Differentiation-dependent Expression of Catecholamine-stimulated Adenylate Cyclase

ROLES OF THE β-RECEPTOR AND G/F PROTEIN IN DIFFERENTIATING 3T3-L1 ADIPOCYTES*

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3T3-L1 Preadipocytes possess an adenylate cyclase system that is highly stimulated (~4.5-fold) by 10 μM GTP in the absence of β-adrenergic agonists and other hormones. During the differentiation of preadipocytes into adipocytes, a sharp decline in this activity precedes the manifestation of catecholamine-stimulated (6- to 10-fold) adenylate cyclase activity. Preadipocytes exhibit 1900 high affinity β-receptors/cell, but the maximal occupation of these receptors by isoproterenol results in only a 2%-fold elevation in the rate of cAMP synthesis. During differentiation, β-receptor number decreases by over 50% to 500 sites/cell, but the 8-receptor stimulation remains unaltered in adipocytes. The adrenergic agonist, isoproterenol, selectively reduces adenylate cyclase activity from preadipocytes to adipocytes, while the GTP-mediated enhancement of this activity remains constant. In adipocyte membranes, the 8-receptor is essentially saturated with isoproterenol, while the GTP-mediated enhancement is still present. This finding suggests that GTP and the G/F regulatory component of the enzyme can interact in a series of β-agonists and 8-receptors, but not β-antagonists, for the regulation of adenylate cyclase activity. Furthermore, determinations of the relative proportions of the two 32P-labeled components of G/F disclosed a preponderance of 32P in the 50,000-dalton species in preadipocyte membranes and nearly equal amounts of the 42,000-dalton and 49,000- to 50,000-dalton polypeptides in adipocyte membranes. These findings raise the possibility that differentiation-associated changes in the regulation and coupling properties of catecholamine-stimulated adenylate cyclase may be determined principally by modulation of these levels, proportions, and/or properties of the constituents of G/F.

The role of β-receptor and G/F protein in the differentiation of 3T3-L1 preadipocytes into adipocytes is discussed. The physiological response to catecholamines in this system is mediated by β-receptors and G/F, and the regulatory component of the enzyme (G/F) is shown to interact with this receptor in a series of agonists. The relative proportions of the two 32P-labeled components of G/F disclosed a preponderance of 32P in the 50,000-dalton species in preadipocyte membranes and nearly equal amounts of the 42,000-dalton and 49,000- to 50,000-dalton polypeptides in adipocyte membranes. These findings raise the possibility that differentiation-associated changes in the regulation and coupling properties of catecholamine-stimulated adenylate cyclase may be determined principally by modulation of the levels, proportions, and/or properties of the constituents of G/F.

The regulation of catecholamine-stimulated adenylate cyclase by hormones and guanine nucleotides depends upon interactions among the β-adrenergic receptor, the guanine nucleotide-binding regulatory protein or G/F, and the catalytic component of the enzyme. Recent investigations employing a combination of biochemical, genetic, and membrane fusion techniques have provided substantial evidence that these three functional components of the adenylate cyclase system are distinct and physically separable proteins. In addition, biochemical characterizations of mutants of S49 lymphoma cells (6-9) and naturally occurring variants of other cells (7, 9) have disclosed that G/F plays a central role in mediating the interactions between the β-receptor and the catalytic component. Physiologically responsive target cells for β-adrenergic agonists are highly specialized and terminally differentiated. Presumably, these cells contain β-receptors, G/F and catalytic protein in appropriate stoichiometries, conformations, and membrane environments to optimize ligand recognition and signal transmission. There is very little known about how these components are integrated to become a hormonally responsive adenylate cyclase system during cell and tissue differentiation.

The murine 3T3-L1 cell line appears to be especially suitable for studying the assembly of a hormonally sensitive adenylate cyclase system. Differentiation of 3T3-L1 preadipocytes into adipocytes can be elicited rapidly and uniformly in cell culture (10). This process is accompanied by the acquisition of a catecholamine-sensitive adenylate cyclase and a lipolytic response to catecholamines (11). The physiological development of the catecholamine-responsive adenylate cys-

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† The abbreviations used are: G/F, the regulatory component of adenylate cyclase which appears to be a site of action of guanine nucleotides and fluoride; [3H]YIP, [3H]iodohydroxybenzylpindolol; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; SDS, sodium dodecyl sulfate.

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class system in differentiated 3T3-L1 cells enables the study of developmental changes in the β-receptor, G/F protein, and catalytic unit and the degree to which these components interact at various stages during differentiation.

In this communication, we (a) report the occurrence of an adenylate cyclase system in 3T3-L1 preadipocytes that is highly stimulated by GTP in the absence of hormones, (b) find that a decline in this activity is coordinated with the appearance of catecholamine-responsive adenylate cyclase during adipocyte differentiation, (c) characterize the specific binding and coupling properties of β-adrenergic receptors in preadipocytes and adipocytes and (d) document large, differentiation-associated changes in the relative proportions and apparent amounts of the 42,000-dalton and 49,000- to 50,000-dalton species of G/F subunits as determined by labeling with cholera toxin and [32P]NAD.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 and 3T3-C2 cell lines were originally obtained from Dr. Howard Green (Harvard Medical School). Cells were grown in 150-mm tissue culture dishes (Lux) or 530-cm² square tissue culture plates (Nunc) as previously described (11). Preadipocytes were seeded at a density of ~2500 cells/cm² and grown to confluence in 4–5 days (approximately four cell divisions).

Murine S49 lymphoma cell lines (wild type and cysteine variants) were kindly provided by Dr. Alfred Gilman (Center for Texas Health Sciences Center, Dallas) and were propagated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, non-essential amino acids, and 2 mM glutamine.

Differential of 3T3-L1 Cells to Adipocytes—Confluent preadipocytes (day 0) were fed with fresh medium containing 0.5 mM 1-methyl-3-isobutylxanthine and 0.25 mM dexamethasone (10). After 48 h (day 2), this medium was aspirated and the cells were refed with fresh standard medium. Cells were fed again by the addition of 30% (v/v) fresh medium 96 h after initiating drug treatment (day 4). Day 6 cells (preadipocytes) were harvested 48 h after the second refedging. By day 6, 80–90% of the cells had differentiated into rounded cells with large lipid droplets.

Preparation of Cell Membranes—All steps were performed at 4°C. Dishes of 3T3-L1 or C2 cells were rinsed twice in buffer A (10 mM potassium phosphate, pH 7.4, and 0.14 M NaCl). Cells were then scraped off the dish in buffer A with a rubber policeman. Cell number was determined and the cells were harvested by centrifugation at 350 × g for 4 min. The cells were then resuspended (~10–15 × 10⁶ cells/ml) in buffer B (5 mM Tris buffer, pH 7.4, containing 5 mM NaCl, 0.1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM dithiothreitol, and 20% glycerol) and heated to a final Mg²⁺ concentration of 0.5 mM. Two min later, the cells were homogenized with 20 strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged at 650 × g for 10 min to remove nuclei and the supernatant fraction was collected. The pellet was washed once and the second supernatant fraction was combined with the first. The combined postnuclear supernatant fraction was centrifuged at 45,000 × g for 15 min. The membrane pellet was washed once in buffer A without added dithiothreitol and resuspended at a final concentration of 2–3 mg of protein/ml.

Preadipocytes have less membrane protein per cell than do adipocytes. Preadipocytes yielded approximately 0.079 ± 0.020 mg of protein/10⁶ cells, while adipocytes yielded 0.208 ± 0.028 mg of protein/10⁶ cells (average of seven sets of differentiating cells).

Membranes from 3T49 lymphoma cells were prepared in a similar manner. Cells were harvested by centrifugation at 350 × g for 5 min in buffer A. They were then resuspended in buffer B and processed as described above.

Adenylate Cyclase Assay—Adenylate cyclase activity was determined as described by Salomon et al. (12). An ATP preparation (Sigma) containing ~20% pyrophosphate was used as substrate. The reaction mixture contained 0.5 mM [γ-32P]ATP (specific activity ~150 cpm/pmol), 5 mM MgCl₂, 20 mM phosphocreatine, 20 units/ml of creatine phosphokinase, 1 mM cAMP, and 0.5 mM dithiothreitol in 25 mM Tris buffer, pH 7.4 (37°C).

Preparation of Membranes—Membranes were incubated with [3H]-HYP at 37°C in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM pyrocatechol, and 1 mM ascorbic acid. Unless otherwise specified, the [3H]-HYP concentration was 300–400 pm. Membranes from

RESULTS

Comments on the Normalization of the Data—3T3-L1 Adipocytes are larger and yield more membrane protein per cell than the preadipocytes from which they have developed. Consequently, comparisons between preadipocytes (day 0) and adipocytes (day 6) can be normalized either to the amount of membrane protein or to the number of cells from which the membranes are derived. Because the relative proportion of plasma membranes (which contain the cyclase system) in crude membrane preparations isolated from adipocytes may be different from that in crude preadipocyte preparations, any normalization of the data to protein could be misleading. Thus, all results were expressed in terms of cell number. Conversion of the data to normalization by amount of protein can be readily approximated from the values given under "Experimental Procedures."

Development of β-Agonist-stimulated Adenylate Cyclase—Adenylate cyclase activity was measured in both homogenates
and partially purified membranes prepared from differentiating 3T3-L1 cells. Activation of adenylate cyclase by the β-adrenergic agonist, isoproterenol, was very limited in membranes from undifferentiated cells (day 0) but increased markedly during adipocyte development (Fig. 1A), reaching a maximum value by day 6 of the differentiation protocol (see "Experimental Procedures"). Typically, isoproterenol stimulated adenylate cyclase activity by 500-900% in membranes from differentiated 3T3-L1 cells but elevated enzyme activity only 20% above values obtained with GTP alone in preadipocyte membranes. In contrast, the degree of stimulation of adenylate cyclase by prostaglandin E₁ remained relatively constant during the conversion of preadipocytes to adipocytes (Fig. 1B). The acquisition of catecholamine-stimulated adenylate cyclase activity was correlated with differentiation rather than drug treatment. 3T3-C2 cells, which do not differentiate after exposure to dexamethasone and methylisobutylxanthine, did not develop catecholamine-stimulated adenylate cyclase activity (Fig. 1A).

The data in Fig. 1 show that isoproterenol elicited very similar levels of adenylate cyclase activation in homogenates and partially purified membranes. Since the fractionation procedure used to isolate membranes apparently does not alter the functional organization of the β-agonist-sensitive adenylate cyclase system and the membranes can be washed free of endogenous GTP, the membrane preparation was judged to be suitable for studies on the effects of guanine nucleotides on the adenylate cyclase system and the characteristics of the β-receptor.

**Characterization of the β-Adrenergic Receptor**—The β-adrenergic receptor of 3T3-L1 cells was characterized during differentiation using the radiolabeled β-adrenergic antagonist, [¹²⁵I]iodohydroxybenzylpindolol (¹²⁵I-HYP). In initial studies carried out on 3T3-L1 adipocyte membranes, ¹²⁵I-HYP binding was found to be rapid and reversible (Fig. 2), saturable, and specifically displaceable by a series of β-adrenergic agonists (Fig. 3A) and antagonists (Fig. 3B). Under the standard assay conditions, equilibrium binding was attained within 20 min at 37 °C and saturation of the specific β-binding sites occurred at a concentration of 2 nM ¹²⁵I-HYP.

The binding of β-adrenergic ligands by the membranes was also highly stereoselective (Fig. 3). The physiologically active (+)-isomers of isoproterenol and propranolol were approximately 100-fold more potent than the corresponding (-)-isomers in competitively inhibiting ¹²⁵I-HYP binding. The β-adrenergic antagonist, phentolamine, did not displace ¹²⁵I-HYP at concentrations up to 100 μM. β-Adrenergic receptors in preadipocytes and adipocytes were compared in equilibrium binding studies. Data from a typical experiment are plotted according to the method of Scatchard (16) in Fig. 4. Membranes from preadipocytes and adipocytes both yielded linear plots, consistent with a single set of noninteracting binding sites. Day 0 cells (preadipocytes) exhibited approximately 1850 sites/cell with a dissociation constant of 0.02 ± 0.03 nM, while day 5 cells (adipocytes) possessed 3050 sites/cell with an apparent KD of 0.22 ± 0.06 nM (averages of four separate sets of differentiating cells).
number of sites per cell varied among different batches of cells, but the proportional change in receptor number between day 0 and day 6 in each batch was consistent.

The kinetics, saturability, and reversibility of binding and the stereoselective displacement of $^{125}$I-HYP by $\beta$-adrenergic agonists and antagonists observed using preadipocyte membranes also paralleled the results obtained with adipocyte membranes (data not shown).

Regulation of $\beta$-Adrenergic Receptor Affinity and Number—Two indices of the ability of the $\beta$-receptor to interact productively with G/F are the down regulation of $\beta$-receptors upon prolonged exposure to agonists (17) and the decrease in receptor affinity for agonists effected by guanine nucleotides (18, 19).

Both 3T3-L1 preadipocytes and adipocytes exhibit the down regulation of their $\beta$-receptors with similar kinetics upon persistent treatment with isoproterenol (Table I). More than one-half of the $\beta$-receptors were lost within 1 h and the number of receptors observed after 6 h closely approached a stable plateau value of approximately 16% of the initial receptor density seen at 20 h and subsequent times.

Guanine nucleotides negatively modulate the affinity of the $\beta$-adrenergic receptor for agonists in membranes that contain fully functional G/F protein (18, 19). Fig. 5 shows that GTP decreases the affinity of the receptor for isoproterenol in both preadipocyte and adipocyte membranes. In contrast to the equally avid binding of $^{125}$I-HYP by both membranes (Fig. 4), adipocyte membranes displayed a slightly higher affinity for isoproterenol and a somewhat larger shift in the presence of GTP. The significance of these small differences is unknown.

Activation of Adenylate Cyclase by Guanine Nucleotides during the Differentiation of 3T3-L1 Adipocytes

Adenylate cyclase activities were determined in membrane preparations isolated during the course of adipocyte differentiation as described under “Experimental Procedures.” The data represent duplicate determinations in a typical experiment using membranes from a single set of cells. GTP and Gpp(NH)p were present at concentrations of 10 μM. The data are normalized to the number of cells from which the membranes were derived.

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<th>Gpp(NH)p</th>
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Fig. 5. Effects of GTP on the isoproterenol displacement of $^{125}$I-HYP binding to $\beta$-receptors in preadipocyte and adipocyte membranes. Membranes from preadipocytes (A) and adipocytes (B) were incubated with $^{125}$I-HYP as described under “Experimental Procedures.” The data are presented as percentages of total specific $^{125}$I-HYP binding remaining in the presence of the indicated concentrations of isoproterenol in the absence (■—■) and presence of 100 μM GTP (●—●) and presence of 100 μM GTP (△—△) and presence of 100 μM GTP (○—○). The data represent the averages of ± S. D. of three separate experiments. The $^{125}$I-HYP concentration was between 0.5 nm and 0.6 nm.
3T3-L1 Adenylate Cyclase

TABLE I


table of guanine nucleotide-activated adenylate cyclase in membranes derived from 3T3-C2 cells and undifferentiated 3T3-L1 cells seem to be quite similar.

The loss of activation by GTP alone was correlated with differentiation. When membranes were isolated from 3T3-C2 cells throughout the course of a standard dexamethasone-methylisobutylxanthine treatment protocol, GTP (10 μM) stimulated adenylate cyclase activity 6- to 8-fold at all stages (Table III). Furthermore, the ratio of cyclase activity in the presence of Gpp(NH)p to that observed in the presence of GTP also remained constant at 1.7 ± 0.2. Thus, the properties of guanine nucleotide-activated adenylate cyclase in membranes from 3T3-C2 cells and undifferentiated 3T3-L1 cells were unsuccessful.

GTP-dependent isoproterenol activation of adenylate cyclase was demonstrated, however, when an alternative ATP-regenerating system, phosphoenolpyruvate/pyruvate kinase, was substituted for phosphocreatine/creatinine phosphokinase. Thus, 3T3-L1 adipocyte membrane adenylate cyclase seems to be highly sensitive to a factor(s) inherent in or generated by phosphocreatine/creatinine phosphokinase that mimics GTP. While the nature of the factor remains unknown, it is unlikely to be a contaminating guanine nucleotide because the purification procedures used should have eliminated such contaminants. The initial reaction volume was 215 μl and contained ~2 μCi/ml of [3H]cAMP. At the indicated times during the incubation, 25-μl aliquots were removed and transferred to tubes containing 100 μl of 2% SDS, 1.4 mM cAMP, and 20 mM ATP. Analyses were performed as described by Salomon et al. (12). The data are from a typical experiment. C——O, Adipocyte membranes incubated with 10 μM Gpp(NH)p; ○ — ○, adipocyte membranes incubated with 10 μM GTP; Δ — Δ, preadipocyte membranes incubated with 10 μM Gpp(NH)p; △ — △, preadipocyte membranes incubated with 10 μM GTP.

**FIG. 6.** Time course of adenylate cyclase activation by GTP and Gpp(NH)p. Adenylate cyclase activity was determined as described under "Experimental Procedures" except that the initial reaction volume was 215 μl and contained ~2 μCi/ml of [3H]cAMP. At the indicated times during the incubation, 25-μl aliquots were removed and transferred to tubes containing 100 μl of 2% SDS, 1.4 mM cAMP, and 20 mM ATP. Analyses were performed as described by Salomon et al. (12). The data are from a typical experiment. C——O, Adipocyte membranes incubated with 10 μM Gpp(NH)p; ○ — ○, adipocyte membranes incubated with 10 μM GTP; Δ — Δ, preadipocyte membranes incubated with 10 μM Gpp(NH)p; △ — △, preadipocyte membranes incubated with 10 μM GTP.

**TABLE III**

Stimulation of adenylate cyclase by guanine nucleotides in 3T3-C2 treated with dexamethasone and 1-methyl-3-isobutylxanthine

Adenylate cyclase activities were determined in membrane preparations isolated from 3T3-C2 cells treated for 48 h with dexamethasone and 1-methyl-3-isobutylxanthine as described in the protocol for the differentiation of 3T3-L1 cells under "Experimental Procedures." Unlike 3T3-L1 cells, the 3T3-C2 cells do not differentiate under these culture conditions. GTP and Gpp(NH)p were present at concentrations of 10 μM. The data are normalized to the number of cells from which the membranes were derived.

<table>
<thead>
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<th>Day</th>
<th>Basal</th>
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<th>GTP/ Basal</th>
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<th>Gpp(NH)p/ Basal</th>
<th>Gpp(NH)p/ GTP</th>
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<td>8.2</td>
<td>61.2</td>
<td>13.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

**Fig. 6.** Time course of adenylate cyclase activation by GTP and Gpp(NH)p. Adenylate cyclase activity was determined as described under "Experimental Procedures" except that the initial reaction volume was 215 μl and contained ~2 μCi/ml of [3H]cAMP. At the indicated times during the incubation, 25-μl aliquots were removed and transferred to tubes containing 100 μl of 2% SDS, 1.4 mM cAMP, and 20 mM ATP. Analyses were performed as described by Salomon et al. (12). The data are from a typical experiment. C——O, Adipocyte membranes incubated with 10 μM Gpp(NH)p; ○ — ○, adipocyte membranes incubated with 10 μM GTP; Δ — Δ, preadipocyte membranes incubated with 10 μM Gpp(NH)p; △ — △, preadipocyte membranes incubated with 10 μM GTP.

The loss of activation by GTP alone was correlated with differentiation. When membranes were isolated from 3T3-C2 cells throughout the course of a standard dexamethasone-methylisobutylxanthine treatment protocol, GTP (10 μM) stimulated adenylate cyclase activity 6- to 8-fold at all stages (Table III). Furthermore, the ratio of cyclase activity in the presence of Gpp(NH)p to that observed in the presence of GTP also remained constant at 1.7 ± 0.2. Thus, the properties of guanine nucleotide-activated adenylate cyclase in membranes derived from 3T3-C2 cells and undifferentiated 3T3-L1 cells seem to be quite similar.

Dose response curves for the effects of guanine nucleotides on adenylate cyclase activation in membranes derived from preadipocytes (day 0) and adipocytes (day 6) disclosed that the decreased ability of GTP to activate the enzyme in adipocytes could not be attributed to a decreased affinity for GTP (Fig. 7A), nor could the increased efficacy of Gpp(NH)p in adipocyte membranes be ascribed to an elevated K_m for the analog (Fig. 7B).

Finally, the foregoing results cannot be explained by a higher rate of nonspecific hydrolysis of GTP by adipocyte membranes during the cyclase assay. When total GTPase activity was determined under the conditions of the adenylate cyclase assay (see "Experimental Procedures"), only ~10% of added [3H]GTP was degraded to GDP and GMP by either preadipocyte or adipocyte membranes.

**GTP-dependent Activation of Adenylate Cyclase by Iso- proterenol**—When adipocyte membrane adenylate cyclase activity was assayed using the standard phosphocreatine/creatinine phosphokinase ATP-regenerating system, enzyme activity was highly and equally stimulated by isoproterenol in the presence (Fig. 1) and absence of GTP. Although unexpected, this result is not unique. Adenylate cyclase activity in highly purified liver plasma membranes is readily activated by glucagon in the absence of added GTP (20, 21). Such observations have been attributed to the presence of contaminating GTP in the membranes or assay reagents, the generation of GTP during the assay, or the occurrence of non-nucleotide activators in the components of the ATP-regenerating system (22). Attempts to unmask GTP-dependent, isoproterenol-stimulated activity by employing highly purified adipocyte membranes isolated by the procedure of Ross et al. (23) or by purifying ATP and the components of the ATP-regenerating system, phosphocreatine and creatine phosphokinase (24), were unsuccessful.
not alter the GTP requirement for isoproterenol stimulation.

Typical results of assays performed with the phosphoenol-
pyruvate/pyruvate kinase-regenerating system were provided
in Table IV. GTP alone increased adipocyte adenylate cyclase
activity very little, but the combination of 6 µM isoproterenol
and 10 µM GTP stimulated the enzyme approximately 10-fold
relative to the basal value. In contrast, preadipocyte adenylate
cyclase was activated by GTP whether or not hormone was
present.

Labeling and Comparison of G/F Subunits in Adipocyte
and Preadipocyte Membranes—The striking changes in hor-
monedependent and -independent activation of adenylate
cyclase by guanine nucleotides during differentiation sug-
gested that qualitative and/or quantitative changes in G/F
may occur. Experiments aimed at characterizing several of
the subunits of G/F were based on the observation that
activated cholera toxin catalyzes the transfer of the [32P]ADP-
ribose moiety of [32P]NAD to the subunits of G/F in a highly
specific manner in several membrane systems (25-27).

Fig. 8 shows an autoradiogram of a 0.1% SDS and 10%
polyacrylamide gel electrophoresis and autoradiography as indicated un-
der "Experimental Procedures." Solubilized membrane protein
from 1.2 × 10^7 3T3-L1 WT cells, 1.2 × 10^7 3T3-L1 mutant
cells, 1.5 × 10^7 3T3-L1 preadipocytes, and 5.2 × 10^7 adipocytes (day 6, lanes 0, 2) were incubated with [32P]NAD plus
cholera toxin, B, in a separate experiment, membranes from 1.3 × 10^7 pread-
ipocytes (day 0, lane 1) and 1.3 × 10^7 adipocytes (day 6, lane 2) were
labeled and analyzed as described in A.

![Fig. 7. Activation of adenylate cyclase by guanine nucleo-
tides. Adenylate cyclase activities in preadipocyte (●/●) and
adipocyte (○/○) membranes were determined as described under
"Experimental Procedures" in the presence of various concentra-
tions of GTP (A) or Gpp(NH)p (B). The points represent the average of
duplicate determinations in a typical experiment.](image)

**Table IV**

Effects of GTP and isoproterenol on adenylate cyclase in
preadipocyte and adipocyte membranes

Adenylate cyclase assays were carried out as described under
"Experimental Procedures" except that the standard ATP regener-
ating system was replaced with 5 mM phosphoenolpyruvate and 25
µg/ml of pyruvate kinase. Incubations were carried out for 15 min at
37°C.

<table>
<thead>
<tr>
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<th>Adenylate cyclase activity (pmol cAMP synthesized/10⁶ cells/15 min)</th>
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<tr>
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<td>Basal</td>
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<td>Preadipocytes</td>
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<td>Adipocytes</td>
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<td>(day 6)</td>
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![Fig. 8. Autoradiogram of [32P]ADP-ribosylated G/F compo-
ments in S49 and 3T3-L1 membranes. A, membranes from S49
WT cells (lanes 1 and 2), S49 cyc- cells (lanes 3 and 4), 3T3-L1
preadipocytes (day 0, lanes 5 and 6), and 3T3-L1 adipocytes (day 6,
lanes 7 and 8) were incubated with 10 µM [32P]NAD in the absence
or presence of 40 µg/ml of preactivated cholera toxin for 45 min at
30°C as described under "Experimental Procedures." Membranes were
then washed, solubilized, and denatured and subjected to SDS-poly-
acrylamide gel electrophoresis and autoradiography as indicated un-
der "Experimental Procedures." Solubilized membrane protein from
1.2 × 10⁷ S49 WT cells, 1.2 × 10⁷ S49 cyc- cells, 1.5 × 10⁷ 3T3-L1
preadipocytes, and 5.2 × 10⁷ adipocytes were applied to the indicated
lanes. Samples in even numbered lanes were incubated with cholera
toxin; samples in odd numbered lanes received [32P]NAD but no
toxin. B, in a separate experiment, membranes from 1.3 × 10⁷ pread-
ipocytes (day 0, lane 1) and 1.3 × 10⁷ adipocytes (day 6, lane 2) were
labeled and analyzed as described in A.](image)
G/F protein isolated by Northup et al. (28).

Examination of the [32P]ADP-ribosylation patterns in membranes from 3T3-L1 preadipocytes and adipocytes revealed significant differences. Fig. 8B compares the relative intensities of labeling of the G/F subunits when membranes from equal numbers of preadipocytes and adipocytes were analyzed. The number of ADP-ribosylation sites available in membranes from differentiated cells is clearly much higher. In Fig. 8A, the amount of preadipocyte membranes was increased to facilitate the determination of the relative amounts of [32P] incorporated into the 42,000-dalton protein and the 49,000- to 50,000-dalton doublet. The relative intensities of labeling of the 42,000-dalton polypeptide and the 49,000- to 50,000-dalton doublet were dramatically altered during differentiation (Fig. 8A, lanes 6 and 8). In preadipocyte membranes, incorporation of [32P] into the higher molecular weight doublet predominated, whereas in adipocyte membranes, the 42,000-dalton polypeptide served as a better acceptor of ADP-ribose than either the 49,000- or 50,000-dalton proteins.

The incorporation of [32P]ADP-ribose into the 42,000-dalton protein and the 49,000- to 50,000-dalton doublet (taken as a sum) was a linear function of the amount of membrane protein exposed to cholera toxin and [32P]NAD over a wide range (Fig. 9). Approximately 13 times more [32P] was incorporated into the 42,000-dalton component of adipocyte G/F than the corresponding subunit of preadipocyte G/F (Fig. 9). In addition, the amount of [32P] in the 49,000- to 50,000-dalton doublet in adipocyte membranes was 4-fold higher than that in membranes from undifferentiated cells. In preadipocyte membranes, the ratio of the labeled doublet to the 42,000-dalton protein was 4.2, while in adipocyte membranes it was 1.3.

The preceding results were independent of the length of the prolonged treatment with isoproterenol (Table I) and membranes; the ratio of the labeled doublet to the 42,000-dalton polypeptide and the 49,000- to 50,000-dalton doublet (taken as a sum) was a linear function of the amount of membrane protein exposed to cholera toxin and [32P]NAD over a wide range (Fig. 9). Approximately 13 times more [32P] was incorporated into the 42,000-dalton component of adipocyte G/F than the corresponding subunit of preadipocyte G/F (Fig. 9). In addition, the amount of [32P] in the 49,000- to 50,000-dalton doublet in adipocyte membranes was 4-fold higher than that in membranes from undifferentiated cells. In preadipocyte membranes, the ratio of the labeled doublet to the 42,000-dalton protein was 4.2, while in adipocyte membranes it was 1.3.

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branes derived from these cells displayed similar susceptibilities to the negative modulation of agonist affinity by GTP (Fig. 5) and Gpp(NH)p. Thus, preadipocyte β-receptors are clearly capable of productive regulatory interactions (direct or indirect) with G/F and the acquisition of catecholamine-sensitive adenylate cyclase activity is not correlated with changes in these interactions.

Further studies of adenylate cyclase activity revealed marked differentiation-dependent changes in responsiveness to guanine nucleotides (Tables II and IV). GTP (10 µM) stimulated preadipocyte adenylate cyclase equally well in the absence or presence of isoproterenol, while the GTP-mediated enhancement of adipocyte membrane adenylate cyclase activity was highly dependent on the presence of the β-adrenergic agonist (Table IV). In contrast, Gpp(NH)p activated the enzyme in the absence of hormone in both preadipocyte and adipocyte membranes. The net stimulatory effect of Gpp(NH)p increased 20-fold (relative to the net activation observed with GTP alone) during differentiation as the result of an increase in catalytic activity and a sharp decline in the effect of GTP alone (Table II). There are several possible explanations for these results.

The model of adenylate cyclase proposed by Cassel and Selinger (29) postulates that the catalytic component of the enzyme is activated by a complex of G/F and GTP. A specific, G/F-associated GTPase terminates stimulation of the enzyme by converting GTP to GDP. In this model, hormone-receptor complexes activate adenylate cyclase by facilitating the displacement of tightly bound GDP by GTP at the regulatory site of G/F. It is possible that the G/F protein in adipocytes requires the presence of hormone to allow GTP to activate the adenylate cyclase (as modeled by Cassel and Selinger (29)), while the G/F protein in preadipocytes may exist in a conformation where GTP can readily displace bound GDP. Alternatively, the specific GTPase activity of the G/F protein may be diminished (or absent) in the preadipocyte adenylate cyclase system. During differentiation, the rate of cleavage of the γ-phosphate bond of GTP may increase sharply at the guanine nucleotide regulatory site, thereby lowering the steady state concentration of G/F-GTP complexes available for catalytic unit activation. Thus, one view of the development of catecholamine responsiveness in 3T3-L1 adipocytes is that a differentiation-dependent alteration in the properties of G/F is responsible for the loss of activity in the presence of GTP and this activity may be transiently restored by interactions between G/F and agonist-occupied β-receptor. Unfortunately, it has not been possible to directly measure G/F-associated GTPase activity in 3T3-L1 cell membranes and membranes derived from other mammalian cells (1, 30–32) because of the presence of high levels of membrane-associated GTP-degrading enzymes that are unrelated to the adenylate cyclase system. In addition, there are no reports documenting hormone-dependent facilitation of the displacement of GDP by GTP at a regulatory site in mammalian cell membranes. Nevertheless, when adipocyte membranes were incubated with cholera toxin and NAD under conditions known to result in the ADP-ribosylation of components of G/F (see Figs. 8–10) and the inactivation of G/F-associated GTPase (33, 34), the stimulation of adipocyte membrane adenylate cyclase by GTP alone rose to a level comparable to that observed in preadipocyte membranes. Moreover, adenylate cyclase activity in the toxin-treated, adipocyte membranes became refractory to further stimulation by isoproteoranol after activation by GTP. These preliminary observations, while not definitive, are consistent with differentiation-dependent changes in the rate of hydrolysis of GTP or the rate of entry of GTP at a regulatory site on G/F. Detailed kinetic studies are in progress to further characterize the system and establish whether or not there is a significant increase in the turn-off rate (35) of the adenylate cyclase reaction during adipocyte development.

The kinetic observations indicating that G/F exhibited different sets of regulatory properties in adipocyte and preadipocyte membranes were correlated with marked differences in the patterns and levels of cholera toxin-catalyzed [32P]ADP-ribosylation of G/F subunits (Figs. 8–10). In adipocyte membranes, 13-fold more 32P was incorporated into the 42,000-dalton component and 4-fold more labeling was observed in the 49,000- to 50,000-dalton doublet than in the corresponding G/F subunits in preadipocyte membranes (Figs. 8 and 9). While the quantitation of the amount of ADP-ribosylation suggests that the concentration of G/F rises sharply during differentiation, we cannot rule out the possibility that qualitative rather than quantitative changes occur. For example, the differences observed in the ADP-ribosylation of G/F may reflect differentiation-directed conformational modifications of the G/F subunits which enhance their capacity as substrates in the cholera toxin-catalyzed reaction. Alternatively, the greater amount of [32P]ADP-ribosylated G/F subunits observed in adipocytes may actually indicate an increase in the number of molecules of G/F protein per cell. A large increase in the amount of G/F, coupled with the small increase in β-adrenergic receptor number described above, could account for the great increase in the responsiveness of the adenylate cyclase system to isoproterenol during the differentiation of 3T3-L1 cells.

Regardless of whether the changes in ADP-ribosylation patterns reflect changes in the amount of G/F or its ability to be labeled, the results show that there is a significant change in G/F constituents during cell differentiation. There is little information on the roles of the individual subunits of the G/F regulatory component. Northup et al. (28) purified rabbit liver G/F to near homogeneity and showed that G/F was probably composed of three peptides of molecular weights 35,000, 42,000, and 52,000 with unknown stoichiometries. The latter two components are substrates for ADP-ribosylation. The 49,000- to 50,000-dalton doublet was evident at the leading edge of the G/F peak during purification on heparin-Sepharose, while the 42,000-dalton protein was prevalent at the trailing portion of the peak and the 35,000-dalton protein was observed throughout (28). Thus, the differential increases in the labeling of the 42,000-dalton polypeptide and the 49,000-50,000 doublet in adipocyte membranes (Figs. 8–10) may reflect the appearance of distinct subspecies of G/F during differentiation. Hudson and Johnson (36) reported that partial proteolytic digests of [32P]ADP-ribosylated 42,000-dalton protein and the 49,000-50,000-dalton doublet yielded identical peptide maps except for a single fragment unique to the larger doublet. These observations suggest that these G/F components are structurally and functionally related and raise the possibility that the 49,000-50,000-dalton doublet serves as a precursor of the 42,000-dalton polypeptide.

Our results suggest that 3T3-L1 adipocytes may acquire catecholamine-sensitive adenylate cyclase activity by altering the properties of G/F to effect the coupling of both pre-existing and newly synthesized β-receptors and catalytic protein. A preponderance of the 49,000-50,000 doublet and a low

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level of 42,000-dalton protein are associated with the G/F of hormone-insensitive, GTP-activated preadipocyte adenylate cyclase. The finding that adipocyte membranes have a very large increase in the labeling of the 42,000-dalton protein relative to the 49,000- to 50,000-dalton doublet, along with reports that avian erythrocyte adenylate cyclase has only the 42,000-dalton G/F subunit and is totally dependent on hormone for GTP activation (25, 37, 38) suggest the speculation that the relative concentrations of the 42,000-dalton and 49,000- to 50,000-dalton proteins or some other factor which alters their relative suitability as substrates for ADP-ribosylation may determine whether the interaction of G/F with GTP requires the presence of agonist occupied β-receptor. Detailed biochemical studies on the nature of the functional and structural changes in G/F subunits during adipocyte differentiation will now be required to explore this working hypothesis.

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REFERENCES