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Changes in the expression of guanine nucleotide-binding proteins during differentiation of 3T3-F442A cells in a hormonally defined medium

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Using specific antisera, the expression of the G protein α subunits, G_s, G_{i1}, G_{i2}, G_{i3} and G_o, were determined in 3T3-F442A cells during their differentiation to adipocytes in a hormonally defined medium. Differentiation caused distinct increases in the expression of two G_s isoforms and decreases in the expression of both G_{i2} and G_{i3}. Differentiation also resulted in a 2- to 4-fold increase in forskolin-stimulated adenylyl cyclase activity and a 15-fold increase in the response of cells to a β-adrenergic agonist. The increase in G_s expression was also observed, to a lesser degree, in cells maintained at confluence under conditions where morphological conversion was negligible and the decreased expression of G_{i2} and G_{i3} and the increased β-adrenergic responsiveness did not occur.

3T3-F442A cell; Adipocyte; Differentiation; G protein; Adenylyl cyclase

1. INTRODUCTION

The ability of certain fibroblast cell lines to differentiate in culture provides an excellent model for the study of adipocyte differentiation in vivo. For example, both 3T3-L1 and 3T3-F442A fibroblasts readily differentiate, in the presence of serum and other factors, into cells possessing the morphological and biochemical properties of adipocytes [1,2]. However, the factors which regulate the differentiation process and which are required to maintain the adipocyte phenotype remain to be determined. Progress towards understanding these mechanisms has been aided by the development of hormonally defined serum-free media which support adipocyte differentiation [3–5].

One of the consequences of 3T3 cell differentiation is the acquisition of an increased responsiveness of adenylyl cyclase (AC) to lipolytic agents [6]. Hormone receptors regulate AC via stimulatory (G_s) and inhibitory (G_i) guanine nucleotide binding (G) proteins [7]. Changes in the expression of G proteins have been examined in 3T3-L1 cells differentiating in serum-containing media [8–11]. However, these studies have produced some conflicting results, perhaps due to differences in the methods used to assess G protein expression. Moreover, the use of serum precludes a detailed assessment of the hormones and other factors necessary to elicit such changes. With the availability of a full range of specific immunological probes, we now report on the expression of those G proteins with a potential for regulating AC during the differentiation of 3T3-F442A cells under hormonally defined, serum-free conditions. Our results indicate that independent changes in the expression of G_s, G_{i2} and G_{i3} accompany the increased responsiveness of differentiated 3T3-F442A cells to lipolytic stimuli.

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Abbreviations: AC, adenylyl cyclase; PBS, phosphate-buffered saline, EGF, epidermal growth factor; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; GPDH, α-glycerophosphate dehydrogenase.

2. MATERIALS AND METHODS

2.1. *Cell culture*

3T3-F442A cells (provided by Dr. Howard Green, Harvard Medical School) were cultured in DMEM containing 10% calf serum (standard medium) as described previously [12]. Confluent cultures (day 5 after seeding, subsequently referred to as JC cells) were washed three times in PBS and incubated for 10–14 days in a defined differentiation medium (DDM) containing growth hormone (2 nM), insulin (1.8 μM), T_3 (0.1 ng/ml), EGF (50 ng/ml) and other factors as described by Guller et al. [3]. Cells cultured under these conditions, in which at least 60% of the cells exhibited adipocyte morphology assessed by light microscopy and Oil Red O staining, are subsequently referred to as DDM cells. Alternatively, the growing medium was replaced at confluence with DMEM containing 10% foetal calf serum and 0.9 μM insulin. Cells cultured under these conditions, in which at least 70–80% of the cells exhibited adipocyte morphology after 10–14 days, are subsequently referred to as FCS cells. As a control, parallel cultures were maintained at confluence for 12 days in standard medium. These cultures, in which adipocyte conversion was always less than 5%, are subsequently referred to as 12dc cells.

2.2 *Preparation of membrane fractions*

Cells were washed once in ice cold PBS then scraped into lysis buffer (50 mM HEPES, pH 7.6, 0.25 M sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulphonylfluoride and 1 μg/ml leupeptin). The

cells were lysed in a tight-fitting Dounce homogeniser followed by centrifugation (1,000 × g, 10 min) to remove nuclei and unbroken cells. The resulting supernatant was centrifuged (100,000 × g, 45 min) and the membrane pellet resuspended in 50 mM HEPES, pH 7.4, 1 mM EDTA at a concentration of 2–3 mg protein/ml.

2.3. *Immunoblotting*

Equal quantities of membrane protein (75 μg) were subjected to SDS-PAGE and immunoblotting as described [13]. Specific antisera to G_s, G_{i1}, G_{i1}/G_{i2}, G_{i3} and G_0 were generously provided by Dr. Allen Spiegel, NIH [14] and Dr. Graeme Milligan, University of Glasgow [13]. Immunoreactive bands were detected using ^{125}I-protein A and quantified by densitometric scanning using a Bio-Rad Model 620 video densitometer.

2.4. *Enzyme assays and expression of results*

Adenylyl cyclase activities and cyclic AMP concentrations were measured by the binding protein method as described [15]. GPDH activity was measured using the method of Wise and Green [16]. In accordance with others [8] we found that the amount of protein per cell was increased by a factor of 2.3 in adipocytes compared with fibroblasts. Data are therefore expressed relative to cell number rather than amount of cellular protein. Data were analysed by Student's *t*-test.

3. RESULTS

Table I shows the effects of differentiation on the ability of the β-adrenergic agent isoproterenol to increase intracellular cyclic AMP levels in 3T3-F442A cells. Responsiveness to isoproterenol increased from 1.5-fold in JC cells to around 15-fold in cells differentiated using either protocol. A much smaller, but significant, increase in responsiveness to isoproterenol occurred in 12dc cells in which differentiation, assessed morphologically, was less than 5%. The amount of AC catalytic subunit, measured in membranes in the presence of forskolin, was significantly elevated (2–4-fold) in differentiated cells (Table II). The levels of GPDH, a marker for adipocyte differentiation [16], increased dramatically during differentiation in either conversion medium but were unchanged, compared to fibroblasts, in 12dc cells (data not shown).

Fig. 1 shows the effects of differentiation on the relative expression of G protein α-subunits, detected using anti-peptide antibodies [13,14] specific for the α-subunits of G_s, G_{i3} and G_0. Initial experiments, with appropriate positive controls, did not detect $G_{i1\alpha}$ in 3T3-F442A cells (data not shown). Consequently, an antibody recognizing both $G_{i1\alpha}$ and $G_{i2\alpha}$ [13] was used specifically to probe $G_{i2\alpha}$ expression in this system.

3T3-F442A adipocytes were found to contain approximately 2.3 times more protein than fibroblasts. Hence, the densitometric values for G protein expression derived from Fig. 1 (based on equivalent amounts of membrane protein) were transformed into values indicating expression per cell (Fig. 2). Thus, on a per-cell basis, the expression of the 42 kDa and 47 kDa forms of G_s increased during differentiation by ~750% and ~125%, respectively, relative to their expression in fibroblasts. The expression of the 42 kDa and 47 kDa forms of G_s also increased in 12dc cells by ~450% and ~100%, respectively. In contrast, G_{i2} expression decreased by 60–70% during differentiation but this decrease did not occur in 12dc cells. Differentiation also decreased the expression of G_{i3} by 70–80% but this change was not observed in 12dc cells. All of the above changes occurred to a similar degree whether differentiation was induced in the presence of foetal calf serum

Table II

Effect of differentiation on adenylyl cyclase activity in 3T3-F442A membranes

	Adenylyl cyclase activity (pmol · min^{-1} per 10^6 cells)			
Addition	JC	12dc	FCS	DDM
None	3.42 ± 0.41	3.33 ± 0.29	7.80 ± 0.68*	7.43 ± 1.49*
Forskolin	54.3 ± 7.41	50.2 ± 4.30	170.6 ± 40.8*	136.5 ± 29.8*

Adenylyl cyclase activity was measured in the presence and absence of 10 μM forskolin in membranes prepared from cells treated or not with differentiation media as described in section 2. Results are means ± S.E.M. from six independent observations.

*Indicates the value is significantly different from the appropriate value for JC cells ($P < 0.05$).

Table I

Changes in the responsiveness to isoproterenol during differentiation of 3T3-F442A preadipocytes

	Intracellular cyclic AMP (pmol/10^6 cells)			
Addition	JC	12dc	FCS	DDM
None	5.6 ± 0.7 (9)	5.5 ± 0.5 (6)	3.7 ± 0.3 (10)*	2.1 ± 0.5 (7)*
Isoproterenol	8.4 ± 1.4 (9)	15.7 ± 1.4 (6)*	47.9 ± 4.3 (10)**	25.9 ± 2.6 (7)**
Fold stimulation	1.5 ± 0.12	2.8 ± 0.13*	15.5 ± 3.5*	15.1 ± 2.6**

Cells were incubated for 10 min in medium containing 0.1 mM isobutylmethylxanthine in the presence or absence of 10 μM isoproterenol. Cyclic AMP levels were then determined as described in section 2. Results are means ± S.E.M. with the number of observations in parentheses.

*,**Indicate that the value is significantly different from the appropriate value for JC cells ($P < 0.02$, $P < 0.001$, respectively)

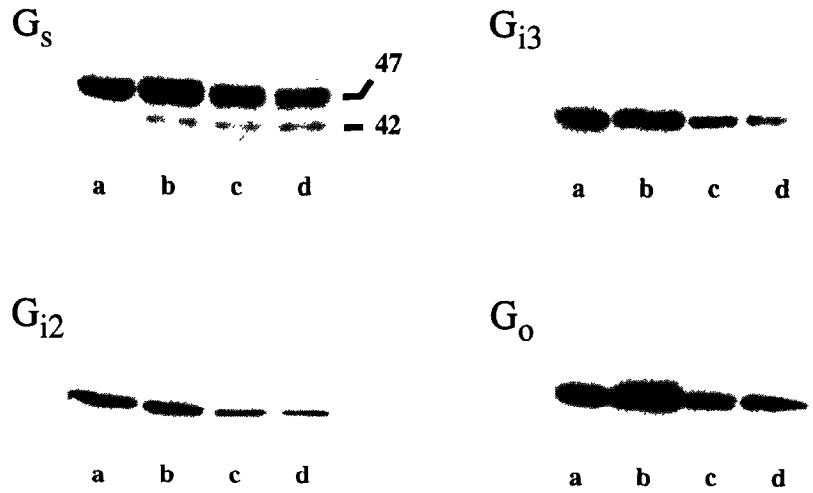

Fig. 1. Expression of G protein α subunits in 3T3-F442A membranes. Equal quantities (75 μg) of membrane protein from confluent fibroblasts (lanes a), 12 day confluent fibroblasts (lanes b), cells differentiated with FCS medium (lanes c) or cells differentiated with DDM medium (lanes d) were immunoblotted with specific G protein antibodies as described in Section 2. The figure is composed of representative blots for each G protein which were performed on at least three separate occasions. Molecular weights of the two isoforms of G_s are shown to the right of the top left panel.

or with the defined serum-free medium. No consistent changes occurred in the expression of G_0 under any condition tested.

4. DISCUSSION

In agreement with studies performed using other models of adipocyte differentiation [6,8,17], our data show that the differentiation of 3T3-F442A cells is associated with an increased ability of β-adrenergic agents to raise intracellular cyclic AMP levels. Previous studies with 3T3-L1 cells reported minimal changes in the levels of β-adrenergic receptor and AC catalytic subunit during serum-induced differentiation and therefore concluded that increased responsiveness to β-adrenergic agents was mediated by changes in the expression of regulatory G proteins [9,11]. Our measurements of forskolin-stimulated AC activity in 3T3-F442A membranes

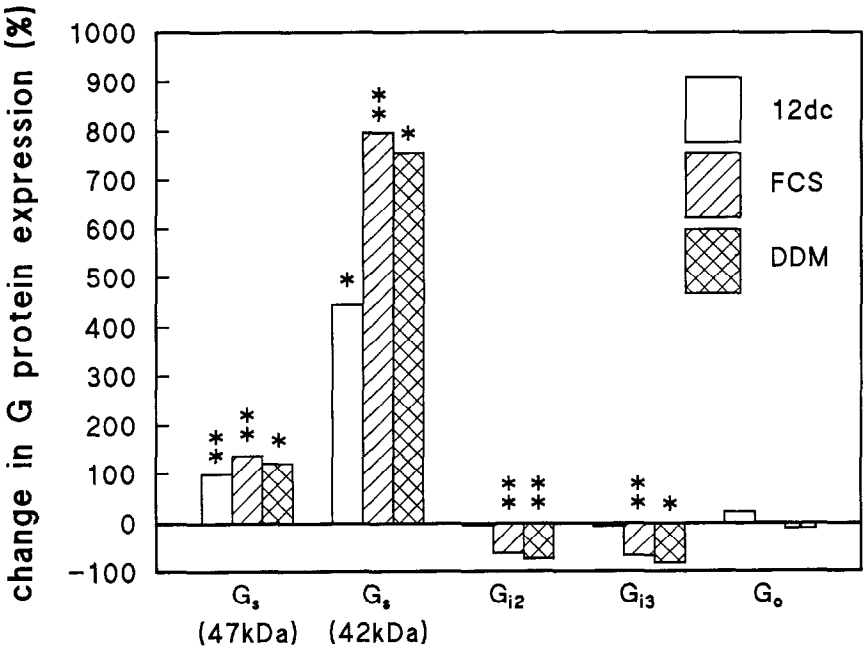

Fig. 2. Changes in the expression of G protein α subunits following differentiation of 3T3-F442A cells. The change in expression of each α subunit following maintenance of cells at confluence for 12 days (12dc) or differentiation in FCS or DDM media is shown relative to its expression in confluent fibroblasts. Data represent average changes in expression from 3–5 separate experiments. Data for G protein expression were calculated on a 'per cell' basis as described in Section 2. Statistically significant changes in expression relative to fibroblasts are indicated as $^*P < 0.05$ and $^{**}P < 0.02$.

indicate that the cellular levels of catalytic subunit increase 2–4-fold during differentiation. Differentiation also resulted in an increase in expression of the two forms of G_s, which have the potential to positively regulate AC [7], and a concomitant decrease in expression of G_{i2} and G_{i3}, both of which have been shown to negatively regulate AC [7,18,19]. Thus it seems likely that the combined changes in AC, G_s and G_i expression account for the large (15-fold) increase in responsiveness of differentiated cells to β-adrenergic agents.

Whilst changes in the level of G protein expression probably contribute to AC regulation, the precise contributions of changes in the levels of particular G proteins remain uncertain. Although the 42 kDa form of G_s increased more dramatically than the 47 kDa form, little is known regarding the individual regulatory roles of different G_s isoforms. Similarly, the individual influences of the decreased expressions of G_{i2} and G_{i3} on AC responsiveness remain to be established, although evidence indicates that AC inhibition is most commonly mediated via G_{i2} with a role for G_{i3} in some cases [7,18,19]. Our data also showed increased G_s expression in cells maintained at confluence for 12 days, where morphological conversion to adipocytes was negligible. These cells exhibited a much smaller increase in responsiveness of AC to isoproterenol compared with maximally differentiated cultures. This implies that other factors, including decreased expression of G_i, are also necessary to elicit maximal changes in responsiveness: G_i levels did not change in 12dc cells.

In addition to their role in the altered responsiveness of adipocytes to hormonal stimuli, it has been suggested recently that G proteins may also regulate the differentiation process per se. Antisense oligonucleotide probes to G_s promote differentiation of 3T3-L1 cells [20] and pertussis toxin, which inactivates certain G proteins, including the G_i family [21], has been shown to enhance the differentiation of another adipogenic cell line [22]. Our finding that G_s expression increased in the absence of morphological changes is consistent with an additional, perhaps regulatory, role in the differentiation process. In support of this, we have found that cholera toxin, which constitutively activates G_s [23], severely retards the differentiation of 3T3-F442A cells in DDM (unpublished results). Interestingly, increased expression of G_s with time in culture has also been observed in 3T3-C2 cells, a subclone which has a very low capacity to undergo adipocyte differentiation [9]. If G_s does inhibit adipocyte differentiation and G_s levels rise in cells maintained at confluence, this may explain a previous report showing a decrease in the ability of 3T3-F442A cells to differentiate with increasing time at confluence [24].

Overall, the results of this study demonstrate that independent changes in the expression of individual G proteins occur during the conversion of 3T3-F442A fibroblasts into adipocytes. Importantly, these changes occur whether differentiation is induced in the presence of foetal calf serum or in a hormonally-defined medium. Furthermore, the increased responsiveness of AC to β-adrenergic agents, shown previously in 3T3-L1 cells differentiated in the presence of serum [6,8], also occurred under defined conditions. The use of defined media to induce differentiation should aid in the identification of the individual factors which regulate the changes in gene expression associated with adipocyte differentiation. Regarding components of the defined medium used in this study, previous work has demonstrated obligatory roles for both growth hormone and insulin in the conversion process with other components such as EGF and T_3 exerting modulatory influences [3]. Nevertheless, further work will be necessary in order to assign roles for individual components in, for example, regulating changes in G protein expression. Such studies are currently in progress.

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REFERENCES

[1] Green, H and Kehinde, O. (1974) Cell 1, 113–116.
[2] Green, H. and Kehinde, O. (1976) Cell 7, 105–113.
[3] Guller, S., Corin, R.E., Mynarcik, D C , London, B.M. and Sonnenberg, M. (1988) Endocrinology 122, 2084–2089.
[4] Schmidt, W., Jordan-Pöll, G. and Löffler, G (1990) J. Biol. Chem 265, 15489–15495.
[5] Hauner, H. (1990) Endocrinology 127, 865–872.
[6] Rubin, C.S., Lai, E. and Rosen, O.M. (1977) J. Biol. Chem. 252, 3554–3557.
[7] Houslay, M.D (1991) Cell. Signal. 3, 1–9.
[8] Lai, E., Rosen, O.M. and Rubin, C.S (1981) J Biol. Chem. 256, 12866–12874.
[9] Watkins, P.A., Moss, J., Pekala, P.H. and Lane, M.D. (1982) J. Biol. Chem. 257, 14719–14722.
[10] Gierschik, P., Morrow, B., Milligan, G., Rubin, C. and Spiegel, A. (1986) FEBS Lett. 199, 103–106.
[11] Watkins, D.C., Northup, J.K. and Malbon, C.C. (1987) J. Biol. Chem 262, 10651–10657.
[12] Anderson, N.G. (1992) Biochem. J. 284, 649–652.
[13] Mitchell, F.M., Griffiths, S.L., Saggerson, E.D., Houslay, M.D., Knowler, J.T. and Milligan, G. (1989) Biochem. J. 262, 403–408.
[14] Spiegel, A.M. (1990) in. G Proteins (Iyengar, I. & Brinbaumer, L. Eds.) pp. 115–143, Academic Press.
[15] Farndale, R.W., Allan, L.M. and Martin, B.R. (1992) in: Signal Transduction: A Practical Approach (G. Milligan, Ed.) Chapter 4, pp. 75–104, IRL Press, Oxford.
[16] Wise, L.S. and Green, H. (1979) J. Biol. Chem. 254, 273–275
[17] Pairault, J. and Lasnier, F. (1987) J. Cell. Physiol. 132, 279–286.
[18] Gerhardt, M A. and Neubig, R.R. (1991) Mol. Pharmacol. 40, 707–711.
[19] Wong, y.H , Conklin, B.R. and Bourne, H R. (1992) Science 255, 339–342
[20] Wang, H.-y., Watkins, D C and Malbon, C.C. (1992) Nature 358, 334–337.
[21] Ui, M. (1984) Trends Pharmacol. Sci. 5, 277–279.
[22] Shinohara, O., Murata, Y.-i. and Shimizu, M. (1992) Biochem. Cell Biol. 70, 650–655.
[23] Gill, D.M. and Meren, R. (1978) Proc. Natl. Acad. Sci. USA 75, 3050–3054.
[24] Morikawa, M. (1986) J. Cell Physiol. 128, 293–298.