Changes in the guanine nucleotide-binding proteins, G_i and G_o, during differentiation of 3T3-L1 cells

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Differentiation of 3T3-L1 cells from fibroblasts to adipocytes is accompanied by increased adenylate cyclase response to lipolytic agents. We used pertussis toxin and specific antibodies to measure the inhibitory guanine nucleotide-binding protein, G_i, and the novel G-protein, G_o, in membranes from 3T3-L1 cells. Pertussis toxin-dependent labeling of a 39–40 kDa protein showed an initial 30% rise, followed by an 80% fall during differentiation. Immunoblots showed that 3T3-L1 cells contain G_o, as well as G_i, and that changes in the former parallel the changes in pertussis toxin labeling. Changes in G_i and G_o may contribute to altered adenylate cyclase response during 3T3-L1 cell differentiation.

Pertussis toxin *Adenylate cyclase* *Signal transduction*

1. INTRODUCTION

3T3-L1 fibroblasts differentiate either spontaneously [1] or after treatment with agents such as DEX + IBMX into adipocytes [2]. Differentiation of 3T3-L1 cells from fibroblasts to adipocytes is accompanied by increased responsiveness of adenylate cyclase to lipolytic agents [1]. Previous studies [2,3] indicate that changes in hormone receptors or in G_s cannot by themselves account for increased cyclase response. Since adenylate cyclase is also subject to inhibitory regulation by a distinct G_i protein [4], we attempted to measure G_i in membranes from differentiating cells. Pertussis toxin-catalyzed ADP-ribosylation, which has been used as a specific probe for G_i [5], is now known to ADP-ribosylate other G-proteins, including G_o, a novel G-protein of unknown function, first purified from brain [6–8]. Therefore, we used specific antibodies against G_i [9] and G_o [10], in addition to pertussis toxin labeling, to measure these proteins in 3T3-L1 cells.

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Abbreviations: G-protein, guanine nucleotide-binding protein; G_s and G_i, the G-proteins associated with stimulation and inhibition, respectively, of adenylate cyclase; G_o, a G-protein of unknown function discovered in brain; DEX + IBMX, dexamethasone + isobutylmethylxanthine

2. EXPERIMENTAL

3T3-L1 and 3T3-C2 cells were cultured as described [2]; confluent cells (day 0) were treated for 48 h with fresh medium containing 0.25 μM dexamethasone and 0.5 mM isobutylmethylxanthine (DEX + IBMX); after 48 h (day 2), cells were refed with fresh medium and harvested at days 4

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and 6 after initial treatment. Cell membranes were prepared as in [2].

G_i and G_o were purified from bovine brain as in [8]. Rabbit antisera against G_i [9] and G_o [10] were prepared and characterized as described. Adenylate cyclase was assayed as in [2]. Pertussis toxin radiolabeling was done as in [9]. Briefly, pertussis toxin (kindly provided by Dr R. Sekura, NICHD) was activated by incubation for 1 h at room temperature in 20 mM Tris-HCl, pH 7.5, with 50 mM dithiothreitol. Activated toxin (35 μg/ml final concentration) was added to buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 2 mM ATP, and 50 μg membrane protein in a final volume of 0.1 ml. To this was added [α-^{32}P]NAD (35 μCi/ml, New England Nuclear); final concentration 10 μM NAD. The reaction mixture was incubated for 1 h at 37°C, and the reaction terminated by addition of SDS-PAGE sample buffer. SDS-PAGE and autoradiography were performed as in [9,10]. M_r estimates are based on standard proteins (Bethesda Research Labs, high-M_r) run in parallel. Immunoblotting was performed as in [9,11] with affinity-purified antibodies [10] and radioiodinated protein A (New England Nuclear). Quantitative immunoblotting of the common β-subunit was performed as in [9,10] with purified transducin β/γ-subunit as standard.

3. RESULTS

We compared 3T3-L1 cells treated with DEX + IBMX to another fibroblast cell line, 3T3-C2, similarly treated. As noted in [2], by day 6 approx. 90% of 3T3-L1 cells have differentiated into adipocytes, whereas virtually no 3T3-C2 cells differentiate. Pertussis toxin-dependent ADP-ribosylation [5] was used to measure G_i in plasma membranes from 3T3-L1 and 3T3-C2 cells (fig.1). A toxin-dependent band of approx. 39–40 kDa was observed on autoradiograms. Densitometric quantitation of this band showed a 30% rise in membranes from L1 cells 2 days after DEX + IBMX followed by a 71 and 83% fall at days 4 and 6, respectively (all values relative to day 0 = 100%). In contrast, C2 cells showed very little change 6 days after DEX + IBMX (77 and 83% respectively for day 0 and day 6 3T3-C2 cells, relative to day 0 3T3-L1 cells).

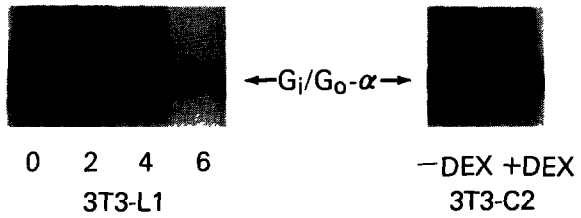

Fig.1. Pertussis toxin-dependent ADP-ribosylation of an approx. 40 kDa protein in membranes derived from 3T3-L1 and 3T3-C2 cells. Membranes were prepared from 3T3-L1 cells before treatment with DEX + IBMX (day 0) and 2, 4 and 6 days after treatment, and from 3T3-C2 cells before treatment (− DEX) and 6 days after treatment (+ DEX). Membranes were treated with [α-^{32}P]NAD and with or without pertussis toxin, and subjected to SDS-PAGE and autoradiography (see section 2). 25 μg membrane protein were loaded per lane. The band shown is the only one labeled in a pertussis toxin-dependent manner and is denoted as G_i/G_o-α based on the immunoblot results in fig.2.

We next used affinity-purified antibodies specific for either G_i [9] or G_o [10] to determine whether the latter is present in 3T3-L1 cells, and to measure changes during differentiation. Both types of antibodies also react with the common (approx. 35 kDa) β-subunit of G-proteins [9–11]. Antiserum RV3 has been shown to react specifically with G_o-α, and not with G_i, G_s, or transducin-α [10]. In contrast, antiserum CW6 was raised against transducin but cross-reacts strongly with G_i-α, and weakly, if at all, with G_o-α [9]. Fig.2A shows the specificity of the antisera used on immunoblot strips containing a mixture of G_i and G_o. Both antisera react with the common β-subunit (although to a different degree). The selectivity of CW6 for G_i-α (fig.2A, lane 2) and of RV3 for G_o-α (lane 3) is reflected in the greater distance on immunoblot between α- and β-bands for CW6 compared with RV3 (as expected if CW6 reacts with the higher G_i-α-band). Previous experiments in which immunoblots of G_i and G_o performed with a mixture of CW6 and RV3 revealed the resolved G_i- and G_o-α doublet provide definitive evidence for the selectivity of these antisera [10].

Immunoblots with CW6 and RV3 (fig.2B,C) show that both G_i-α and G_o-α decrease substantially by day 6 in 3T3-L1 cells (by 64 and 80%, respectively, compared with day 0 cells). At day 2, G_i-α was relatively unchanged (7% reduction) but G_o-α

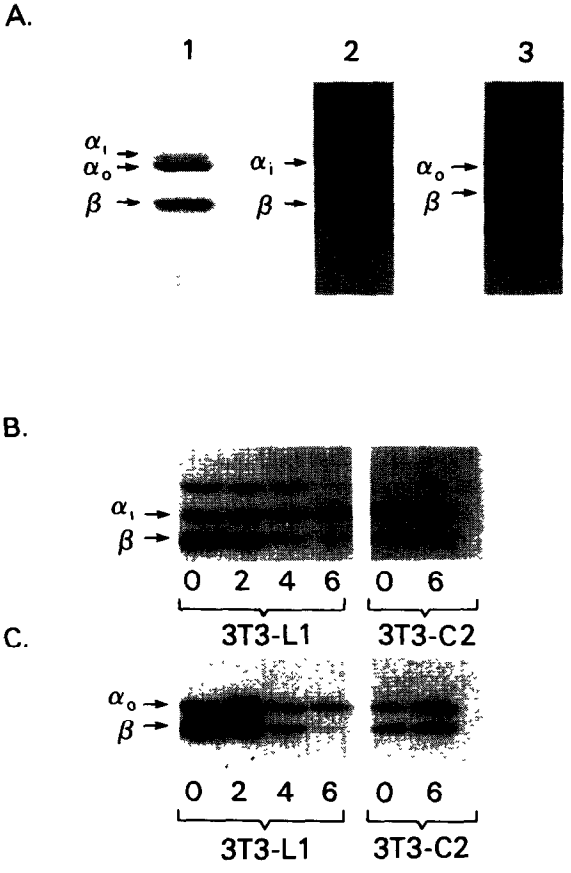

Fig.2. Immunoblots of purified G_i and G_o (A) and of 3T3-L1 and 3T3-C2 cell membranes (B,C) with affinity-purified antibodies specific for G_i and G_o. (A) G_i and G_o purified from bovine cerebral cortex were subjected to SDS-PAGE and either stained with Coomassie blue (lane 1, 4 μg), or immunoblotted with antibodies specific for G_i-α and β (lane 2, 1.5 μg) or with antibodies specific for G_o-α and β (lane 3, 0.8 μg). In B and C, plasma membranes prepared from 3T3-L1 and 3T3-C2 cells treated with DEX + IBMX (as in fig.1 legend) were subjected to SDS-PAGE (150 μg/lane) and immunoblotting with antibodies specific for G_i (B) or G_o (C). The arrows indicate the α-subunits of G_i and G_o and the common β-subunit. The identity of the protein above G_i-α that reacts with G_i-specific antibodies is unknown (B). No other bands were stained on the immunoblots.

increased by 48% compared with day 0 3T3-L1 cells. Neither G_i nor G_o-α declined by day 6 in 3T3-C2 cells. For the experiment shown, both subunits actually increased by day 6 after DEX + IBMX but this was exceptional, and in most experiments (cf. fig.1) no difference between day 0 and day 6 3T3-C2 cells was observed. In contrast, 3T3-L1 cells reproducibly (at least 2 separate experiments on 3 independently differentiated sets of cells) showed a marked decline in G_i and G_o by day 6, and a rise in G_o at day 2.

The common β-subunit also decreases in membranes of differentiated 3T3-L1 cells (fig.2). In separate quantitative immunoblots using purified transducin β/γ-subunit as standard, we determined that there is approx. 1.4 μg β/mg total membrane protein in day 0 3T3-L1 cells, and that β decreases by 21, 74 and 83% at days 2, 4 and 6, respectively. Again, no decline was seen in 3T3-C2 cell membranes treated with DEX + IBMX.

Since 3T3-L1 cells increase in size during differentiation, the yield of membrane protein/cell increases (by a factor of 2.7) from day 0 to day 6 [2]. In figs 1 and 2, equal amounts of membrane protein are compared. If the data are normalized for cell number there is a 50% decrease in β-subunits and in G_o-α, but no change in G_i-α from day 0 to day 6 in 3T3-L1 cells.

In agreement with [2], adenylate cyclase activity in membranes from the cells shown in figs 1 and 2 shows two major changes during differentiation: (i) increased response to the β-adrenergic agonist, isoproterenol, in day 6 compared with day 0 3T3-L1 but not 3T3-C2 cells; (ii) lowest absolute activity in response to all stimulators (isoproterenol or guanine nucleotides) at day 2 (not shown).

4. DISCUSSION

Adenylate cyclase activity is regulated by both stimulatory and inhibitory G proteins [4]. Although differentiation of 3T3-L1 cells is accompanied by increased G_s (measured by cholera toxin radiolabeling) this change alone is unlikely to explain increased adenylate cyclase response, since a similar rise in G_s was observed in 3T3-C2 cells treated with DEX + IBMX [3]. 3T3-C2 cells, however, do not differentiate to adipocytes in response to DEX + IBMX, nor do they show increased adenylate cyclase response after treatment [2,3].

Our data suggest that changes in inhibitory G-proteins may contribute to altered adenylate cyclase activity during 3T3-L1 cell differentiation. The mechanism of inhibition of adenylate cyclase

by G_i is unclear. Although G_i-α appears capable of inhibition under certain conditions [12], most evidence points to a predominant role for the β/γ-subunit [13]. The function of G_o is unknown (see below) but since it appears to contain the common β/γ-subunit, G_o may also play a role in adenylate cyclase inhibition [6]. Thus, the increase in G_o at day 2 (and an increase in β-subunit when expressed per cell number) may be relevant to the decline in absolute adenylate cyclase activity at day 2. The decline in both G_i and G_o by day 6 in 3T3-L1 but not 3T3-C2 cells may also help explain the increase in agonist-stimulated activity at day 6 in L1 but not C2 cells. Additional factors, such as a concomitant increase in G_s [2,3], or changes in other regulatory factors could also be involved.

Our data on G_o provide clearcut evidence for the occurrence of this novel G-protein in a non-neural cell. Previous reports of two pertussis toxin substrates in fat cells [14,15] left unclear the specific identity of these proteins. Identification of G_o in tissues other than brain, e.g. heart [6,16], also did not indicate the specific G_o-containing cell type. If one can extrapolate from 3T3-L1 cells, our data suggest that normal fibroblasts and adipocytes may contain G_o.

Although the specific function of G_o is unknown, its location in the plasma membrane and heterotrimeric structure, analogous to that of other G-proteins [4], suggest a role in signal transduction. The rise in G_o observed in 3T3-L1 cells at day 2 after DEX + IBMX and subsequent decline with differentiation are of interest with respect to the possible function of G_o. Treatment of 3T3-L1 cells with DEX + IBMX is followed initially by cell proliferation which is completed by day 2 when DEX + IBMX are removed from the medium [17]. Differentiation follows thereafter. This leads us to speculate that G_o could be involved in a signal transduction pathway related to control of cell division. The relatively high concentrations of G_o (approx. 1% of total membrane protein) in fully differentiated, non-dividing neural cells [6,10] would appear to argue against a role for G_o in control of cell proliferation, but it should be noted that other proteins ordinarily associated with cell proliferation such as the product of the *src* proto-oncogene are also abundant in neural cells [18]. Cultured 3T3-L1 cells should prove useful in helping elucidate the function of G_o, and as a model system for studying alterations in G-protein subunit turnover.

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