1 cells differentiate to adipocytes (Green and Kehinde, 1974, 1975; Green and Meuth, 1974), in response to inducers such as insulin (Russell and Ho, 1976) or dexamethasone and methylisobutylxanthine (DEX + MIX) in combination (see Wang et al., 1992, and Refs. therein). In addition to mediating signaling from a populous class of plasma membrane receptors to a less populous group of effectors, including adenylyl cyclases, phospholipase C\theta, and various ion channels (Gilman, 1987; Birnbaumer et al., 1990; Bourne et al., 1990), G-proteins participate in more complex biological responses, including oncogenesis (Bourne et al., 1990), early (Wang et al., 1992) and neonatal (Mocham et al., 1993) mouse development, and cellular differentiation (Strittmatter et al., 1994; Wang et al., 1992). The G-protein G\textsubscript{sa} is implicated as playing a critical role in adipogenesis based on the following observations: (i) G\textsubscript{sa} levels decline during differentiation; (ii) metabolic labeling with \textsuperscript{14}C)methionine reveals a sharp decline in the rate of synthesis of G\textsubscript{sa} during differentiation; (iii) cholera toxin activation of G\textsubscript{sa} blocks differentiation; (iv) oligodeoxynucleotides antisense to G\textsubscript{sa} accelerate differentiation in response to dexamethasone and methylisobutylxanthine; (v) oligodeoxynucleotides antisense to, but neither sense nor sense to, G\textsubscript{sa} are themselves inducers of differentiation; (vi) overexpression of the counterregulatory G-protein G\textsubscript{sib} induces adipogenesis in the absence of inducers; and (vii) constitutive expression of G\textsubscript{sa} blocks the ability of inducers to promote adipogenesis (Wang et al., 1987, 1989a, 1989b; Wang et al., 1992, Su et al., 1993).

Cholera toxin blocks differentiation, yet agents that raise intracellular cyclic AMP levels such as forskolin or pertussis toxin, or addition of dibutyl cyclic AMP fail to influence adipogenesis (Wang et al., 1992). These observations suggest that some effector other than adenylyl cyclase may be responsible for propagating the effects of G\textsubscript{sa} and G\textsubscript{sib} on differentiation. In the current work, we make use of the ability of constitutive expression of G\textsubscript{sa} to block induction of adipogenesis, creating chimeras in which regions of G\textsubscript{sa} are replaced with G\textsubscript{sib} and evaluating their ability to block differentiation. The results identify regions of G\textsubscript{sa} critical to regulation of differentiation, mapping to a region of the molecule including switch I and II regions (Lambright et al., 1994) but distinct from the domains regulating adenylyl cyclase (Coleman et al., 1994).

Experimental Procedures

Mouse embryo fibroblast 3T3-L1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in culture in 100-mm Petri dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. The protocols for stable transfection were described previously (Wang et al., 1992; Watkins et al., 1992). Transfectant clones selected in G418 were identified and replicate plated in 8-well chamber slides as well as 24-well plates that were sparsely seeded. Cells in 24-well plates were maintained for propagation. Protocols for indirect immunofluorescence and histochemical staining techniques are described elsewhere (Wang et al., 1992; Watkins et al., 1992; Su et al., 1993). Construction of chimeras within expression vector pCW1 and structure-function analysis of control of adenylyl cyclase were as described earlier (Osawa et al., 1990; Gupta et al., 1990). The clones treated with DEX + MIX were harvested at day 7. For clones not treated with DEX + MIX, cultures were examined at day 7 but routinely harvested at day 18 to provide the fullest opportunity to detect adipogenic conversion in the absence of inducers. In all cases, the results obtained with clones not treated with DEX + MIX were identical at days 7 and 18 (not shown). For Northern blot RNA hybridization, cells were harvested by phosphate-buffered saline and EDTA treatment and low speed centrifugation. Total RNA was extracted using RNA Stat-60 reagent (TEL-TEST B, Inc., Friendswood, TX). RNA (30 μg/ lane) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes in the presence of 20 × SSC. Membranes were hybridized for 20 h at 42 °C with the aP2 cDNA, which was labeled by
CONSTRUCTS

**WT Expression Only**

**pCW1 Empty Vector Alone**

| Gαs, Q227L | Q227L | 394 S |
|-------------|-------|-------|
| Gαs         |       | 394 S |
| i(122)/s    | 145   | 394 S |
| i(212)/s    | 212   | 394 S |
| Gα2         |       | 355 S |
| Gα2, Trun   |       | 356 S |
| s(356)/i    | 320   | 355 S |
| Gα2, Q205L  | Q205L | 355 S |

**Fig. 1. Schematic of G-protein chimeras and mutants used to analyze the control of adipogenesis by Gαs.**

Random priming with [32P]dCTP. The blots were hybridized in the presence of 50% formamide and then washed with 1× SSC three times at 37 °C before exposure to film.

**RESULTS**

Constitutive expression of Gαs blocks the ability of dexamethasone and methylisobutylxanthine (DEX + MIX) to induce adipogenesis, whereas expression of Gα2 itself induces adipogenesis in the absence of DEX + MIX. The strategy adopted to identify the domain(s) controlling this adipogenesis was to express chimeras of Gαs and Gα2 and to evaluate their influence on adipogenesis in both the absence and presence of inducers. Chimeras with variable N-terminal substitution of Gα2 for Gαs, a C-terminal 38-residue substitution of Gα2 for Gαs, a C-terminal truncation of Gαs lacking 38 residues, and constitutively activated mutants of Gαs and Gα2 allowed diminished intrinsic GTPase activities were prepared and used to stably transfect 3T3-L1 cell cultures (Fig. 1).

Stably transplanted clones harboring the neomycin resistance gene were selected in the neomycin analog G418 and examined for G-protein subunit expression by indirect immunofluorescence microscopy (Fig. 2A). In the absence of the primary (1st Ab) or secondary (2nd Ab) antibodies, no epifluorescence signal was detected. Immunostaining with a primary antibody raised against the C-terminal peptide of Gαs (CM-112) revealed faint staining of endogenous subunits in the cultures transfected with an empty expression vector alone, pCW1. Cultures stably transfected with pCW1 harboring the Gαs(356)/Gα2 chimera displayed a strong epifluorescence signal following staining with the anti-Gα2 antibodies. Expression of the Gα2(122)/Gαs and Gα2(212)/Gαs chimera, the Q227L constitutively activated mutant of Gαs, and Gαs itself was established by staining the cultures with anti-Gαs antibody (CM-129) raised against the C-terminal decapeptide of the G-protein subunit (Fig. 2B). Expression of the C-terminal truncated mutant of Gαs was established by staining with antibodies raised against the holoprotein rGαs (CM-474). Immunoblotting of crude cell extracts confirmed these results, obtained by indirect immunofluorescence (Fig. 2C, a–c). Expression of G-protein α subunits in the stable transfectant clones was approximately 1.5-fold over endogenous Gαs levels. As shown earlier (Wang et al., 1992), endogenous levels of Gαs decline during differentiation in response to DEX + MIX, whereas levels of the vector-driven expression of α subunits remains relatively constant (Fig. 2C, d).

In wild-type cells and cells stably transfected with the empty expression vector, no differentiation was observed in the absence of the inducers DEX + MIX, as detected by staining for lipid with oil red O (Fig. 3). Nuclei were made visible by counterstaining with hematoxylin. When exposed to DEX + MIX, cultures displayed nearly complete differentiation, as typified by lipid accumulation, the hallmark of adipocytes. Cultures stably transfected with the empty vector alone displayed robust differentiation in response to DEX + MIX also. Expression of either wild-type Gαs or the GTPase-deficient, activated Q227L mutant of Gαs resulted in cultures that failed to respond to DEX + MIX, unable to differentiate into adipocytes. Much like the short term effects of cholera toxin activating Gs and Gα2 (Wang et al., 1992), stable expression of Gαs blocks adipogenesis.

The role of the N-terminal domain of Gαs was analyzed using a series of chimeras with increasing degree of substitution with Gα2 (Fig. 1). N-terminal substitutions of Gαs to residue 145 with analogous regions of Gα2 were largely unremarkable (Fig. 4). Stable expression of the Gα2(122)/Gαs chimera or the Gα2(54)/Gαs chimera (not shown) effectively blocked the ability of the inducers to provoke adipogenesis, much like constitutive expression of Gαs. When the region of Gαs substitution for Gα2 increased from 122 to 212, corresponding to region 145–235 in Gαs, the phenotype changed dramatically. Whereas expression of Gα2 or chimeras with N-terminal substitutions to 145 blocked DEX + MIX-induced adipogenesis, expression of Gα2(212)/Gαs chimera no longer blocked adipogenesis, relieving the repression of differentiation by Gαs, much like diglucosyloxyxynucleotides antisense to Gαs (Wang et al., 1992). Constitutively expressed Gα2 provoked adipogenesis in either the absence or presence of the inducers, whereas expression of the Gα2(212)/Gαs chimera does not induce adipogenesis in the absence of DEX + MIX. Thus, it is not the presence of the additional region of Gα2 but, rather, the loss of the corresponding region of Gαs that ablates the block of differentiation.

The role of the C-terminal domain of Gαs was explored through comparison of the effects of wild-type compared with a mutant of Gαs in which the C-terminal 38 residues have been removed. As shown in Fig. 5, constitutive expression of either the wild-type or truncated version of Gαs blocks the ability of DEX + MIX to induce adipogenesis. Although these data suggest no major role of the C-terminal 38 residues in the ability of Gαs to repress adipogenesis, expression of a chimera in which the C-terminal 38 residues of Gαs (356–394) are replaced with the analogous region of Gα2 (320–355) reveal a more complex picture. Expression of the Gαs(356)/Gα2 chimera itself does not induce adipogenesis but does derepress the block of adipogenesis in response to DEX + MIX in 20–25% of the culture. Examination of the cultures for 4–10 days after induction reveals this rather constant percentage of the cells progressing to adipocytes when the Gαs(356)/Gα2 chimera is stably expressed.

α2P is an early marker expressed when the embryonic fibroblasts commit to the adipocyte phenotype. In the 3T3-L1 cells,
FIG. 2. Stable expression of $G_{sa}$ and mutant forms of $G_{sa}$ in mouse embryo fibroblast 3T3-L1 cells: identification of high expressing clones by indirect immunofluorescence and immunoblotting. A and B, cells grown in eight-well chamber slides were fixed with 3% paraformaldehyde and prepared for indirect immunofluorescence (Su et al., 1993) using antibodies specific for the C terminus of $G_{sa}$ (CM-129; NULL (−2nd Ab), NULL, $\alpha$s, $\alpha$sQ227L, $\alpha$2(112)/s, and $\alpha$2(212)/s), the C terminus of $G_{ia}$ (CM-112; NULL and $\alpha$s(356)/i), and recombinant $G_{sa}$ (CM-474, data not shown). Clones were selected on the basis of expression relative to wild type. The results from the indirect immunofluorescence
the expression of aP2 mRNA was analyzed by RNA blotting (Fig. 6A). Within 48 h of exposure to DEX + MIX, aP2 mRNA expression increases dramatically. By day 3, aP2 mRNA levels have peaked and are sustained in the mature phenotype. The expression of this early marker for differentiation was analyzed in cells stably expressing the chimera and mutant forms of Gsα and Giα2 (Fig. 6B). Expression of Gsα, two GTPase-deficient mutants of Gsα (G225T and Q227L), the G1α2(122)/Gsα chimera, and Gsα with the truncated C terminus all displayed no significant induction of aP2 mRNA in response to DEX + MIX. Expression of Gsα, Giα2(122)/Gsα, Giα2(212)/Gsα, Giα2(122)/Gsα(235)/Giα2, and Giα2 (Fig. 6B). Expression of Gsα, two GTPase-deficient mutants of Gsα (G225T and Q227L), the G1α2(122)/Gsα chimera, and Gsα with the truncated C terminus all displayed no significant induction of aP2 mRNA in response to DEX + MIX. Ex-

were confirmed independently by immunoblotting of whole cell extracts prepared from the clones selected for further study (not shown). In each case, three to five independent clones were propagated for study, all yielding the same phenotype with respect to adipogenenic conversion. PC, phase-contrast microscopy; EPI, epifluorescence microscopy. C, immunoblots of crude cell membranes (a and b) and whole cell extracts (c and d) of stable transfectant clones. Samples (50 μg protein/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, probed with the anti-subunit-specific rabbit antisera (1:200 dilution) indicated, and stained with calf alkaline phosphatase-linked goat anti-rabbit IgG (Watkins et al., 1987). The levels of expression in the stable transfectants relative to endogenous Gsα levels (set at 1.0) were as follows: empty vector, 1.0; Gsα, 1.6; Gsα Q227L, 1.7; Gsα G225T, 1.24; Gsα(122)/Gsα, 1.3; Gsα(212)/Gsα, 1.4; G1α2(122)/Gsα(235)/Giα2, 1.7; Gsα(356) truncate (TRUN), 1.25; and Gsα(356)/Giα2, 2.1.

Fig. 3. Expression of Gαs, constitutively active mutants of Gαs, blocks induction of adipogenesis: analysis by staining of lipid with oil red O. Wild-type 3T3-L1 cells (NULL) and clones transfected with empty vector (VECTOR), as well as those transfected stably expressing Gαs (αs) and Gαs Q227L, transfected (αsQ227L) were plated on coverslips and propagated in 24-well culture plates. At confluence (day 0), one set of cells were treated with dexamethasone and methylisobutylxanthine (+INDUCERS). DEX + MIX were removed after incubation for 2 days, and the cells were maintained in DMEM containing 10% fetal bovine serum for 7 days. A second replicate set of cells was maintained in DMEM containing 10% fetal bovine serum in the absence of DEX + MIX. At day 7, cells were fixed by 3% paraformaldehyde for 5 min and stained with oil red O for 10 min. Hematoxylin (1%) was used to stain nuclei. Adipogenesis was examined under a Zeiss Axiophot microscope. The darkly stained bodies of the cytosol are oil droplets. Bar, 100 μm.

Fig. 4. Expression of Gαs and the Giα2(122)s but not the Giα2(212)s, chimeras blocks induction of adipogenesis: analysis by oil red O staining. Stably transfected clones expressing Gαs, Gαs/Giα2 chimeras, and Giα2 were plated on coverslips and propagated in 24-well culture plates. At confluence (day 0), one set of cells were treated with dexamethasone and methylisobutylxanthine (+INDUCERS). DEX + MIX were removed after incubation for 2 days, and the cells were maintained in DMEM containing 10% fetal bovine serum for 7 days. A second replicate set of cells was maintained in DMEM containing 10% fetal bovine serum in the absence of DEX + MIX. At day 7, cells were fixed by 3% paraformaldehyde for 5 min and stained with oil red O for 10 min. Hematoxylin (1%) was used to stain nuclei. Adipogenesis was examined under a Zeiss Axiophot microscope. The darkly stained bodies of the cytosol are oil droplets. Bar, 100 μm.
pression of the Gsα(212)/Gsα and Gsα(356)/Gsα chimeras, in sharp contrast, removed the block of DEX+MIX-induced adipogenesis caused by Gsα, as depicted by the dramatic increase in aP2 mRNA.

The extent of differentiation was quantified by scoring either positive or negative for adipogenesis. Individual cells were maintained in DMEM containing 10% fetal bovine serum in the absence of DEX+MIX. At day 7, cells were fixed with 3% paraformaldehyde for 5 min and stained with oil red O for 10 min. Hematoxylin (1%) was used to stain nuclei. Adipogenesis was examined under a Zeiss Axiophot microscope. The dark stained bodies of the cytosol are oil droplets. Bar, 100 µm.

To define further the nature of the repressor domain of Gsα for adipogenesis, additional chimeras were constructed and stably expressed in the 3T3-L1 cells (Fig. 8A). Because expression of Gsα(1–394), Gsα(356)/Gsα, and Gsα(235)/Gsα chimeras blocks adipogenesis in response to inducers, but Gsαα(212)/Gsα does not, we explored whether the 145–235 region of Gsα embedded within Gsα would repress or permit induction of adipogenesis. As shown by oil red O staining of the clones (Fig. 8B), expression of the Gsαα(122)/Gsα/235/Gsαα(212)/Gsα chimera suppresses the adipogenic response to DEX+MIX. Expression of the Gsαα(212)/Gsαα chimera devoid of the 145–235 region permits a robust adipogenic response, as made more clear by color (Fig. 8B) compared with black-and-white (Fig. 4) images of the oil red O staining. The fact that expression of the Gsαα(235)/Gsαα chimera fully represses DEX+MIX-induced adipogenesis suggests no simple interpretation of the inability of the Gsαα(356)/Gsαα to block adipogenesis completely (Fig. 7).

### DISCUSSION

G-proteins now are recognized as critical elements in a variety of complex biological processes. G-proteins have been shown to be key regulators of oncogenesis (Pace, et al., 1991; Voyno-Yasenetskaya et al., 1994; Wong et al., 1995), neonatal development (Moxham et al., 1993), early mouse development (Watkins et al., 1992), and cellular differentiation (Strittmatter et al., 1990, 1994; Wang et al., 1992; Su et al., 1993). Activating mutants of Gsα and Giβ2 are associated with a number of cancers arising in endocrine tissues such as the thyroid, pituitary, and ovary (Landis et al., 1989; Lyons et al., 1990). In neonatal development, targeted elimination of Giβ2 in liver and adipose tissue at birth generates transgenic mice with a runted phenotype (Moxham et al., 1993). In early mouse development, probing in totipotent mouse F9 teratocarcinoma stem cells, the morphogen retinoic acid induces a sharp reduction in Giβ2 as the cells commit to primitive endoderm (Galvin-Parton et al., 1990). Suppression of Giβ2 by constitutive expression of RNA...
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The $G_{sa}/G_{ia2}$ axis controlling adipogenesis was exploited in the current work to identify regions of the $G_{sa}$ molecule that are involved in this regulation. Expression of wild-type $G_{sa}$ effectively blocks the induction of differentiation. Constitutively active mutants of $G_{sa}$, G225T and Q227L, both blocked the ability of dexamethasone and methylisobutylxanthine to induce differentiation. Thus, constitutively active mutants of $G_{sa}$ block and those of $G_{ia2}$ induce differentiation. Mutations of this nature have been associated with tumorigenesis of several endocrine tissues (Bourne et al., 1990). Substitution of sequences of the N terminus of $G_{sa}$ with analogous ones of $G_{ia2}$ proved revealing with respect to the domain(s) involved in controlling adipogenesis. Whereas substitution of the N-terminal 145 amino acids of $G_{sa}$ with the first 122 amino acids of $G_{ia2}$ produced no obvious effects on the ability of chimera to suppress differentiation, substitution of the N-terminal 235 residues of $G_{sa}$ with the first 212 residues of $G_{ia2}$ had a profound effect. Expression of the $G_{ia2}(212)/G_{sa}$ construct abolishes the ability of the chimera to suppress induction of adipogenesis in response to DEX + MIX, whereas expression of a chimera in which the $G_{sa}$146–235 region is embedded in $G_{ia2}$ effectively blocks DEX + MIX-induced adipogenesis. These data clearly demarcate region 146–235 of $G_{sa}$ as critical to its ability to repress adipogenesis.

Careful comparison of the aligned protein sequences of $G_{sa}$ and $G_{ia2}$ reveals the control domain to be restricted to region 146–220, as the 221–235 region of $G_{sa}$ includes only one conservative substitution (R231K) and one nonconservative substitution (D229S). Analysis by mutagenesis revealed that this (D229S) substitution is not critical to repressor activity (not shown). Analysis of the C-terminal region of $G_{sa}$ was approached using truncation of the final 38 residues, which in this model of differentiation had equal capability as full-length $G_{sa}$ or constitutively activated $G_{sa}$ mutants to suppress 3T3-L1 cell differentiation. Interestingly, the substitution of the C-terminal 36 residues of $G_{ia2}$ for the corresponding 38 residues of $G_{sa}$ chimera $G_{sa}(356)/G_{ia2}$ resulted in a small, but significant, release from the repression observed with constitutive expression of native $G_{sa}$ or the truncated 1–356 version. The ability of the $G_{sa}(235)/G_{ia2}$ chimera to fully repress the adipogenic response suggests that the effects of the $G_{sa}(356)/G_{ia2}$ chimera are not simply related to substitution of the $G_{ia2}$ sequence into the C terminus of $G_{sa}$.

With structural features of the $\alpha$ subunits of several heterotrimeric G-proteins deduced from x-ray diffraction, it is now possible to speculate as to the regions of $G_{sa}$ involved in effector regulation (Lambright et al., 1994). Projections of $G_{sa}$ and $G_{ia2}$ sequences on the deduced structure of the GTP-ligated form of $G_{ia2}$ (Coleman et al., 1994) (red) were used to identify the regions implicated in the control of adenylyl cyclase (yellow) in the space-filling model displayed in Fig. 9. The GTP molecule is rendered in white. Highlighted in blue is the region of $G_{sa}(122–212)$, which is corresponding to the amino acid sequence 145–235 of $G_{sa}$, shown to be critical to the ability of the chimera to repress adipogenesis. Interestingly, this region of $G_{sa}$ includes switch domains I and II (green), but not III (cyan), which change conformation on activation of the molecule by GTP binding. The colorization of the sequences displays not only the overlap of the control domain with switches I and II, but also the spatial separation of the domain from that implicated in adenylyl cyclase regulation.

It is the loss of the controlling domain of $G_{sa}$ or the addition of the analogous region of $G_{ia2}$ that is responsible for the effects on adipogenesis? The fact that expression of native or consti-

antisense to $G_{ia2}$ mRNA mimics the decline observed in response to retinoic acid and induces differentiation to primitive endoderm in the absence of the morphogen (Gao and Malbon, 1996).

3T3-L1 embryonic fibroblasts are a useful model of cellular differentiation, in which inducers such as dexamethasone in combination with methylisobutylxanthine promote a highly differentiated state in which the cells accumulate lipid. The adipocyte phenotype requires the activation of an array of genes necessary for lipid synthesis. The capacity to stain the cells for lipid with oil red O provides a facile determination of differentiation. Earlier it was shown that cholera toxin activation of $G_{sa}$ effectively blocks induced differentiation and that $G_{sa146–220}$, as the 221–235 region of $G_{sa}$, resulted in a small, but significant, release from the repression observed with constitutive expression of native $G_{sa}$ or the truncated 1–356 version. The ability of the $G_{sa}(235)/G_{ia2}$ chimera to fully repress the adipogenic response suggests that the effects of the $G_{sa}(356)/G_{ia2}$ chimera are not simply related to substitution of the $G_{ia2}$ sequence into the $G_{sa}$ C terminus.

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Fig. 7. Expression of $G_{sa}$, constitutively active mutants of $G_{sa}$, and the $G_{ia2}(122)/s$ but not the $G_{ia2}(212)/s$ chimeras blocks induction of adipogenesis: quantitation. Wild-type 3T3-L1 cells (Null) and clones transfected with empty vector (Vector alone), as well as those transfected stably expressing various $\alpha$ subunits were plated on coverslips and propagated in 24-well culture plates. At confluence (day 0), one set of cells was treated with dexamethasone and methylisobutylxanthine (+ D/M). DEX + MIX were removed after incubation for 2 days, and the cells were maintained in DMEM containing 10% fetal bovine serum for 7 days. A second replicate set of cells was maintained in DMEM containing 10% fetal bovine serum in the absence of DEX + MIX. At day 7, cells were fixed by 3% paraformaldehyde for 5 min and stained with oil red O for 10 min. Hematoxylin (1%) was used to stain nuclei. Adipogenesis was examined under a Zeiss Axiophot microscope. Fields of 100–200 cells were scored either positive or negative for lipid accumulation. Percentages are mean values from at least six individual experiments, each with independent clones quantified from multiple fields. Bars, S.E.
**FIG. 8. Expression of Gsα, Gsα(122)/Gαs, and the Gsα(122)/Gαs(235)/Gαs blocks induction of adipogenesis: staining of lipid accumulation.** Cloning, induction of adipogenesis, and staining of lipid accumulation with oil red O were performed as described in the legend to Fig. 4. A, constructs analyzed. B, oil red O staining of clones. +, adipogenesis in response to DEX + MIX.

| CHIMERAS                  | ADIPOGENESIS |
|---------------------------|--------------|
| Gsα2                      | -            |
| e(235)/f                  | -            |
| hi22b vs                  | -            |
| k(122)/s(235)/j           | -            |
| i(212)/s                  | +            |
| Gsα2                      | +            |

B

- NULL
- VECTOR
- + INDUCERS

[Image of stained cells showing adipogenesis]
tively active Gs2 induces differentiation in the absence of dexamethasone and methylisobutylxanthine (Su et al., 1993) and the inability of the Gs2(212)s chimera to alter differentiation in the absence of the inducers, suggests the former rather than the latter interpretation. Thus, we have identified a domain of Gs required for regulation of adipogenesis as separate and distinct from those controlling adenyl cyclase. Consistent with the inability of cAMP to influence this differentiation process (Wang and Malbon, 1996), identification of the region controlling adipogenesis provides the boundaries for more detailed mutagenesis and a basis from which to initiate the search for the effector(s) through which Gs exerts its control of adipogenesis.

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FIG. 9. Mapping controlling region 145–220 of Gs onto the deduced structure of Gs; identification of regions regulating adenyl cyclase (yellow) and adipogenesis (blue), with respect to switches I, II (green), and III (cyan). Top left panel, the landmarks for Gs were projected on the deduced crystal structure Gs with the space-filling model (red), the region controlling adipogenesis (blue), and the region controlling adenyl cyclase (yellow). Bottom left panel, switch III region (cyan) does not overlap with the regions controlling adipogenesis (blue) or adenyl cyclase (yellow). Top right panel, the switch I region (green) is contained within the region controlling adipogenesis (blue) but does not overlap with the region controlling adenyl cyclase (yellow). Bottom right panel, switch I and II regions (green) are contained within the region controlling adipogenesis (blue) but do not overlap with the region controlling adenyl cyclase (yellow).
Repression of Adipogenesis by Adenylyl Cyclase Stimulatory G-protein α Subunit Is Expressed within Region 146-220
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