Journal of Cellular Biochemistry 52:463–475 (1993)

Identification and Localization of G-Proteins in the Clonal Adipocyte Cell Lines HGFu and Ob17

Norma McFarlane-Anderson, Monique Cadrin, and Nicole Bégin-Heick

Departments of Biochemistry (N.M.-A., N.B.-H.) and Pathology (M.C.), University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

Abstract HGFu and Ob17 are cell lines derived from adipose tissue of lean (+/?) and ob/ob mice, respectively Neither adenylyl cyclase activity nor G protein abundance and subcellular distribution have been assessed previously in these cells Cyclase activity was low and resistant to catecholamine stimulation in both cell lines However, the enzyme could be stimulated to high levels by forskolin and Mn^{2+} $G_s\alpha$ (largely the long isoform), $G_i\alpha 2$, and Gβ were the major G protein subunits identified. The levels of G protein mRNA expression were similar in both cell lines and, unlike actin expression, did not change as a result of differentiation Immunoblotting and ADP-ribosylation of the G peptides corroborated these results Assessment of the subcellular localization of the subunits by indirect epifluorescence and scanning confocal microscopy showed that each of the subunits had a characteristic subcellular pattern $G_s\alpha$ showed vesicular cytoplasmic and nuclear staining, $G_i\alpha 2$ colocalized with actin stress fibers and disruption of these structures altered the distribution of $G_i\alpha 2$; β subunits showed some colocalization with the stress fibers as well as a cytoplasmic vesicular and nuclear pattern. As a result of differentiation, there was reorganization of the actin, together with the $G_i\alpha 2$ and β fibrous patterns. Both cell lines showed similar modifications. The induction of differentiation in these cells is therefore not associated with changes in adenylyl cyclase activity nor of the abundance of G-protein subunits, although reorganization of some of these subunits does accompany actin reorganization

Key words: preadipocytes, differentiation, $G_s\alpha$, $G_i\alpha$, G_β, actin, stress fibers, subcellular localization, confocal microscopy, antibodies

Effector systems such as adenylyl cyclase, phospholipase C, and several ion channels are regulated by G-proteins [Birnbaumer et al., 1990]. These proteins are considered to be associated with the plasma membrane allowing them to interact with both membrane receptors and effectors [Gilman, 1987] and their presence in tissues is normally assessed in membrane preparations.

The nature of the association of G-protein subunits with the membrane is not well understood but it is now evident that some of these subunits need to be modified [Buss et al., 1987; Lai et al., 1990; Maltese, 1990; Maltese and Robishaw, 1990; Muntz et al., 1992] prior to membrane interaction. Recent evidence also indicates that there is translocation of the peptides upon activation [Iyengar et al., 1988; Rotrosen et al., 1988; Crouch et al., 1989; Premont and Iyengar, 1989; Haraguchi and Rodbell, 1990] and during cell division [Crouch, 1991].

Recent immunocytochemical studies have suggested that some of the peptides are associated with other cellular structures as well as with plasma membranes. For example, β subunits have been found in the cytoplasm [Wang et al., 1989; Muntz et al., 1992], $G_i\alpha$ subunits in cytoplasm and lysosomes [Lewis et al., 1991], $G_o\alpha$ in the cytoplasm [Brabet et al., 1988], $G_i\alpha$ in the Golgi apparatus [Ercolani et al., 1990; Wilson et al., 1992], and $G_i\alpha 2$ with basolateral membranes [Holtzman et al., 1991].

Previous studies have demonstrated that in membranes derived from mature epididymal adipocytes of obese mice, there is anomalous adenylyl cyclase activity [Bégin-Heick, 1985] and lower abundance of G-protein [Bégin-Heick, 1990; Gettys et al., 1991] compared to adipocytes derived from lean (+/+) mice. So far, no studies of adenylyl cyclase regulation or G-pro-

Abbreviations F-actin, filamentous actin, G-actin, globular actin, G-protein, guanine nucleotide-binding regulatory protein, HGFu/Ob17 cells, preadipocyte cells derived from epididymal fat pads of the C57Bl/7 (+/?) and (ob/ob) mice respectively, T3, 3,3 ,5-triiodo-L-thyronine, WGA, wheat germ agglutinin

Received January 19, 1993, accepted March 25, 1993

tein abundance and distribution have been carried out in the clonal cell lines, HGFu and Ob17, derived from adipose tissue of lean (+/?) and obese (ob/ob) mice, respectively [Négrel et al., 1978; Forest et al., 1983]. However, changes in expression and cellular distribution of G-proteins in differentiating 3T3 cells have been described [Lai et al., 1981; Watkins et al., 1989] and more recently Wang et al. [1992] have suggested a role for G-proteins in differentiation.

The purpose of the present study was twofold: first, to assess adenylyl cyclase activity and G-protein levels in HGFu and Ob17 cells and find out whether there are variations during differentiation. Second, to examine the intracellular localization of G-proteins in these cells and to determine whether differentiation is associated with changes in their distribution.

MATERIALS AND METHODS

Materials

[α-^{32}P]-ATP, [^{3}H]-cAMP, [^{3}H]-AMP, and [^{32}P]-NAD were obtained from New England Nuclear Corporation (Lachine, Quebec, Canada). [γ-^{32}P]-ATP, [α-^{32}P]-dCTP, [^{125}-I]-goat anti-rabbit IgG, Hybond nylon membrane, and the multiprime labeling kit were from Amersham Canada, Ltd. (Oakville, Ontario, Canada). Caffeine, ATP (cat. number A-2383, prepared by the phosphorylation of adenosine), cAMP, creatine phosphate (Tris salt), creatine phosphokinase, myokinase, (−)-isoproterenol, salmon sperm DNA, dextran sulphate, cytochalasin D, and demecolcine were from the Sigma Chemical Co. (St. Louis, MO). GTP was from PL Biochemicals, (Milwaukee, WI). Cholera and pertussis toxins were from List Biochemicals (Campbell, CA). Universol™ was from ICN Biomedicals Corp. (Montreal, Quebec, Canada). Cell culture reagents were from GIBCO/BRL, Life Technologies, Inc. (Burlington, Ontario, Canada). The G-protein antibodies specific for $G_i\alpha1/G_i\alpha2$ (NEI-801), $G_s\alpha$ (NEI-805), and Gβ (NEI-807) were from NEN, Dupont Canada (Mississauga, Ontario, Canada). TRITC conjugated phalloidin was from Molecular Probes, Inc. (Eugene, OR). Con A-FITC or -TRITC and WGA-FITC or -TRITC were from Dimension Lab., Inc. (Mississauga, Ontario, Canada) and FITC-goat anti-rabbit were from Bio/Can scientific, (Toronto, Ontario, Canada).

Cell Lines

Ob17 and HGFu cell lines were generously provided by Dr. U. Kozak, Jackson Laboratories, Bar Harbor, ME. The cells were grown in Dubelco-modified Eagle's medium (DME) containing 10% fetal calf serum supplemented with penicillin/streptomycin (200 U/100 μg/ml, respectively) and fungizone (0.25 μg/ml). Cells were induced to differentiate by adding insulin (17 nM) and T_3 (2 nM) to the cultures at confluence. The experiments were conducted with cultures at confluence (undifferentiated) or 6 days after the addition of insulin/T_3 (differentiated). Some experiments were also conducted in cells exposed to isobutylmethylxanthine + dexamethasone for 48 h, following the induction of differentiation with insulin/T_3. The results obtained with such cells were identical to those shown for insulin/T_3 alone.

For RNA and membrane preparations, cells were grown in 100 mm dishes. For the immunocytochemical studies, they were grown on glass cover slips in 35 mm dishes. In some experiments, cells were grown in the presence of 100 ng/ml pertussis toxin for 1–24 h. Cells treated with cytochalasin B or colchicine (2 μg/ml) were exposed to these compounds for 4 h.

Membrane Preparation

The cells were scraped from the plates, washed in 10 mM potassium phosphate buffer pH 7.4 containing 1 mM EDTA and 1 mM DTT, and homogenized by hand. The homogenate was centrifuged for 15 min at 15,000*g*. The pellet was washed and rehomogenized three times and the final pellet resuspended in 10 mM potassium phosphate pH 7.4 containing 1 mM EDTA, 1 mM DTT, 40 μM leupeptin, and 1 μg/ml trypsin inhibitor and aliquots frozen at −70°C.

Preparation of Nuclei

This was done essentially by the method described by Leach et al. [1989]; 25 μg of nuclear protein was subjected to electrophoresis and immunodetection as described below.

Protein Determination

The protein content of the membrane fractions was determined by the Coomassie Blue method, using bovine serum albumin as the standard [Bradford, 1976].

Adenylyl Cyclase Assay

This was carried out essentially as described by Bégin-Heick [1985].

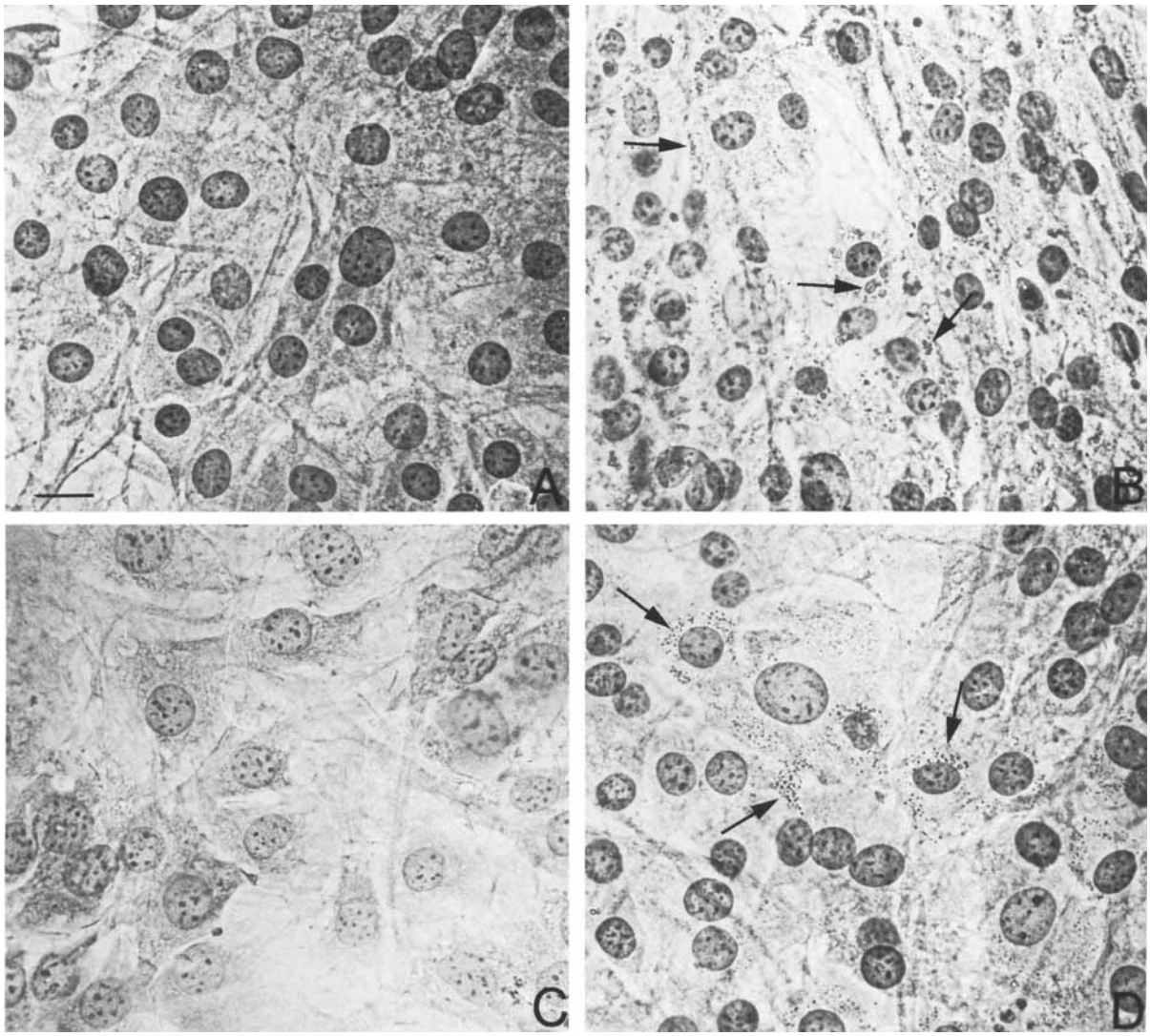


Fig. 1. Lipid accumulation in differentiating HGFu and Ob17 cells HGFu and Ob17 cells were grown to confluence (**A** and **C**) and cultured for 7 days in medium containing 17 nM insulin and 2 nM T_3 (**B** and **D**). The cultures were stained with Oil Red O as described in the Methods section Arrows indicate lipid droplets

ADP-Ribosylation of Membranes With Cholera and Pertussis Toxins

This was done essentially according to the method of Ribeiro-Neto et al. [1987] as described in detail recently [Bégin-Heick, 1990].

SDS-PAGE and Immunoblotting

Proteins were separated in 10% (w/v) acrylamide gels. To resolve the pertussis toxin substrates, urea-containing gels were employed [Bégin-Heick, 1992]. Immunoblotting was carried out as previously described [McFarlane-Anderson et al., 1992]. The antibodies used for immunoblotting and immunocytochemistry (see Immunofluorescene Staining, page 466) were well-characterized commercial antibodies that had been shown to be specific for the G-proteins tested [Goldsmith et al., 1988; Simonds et al., 1989].

RNA Preparation and cDNA Hybridization

Total RNA prepared by the guanidinium-thiocyanate method [Chomczynski and Saachi, 1987] was used to prepare Northern blots. The mouse Gβ [McFarlane-Anderson et al., 1992]; $G_i\alpha 1$, $G_i\alpha 3$, and $G_o\alpha$ a gift from Dr. R. Reed [Jones and Reed, 1987] and $G_s\alpha$ and $G_i\alpha 2$ cDNAs [Sullivan et al., 1986] a gift from Dr. S. Masters (University of California, San Francisco, CA) were used in multiprime DNA labeling reactions

TABLE I. Adenylyl Cyclase Activity in HGFu and Ob17 Clonal Cells*

	HGFu		Ob17	
	Confl	Diff	Confl	Diff
Basal (pmol/mg) fold stimulation by:	2.3 ± 0.26	2.4 ± 0.60	2.2 ± 0.26	2.1 ± 0.42
0.1 mM GTP	1.3 ± 0.1	1.6 ± 0.3	1.8 ± 0.4	1.4 ± 0.3
0.1 mM Gpp(NH)P	6.1 ± 0.7	9.9 ± 1.2	6.9 ± 1.0	10.2 ± 0.7
0.01 mM GTPγS	12.2 ± 0.2	14.0 ± 1.2	13.9 ± 2.0	15.2 ± 3.2
0.001 mM INA + GTP	1.2 ± 0.2	1.5 ± 0.1	1.5 ± 0.1	1.6 ± 0.4
0.1 mM INA + GTP	1.7 ± 0.4	2.2 ± 0.4	2.2 ± 0.2	2.3 ± 0.5
10 mM NaF	7.6 ± 0.9	5.3 ± 1.0	7.3 ± 3.0	4.5 ± 0.8
NaF + Al^3	66.4 ± 4.6	35.3 ± 2.0	41.8 ± 12.0	35.5 ± 8.9
5 mM Mn^{2+}	6.5 ± 0.8	3.6 ± 0.1	6.0 ± 0.5	4.1 ± 0.7
0.1 mM Forskolin	42.6 ± 3.0	29.3 ± 2.8	30.2 ± 11.5	18.9 ± 3.5
Forskolin + Mn^{2+}	293 ± 25.5	203 ± 35.3	192 ± 21.9	157 ± 33.8

*Adenylyl cyclase activity was measured in membrane preparations of confluent and differentiated cells, as described in the method section. Basal values are expressed as pmol cAMP formed/mg protein. The other values are expressed as fold stimulation above basal values. Confl and Diff refer to confluent (undifferentiated) and differentiated cells, respectively. Data are means ± SE for 3–7 observations with different membrane preparations.

as previously described [McFarlane-Anderson et al., 1992].

Oil Red O Staining

Cells were fixed in buffered formalin and stained with 0.25% Oil Red O in 50% isopropanol.

Immunofluorescence Staining

Cells were fixed in ethanol for 10 min at −20°C as previously described [Kawahara et al., 1990] or in 4% paraformaldehyde followed by permeabilization in 0.2% Triton X-100. Similar results were obtained with both methods, but cold ethanol fixation gave clearer images [cf. Akner et al., 1990] and therefore was used routinely. For G-protein labeling, coverslips were incubated for 45 min with the appropriate antibody, diluted 1/50 in PBS containing 0.5% skim milk. After rinsing in PBS, coverslips were incubated with goat anti–rabbit IgG conjugated to FITC (1/25). For visualization of F-actin and in double labeling experiments, fixed cells were first incubated with TRITC-phalloidin (1/20) for 20 min at room temperature and then incubated with the appropriate primary and secondary antibodies. Similar treatment of fixed cells with Con A-TRITC or WGA-TRITC was used to visualize the ER and the Golgi system, respectively [Virtanen et al., 1980; Tartakoff and Vassali, 1983; Lipsky and Pagano, 1985; Rabinowitz et al., 1992; Wong et al., 1992]. After staining, cover slips were mounted (0.1% p-phenylenediamine in 50% glycerol/PBS) on glass slides and viewed by conventional epifluorescence microscopy on a Zeiss Axiophot Photomicroscope or by scanning with a Leica Confocal Laser Scanning Microscope. For controls, the first antibody was omitted from the labeling reaction. In such cases no staining was observed.

RESULTS

Differentiation

To determine if addition of insulin/T3 induced the conversion from fibroblasts to adipocytes, the morphology of the cells and their ability to accumulate lipids were monitored. Figure 1 shows that the addition of insulin/T_3 induced lipid accumulation and morphological changes in both HGFu and Ob17 cells. In HGFu cells, the hormonal treatment induced accumulation of small lipid droplets and the cells had a thicker and more rounded appearance than undifferentiated cells (compare A and B). Differentiating Ob17 cells also appeared to be thicker and more rounded with the lipid accumulation confined to groups of cells (compare C and D). It is therefore clear that the hormones are capable of inducing differentiation and lipid accumulation in both HGFu and Ob17 cells.

Adenylyl Cyclase Activity

Both clonal lines have very low basal adenylyl cyclase activity. The enzyme could be activated by forskolin and Mn^{2+} independently and to high levels by adding both together. Similarly, NaF alone or with Al^{+3} stimulated the enzyme. GTP alone or together with isoproterenol produced low levels of activation whereas Gpp(NH)p and GTPγS activated several fold. Therefore, in these cells, while adenylyl cyclase is

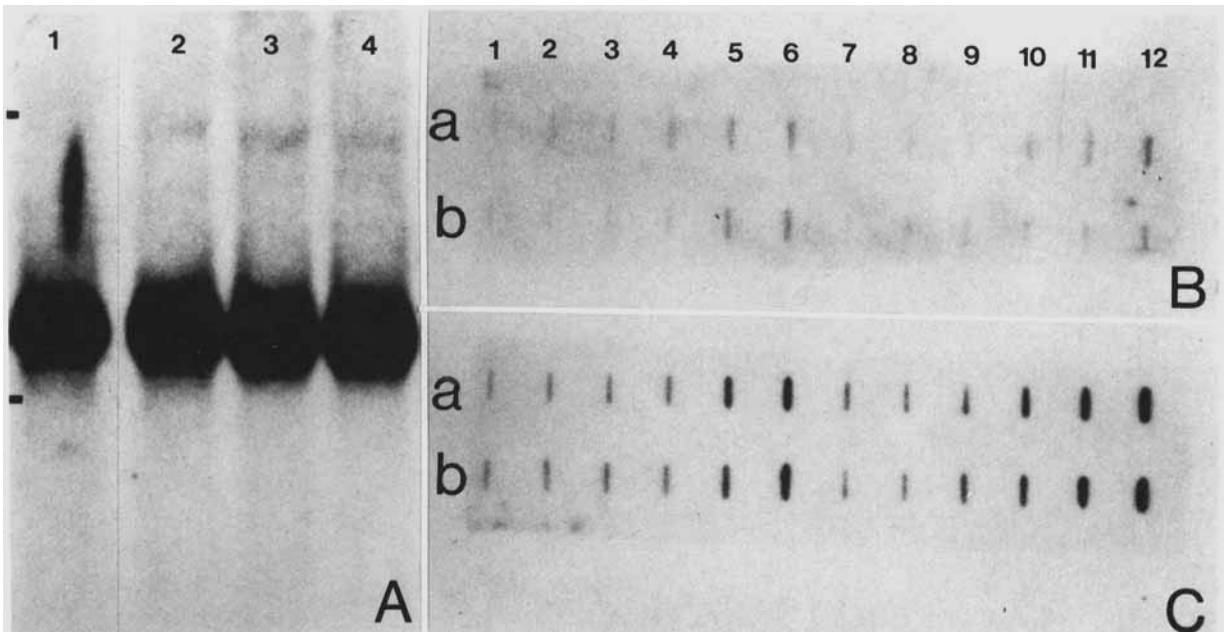


Fig. 2. Expression of G-protein subunits in HGFu and Ob17 cells. **(A)** Northern blot of 10 μg of total RNA was probed with $G_s\alpha$ cDNA. Lanes 1 and 3 represent HGFu cells and lane 2 and 4 Ob17 cells. 1 and 2, confluent cells, 3 and 4, differentiated cells Bars represent RNA markers of 4 7 kb and 1 8 kb, respectively. **(B)** and **(C)** Slot blots of 0.125, 0.20, 0 25, 0.50, 1.0, and 2.0 μg of RNA probed with $G_i\alpha 2$ and Gβ, respectively. Slots 1–6, HGFu; Slots 7–12, Ob17. (a) confluent cells; (b) differentiated cells.

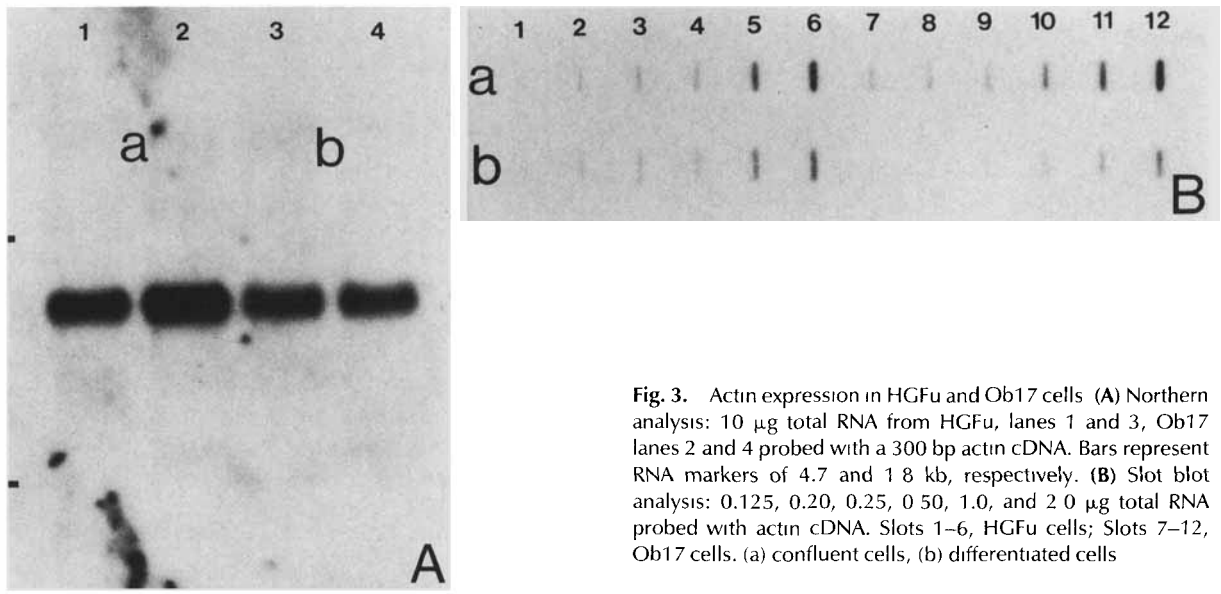
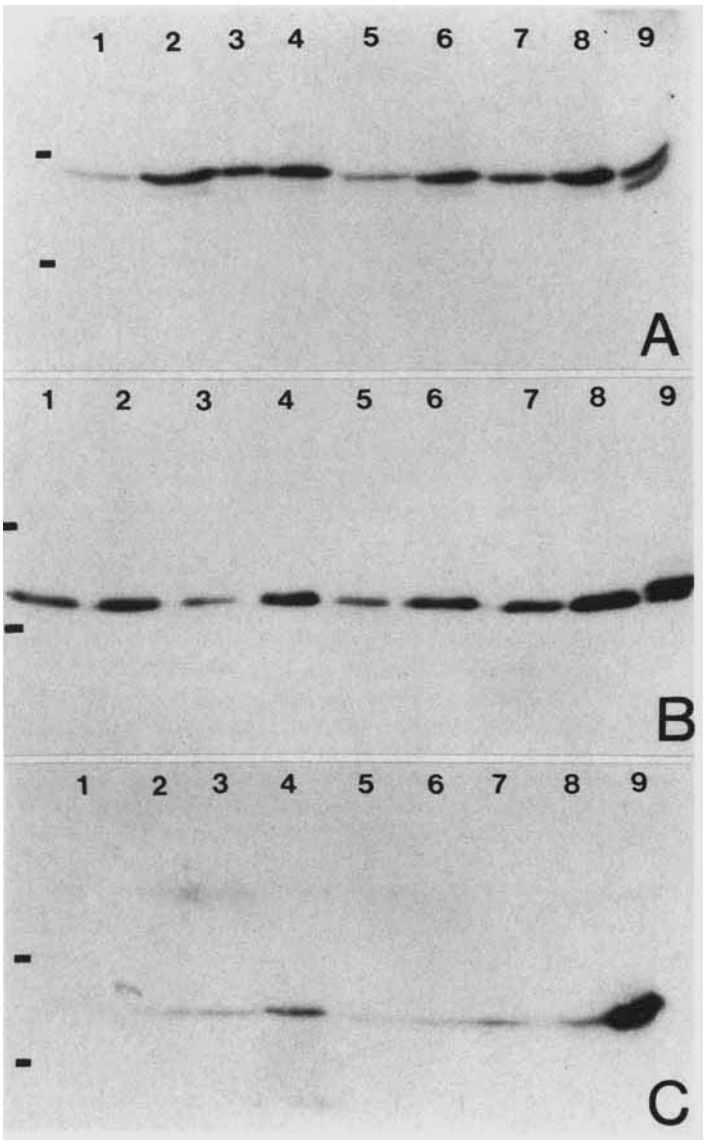


Fig. 3. Actin expression in HGFu and Ob17 cells **(A)** Northern analysis: 10 μg total RNA from HGFu, lanes 1 and 3, Ob17 lanes 2 and 4 probed with a 300 bp actin cDNA. Bars represent RNA markers of 4.7 and 1 8 kb, respectively. **(B)** Slot blot analysis: 0.125, 0.20, 0.25, 0 50, 1.0, and 2 0 μg total RNA probed with actin cDNA. Slots 1–6, HGFu cells; Slots 7–12, Ob17 cells. (a) confluent cells, (b) differentiated cells

present, the coupling between the β-adrenergic receptor and the enzyme is altered. There was no difference between the two cell lines and the activities were not significantly affected by differentiation (Table I).

Expression of G-Protein Subunits

RNA from confluent and differentiating cells was hybridized with probes for $G_s\alpha$, $G_i\alpha 1$, $G_i\alpha 2$, $G_i\alpha 3$, $G_o\alpha$, and Gβ mRNAs. Of the alpha subunits, $G_i\alpha 1$ and $G_o\alpha$ were either not expressed or at levels that were below the detection limits of the assay. Figure 2 shows that the expression of the $G_s\alpha$ (2A), $G_i\alpha 2$ (2B), and Gβ (2C) subunits was the same in both cell lines (compare slots 1–6 to slots 7–12) in each of the blots. Differentiation from a fibroblast-like preadipocyte cell type into a lipid containing adipocyte was not associ-



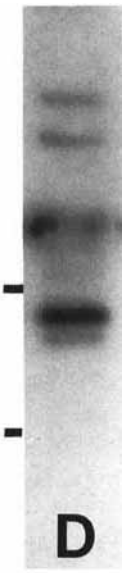


Fig. 4. Immunoblotting of $G_s\alpha$, $G_i\alpha2$, and Gβ subunits in HGFu and Ob17 cells. In each case, lanes 1–4 represent HGFu cells and lanes 5–8, Ob17 cells and lane 9, 25 μg protein from brain membranes. Lanes 1, 2, 5, and 6: confluent cells; lanes 3, 4, 7, and 8: differentiated cells; even number lanes contained twice the amount of membrane proteins as odd number lanes. **(A)** $G_s\alpha$ subunit (NEI-805), 20 & 40 μg protein, exposure time 60 h; **(B)** $G_i\alpha2$ (NEI-801), 20 and 40 μg protein, 20 h; **(C)** Gβ (NEI-807) 15 and 30 μg protein, 68 h. Bars represent molecular mass markers of 46 kDa and 30 kDa, respectively. **(D)** $G_s\alpha$ subunits (NEI-805) in nuclear preparations (25 μg protein) from HGFu cells.

ated with a difference in expression of the G-protein mRNA [compare lanes marked (a) with those marked (b)]. In contrast, Figure 3 shows that actin mRNA levels were markedly reduced in cells induced to differentiate by insulin/T_3 (lanes marked b) compared to confluent cells (lanes marked a). This decrease was observed in both cell lines.

Immunoblotting of $G_s\alpha$, $G_i\alpha2$, and Gβ

The antibody specific for $G_s\alpha$, which has similar affinity for the short and the long form of the subunit, revealed that the 45 kDa isoform predominates in these cells while the 42 kDa isoform is barely detectable (Fig. 4A). The antibody directed against $G_i\alpha1$ and $G_i\alpha2$ revealed that only $G_i\alpha2$ is present in detectable amounts (Fig. 4B). Low levels of Gβ were detected, compared to the level found in brain membranes (Fig. 4C). There was no difference in the abundance of any of the subunits between the two cell lines. The process of differentiation had little if any effect on the amount of these proteins.

ADP-Ribosylation of $G_s\alpha$ and $G_i\alpha$ peptides

The ADP-ribosylation patterns obtained with cholera (A) and pertussis toxins (B) are shown in Figure 5. They confirm the results found by immunoblotting with the $G_s\alpha$ (Fig. 4A) and $G_i\alpha$ (Fig. 4B) antibodies. Again, no difference was observed between the two cell lines or as a result of differentiation.

Subcellular Distribution of $G_s\alpha$, $G_i\alpha2$, and Gβ

The cellular pattern of distribution was different for each of the subunits, but the association

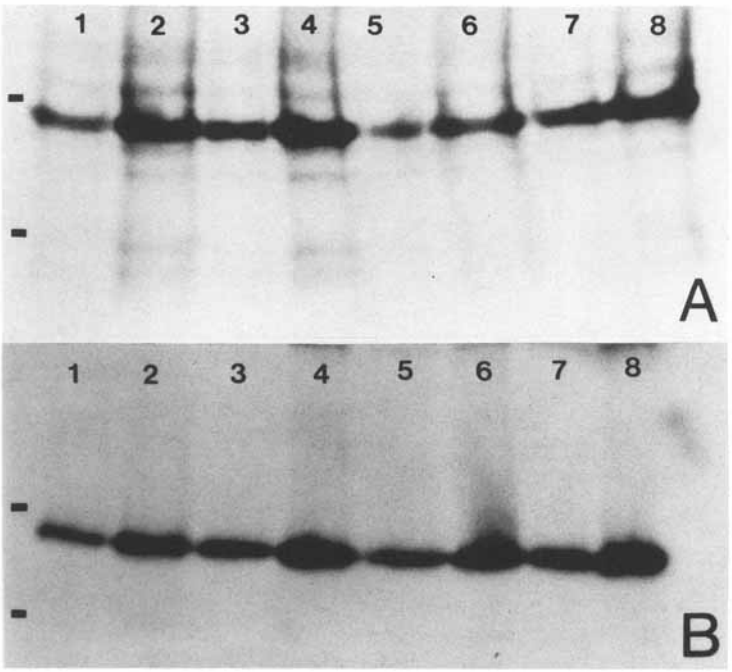


Fig. 5. ADP-ribosylation of $G_s\alpha$ and $G_i\alpha 2$ with cholera and pertussis toxins Membranes were activated and treated with (**A**) cholera toxin and (**B**) pertussis toxin Lanes 1–4, HGFu cells, lanes 5–8, Ob17 cells, lanes 1–2, 5–6, confluent cells, lanes 3–4, 7–8, differentiated cells even number lanes contained twice the amount of membrane proteins as odd number lanes (A) cholera toxin substrates 20 and 40 μg protein, exposure 60 h, (B) pertussis toxin substrates 7 5 and 15 μg protein, exposure 22 h Bars represent molecular mass markers of 46 kDa and 30 kDa, respectively

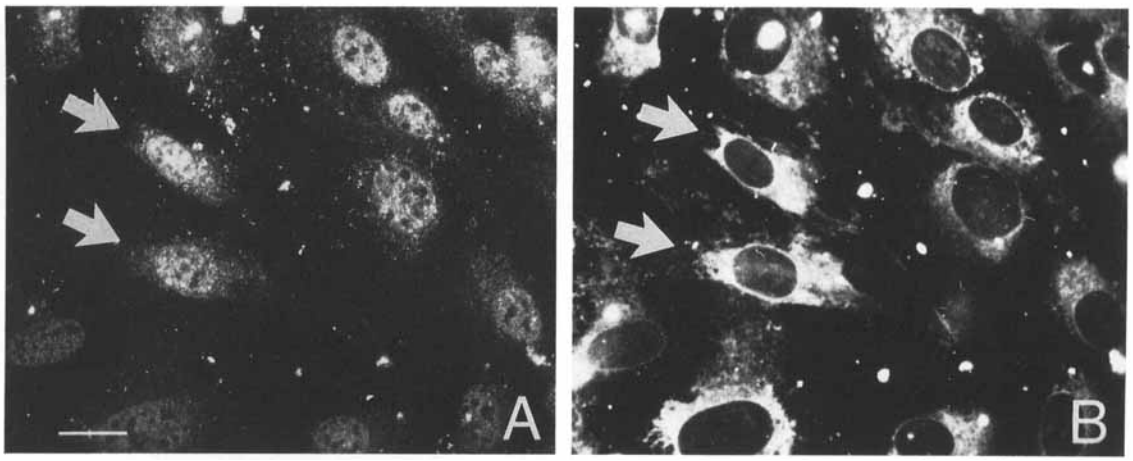


Fig. 6. Distribution pattern of $G_s\alpha$ in confluent HGFu cells Cells were double-labeled with anti-$G_s\alpha$ (**A**) and Con A (**B**) and the immunofluorescence pattern was assessed Arrows indicate the apparent co-localization of $G_s\alpha$ and the ER Bar indicates 20 μm

of a given subunit with particular cellular organelles was the same in both cell types and did not change as a result of differentiation.

The staining pattern obtained with the $G_s\alpha$ antibody was punctate cytoplasmic and nuclear (Fig. 6A). To characterize the cytoplasmic elements recognized by the antibody, the cells were double-stained with the anti-$G_s\alpha$ and Con A, a lectin that binds predominantly to the rough ER, as well as with WGA, which has been shown by Rabinowitz et al. [1990] to bind to components of the Golgi system (trans-cisternae, endosomes, endosomal tubuloreticular compartment, and its associated vesicles). No evidence for co-localization of $G_s\alpha$ with the Golgi system was found (not shown), but there did appear to be

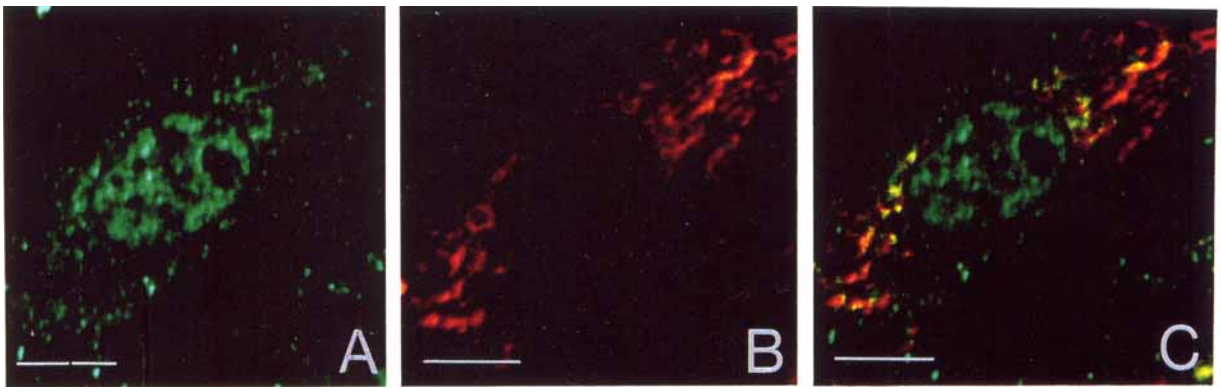


Fig. 7. Confocal microscopic analysis of $G_s\alpha$ and ER in confluent HGFu cells. Cells were double-labeled with anti-$G_s\alpha$ and Con A and confocal imaging of 0.5 μm sections was used to determine colocalization. To determine if there was colocalization, separate anti-$G_s\alpha$ (**A**) and Con A (**B**) images were obtained and superimposed (**C**). Green marks $G_s\alpha$; red, Con A and yellow, co-localization. Note that most of the $G_s\alpha$ is in vesicles close to the ER. Nuclear staining is also evident. Bar indicates 10 μm.

some colocalization with the ER (Fig. 6B). Scanning by confocal microscopy and reconstitution of the image revealed that the $G_s\alpha$ antibody was present in vesicular structures around the nucleus (Fig. 7A). ConA staining is associated with the ER (Fig. 7B). The separation of color obtained when the images were superimposed (Fig. 7C) indicated that the structures recognized by $G_s\alpha$ were not part of the ER. When the microtubule network was disrupted by colchicine treatment, there was a redistribution of $G_s\alpha$ (Fig. 8), suggesting that the structures to which $G_s\alpha$ binds are associated with the microtubule network.

To find out if the nuclear staining was due to peptides associated with the nucleus, immunodetection was carried out on homogenates of purified, washed nuclei. The blots show clearly that $G_s\alpha$ was present in these preparations (Fig. 4D), suggesting a strong association.

The antibody to $G_i\alpha2$ labeled filamentous structures that resemble the actin stress fiber pattern usually observed in cells growing in culture (Fig. 9). Double labeling with the anti-$G_i\alpha2$ and TRITC-phalloidin, which recognizes F-actin, showed that the fibrous pattern of $G_i\alpha2$ and F-actin colocalized (Fig. 9A,B). Treatment of the cells with cytochalasin B, which prevents actin polymerization by binding to G-actin [Särndahl et al., 1989], also resulted in $G_i\alpha2$ reorganization (Fig. 9C,D). These data therefore confirm the colocalization of $G_i\alpha2$ with actin stress fibers.

The antibody against the β-subunit labeled fibrous as well as cytoplasmic and some nuclear structures (Fig. 10). The fibrous structures were

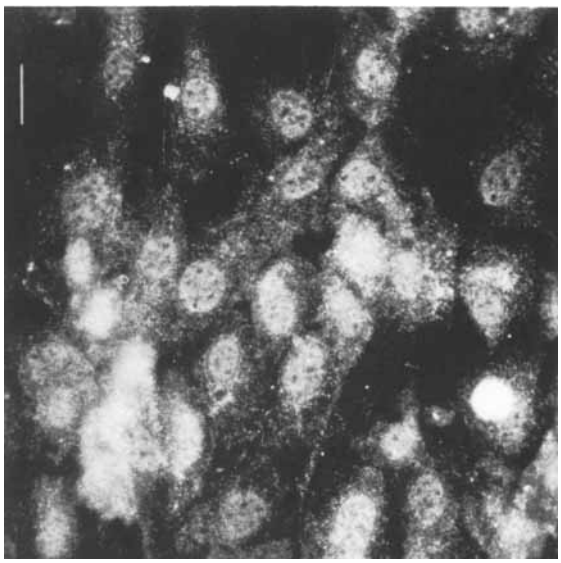


Fig. 8. Effect of colchicine on $G_s\alpha$ distribution in confluent HGFu cells. Cells were incubated with 2 μM colchicine for 4 h and then subjected to indirect immunofluorescent microscopy with anti-$G_s\alpha$. Note the disruption of the perinuclear pattern and the distribution of label throughout the cytoplasm. Bar indicates 20 μm.

identified as actin stress fibers by TRITC-phalloidin. This was confirmed by treatment of the cells with cytochalasin B, which caused the disassembly of the fibrous β subunit pattern (not shown). Cells were double-stained with anti-Gβ and Con A or WGA and analyzed by confocal microscopy (Fig. 11). The perinuclear cytoplasmic staining pattern obtained with the Gβ antibody partially colocalized with a portion of the Golgi apparatus stained with WGA (Fig. 11A). There was no evidence of Gβ staining in

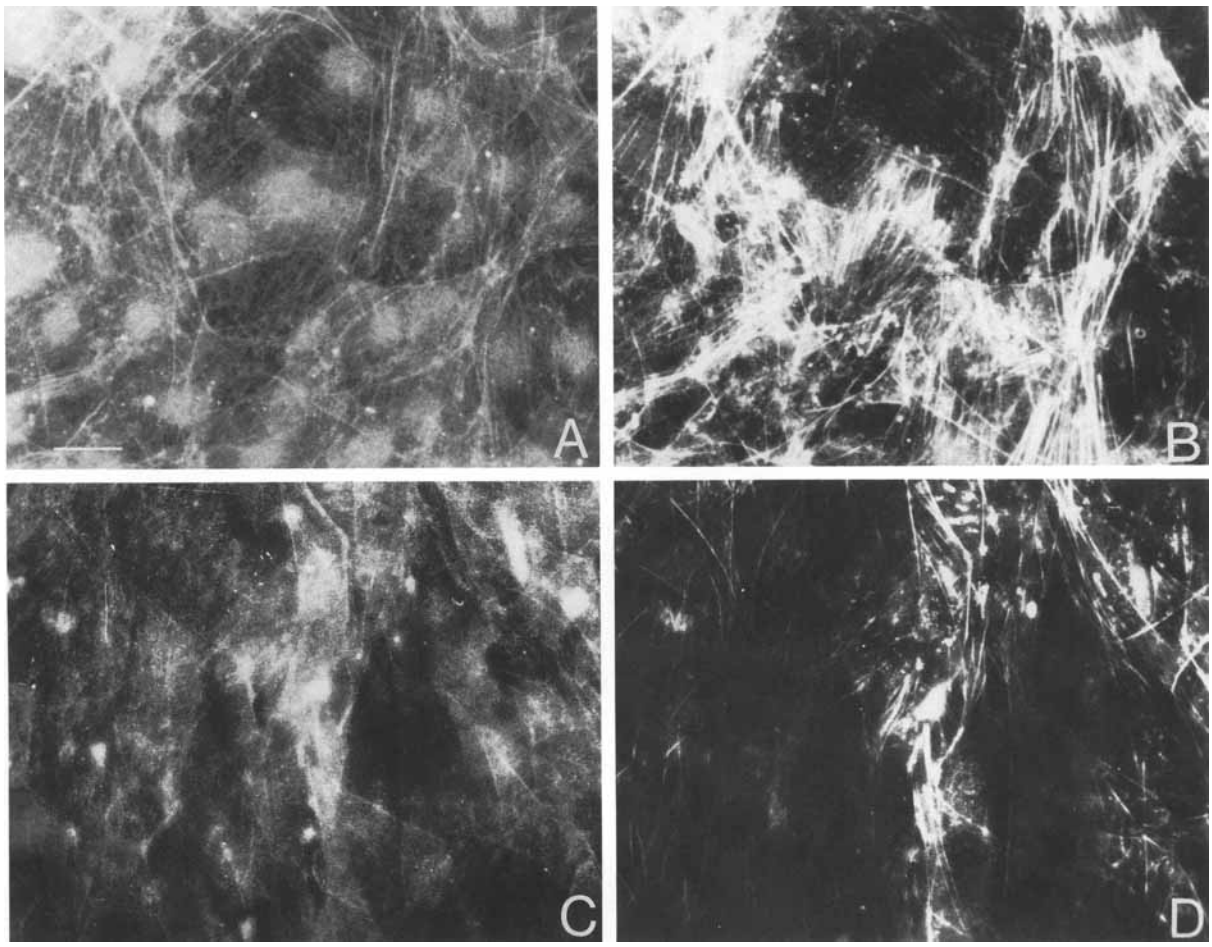


Fig. 9. Distribution pattern of $G_i\alpha 2$ in confluent HGFu cells Double-labeling for $G_i\alpha 2$ **(A,C)** and actin **(B,D)** shows that $G_i\alpha 2$ partially colocalizes with actin (C) and (D) show the effect of cytochalasin D (4 μM for 4 h) on the $G_i\alpha 2$ and actin distribution patterns The disruption pattern of the actin stress fibers and $G_i\alpha 2$ is similar Bar indicates 20 μm Ob17 cells (not shown) showed the same distribution pattern

the area of the ER that is stained by Con A where $G_s\alpha$ was found (Fig. 11B). In general therefore, the localization of Gβ subunits is consistent with the known function of this subunit.

Effect of Differentiation on G-Protein Distribution

TRITC-phalloidin staining revealed a modification of the actin pattern in the differentiating cells (Fig. 12). As a result of differentiation, actin filaments seem to reorganize and become located at the periphery of the cells, in close association with the plasma membrane. This is consistent with the more rounded appearance of the cells, as shown in Figure 1. Staining with the anti-$G_i\alpha 2$ antibody showed that, with the loss of organization of actin stress fibers, there was a concomitant change in the distribution of the $G_i\alpha 2$ subunit. There was also a similar reorganization of the fibrous pattern of the β subunit while the cytoplasmic pattern remained unchanged (results not shown). No change in distribution of the $G_s\alpha$ subunits was observed as a result of differentiation (results not shown).

Treatment With Pertussis Toxin

Exposure of cells or membranes to pertussis toxin results in persistent stimulation of adenylyl cyclase activity due to inactivation of $G_i\alpha$ [Gilman, 1987] and also induces preadipocyte differentiation [Shinohara et al., 1991]. Treatment with pertussis toxin resulted in reorganization of actin stress fibers paralleled by the loss of the typical fibrous patterns for $G_i\alpha$ and Gβ subunits but was without effect on the $G_s\alpha$ pattern (data not shown).

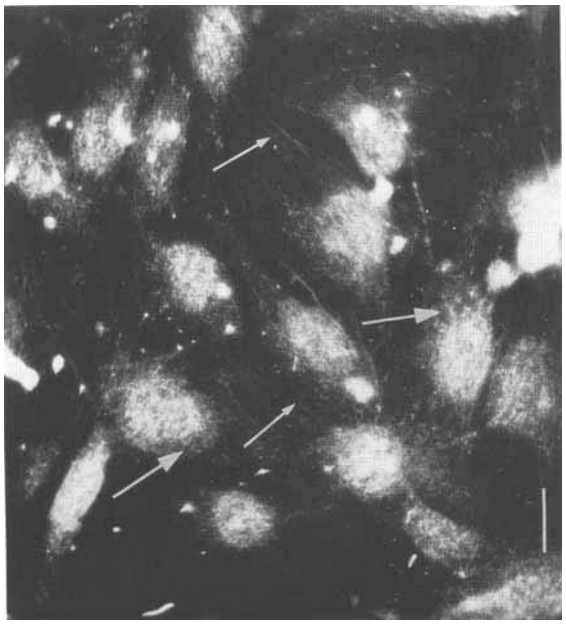


Fig. 10. Analysis of the β subunit distribution in confluent HGFu cells. The cells were labeled with an anti-β subunit antibody. Note the fibrous pattern similar to that of $G_i\alpha 2$ and the perinuclear cytoplasmic pattern which resembles the $G_s\alpha$ pattern. Bar indicates 20 μm.

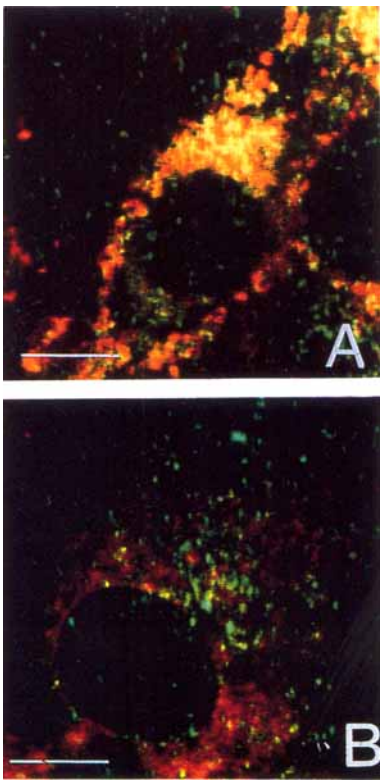


Fig. 11. Analysis of the β subunit perinuclear cytoplasmic distribution in confluent HGFu cells. The cells were double-labeled with anti-Gβ and either (A) WGA or (B) Con A and the distribution pattern assessed by confocal imaging of 0.5 μm sections. Green indicates Gβ staining; red, Con A or WGA staining and yellow, colocalization. Note the colocalization of Gβ with WGA but not with Con A. Bar indicates 10 μm.

DISCUSSION

Abundance of G-Protein Subunits and Adenylyl Cyclase Activity

The patterns of G-protein found in the clonal lines HGFu and Ob17 differ substantially from those seen in adipocytes of the lean and ob/ob mice from which they are derived [Bégin-Heick, 1990, 1992, Gettys et al., 1991]. Thus, in the clonal cells, the two major G-protein alpha subunits are $G_s\alpha$ [with a predominance of the long (45 kDa) form] and $G_i\alpha 2$, whereas in normal (+/+) adipocytes the 42 and 45 kDa forms of $G_s\alpha$ are equally abundant, as are $G_i\alpha 1$ and $G_i\alpha 2$. This indicates that factors controlling the normal expression of these G-proteins are either absent or inactive in the clonal lines. Although adenylyl cyclase was stimulated 10–15-fold by guanine nucleotides and 150–200-fold by agents that activate the catalytic unit directly, it was resistant to activation by isoproterenol. Such resistance to hormonal activation was noted previously in two systems where the 45 kDa isoform of $G_s\alpha$ is the predominant peptide [McFarlane-Anderson and Bégin-Heick, 1991; Walseth et al., 1989]. Together, these results constitute evidence that the short (42 kDa) isoform of $G_s\alpha$ is important for coupling hormone receptors to the transduction of hormonal signals [Walseth et al., 1989], although both the 42 and 45 kDa isoforms have been reported to be effective in the transduction of adenylyl cyclase activity [Freissmuth et al., 1989]. No data are yet available on the β-adrenergic receptor activity in HGFu and Ob17 cells and how this activity may be affected by differentiation.

Intracellular Localization

The data presented above show that, in HGFu and Ob17 cells, G-proteins are not solely confined to the plasma membranes but are associated with other cellular organelles. This is in agreement with recent findings of others to the effect that the G-proteins may have a diverse intracellular distribution [Brabet et al., 1988; Wang et al., 1989; Ercolani et al., 1990; Holtzman et al., 1991; Lewis et al., 1991; Muntz et al., 1992].

Actin reorganization is a feature of differentiating cells [Grant and Aunis, 1990]. The associa-

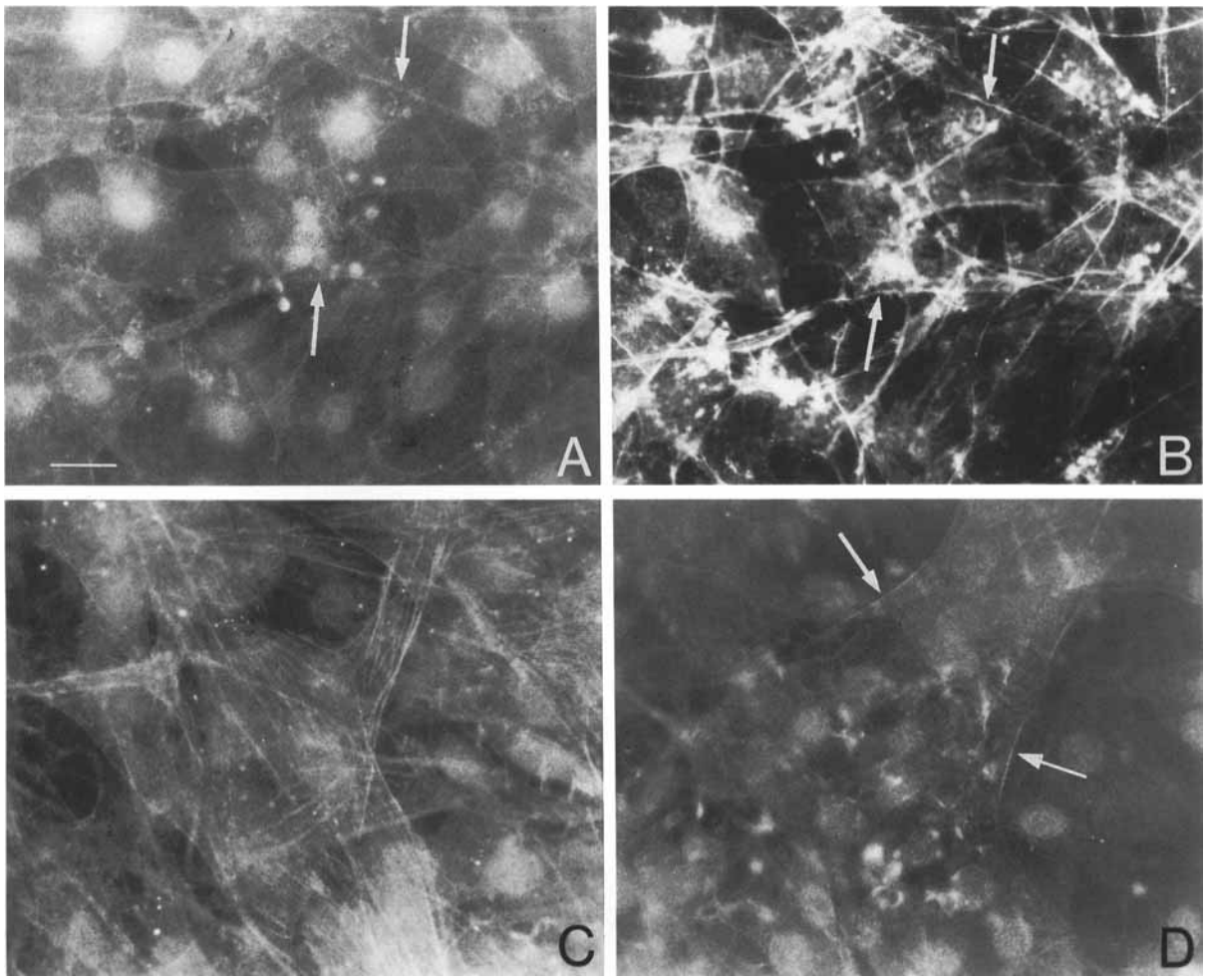


Fig. 12. Effect of differentiation on $G_i\alpha 2$ distribution Double immunofluorescence staining of $G_i\alpha 2$ (**A**) and actin (**B**) in differentiating HGFu cells Note that in differentiating cells, actin and $G_i\alpha 2$ are located mostly at the periphery of the cells and stress fibres are reduced In (**C**) Ob17 confluent cells stained with anti-$G_i\alpha 2$ showing the typical stress fibre pattern is present In (**D**) differentiating Ob17 cells stained with anti-$G_i\alpha 2$ there is reorganization of the peptide at the cell periphery Bar indicates 20 μm

tion of $G_i\alpha$ and Gβ with actin in confluent cells and their simultaneous reorganization with actin during differentiation suggests an involvement in the cellular functions that involve actin. Studies in other cells have suggested association of $G_i\alpha$ and Gβ with actin [Carlson et al., 1989; Peraldi et al., 1989], although the nature of the interaction is not known.

The disruption of the $G_s\alpha$ subunit pattern by colchicine is consistent with the suggestion by Wang et al. [1990] that $G_s\alpha$ is associated with tubulin. The fact that Gβ, and not $G_s\alpha$, partly colocalized with vesicles that are stained by WGA indicates that Gβ resides in subcellular compartments closely associated with, but not identical to those with which $G_s\alpha$ is associated.

The results showing that the peptides are detected in purified and washed nuclei indicate that the immunocytochemical detection of the peptides in association with the nucleus is not an artefact of preparation. Furthermore, they support the findings of Crouch [1990] that $G_i\alpha$ is associated with chromatin during mitosis and of Takei et al. [1992] who described a pertussis toxin sensitive G-protein in rat liver nuclear preparations.

Recent data from our laboratory using the same antibodies and the same fixation techniques (Cadrin et al., unpublished data) show that in liver sections, G-proteins are found largely, although not entirely, associated with the plasma membrane. Whether the association

of G-protein subunits with organelles other than the plasma membrane, as reported here and described by others, is a special characteristic of clonal lines or cells in culture is not known at present. However, their association with different cellular organelles indicate that they transduce signals at other cellular locations and their differential localization reinforces the notion that they are individually regulated.

ACKNOWLEDGMENTS

This work was supported by a grant from the Medical Research Council of Canada. The authors are grateful to Pascale Reinhardt-Poulin for expert technical assistance and Andrew Vaillant for help with the confocal microscopy.

REFERENCES

Akner G, Sundqvist K-G, Denis M, Wikstrom A-C, Gustafsson J-A (1990) Immunocytochemical localization of glucocorticoid receptor in human gingival fibroblasts and evidence for a co-localization of glucocorticoid receptor with cytoplasmic microtubules Eur J Cell Biol 53 390–401

Begin-Heick N (1985) Absence of the inhibitory effect of guanine nucleotides on adenylate cyclase activity in white adipocyte membranes of the ob/ob mouse J Biol Chem 260 6187–6193

Begin-Heick N (1990) Quantification of the α and β subunits of the transducing elements (G_s and G_i) of adenylate cyclase in adipocyte membranes from lean and obese (ob/ob) mice Biochem J 268 83–89

Begin-Heick N (1992) α-subunits of G_s and G_i in adipocyte membranes of genetically diabetic (db/db) mice Am J Physiol 263 (Cell Physiol 32) C121–C129

Birnbaumer L, Abramowitz J, Brown AM (1990) Receptor-effector coupling by G-proteins Biochim Biophys Acta 1031 163–224

Brabet P, Dumuis A, Sebben M, Pantaloni C, Bockaert J, Homburger V (1988) Immunocytochemical localization of the guanine nucleotide-binding protein G_o in primary cultures of neuronal and glial cells J Neurosci 8 701–708

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal Biochem 72 248–254

Buss JE, Mumby SM, Casey PJ, Gilman AG (1987) Myristoylated α subunits of guanine nucleotide-binding regulatory proteins Proc Natl Acad Sci USA 84 7493–7497

Carlson KE, Woolkalis MJ, Newhouse MG, Manning DR (1986) Fractionation of the β subunit common to guanine nucleotide-binding regulatory proteins with the cytoskeleton Mol Pharmacol 30 463–468

Chomczynski P, Saachi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction Analyt Biochem 162 156–159

Crouch M, Winegar DA, Lapetina EG (1989) Epinephrine induces changes in the subcellular distribution of the inhibitory GTP-binding protein $G_{i\alpha\,2}$ and a 38-kDa phosphorylated protein in the human platelet Proc Natl Acad Sci USA 86 1776–1780

Crouch M (1991) Growth factor-induced cell division is paralleled by translocation of $G_{i\alpha}$ to the nucleus FASEB J 5 200–206

Ercolani L, Stow JL, Boyle JF, Holtzman EJ, Lin H, Grove JR, Ausiello DA (1990) Membrane localization of the pertussis toxin-sensitive G-protein subunits $\alpha_{i\,3}$ and the expression of a metallothionein-$\alpha_{i\,2}$ fusion gene in LLC-PK_1 cells Proc Natl Acad Sci USA 87 4635–4639

Forest C, Czeruka D, Grimaldi P, Vannier C, Négrel R, Ailhaud G (1983) In Angel A, Hollenberg CH, Roncari DAK (eds) "The Adipocyte and Obesity Cellular and Molecular Mechanisms" New York Raven Press, pp 53–64

Freissmuth M, Casey PJ, Gilman AG (1989) G proteins control diverse pathways of transmembrane signalling FASEB J 3 2125–2131

Gettys T, Ramkumar V, Uhing RJ, Seger LE, Taylor IL (1991) Alterations in mRNA levels, expression, and function of GTP-binding regulatory proteins in adipocytes from obese mice (C57BL/6J-ob/ob) J Biol Chem 266 15949–15955

Gilman AG (1987) G proteins Transducers of receptor-generated signals Annu Rev Biochem 56 615–650

Goldsmith P, Rossiter K, Carter A, Simonds W, Unson CG, Vinitsky R, Spiegel AM (1988) Identification of the GTP-binding protein encoded by G_{i3} complementary DNA J Biol Chem 263 6476–6479

Grant NJ, Aunis D (1990) Effects of phorbol esters on cytoskeletal proteins in cultured bovine chromaffin cells induction of neurofilament phosphorylation and reorganization Eur J Cell Biol 52 36–46

Haraguchi K, Rodbell M (1990) Isoproterenol stimulates shift of G proteins from plasma membrane to pinocytic vesicles in rat adipocytes A possible means of signal dissemination Proc Natl Acad Sci USA 87 1208–1212

Holtzman EJ, Soper BW, Stow JL, Ausiello DA, Ercolani (1991) Regulation of the G-protein $\alpha_{i\,2}$ subunit gene in LLC-PK_1 renal cells and isolation of porcine genomic clones encoding the gene promoter J Biol Chem 3 1763–1771

Iyengar R, Rich KA, Herberg JT, Premont RT, Codina J (1988) Glucagon receptor-mediated activation of G_s is accompanied by subunit dissociation J Biol Chem 263 15348–15353

Jones DT, Reed RR (1987) Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium J Biol Chem 262 14241–14289

Kawahara H, Cadrin M, Perry G, Autilo-Gambetti L, Swierenga SHH, Metuzals J, Marceau N, Frenc SW (1990) Role of cytokeratin intermediate filaments in transhepatic transport and canalicular secretion Hepatology 11 435–448

Lai E, Rosen OM, Rubin CS (1981) Differentiation-dependent expression of catecholamine-stimulated adenylate cyclase J Biol Chem 256 12886–12874

Lai RK, Perez-Sala D, Canada FJ, Rando RR (1990) The γ subunit of transducin is farnesylated Proc Natl Acad Sci USA 87 7673–7677

Leach VL, Powers EA, Ruff VA, Jaken S, Kaufmann S (1989) Type 3 protein kinase C localization to the nuclear envelope of phorbol ester-treated NIH 3T3 cells J Cell Biol 109 685–695

Lewis JM, Woolkalis MJ, Gerton GL, Smith RM, Jarett LT, Manning DR (1991) Subcellular distribution of the α subunit(s) of G_i Visualization by immunofluorescent and immunogold labelling Cell Reg 2 1097–1113

Lipsky NG, Pagano RE (1985) A vital stain for the Golgi apparatus Science 228 745–747

McFarlane-Anderson N, Begin-Heick N (1991) mRNA and protein levels of the stimulatory guanine nucleotide regulatory protein G_s are lower in the testis of obese (ob/ob) mice Cell Signal 3 233–241

McFarlane-Anderson N, Bailly J, Begin-Heick N (1992) Levels of G-proteins in liver and brain of lean and obese (ob/ob) mice Biochem J 282 15–23

Maltese WA (1990) Posttranslational modification of proteins by isoprenoids in mammalian cells FASEB J 4 3319–3328

Maltese WA, Robishaw JD (1990) Isoprenylation of C-terminal cysteine in a G-protein γ subunit J Biol Chem 265 18071–18074

Muntz KH, Sternweis PC, Gilman AG, Mumby SM (1992) Influence of γ subunit prenylation on association of guanine nucleotide-binding regulatory proteins with membranes Mol Biol Cell 3 49–61

Negrel R, Grimaldi P, Ailhaud G (1978) Establishment of a preadipocyte clonal cell line from the epididymal fat pad of ob/ob mouse that responds to insulin and lipolytic hormones Proc Natl Acad Sci USA 75 6054–6058

Peraldi S, Nguyen Than Dao B, Brabet P, Homburger V, Toutant M, Bouille C, Assenmacher I, Bockaert J (1989) Apical localization of the alpha subunit of the GTP-binding protein G_o in choroidal and ciliated ependymocytes J Neurosci 9 806–814

Premont RT, Iyengar R (1989) Heterologous desensitization of the liver adenylyl cyclase Analysis of the role of G-proteins Endocrinology 125 1151–1160

Rabonowitz S, Horstmann H, Gordon S, Griffiths G (1992) Immunocytochemical characterization of the endocytic and phagolysomal compartments in peritoneal macrophages J Cell Biol 116 95–112

Ribeiro-Neto F, Mattera R, Grenet D, Sekura RD, Birnbaumer L, Field JB (1987) Adenosine diphosphate ribosylation of G proteins by pertussis and cholera toxins in isolated membranes Different requirements for and effects of guanine nucleotides and Mg^{+2} Mol Endocrinol 1 472–481

Rotrosen D, Gallin JI, Spiegel AM, Malech HL (1988) Subcellular localization of G_i alpha in human neutrophils J Biol Chem 263 10958–10964

Sarndahl E, Lindroth M, Bengtsson T, Fallman M, Gustavsson J, Stendahl O, Andersson T (1989) Association of ligand-receptor complexes with actin filaments in human neutrophils A possible regulatory role for a G-protein J Cell Biol 109 2791–2799

Shinohara O, Murata Y, Kubota C (1991) Enhancement of differentiation of rat adipocyte precursor cells by pertussis toxin Biochem Med Metabol Biol 46 85–92

Simonds WF, Goldsmith PK, Codina J, Unson CG, Spiegel AM (1989) G_i2 mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes In situ identification with G_α C-terminal antibodies Proc Natl Acad Sci USA 86 7809–7813

Sullivan KA, Liao YC, Alborzi A, Beiderman B, Chang FH, Masters S, Levison AD, Bourne HR (1986) Inhibitory and stimulatory G proteins of adenylate cyclase cDNA and amino acid sequences of the α chains Proc Natl Acad Sci USA 83 6687–6691

Takei Y, Kurosu H, Takahashi K, Katada T (1992) A GTP-binding protein in rat liver nuclei serving as the specific substrate of pertussis toxin-catalyzed ADP-ribosylation J Biol Chem 267 5085–5089

Tartakoff AM, Vassalli P (1983) Lectin-binding sites as markers of Golgi subcompartments Proximal-to-distal maturation of oligosaccharides J Cell Biol 97 1243–1248

Virtanen I, Ekblom P, Laurila P (1980) Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells J Cell Biol 85 429–434

Walseth TF, Zhang HJ, Olson LK, Schroeder WA, Robertson RP (1989) Increase in Gs and cAMP generation in HIT cells Evidence that the 45 kDa α-subunit of Gs has greater functional activity than the 52 kDa α-subunit J Biol Chem 264 21106–21111

Wang H, Berrios M, Malbon CC (1989) Indirect immunofluorescence localization of β-adrenergic receptors and G-proteins in human A431 cells Biochem J 263 519–532

Wang H, Watkins DC, Malbon CC (1992) Antisense oligonucleotides to G_s protein α-subunit sequence accelerate differentiation of fibroblasts to adipocytes Nature 358 334–337

Wang N, Yan K, Rasenik MM (1989) Tubulin binds specifically to the signal-transducing proteins, $G_{s\alpha}$ and $G_i\alpha 1$ J Biol Chem 265 1239–1242

Watkins DC, Rapiejko PJ, Ros M, Wang HY, Malbon CC (1989) G-protein mRNA levels during adipocyte differentiation Biochem Biophys Res Commun 165 929–934

Wilson BS, Hermouel S, de Mazancourt P, Spiegel AM, Farquhar MG (1992) Localization of G_{i3} alpha subunit is dependent on cell type and level of expression Implications for Golgi function Mol Biol Cell (Abstr) 3 34a

Wong SH, Low SH, Hong W (1992) The 17-residue transmembrane domain of β-galactoside α2,6-sialyltransferase is sufficient for Golgi retention J Cell Biol 117 245–258