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Subcellular Localization of G-Proteins in Primary-Cultured Mouse Preadipocytes and Adipocytes

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Abstract The subcellular localization of $G_s\alpha$, $G_i\alpha1\&2$, $G_i\alpha3$, and Gβ was studied in primary-cultured undifferentiated and differentiated, lipid replete, adipose cells. The results show a distinct distribution for each of these G-proteins and differences between differentiated and undifferentiated cells. All the G-proteins examined had a cytoplasmic localization; only $G_i\alpha1$ and 2 showed a significant colocalization with the plasma membrane and this only in differentiated cells. Most studies using cells in culture have reported an intracellular localization for G-proteins, whereas in tissue sections the localization has been reported to be largely with the plasma membrane, with some intracellular localization. The results suggest that the cell-cell interactions or the specific geometry imposed by culture conditions favor the intracellular compared to peripheral localization of G-proteins. Alternately, the posttranslational modifications necessary for G-protein insertion in the plasma membrane may be deficient in cultured cells. J. Cell. Biochem. 65:259–266. © 1997 Wiley-Liss, Inc.

Key words: differentiation; $G_s\alpha$; $G_i\alpha$; Gβ; actin; stress fibres; vimentin; subcellular localization; immunofluorescence microscopy; adenylyl cyclase; antibodies; lipid; fat accumulation; obese; obesity

Heterotrimeric G-proteins are well known to be involved in signalling via plasma membrane receptors. The G-proteins regulate effector systems such as AC, phospholipase C, and several ion channels [Birnbaumer and Birnbaumer, 1994]. Their association with the plasma membrane allows them to interact with both membrane receptors and effectors [Hepler and Gilman, 1992]. These peptides are also associated with various cellular organelles [Brabet et al., 1988; Gabrion et al., 1989; Wang et al., 1989; Ercolani et al., 1990; Lewis et al., 1991; Stow and de Almeida, 1993; McFarlane-Anderson et al., 1993; Cadrin et al., 1993; Saffitz et al., 1994; Wilson et al., 1994], and there is now evidence that they can modulate responses in cellular compartments other than the plasma membrane [Mélançon et al., 1987; Leyte et al., 1992; Donaldson et al., 1991; Hidalgo et al., 1995; Konrad et al., 1995; Kowluru et al., 1994, 1996; Aridor et al., 1993; Wedegaertner et al., 1995].

In adipose tissue plasma membranes, G-proteins regulate the lipolytic response to hormones such as catecholamines by modulating the AC-mediated production of cAMP. In previous studies of isolated adipocyte membranes, $G_i\alpha/G_i\alpha2$, $G_i\alpha3$, and $G_s\alpha$ were found to be the major α subunits present in mouse adipose tissue on the basis of the results of ADP ribosylation with bacterial toxins and of Western blot analysis; Gβ subunits were also detected by these methods [Bégin-Heick, 1990, 1992; Gettys et al., 1991, 1995].

We have previously examined the localization of G-proteins in the adipocyte cell lines Ob17 and HGFu, in conjunction with the expression of the various subunits at the mRNA level,

Abbreviations used: AC, adenylyl cyclase; F-actin, filamentous actin; FITC, fluorescein isothiocyanate; G-actin, globular actin; G-protein, guanine nucleotide-binding regulatory protein; $G_s\alpha$, $G_i\alpha1$, $G_i\alpha2$, $G_i\alpha3$, Gβ, the various subunits of G-proteins.

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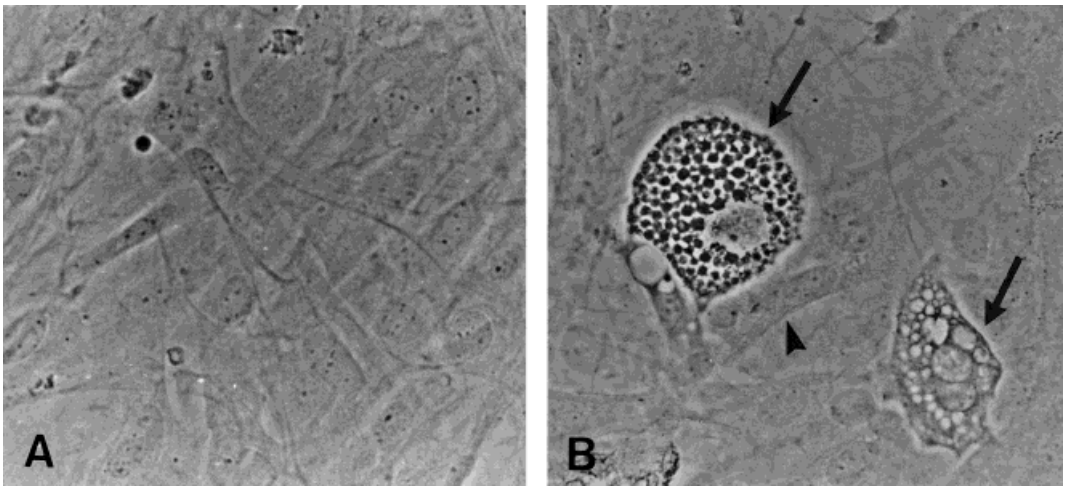

Fig. 1. **A:** Phase-contrast image of confluent preadipocytes showing a fibroblastic appearance. **B:** Phase-contrast photograph of preadipocytes induced to differentiate in the presence of corticosterone, insulin, and MIX, as described in Materials and Methods. Some nondifferentiated (arrowhead) and lipid-containing differentiated adipocytes (arrows) are observed.

their abundance in membrane preparations, and the ability of various agents to stimulate AC [McFarlane-Anderson et al., 1993; Cadrin et al., 1993]. In these studies, we found that the G-proteins were mostly associated with intracellular structures, although some membrane localization was also observed. However, while they showed signs of differentiation, such as actin microfilament reorganization, these clonal cells did not accumulate significant amounts of triglicerides.

The purpose of the current investigation was to determine 1) whether the localization of the G proteins in primary-cultured adipose cells was consistent with their role as plasma membrane signal transducers and 2) whether the localization of the G-proteins changed as a result of preadipocyte maturation into lipid-containing cells.

MATERIALS AND METHODS

Materials

[α-^{32}P]-ATP, [^{3}H]-cAMP, and Formula-989 scintillation fluid were obtained from NEN-Dupont Canada (Mississauga, ON). Caffeine, ATP (cat. #A-2383, prepared by the phosphorylation of adenosine), cAMP, type IV collagenase, creatine phosphate (Tris salt), creatine phosphokinase, myokinase, (−)-isoproterenol, dextran sulphate, trypsin inhibitor, leupeptin, dithiothreitol (DTT), and demecolcine were from Sigma Chemical Co. (St. Louis, MO). GTP was from PL Biochemicals (Milwaukee, WI). Cell culture reagents were from GIBCO/BRL, Life Technologies Inc. (Burlington, ON). G-protein antibodies were from NEN-Dupont Canada. They were specific for $G_i\alpha1/G_i\alpha2$ (NEI-801), $G_i\alpha3/G_o\alpha$ (NEI-803), $G_s\alpha$ (NEI-805), and Gβ (NEI-807), as demonstrated previously [McFarlane-Anderson et al., 1993; Cadrin et al., 1994] using antibodies from the same batch. Rhodamine-conjugated phalloidin was from Molecular Probes Inc. (Eugene, OR). FITC-goat antirabbit antibody was from Bio/Can Scientific (Toronto, ON, Canada). Mesh (25 and 250 µm) nitex filters were from B and SH Thompson (Scarborough, ON, Canada).

Animals

Male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). They were kept in a temperature-controlled room (23 ± 1°C) with 12 h light cycles. The animals were fed chow and water ad libitum; they were used in the experiments at the age of 5–7 weeks.

Preparation of Preadipocytes and Cell Culture

Mice were killed by cervical dislocation, and epididymal fat pads were excised aseptically. Preadipocytes were isolated as described by Shillabeer et al. [1990]. Briefly, the fat tissue was minced and digested with 1 mg/ml collagenase (type IV) for 1 h at 37°C. The cells were filtered once through a 250 µm mesh nitex filter

and once through a 25 μm mesh filter, and a sample was counted using a Coulter counter (Coulter Electronics, Burlington, ON, Canada). The remaining cells were suspended in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin (S-α-MEM). Cells were seeded on either 24-well plates at a density of ~15,000 cells per well or on glass coverslips and grown in S-α-MEM until confluence. At confluence, some of the cultures were exposed to differentiation medium consisting of S-α-MEM containing 0.1 μM corticosterone, 0.01 μM insulin, and 0.5 mM 1-methyl-3-isobutylxanthine (MIX); after 48 h, the medium was replaced with fresh medium containing corticosterone and insulin but no MIX (HS-α-MEM); under these conditions preadipocytes differentiated and accumulated significant amounts of lipid [cf. Black and Bégin-Heick, 1995]. The remaining cultures were maintained in S-α-MEM throughout the experiments and served as undifferentiated controls.

Immunofluorescence Staining

Cells growing on coverslips were fixed for 10 min in −20°C ethanol and processed for immunofluorescence as previously described [McFarlane-Anderson et al., 1993]. For G-protein labeling, coverslips were incubated for 45 min with the appropriate antibody, diluted 1/50 in PBS containing 0.5% skim milk, rinsed in phosphate buffered saline (PBS) and incubated with FITC-conjugated goat-antirabbit IgG (1/25). For double-labeling for the visualization of F-actin (filamentous actin) and G-proteins, fixed cells were first incubated with TRITC-phalloidin (1/20) for 20 min and then incubated with the appropriate primary and secondary antibodies. After staining, coverslips were mounted (0.1% p-phenylenediamine in 50% glycerol/PBS) on glass slides and viewed by conventional epifluorescence microscopy on a Zeiss Axiophot Photomicroscope (Zeiss, Thornwood, NY). For controls the first antibody was omitted, and no staining was observed.

Adenylyl Cyclase Assays

Adenylyl cyclase was measured directly in culture dishes according to the method of Themmen et al. [1993] except that the assay medium was that described previously [Bégin-Heick, 1990]. GTP and adrenergic and other agents were added as specified in Table I. The medium also contained 50 μg/ml saponin and 5 mM p-nitrophenylphosphate. The other conditions were identical.

TABLE I. Adenylyl Cyclase Activity in Cultured Adipocytes and Preadipocytes*

Conditions	Control (cAMP [fmol/h/10^5 cells])	Differentiated (cAMP [fmol/h/10^5 cells])
Basal	90 ± 33	160 ± 11
GTP, 100 nM	90 ± 11	145 ± 6
GTP, 100 nM + ISO, 100 nM	245 ± 33	358 ± 115
GTP, 100 nM + CL, 100 nM	120 ± 22	300 ± 55
Forskolin, 100 μM + Mn^{2+}, 5 mM	1,750 ± 290	1,910 ± 70

*Cells were grown for 5 days to confluence in S-α-MEM and then exposed to either S-α-MEM medium (Control) or to differentiation medium (Differentiated), as described in Materials and Methods. Enzyme activity was measured on day 5 postconfluence. Cyclase assays were done directly in the culture dishes, as described in Materials and Methods. The results are means ± SE of at least three replicate measurements with different cultures. CL, the selective β_3-adrenergic agonist, CL 316,243; ISO, the nonselective β-adrenergic agonist, isoproterenol.

RESULTS

Differentiation and Maturation of Preadipocytes

As seen by phase-contrast microscopy, preadipocytes growing on glass coverslips form a compact cell monolayer. When induced to differentiate as described in Materials and Methods, some cells lose their fibroblastic appearance, round up, and fill with lipids (Fig. 1A,B). Whereas in adipocytes in situ the triglycerides form a monolocular lipid droplet, in cultured cells the lipid remains multilocular, although, as the cells mature, the lipid droplets become larger. As a result of differentiation, lipid synthesis enzymes such as glycerophosphate dehydrogenase (GPDH) are induced. In undifferentiated confluent cells such as those shown in Figure 1A, the GPDH level was of the order of 5 munits/mg protein. By contrast, the GPDH level increased thirtyfold, to 150 munits/mg protein, in cells exposed to differentiation medium and maintained for 5 days postconfluence, such as those shown in Figure 1B. Under the conditions used, approximately 15% of the cells acumulate lipids [Black and Bégin-Heick, 1995].

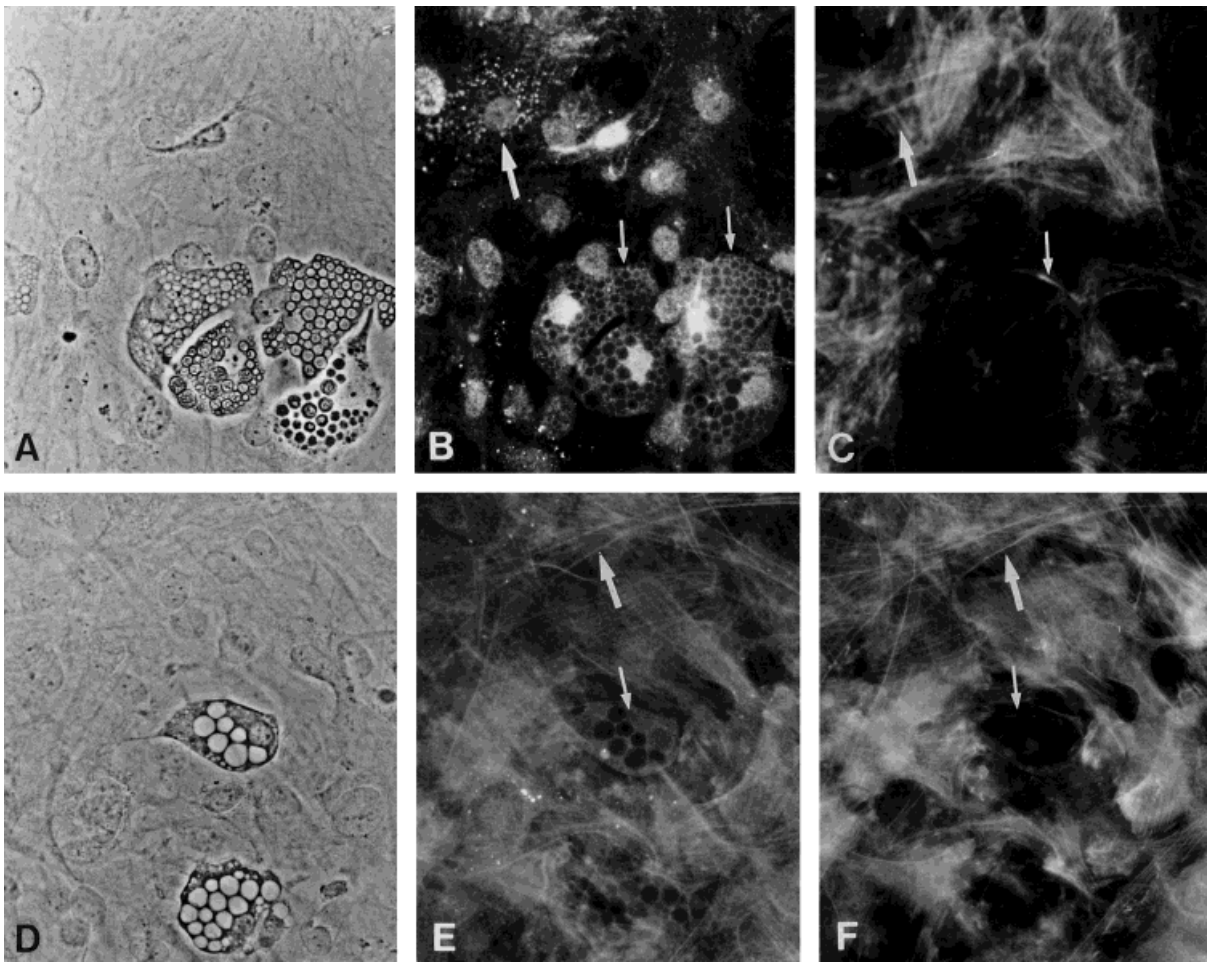

Fig. 2. Localization of G_sa (**B**), $G_i\alpha1/G_i\alpha2$ (**E**), and actin (**C,F**) in preadipocytes and differentiated lipid-containing adipocytes. By immunofluorescence, $G_s\alpha$ (B) shows a nuclear as well as a punctate cytoplasmic staining in undifferentiated preadipocytes (large arrow). In differentiated adipocytes (small arrows), the nuclei are more heavily stained than in undifferentiated cells, and some staining is present at the periphery of each lipid droplet. Actin (C) is in the form of stress fibers in undifferentiated preadipocytes (large arrow) and localized at the membrane in differentiated cells (small arrow). $G_i\alpha1/G_i\alpha2$ (E) has a filamentous appearance in undifferentiated preadipocytes (large arrow) and colocalizes with actin stress fiber (large arrow in F). In differentiated cells (small arrow in E), $G_i\alpha1/G_i\alpha2$ relocalizes in the cytoplasm. Some of the membrane staining colocalizes with actin (small arrow in E and F). Phase-contrast images for each field are shown in **A** and **D.**

Subcellular Distribution of Marker Proteins and of G-Proteins

Labeling of cells with TRITC-phalloidin showed a reorganization of F-actin coincident with the accumulation of lipid in adipocytes. In undifferentiated cells, F-actin formed stress fibers (Figs. 2C,F, 3C,F). As cells differentiated and rounded up, stress fibers disappeared and F-actin reorganized around the cell periphery (Figs. 2C,F, 3C,F).

The subcellular pattern of distribution was different for each of the G-protein subunits. Moreover, a change in their localization was observed following differentiation and accumulation of triglycerides in cells. In undifferentiated cells $G_s\alpha$ gave a pattern that was mostly nuclear. Some punctate cytoplasmic staining was also observed, indicating that $G_s\alpha$ is associated with vesicular structures in these cells (Fig. 2B). In cells that were fully mature, the nucleus tended to be more heavily stained than was observed in undifferentiated cells (Fig. 2B). Furthermore, some staining colocalized with actin at the cell membrane, and $G_s\alpha$ was also found around each each lipid droplet (Fig. 2B).

Double-staining with the anti-$G_i\alpha1/G_i\alpha2$ and rhodamine phalloidin for the detection of F-actin showed that in undifferentiated cells there was partial colocalization of these G-proteins with actin (Fig. 2E,F). In differentiated cells, where

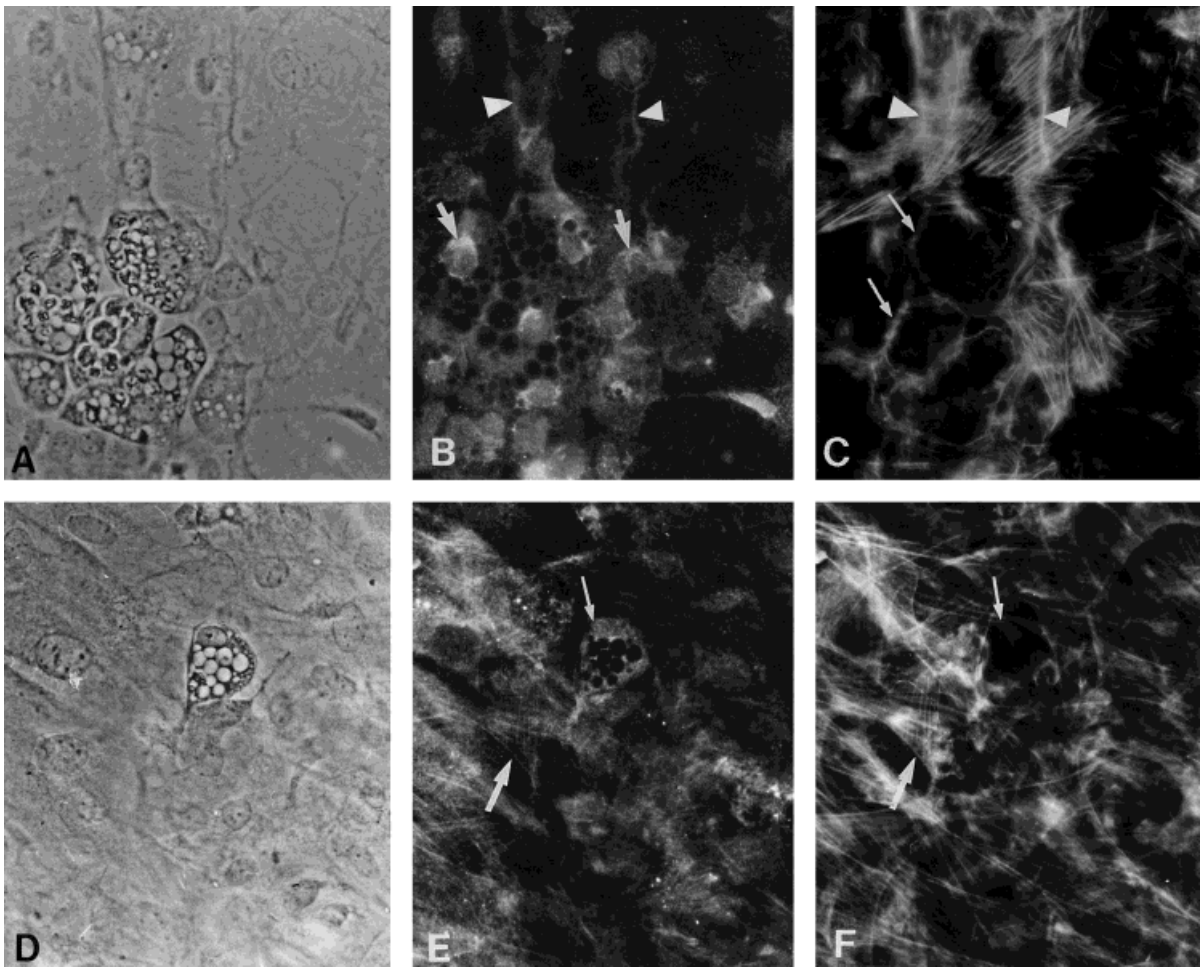

Fig. 3. Localization of $G_i\alpha3$ (**B**), Gβ (**E**), and actin (**C,F**) in preadipocytes and differentiated lipid-containing adipocytes. By immunofluorescence, $G_i\alpha3/G_o\alpha$ (B) in nondifferentiated cells gives a diffuse cytoplasmic staining and some nuclear staining. Staining that colocalizes with actin is also observed at the plasma membrane of some cells (arrowheads in B and C). In differentiated cells, in addition to the cytoplasmic and nuclear staining, a perinuclear staining is observed (B, arrows). Arrows in C show the peripheral localization of actin in differentiated cells. Gβ (E) shows a filamentous staining that colocalizes with actin stress fiber (F) (large arrows in E and F). In differentiated cells (small arrow), Gβ localizes in the cytoplasm (small arrows in E and F); also, some plasma membrane staining that colocalizes with actin is observed.

F-actin is localized mostly at the cell periphery, the fibrous pattern of the $G_i\alpha1/G_i\alpha2$ disappeared, and some labeling colocalized with F-actin (Fig. 2E,F). Figure 2E also shows cytoplasmic staining located around lipid droplets for $G_i\alpha1/G_i\alpha2$, as was the case for $G_s\alpha$. No nuclear staining was observed.

In nondifferentiated confluent cells, the $G_i\alpha3$ antibody (Fig. 3B) gave diffuse cytoplasmic and some nuclear staining. Little if any staining of the plasma membrane was noted, and there appeared to be no colocalization with actin (compare Fig. 3B and 3C). In differentiated cells (Fig. 3B), a perinuclear marking appeared, in addition to cytoplasmic and nuclear staining. We have previously shown that this intense perinuclear pattern corresponds to the Golgi system [Cadrin et al., 1994]. Since the staining pattern is similar to that observed in the clonal line OB17, which does not express significant amounts of $G_o\alpha$, the pattern observed in differentiated primary-cultured adipocytes is probably also due to $G_i\alpha3$.

In nondifferentiated confluent cells, Gβ (Fig. 3E) followed a pattern similar to that described for $G_i\alpha1/G_i\alpha2$ (see Fig. 2E): Gβ colocalized with the actin stress fibers, and there was none of the nuclear or cytoplasmic staining observed with $G_s\alpha$ (compare Fig. 3E with Fig. 2B). In differentiated cells, the Gβ staining (Fig. 3E) followed closely the distribution of $G_i\alpha1/G_i\alpha2$; that is, staining was found at the plasma mem-

brane and in the cytoplasm around the lipid droplets (compare Fig. 3E with Fig. 2E).

Adenylyl Cyclase Activity

The adenylyl cyclase activity of control and differentiated cultures was measured in situ. The results show (Table I) that β-adrenergic agonists, in conjunction with GTP, can activate cyclase in both control and differentiated cells. The stimulation by agonists (approximately 1.5-fold) is considerably less than maximal activity produced by a combination of forskolin and Mn^{2+} (100-fold) and also considerably less than that achieved in membranes from freshly isolated adipocytes (tenfold [cf. Bégin-Heick, 1995]) with assay media of similar composition. This suggests that, while G_s was not detected in the membrane of cultured cells, there may be a small amount present. There was no significant difference between control and differentiated cultures in the basal or stimulated cyclase activity. However, only 15% of cells have accumulated lipids at the time of the experiment [cf. Black and Bégin-Heick, 1995].

DISCUSSION

In mouse adipocyte membranes, the major G-proteins identified by Western blotting, ADP ribosylation with bacterial toxins, and Northern blotting are the long and short forms of $G_s\alpha$ and $G_i\alpha 1$, -2, and -3 [Bégin-Heick, 1990, 1992; Gettys et al., 1991, 1995]. By contrast, in the clonal adipocyte lines HGFu, Ob17 [McFarlane-Anderson et al., 1993] and 3T3-F442A cells [Kilgeour and Anderson, 1993], $G_i\alpha 1$ was not detected.

One of the notable findings of this study is that little if any of each of the G-protein subunits is found in association with the plasma membrane in undifferentiated adipocytes and that a remarkably small proportion is localized to the plasma membrane in differentiated cells. Moreover, in differentiated primary-cultured adipocytes, all the G-proteins that we examined appeared to localize at the periphery of the lipid droplets; this is reminiscent of the distribution of vimentin in lipid-containing tissue. Whether this distribution represents an intimate association of the G-protein with the droplet, as has been reported for perilipin [Greenberg et al., 1991; Blanchette-Mackie et al., 1995], or merely a relocalization of G-proteins to the cytoplasm without an intimate association with the lipid droplet is not known at present. However, since G-proteins have a cytoplasmic localization, it is most likely to be due to the compression of the cytoplasm by the lipid droplets.

Since hormone-sensitive lipase, the enzyme responsible for the mobilization of triglycerides, is translocated to the lipid storage droplet as a result of hormone-stimulated lipolysis [Egan et al., 1992], a process requiring G-proteins transduction, it is possible that the cytoplasmic localization of G-proteins in the adipocyte represents a physiological role. Alternately, it is possible that the culture process, perhaps because it does not duplicate the normal cell:cell interactions found in intact tissue, somehow alters the subcellular distribution and expression of the G-proteins. We have shown previously [Cadrin et al., 1996] that the localization of G-proteins in cultured hepatocytes and liver sections differs substantially, although the type of G-protein subunits present is similar in both preparations. For example, the localization of $G_i\alpha 3$ is predominantly at the Golgi apparatus in cultured hepatocytes, while in liver sections it was found at the plasma membrane as well as in the Golgi. This is similar to the findings of Wilson et al. [1994] with pituitary gland sections and GH3 cells. In clonal [Cadrin et al., 1994] and both undifferentiated and differentiated primary-cultured adipocytes (results presented in Results section and in Fig. 3B), $G_i\alpha 3$ was also associated primarily with the Golgi apparatus, which is in keeping with the notion of adipocytes as secretory cells [Ailhaud et al., 1992], which has been further confirmed by the discovery that leptin, the product of the *ob* gene, is produced by the adipocyte and secreted into the blood [Zhang et al., 1994].

It is notable that most reports of G-protein localization with intracellular structures come from studies on clonal or primary cell cultures [Brabet et al., 1988; Gabrion et al., 1989; Wang et al., 1989; Ercolani et al., 1990; Lewis et al., 1991; Stow and de Almeida, 1993; McFarlane-Anderson et al., 1993; Cadrin et al., 1993; Saffitz et al., 1994; Wilson et al., 1994]. However, few studies have examined the in situ distribution of G-proteins in tissue sections and primary cultures of cells derived from the same tissue. Our findings [Cadrin et al., 1996] suggest that the process of cell culture itself or the cellular dynamics imposed by the culture substratum may influence the subcellular distribution of G-proteins. Alternately, the post-

translational modifications, which have been demonstrated to be essential to the membrane insertion of some G-proteins [Muntz et al., 1992], may be only partial or absent in cultured cells.

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