THE CYCLIC AMP SIGNALLING SYSTEM AS A REGULATOR OF PREADIPOCYTE DIFFERENTIATION

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by

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This Thesis is Dedicated to my Mother and Sister,
Cathleen and Claire

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SUMMARY

A study was carried out on the fibroblastic 3T3-F442A preadipocyte cell system to investigate the role of the adenylate cyclase signalling system in modulating their adipocyte differentiation. Three areas were investigated:

1) The expression of heterotrimeric guanine nucleotide binding protein (G-protein) subunits during individual stages of cellular differentiation.
2) The role of cyclic AMP in modulating preadipocyte differentiation.
3) Interactions occurring between the cyclic AMP and MAP kinase signalling cascades during the differentiation process.

The differentiation of 3T3-F442A preadipocytes was shown, under serum-free conditions, to be divided into at least two stages. The first stage is dependent on the presence of growth hormone (GH) which primes cells to the action of other differentiative agents. The second, maturation, stage involved a combination of insulin, EGF and T_3 which acted on GH-primed cells to promote terminal differentiation. Terminal differentiation was determined by two criteria; morphologically by the accumulation of Oil Red O-positive triacylglycerides in the cell cytoplasm and biochemically, by the emergence of the specific activity of an adipocyte-specific enzyme marker, α-glycerophosphate dehydrogenase.

A range of specific anti-peptide antisera were generated and used to quantify specific changes in the expression of stimulatory- and inhibitory- G-proteins during the two-stages of 3T3-F442A preadipocyte differentiation. Stimulatory-G-proteins (G_s) couple adenylate cyclase to positive-acting extracellular stimuli, whereas inhibitory-G-proteins (G_i) mediate the actions of negative-acting stimuli. Undifferentiated 3T3-F442A preadipocytes were found to express detectable levels of the G_s α-subunits, $G_{s\alpha 42}$, $G_{s\alpha 44}$, and the G_i α-subunits, $G_{i2\alpha}$ and $G_{i3\alpha}$ in their cell membranes, together with the 36 kDa β-subunit ($G_{\beta 36}$) which is subunit common to both G_s and G_i. During the GH-priming stage of adipocyte differentiation significant increases in protein expression were observed for $G_{s\alpha 42}$, $G_{s\alpha 44}$, $G_{i2\alpha}$ and $G_{\beta 36}$, but not $G_{i3\alpha}$. Increased levels of $G_{s\alpha 44}$ and $G_{\beta 36}$ were sustained during the first two days of maturation and then fell, in terminally differentiated cells, to levels observed in undifferentiated 3T3-F442A fibroblasts. Levels of $G_{i2\alpha}$ were also sustained during the initial stages of maturation, but then fell, together with $G_{i3\alpha}$, to levels significantly lower then those found in undifferentiated fibroblasts. In contrast, levels of $G_{s\alpha 44}$ were maintained at a significantly elevated level in terminally differentiated cells when compared to undifferentiated fibroblasts. Changes in $G_{s\alpha}$ subunit expression were also observed in

non-differentiating control cultures, in the absence of differentiative agents. This G-protein may play a role in modulating preadipocyte differentiation. Indeed, treatment of cell with cholera toxin, which constitutively activates G_s and adenylate cyclase, dramatically inhibited differentiation of 3T3-F442A cells by ≈90%.

Adenylate cyclase catalyses the formation of the intracellular second messenger cyclic AMP which then binds to and activates protein kinase A. Direct activation of adenylate cyclase with forskolin (50μM) or treatment with the cyclic AMP analogue CPT-cyclic AMP (0.25mM) was found to potently inhibit the adipose conversion of 3T3-F442A preadipocytes induced with foetal calf serum and insulin (FCS/insulin; ≈90% inhibition) or with a serum-free hormonally defined medium (DDM; ≈70% inhibition). In contrast, treatment of cells with the cyclic AMP phosphodiesterase inhibitor, IBMX, or with low concentrations of CPT-cyclic AMP (10nM) or forskolin (10nM) potentiated cellular differentiation induced with FCS/insulin (≈80-99% increase) or DDM (≈30-40% increase). Both IBMX and 10nM forskolin induced small and relatively transient increases in intracellular cyclic AMP (≈8 and ≈3 fold maximal increase), whereas those induced by 50μM forskolin were much larger and more prolonged (≈120 fold maximal increase). This suggests that the differential effects of cyclic AMP on the adipose conversion of 3T3-F442A cells could be attributable to interactions occurring at different stages of the differentiation program. Indeed, inclusion of 10nM forskolin or IBMX during the GH-priming stage of differentiation synergistically enhanced GH-promoted differentiation (≈90% and ≈130% increase, respectively). In contrast, when included during the maturation stage, these agents were found to inhibit terminal differentiation (≈60% and ≈70% inhibition, respectively).

A potential mechanism underlying the effects of cyclic AMP on cell growth is through functional interplay with the MAP kinase signalling cascade. MAP kinases were potently activated in 3T3-F442A preadipocytes by the differentiative factors GH (≈5 fold activation), insulin (≈5 fold activation) and EGF (≈20 fold activation). Antisense depletion of MAP kinase was found to severely retard the differentiation of 3T3-F442A cells with serum or DDM by approximately 95%, demonstrating that adipose conversion of these cells displays an overall requirement for MAP kinases. Cyclic AMP was found to potentiate GH- (by ≈4 fold), but not EGF/Ins/T_3-activated MAP kinase. In addition, cyclic AMP alone activates MAP kinase (≈2 fold activation). This suggested that the cooperativity between cyclic AMP and GH could be at the level of MAP kinase but MAP kinase was not the site of action of cyclic AMP in inhibiting maturation. However, the availability of the inhibitor of MAP kinase activation, PD098059, enabled the testing of the MAP kinase requirement during priming and this showed a lack of MAP kinase requirement at this stage.

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ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
Bisbenzimide	{2-[2-(4-hydroxyphenyl)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl)-benzimidazole} trihydrochloride
BSA	bovine serum albumin
CPT-cAMP	chlorophenylthio-cAMP
CS	Calf Serum
Cyclic AMP	Adenosine 3', 5'-cyclic monophosphate
DHAP	dihydroxyacetone phosphate
1, 9-Dideoxyforskolin	7β-acetoxy-6β-hydroxy-8, 13-epoxy-labd-14-en-11-one
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetracetic acid
EGF	epidermal growth factor
EGTA	Ethylene glyco-bis (beta-aminoethyl ether) N,N,N',N'-tetracetic acid
H-89	{N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCL}
FCS	foetal calf serum
Forskolin	7β-acetoxy-1α, 6β, 9α-hydroxy-8, 13-epoxy-labd-14-en-11-one
IBMX	3-isobutyl-1-methylxanthine
KLH	keyhole limpet haemocyanin
MAP kinase	Mitogen Activated Protein Kinase
MBP	myelin basic protein
NADH	β-nicotinamide adenine dinucleotide
PBS	phosphate buffered saline
PD098059	2'-amino-3'-methoxyflavone
Ro-20-1724	[4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone]
Rp-cAMPS	8-bromoadenosine-3', 5'-cyclic monophosphate (*Rp* isomer)
SDS	sodium dodecyl sulfate
T_3	triiodothyronine
Tris	2-amino-2hydroxymethyl-propane-1, 3-diol

Chapter One

Literature Review

1.1) INTRODUCTION

White adipocytes (fat cells) are a specialised cell type that allow vertebrates to synthesise and store large amounts of metabolic energy, primarily in the form of triglycerides, in times of nutritional abundance. In times of need, triglycerides are hydrolysed to release unesterified fatty acids. The mature adipose cell is characterised by a spherical or near-spherical shape with approximately 95% of its volume being made up of a single triglyceride droplet (Slavin, 1985). Adipocytes represent between one and two thirds of the total number of cells in adipose tissue, the rest are various blood cells, endothelial cells, fibroblasts, pericytes and adipose precursor cells (preadipocytes) of varying degrees of differentiation (Gèleon *et al*, 1989). Adipocyte differentiation occurs in late prenatal and early post-natal development in distinct anatomical locations. Hypertrophic growth of adipose cells can occur at virtually any time in response to marked overfeeding (Ailhaud *et al*, 1990). This suggests that the committal of mesenchymal cells to become preadipocytes, and the further differentiation of preadipocytes into adipocytes (adipogenesis) is governed, both temporally and spatially, by specific hormonal and nutritional signals.

A complete understanding of adipose tissue development will not be gained until each level of control is assimilated and the signal transduction processes, whereby extracellular stimulation ultimately leads to changes in gene expression associated with phenotypic differentiation, are delineated. Because of the central role of the adipose cell in pathological disorders of systemic energy balance, most notably obesity, obesity-linked diabetes and cachexia (fat and muscle wasting) (Spiegelman and Hotamisigil, 1993), the study of gene control may lead to a better understanding of these disorders and reveal possible targets for therapeutic action. In addition, the ability to control adipose tissue development would offer the possibility of producing, commercially, animals with a higher muscle:fat carcass composition.

1.2) FUNCTIONAL ROLES OF ADIPOCYTES *IN VIVO*

White adipose tissue is found in amphibians, reptiles, birds and mammals (Nir *et al*, 1988; Pond, 1978; Pond, 1986) and is the basis of an abundant, diffuse organ dedicated to the deposition and mobilisation of triglycerides (lipids) (Pond, 1986). The site of these specialised functions is the adipocyte cell (Rodbell, 1964).

1.2.1) Lipid Metabolism

Adipocytes contain an array of stringently regulated enzymes which are responsible for the synthesis of lipids from carbohydrates and their subsequent hydrolysis (lipolysis) (Feller, 1954; Hausberger *et al*, 1954;). Lipid synthesis (lipogenesis) requires a source of fatty acids and glycero-3-phosphate both of which are synthesised from glucose (Fig 1.1; Vernon, 1992). Fatty acids may also be obtained from triglycerides contained in very low density lipoproteins (VLDLs) and chylomicrons in the plasma through the action of the secreted adipocyte enzyme, lipoprotein lipase (Vernon, 1992). Lipolysis is achieved by the action of hormone-sensitive lipase with the eventual production of glycerol and fatty acids. Glycerol is released from the cell whereas a portion of the fatty acids are re-esterified (Vernon, 1992).

Lipogenesis and lipolysis are occurring continuously, therefore the net lipid deposition or mobilisation depends on the relative rate of both mechanisms. To organise these processes of energy storage and release, highly integrated systems have evolved which operate at several physiological levels. As for many complex physiological processes the brain provides an essential coordinating role (Fig 1.2).

Most important are the hypothalamic centres that coordinate energy homeostasis through regulation of food intake (hunger and satiety), energy expenditure (thermogenesis) and the secretion of hormones that regulate substrate interconversion, storage and mobilisation (Hubel, 1979). The hormone which has the major role in promoting fat deposition in mammals is insulin which is secreted from β-cells in the pancreas (Vernon, 1992). Insulin stimulates glucose utilisation, promotes fatty acid synthesis and activates acetyl-CoA carboxylase (ACC), a key enzyme of fatty acid synthesis (Vernon, 1992). In mammals lipolysis is stimulated by glucagon (secreted form pancreatic α-cells) and catecholamines (from the adrenal medulla and sympathetic nerve endings) (Vernon, 1992). Pituitary growth hormone (GH) (Issakson *et al*, 1985) and glucocorticoids (a product of the adrenal cortex) can also antagonise insulin action on lipid metabolism by indirect mechanisms (Katzenellenbogen, 1980).

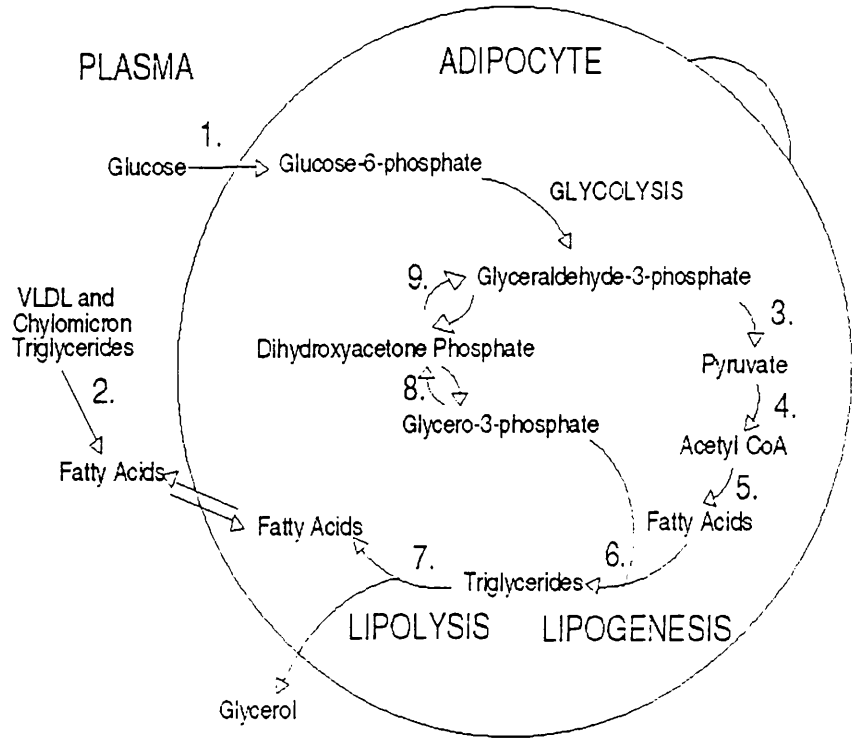


Fig 1.1) Lipogenesis and Lipolysis in the Adipocyte
The key features of adipocyte metabolism are the synthesis of triglycerides (lipogenesis) and their subsequent hydrolysis (lipolysis). The synthesis of lipids requires a source of fatty acids from chylomicron or very low density lipoprotein (VLDL) triglycerides or synthesised from glucose by the sequential actions of the glycolytic and biosynthetic pathways. The startingpoint of lipid synthesis requires *glycerol-3-phosphate* which is formed mainly by the reduction of *dihydroxyacetone phosphate* (DHAP), catalysed by α-*glyero-3-phosphate dehydrogenase* (8). DHAP is an isomer of *glyceraldehyde-3-phosphate* (GAP), an intermediate on the glycolytic pathway, and their interconversion is catalysed by *triose phosphate isomerase* (9). Pyruvate is formed from GAP by the sequential actions of *phosphoglyceraldehyde dehydrogenase*, *phosphoglycerate kinase*, *phosphoglyceromutase*, *enolase* and *pyruvate kinase* (3). The formation of *acetyl coenzyme A* (*acetyl CoA*) from pyruvate is catalysed by pyruvate dehydrogenase (4) and subsequent incorporation into fatty acids is due to the combined actions of *fatty acid synthase* and *acetyl CoA carboxylase* (5). Lipids are synthesised from *glycero-3-phosphate* and fatty acids in multi-sequential reactions catalysed by the *triglyceride synthase complex* (6). Adipocyte triglycerides are hydrolysed by the action of hormone-sensitive lipase (7). with the eventual production of glycerol and fatty acids. Other key enzymes in the scheme are indicated; *hexokinase* (1) and *lipoprotein lipase* (2)

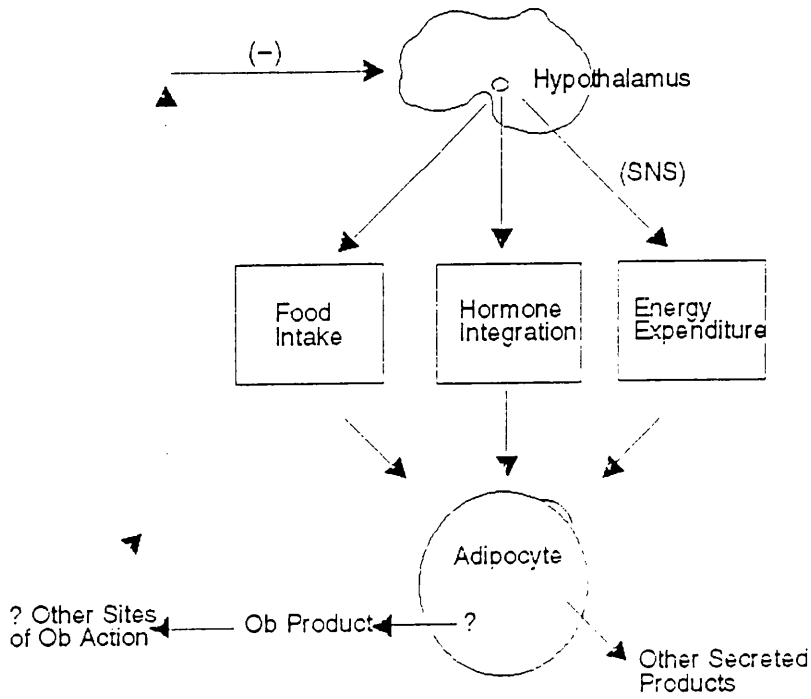


Fig 1.2) Major Components of the Body Weight Regulatory System
The brain, working primarily through hypothalamic centres influences body weight through effects on hunger (satiety), integration of key hormones, such as insulin, and energy expenditure, including actions to regulate thermogenesis in brown adipose tissue *via* sympathetic nervous system (SNS) innervation. These combine to determine the state of energy storage in adipose cells. The adipocyte plays an active role in the process through secretion of a number of secreted products. These include the product of the *ob* gene, which is predicted to regulate hypothalamic function through direct or indirect feedback.

1.2.2) The *Ob* Gene Product (Leptin)

In addition to being the major site for lipid metabolism there is now compelling evidence that adipocytes also act as endocrine/secretory cells. For example, adipocytes are known to secrete adipsin and other components of the alternative complement pathway (Choy *et al*, 1992; Sniderman *et al*, 1994), angiotensinogen (McGhee *et al*, 1993), prostaglandins (Darimont *et al*, 1994) and tumour necrosis factor (TNF) α (Hotamisligil *et al*, 1993). In addition, recent advancements in the field of adipocyte development suggest that adipocytes may play an active role in regulating energy homeostasis and body composition by producing a blood-borne factor (leptin) (Ahima *et al*, 1996, Campfield *et al*, 1995; Halaas *et al*, 1995; Pellymounter *et al*, 1995;). Leptin is the product of a 4.5 kbp mRNA, the product of the *ob* gene, which is apparently expressed solely in adipose tissue, presumably in adipocytes (Zhang *et al*, 1994). The predicted protein product is composed of 167 amino acids and has no homology to other known proteins (Zhang *et al*, 1994). Leptin is present in the plasma of normal mice and is absent in the plasma of *ob/ob* mice, resulting in profound obesity, often accompanied by diabetes and a decrease in energy expenditure (Friedman and Leibel, 1992; Halaas *et al*, 1995). Leptin appears to compensate for these homeostatic aberrations in normal mice by increasing energy expenditure, inducing weight loss and normalising metabolic parameters such as insulin and glucose levels (Halaas *et al*, 1995; Campfield *et al*, 1995; Pelleymounter *et al*, 1995).

The precise mechanisms of leptin action are not known. The most likely may be a direct action on the hypothalamus (Fig 1.2; Lee *et al*, 1996; Targaglia *et al*, 1995) and neuropeptide Y is a potential mediator of its effects (Stephens *et al*, 1995; Ahima *et al*; 1996). A complementary mechanism may be through a direct action of the protein on adipocyte metabolism. *Ob* gene expression in cultured fat cells suppresses ACC gene expression, fatty acid synthesis and lipid synthesis (Bai *et al*, 1996).

Recent studies demonstrated that the expression of the *ob* gene is itself controlled by the nutritional status of the animal. Fasting reduces expression, whereas food intake increases expression (Frederich *et al*, 1995; MacDougald *et al*, 1995b; Saladin *et al*, 1995), an effect that is accounted for by changes in insulin levels (MacDougald *et al*, 1995; Saladin *et al*, 1995). Glucocorticoids have also been shown to regulate *ob* gene expression (DeVos *et al*, 1995). The expression of the *ob* gene is subject to transcriptional control during preadipocyte differentiation by an important adipocyte transcription factor C/EBPα (MacDougald *et al*, 1995a). The expression of C/EBPα has also been shown to be regulated by hormonal stimulants, such as glucocorticoids and insulin (Cao *et al*, 1991; Yeh *et al*, 1995). Therefore, the expression of the *ob* gene

is not only influenced by adipocyte transcription factors such as C/EBPα during adipogenesis, but appears to be under hormonal control. The study of the signalling mechanisms underlying the hormonal control of adipocyte development could enable discovery of potent new drugs, capable of not only regulating fat cell number *in vivo* but also, by effecting leptin production, exerting systemic effects to combat pathological conditions such as obesity and obesity-linked diabetes.

1.3) ADIPOCYTE DEVELOPMENT *IN VIVO*

Adipocytes acquire specialised functions (section 1.2) during the late stages of embryonic development to prepare for the post-natal period (Cook and Kozac, 1982; Slavin, 1979). Further fat pad development is delayed until shortly after birth and is characterised by expansion of the newly formed white adipocyte population (Cook and Kozac, 1982; Slavin, 1979). These increases in adipose mass equips the newborn with additional adipose lipid stores which can then be mobilised during fasting periods.

1.3.1) Embryonic Development of Adipocytes

All of the cell types which form a mature animal are derived from multi-potential embryonic stem cells (Watt, 1991). It is generally accepted that adipose precursor, muscle and cartilage cells arise from totipotent stem cells of a mesenchymal origin (Konieczny and Emerson, 1984). Activation of a small number of regulatory genes by hypomethylation appears sufficient to commit these stem cells to a specific lineage, ie either the adipocyte, myocyte or chondrocyte lineages (Konieczny and Emerson, 1984; Lassar *et al*, 1986; Taylor and Jones, 1979). From an embryological perspective, the process of determination from multi-potential stem cells leads to the sequential formation of early-marker expressing preadipocytes (Gèloen *et al*, 1989a; Gèloen *et al*, 1989b). In the developing embryo, preadipocytes arise from primitive organs, a dense mass of vascularised mesenchymal cells which forms a supporting framework for adipogenesis (Gèloen *et al*, 1989a; Gèloen *et al*, 1989b). Primitive fat lobules are formed at around the second stage of human gestation and these are composed of densely packed fat cells adjacent to capillaries (Poissonet *et al*, 1988). In later stages, growth of adipose tissue is mainly due to an increase in size of fat cells and condensation and thickening of the mesenchyme, which forms septa among fat cell clusters (Burdi *et al*, 1985; Poissonet *et al*, 1984; Poissonet *et al*, 1988). In the embryo adipose tissue develops as distinct pads (or depots) which are distributed in a variety of locations throughout the body where it remains after birth (Burdi *et al*, 1985; Poissonet *et al*, 1984; Poissonet *et al*, 1988; Pond, 1986). The factors determining the time and site of fat pad development are not known. However, the distribution of fat depots is virtually the same amongst a wide range of mammalian species and suggests that conserved genetic factors may be involved (Pond, 1984). The importance of tissue vascularisation in primitive fat organs implies that fat depot development will be heavily influenced by systemic hormonal and circulatory factors (Poissonet *et al*, 1988).

1.3.2) Postnatal Development of Adipose Tissue

The accumulated data from a large number of studies clearly indicate that the cellular development that accompanies postnatal adipose tissue expansion involves both cell hyperplasia and hypertrophy at virtually all stages of life (Cook and Kozac, 1979; Greenwood and Hirsch, 1974; Kirkland and Gurr, 1979; Lemmonier, 1981). The hypertrophic aspect of cell growth and tissue expansion clearly relates closely to the metabolic features of the mature adipocyte and the balance of lipid accretion and mobilisation from it (section 1.2). Although the lipid filling of mature adipocytes is of undoubted importance to the expansion of adipose tissue, the process of hyperplastic growth has recently received a great deal of attention. The major proliferative capacity in adipose tissue is in the interstitial pool of cells which includes preadipocytes (Cook and Kozac, 1982). During early postnatal growth, expansion of interstitial cell types has been observed on many occasions to precede the expression of adipocyte phenotypes and adipocyte-specific genes (Cook and Kozac, 1982; Pilgrim, 1971). Thus it appears that adipose precursor cells undergo mitoses before terminal differentiation takes place. The proliferative and differentiative capacity of precursor cells is dependent on a number of factors, including sex, age and localisation of fat depot (Faust and Miller, 1983; Kirkland *et al*, 1990; Pilgrim, 1971; Wang *et al*, 1989). For example, preadipose cells from rat perirenal and epididymal fat depots show varying capacities for replication, with perirenal preadipocytes displaying a higher frequency of proliferation and differentiation (Djian *et al*, 1983; Kirkland *et al*, 1990; Wang *et al*, 1989). Whereas in humans, in agreement with clinical observations regarding adipose tissue development, preadipocytes from abdominal fat show a higher capacity for differentiation than those of the femoral depot (Hauner and Entenmann, 1991). Part of the explanation for different fat depots having differential hyperplastic capacities probably lies at the cell level and further research into the mechanisms of preadipocyte proliferation and differentiation will be required to gain further insight.

1.4) CELL CULTURE MODELS OF ADIPOCYTE DIFFERENTIATION

Cellular models, including primary cultures of preadipocytes and established cell lines capable of converting to an adipocyte phenotype, have allowed an *in vitro* approach to the study of preadipocyte differentiation. Among the most obvious advantages of *in vitro* studies are:-

1) The ability to control the extracellular environment.

2) The possibility of studying biochemical and morphological events occurring throughout the conversion process.

Of particular relevance to studies of endocrine control are the development of chemically defined, serum-free culture media which support biochemical and morphological adipocyte differentiation (Ailhaud *et al*, 1990). With the application of serum-free culture techniques to an increasing number of cellular models, the factors that control the proliferation and differentiation of preadipocytes have become a little clearer (see section 1.7). However, care must be taken in interpreting the cumulative data since the hormonal requirements of different cellular models can be widely different, particularly when cloned cell lines are compared with preadipocyte cells in primary culture, and when cultures containing serum are compared with serum free conditions. For example, fibroblast growth factor (FGF) is essential for growth of the Ob17 cell line (Gailard *et al*, 1984), whereas FGF inhibits growth and differentiation of rat preadipocytes (Deslex *et al*, 1987). Discrepancies such as these are probably attributable to interspecies variation and the fact that isolated cell lines and primary cultures represent adipocyte precursors of different stages of development (Ailhaud *et al*, 1990).

Most clonal preadipocyte cell lines have been established from mouse embryonic tissues (Doglio *et al*, 1986; Ailhaud *et al*, 1990). The first established adipogenic cell line was 3T3-L1 (Green and Meuth, 1974), a clonal subline from a heterogeneous population of the established fibroblast line 3T3. The 3T3 clone was originally isolated from the disaggregated cells of late foetuses of Swiss mice (Green and Meuth, 1974). The 3T3-F442A subclone was obtained from the 3T3-L1 line and exhibits an even higher susceptibility to adipose conversion (Green and Kehinde, 1976).

Cell lines, despite aneuploid karyotypes, present stable phenotypic properties through a large number of generations (Green and Meuth, 1974). The relevance of studies on 3T3 cells lines has been attested by a number of significant observations. Importantly,

subclones are selected on the basis of good contact inhibition and on inability to form foci indicative of transformed cells (Green and Meuth, 1974). Prior to differentiation in cell culture 3T3-L1 and 3T3-F442A preadipocytes are morphologically similar to the fibroblastic preadipose cells in the stroma of adipose tissue (Green and Meuth, 1974; Green and Kehinde, 1976). When induced to differentiate 3T3 preadipocytes lose their fibroblastic character, assume a rounded appearance and acquire the morphological and biochemical characteristics of adipocytes. The striking change in cell shape is independent of lipid accumulation and is due to alterations in synthesis and assembly of cytoskeletal proteins (Spiegelman and Farmer, 1982). Detailed electron micrographic studies by Novikoff *et al* (1980) revealed that mature 3T3-L1 adipocytes possess virtually all of the ultrastructural features of adipocytes *in situ*. Soon after the induction of differentiation, cytoplasmic triglyceride-containing vacuoles appear. After an extended period in culture, the vacuoles coalesce and become unilocular, causing the typical signet ring appearance of mature white adipocytes (Green and Meuth, 1974).

The cells also acquire virtually all of the enzymes for *de novo* fatty acid biosynthesis, lipogenesis and lipolysis and become responsive to the lipogenic and lipolytic hormones (Colman *et al*, 1978; Rubin *et al*, 1978; Spooner *et al*, 1979; Reed and Lane, 1980; Bernholer *et al*, 1985; Cook *et al*, 1985; Guest *et al*, 1990) Indeed, subcutaneous injection of 3T3-F442A cells into nude, athymic mice gives rise to fully vascularised, non-malignant fat pads which are indistinguishable from normal adipose tissue (Green and Kehinde, 1979).

Other cell lines available for the study of adipocyte development include Ob17 and Ob1771 (Doglio *et al*, 1986), isolated from obese mice (genotype *ob/ob*), and TA1 (Chapman *et al*, 1984) and 30A5 (Konieczny *et al*, 1984) isolated from an established mouse fibroblast line treated with a nucleotide analogue. Cell strains that behave reproducibly in culture have also been obtained from the stromal fraction of adipose tissue from a variety of species (Ailhaud, 1982). These putative precursor cells, although diploid, have a limited life-span and eventually enter a period of senescence characterised by increased cell doubling time and ultimately death. In addition it has been suggested that cell strains derived by this technique do not represent a homogeneous population (Ailhaud, 1982). For these reasons, most of the information on adipocyte differentiation to date has been gained from the study of established cell lines, such as 3T3-L1 and 3T3-F442A. However, in order to gain an overall picture of the control of adipocyte differentiation, results should be verified between a number of species and cellular models, and fat depot-specific aspects should be investigated using primary cell cultures. Another important approach for verification in a physiological

context, which has been employed to a limited extent for adipose development, is the use of transgenic animals (Graves *et al*, 1992).

1.5) THE ADIPOCYTE DEVELOPMENT PROGRAM

Information acquired in cell culture studies on adipocyte development has enabled investigators to identify key events that are likely to be important in this process (Fig 1.3). As with most systems of mammalian development, the acquisition of the morphological and biochemical characteristics of mature adipocytes requires extensive reprogramming of gene expression.

The expression of adipocyte specific genes occurs in distinct temporal stages; the progress of differentiation can be monitored by the progressive accumulation of individual differentiation-specific phenotypes or markers. Early electrophoretic studies revealed major quantitative changes in at least 100 protein species (Sidhu, 1979) and 40% of soluble adipocyte proteins represent newly synthesised material (Spiegelman and Green, 1980). In virtually every case so far examined, new protein synthesis in the adipocyte is accompanied by increases in the related mRNA levels (Table 1.1). Changes in transcription are an important factor in altered mRNA abundance (Bernholer *et al*, 1985). However, it seems likely that other mechanisms, especially changes in mRNA stability, may also be important (Doglio *et al*, 1986).

During proliferation, cultured preadipocytes are biochemically and morphologically indistinguishable from fibroblasts (Hiragun, 1985). Since the proliferation and differentiation of cells are thought to be mutually-exclusive processes (Freytag and Geddes, 1992), the initiation of differentiation and the expression of adipocyte specific markers requires exit from the mitotic cell cycle.

1.5.1) Early Events

Eukaryotic cellular growth is known to be regulated at specific points in the cell cycle, termed G1-, S-, G2- and M-phases (Xiong *et al*, 1991). The two major events common to all cell cycles are S-phase, when chromosomes are replicated, and M-phase, when the replicated chromosomes are segregated into two daughter cells (Nurse, 1990). G1 can also be subdivided into early and late phases (Pardee, 1989). Early G1 cells can either embark on a new cell cycle (late-phase progression) or enter a quiescent, growth arrested state (G0-phase) characterised by an unreplicated DNA content and decreased macromolecular synthesis (Sherr, 1994). Commitment to DNA replication (S-phase entry) occurs at a particular restriction point late in G1 and is dependent on appropriate mitogenic stimuli (Pardee, 1989). From *in vitro* studies, it appears that growth arrest during G1 (G0-entry), rather than contact among arrested cells, is necessary to trigger preadipose cells to enter the differentiation program

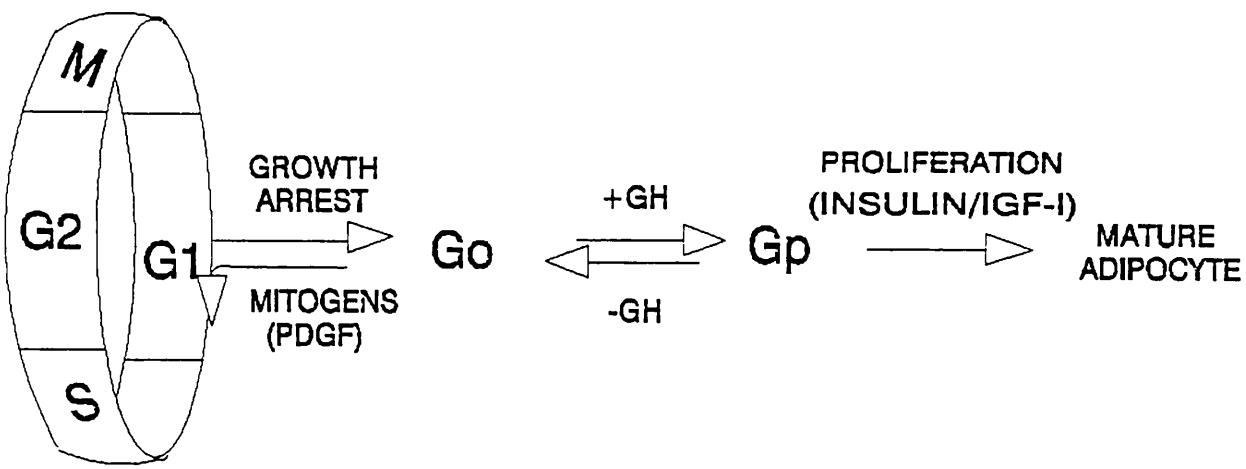


Fig 1.3) Hypothetical Model of 3T3-F442A Preadipocyte Differentiation Initially, cell cycle exit allows cells to enter a state of growth arrest, termed G0. Cells at G0 are then substrates for GH, whch reversibly induces an anti-mitogenic, primed state (Gp). Reversal requires removal of GH and addition of mitogens, such as platelet derived growth factor (PDGF). Subsequently, insulin, in an irreversible step, promotes fat accumulation. Terminal differentiation is preceeded by limited clonal expansion under the control of the insulin-like growth factor- (IGF-) I receptor.

Table 1.2) Multiple Stages of Adipose Cell Differentiation

Stage	mRNA/protein induced	Emerging phenotypes
1	Unidentified	Proliferation
2	FAAR mRNA[m] PPARγ mRNA[n] pGH_3 mRNA[h] Lipoprotein lipase[j] pOb24 mRNA[h]	1) Growth arrest[h] 2) Emergence of very early and early markers 3) Fatty acid transport initiated[a]
3	IGF-I mRNA (Ob1771)[g]	1) Increased response to lipolytic hormones and insulin[b,c] 2) Increased IGF-I response[e]
4	Acetyl CoA carboxylase[d] aP2 fatty acid synthase[k] Hormone sensitive lipase[b] Malic enzyme[i] α-glycero-3-phosphate dehydrogenase[c]	1) DNA synthesis and clonal expansion[e] 2) Emergence of late markers
5	Phosphoenol pyruvate carboxykinase[l] Adipsin[f] Leptin[o]	1) Mature adipose cell 2) Emergence of very late markers 3) Lipid accumulation

This scheme is based on data obtained from 3T3-L1, 3T3-F442A, Ob17 and Ob1771 cells as well as with rodent adipose precursor cells; a) Abumrad *et al*, 1991, b) Kawamura *et al*, 1981, c) Wise and Green, 1979, d) Park and Kim, 1991, e) Ashcom *et al*, 1992, f) Cook *et al*, 1985, g) Doglio *et al*, 1987, h) Amri *et al*, 1986, i) Spiegelman *et al*, 1983, j) Kurten *et al*, 1988, k) Paulauskis and Sul, 1988, l) Dani *et al*, 1988, m) Amri *et al*, 1995 and n) Hwang *et al*, 1996.

Table 1.1) A Selection of Differentiation-dependent Genes Expressed by Adipocytes (N.D.= Not Determined)

mRNA Size (Kbp)	Protein Gene-product	Protein Size (KDa)	mRNA	Transcriptional Activity	Cell Type	Reference
2.15	Actin	42.0	-	-	3T3-F442A Ob1771	Cook *et al.* 1988
0.65	aP2	13.0 14.6	+ + +	+ + +	3T3-F442A 3T3-L1 Ob1771	Cook *et al.* 1988
1.075	Adipsin	28.0	+	+	3T3-F442A	Cook *et al.* 1988
N.D.	Aldolase	40.0	+	+	3T3-F442A	Cook *et al.* 1988
8.2	Fatty acid synthase	250.0	+	N.D.	3T3-L1	Paulauskis and Sul. 1988
1.4	Glyceraldehyde 3-phosphate dehydrogenase	37.0	+	N.D.	3T3-F442A	Doglio *et al.* 1986
3.5	αGlycero-3-phosphate dehydrogenase	34.0	+ +	+ +	3T3-F442A Ob1771	Cook *et al.* 1985
0.8, 1.5, 7.5, 15.0	Insulin-like growth factor	N.D.	+	+	Ob1771	Doglio *et al.* 1987
N.D.	Lactate dehydrogenase	35.0	+	N.D.	3T3-F442A	Spiegelman and Green. 1986
2.8	Phosphoenol pyruvate carboxykinase	N.D.	+ +	+ +	3T3-F442A Ob1771	Dani *et al.* 1986
4.0	Lipoprotein lipase	N.D.	+	N.D.	TAI	Kurten *et al.* 1988
1.8, 2.2, 3.2	Cyclic AMP-dependent protein kinase	N.D.	+	N.D.	TAI	Kurten *et al.* 1988
4.9	Stearoyl-CoA desaturase	N.D	+	+	3T3-L1	Cook *et al.* 1985
1.75	Tubulin (α)	N.D.	-	N.D.	3T3-F442A	Amri *et al*, 1985
5.0 pGH3	Unidentified	N.D.	+	N.D.	Ob1771	Amri *et al*, 1985

(Ailhaud *et al,* 1989). Commitment is characterised by the emergence of mRNAs for the α2 chain of type 2 collagen and LPL (Amri *et al*, 1986). In addition, there is an increase in selective long chain fatty acid uptake and mRNA for putative mediators of the transcriptional effects of fatty acids (Abumrad *et al*, 1991; Amri , 1995). At this stage mitogens, such as platelet derived growth factor (PDGF), can block adipose conversion by stimulating cell-cycle re-entry (Corin *et al*, 1990). Therefore, progression to the terminal stages of differentiation requires that G0 cells become refractory to the action of competence factors for mitogenesis (ie PDGF) (Corin *et al*, 1990). This is achieved by the induction of a reversible anti-mitogenic state in growth arrested cells by growth hormone (GH) (Corin *et al*, 1990). G0 cells treated with GH enter a primed state (Gp) in which they become responsive to the promoters of terminal differentiation.

The mechanisms by which mitogens, such as PDGF, and anti-mitogens, such as GH, control cell cycle entry and exit are not yet fully understood but probably involve modulation of G1-phase regulators (Sherr, 1994). Key regulators of G1 progression in mammalian cells include three D-type cyclins (D1, D2 and D3), the expression of which is greatly increased when re-entering S-phase (reviewed by Sherr, 1994). Neutralisation of cyclin D in cells micro-injected with anti-cyclin D antibodies causes cell growth arrest during the G1-phase (Ohtsubo and Roberts, 1993). Cyclin D expression is suppressed in 3T3-F442A preadipocytes (Timchenko *et al*, 1996) treated with GH which may contribute to the priming action of GH in these cells (Baldin *et al*, 1993).

1.5.2) Late Events

The process of terminal differentiation is characterised by the induction of late and very late markers, including the enzymes responsible for lipogenesis and triglyceride synthesis, which result in the characteristic lipid droplet accumulation associated with full differentiation (Table 1.2). Although the degree and time course of induction vary among these enzymes, in general the increase in activity is due to a corresponding increase in the synthesis of the respective catalytic subunit (Wise *et al*, 1984). In contrast, synthesis and assembly of major cytoskeletal proteins, such as actin and tubulin, decrease as cells change in morphology (Spiegelman and Farmer, 1982).

The expression of late and very late markers is associated with limited growth resumption of primed (Gp), early marker-expressing cells (Kuri-Harcuch and Marsch-Moreno, 1983; Schmidt *et al*, 1987; Zezulak and Green, 1986). At least one cell doubling has been consistently observed by various investigators using different cell

lines and different culture media, and this process of clonal amplification of committed cells (defined as post-confluent mitoses) is limited both in magnitude and duration (Ailhaud *et al*, 1989; Gamou and Shimizu, 1986; Kuri-Harcuch and Marsch-Moreno, 1983). Clonal expansion of primed preadipose cells appears to be essential for terminal differentiation (defined by the emergence of GPDH activity) and is stimulated by IGF-I and/or insulin (active at least in part by binding to the IGF-I receptor) (Hauner, 1990; Schmidt *et al*, 1987; Zezulak and Green, 1986). These observations made *in vitro* are in agreement with those made *in vivo* by Pilgrim (1971) and Cook and Kozac (1982) concerning the relationships in rodent adipose tissue between cell proliferation and differentiation. As clonal expansion slows the expression of proteins that give rise to the adipocyte phenotype is initiated (Table 1.2). Among the first of these proteins to be expressed is C/EBPα, a nuclear DNA-binding protein that has been implicated in the coordinate transcriptional activation of adipose-specific genes (reviewed by Vasser-Cognet and Lane, 1993; see also section 1.6). In addition, C/EBPα is anti-mitogenic (Tang *et al*, 1995) and has been implicated in the termination of clonal expansion and maintenance of the terminally differentiated cell (Freytag and Geddes, 1992).

1.6) TRANSCRIPTIONAL CONTROL OF PROTEIN EXPRESSION DURING PREADIPOCYTE DIFFERENTIATION

The promoters of many adipocyte specific genes have now been characterised and it is becoming increasingly evident that a number of common features are important for coordinating changes in their transcriptional activity during preadipocyte differentiation. Two families of transcription factors, the C/EBP (CCAAT/enhancer binding protein) family and the PPAR (peroxisome proliferator activator receptor) family (Amri *et al*, 1995; Tontonez *et al*, 1994a) are known to interact with the promoter elements of a number of the best characterised adipocyte genes (Tontonez *et al*, 1995). The temporal and hormonal regulation of transcriptional regulators, such as C/EBPs and PPARs, are thought to contribute critically to the coordination of the adipocyte program (Fig 1.4).

1.6.1) Expression of Transcription Factors During Adipogenesis

Three PPAR genes, PPARα (Gearing *et al*, 1994), PPARδ (Schmidt *et al*, 1992) and PPARγ (Tontonez *et al*, 1994a Zhu *et al*, 1993) encode different members of the superfamily of orphan nuclear hormone receptors. PPARγ1 and PPARγ2 are two splice variants of the PPARγ gene which are abundantly expressed in white adipose tissues (Vidal-Puig *et al*, 1996; Zhu *et al*, 1995). Expression of PPARγ1 mRNA is detectable in the stromal fraction of the tissue, presumably in undifferentiated preadipocytes, whereas both PPARγ1 and PPARγ2 are expressed in mature adipocytes (Vidal-Puig *et al*, 1996). PPARγ1 and γ2 expression in murine white adipose tissue is down-regulated during fasting, whereas a high-fat diet induces expression (Vidal-Puig *et al*, 1996). Both PPARγ isoforms are induced very early in several cell culture models of adipogenesis (Amri *et al*, 1995; Chawla *et al*, 1994) and ectopic expression of PPARγ1 or PPARγ2 in fibroblasts is sufficient to drive the determination of an adipocyte cellular lineage (Tontonez *et al*, 1994b). Taken together, these observations suggest that both PPARγ1 and PPARγ2 might play an important role in the initiation or determination of the adipocyte program *in vivo*. C/EBPα, while expressed in many tissues *in vivo*, is also induced during adipogenesis, albeit later in the time-course than PPARγ (Christey *et al*, 1989). Forced expression of C/EBPα can trigger differentiation in preadipocytes and cause adipogenesis in several fibroblastic cell types (Freytag *et al*, 1994). When C/EBPα mRNA is expressed at, or near fat cell levels, it powerfully synergises with PPARγ to stimulate adipogenesis (Tontonez *et al*, 1994b). The important role of C\EBPα in adipogenesis was confirmed in mice in which the C\EBPα gene was disrupted by homologous recombination (Wang *et al*, 1995); lipid accumulation was found to be severely retarded in adipose tissue from mutant mice.

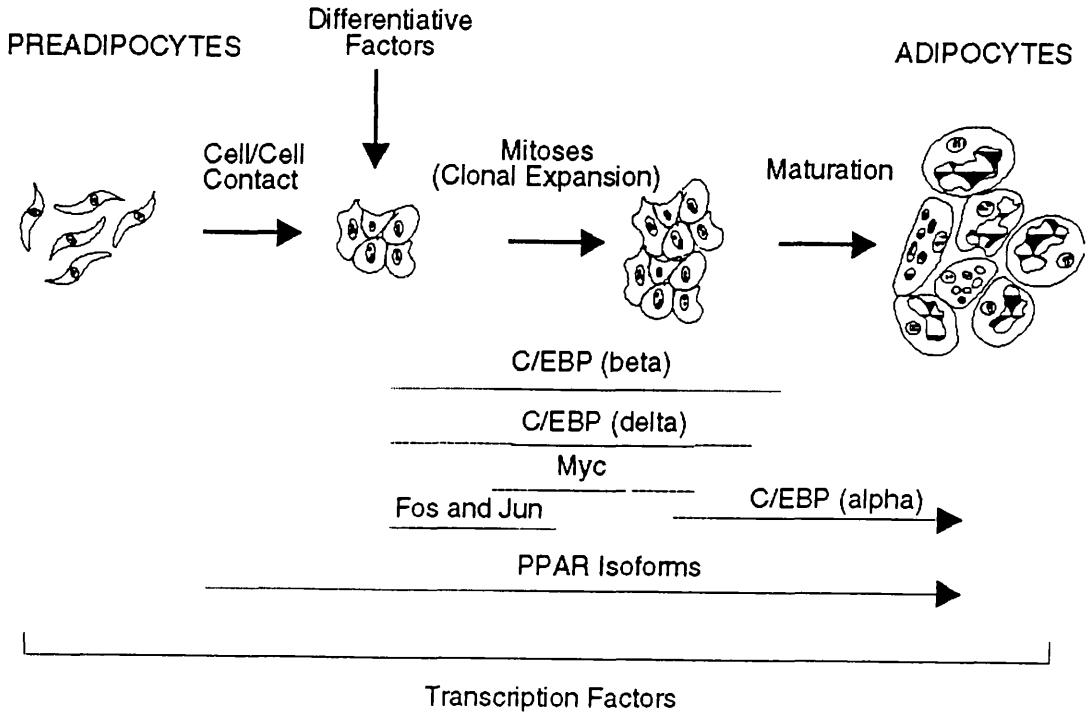


Fig 1.4) Expression of Transcription Factors During Adipocyte Development

This scheme is based upon data with 3T3-L1 and 3T3-F442A adipose precursor cells. The acquisition of the adipocyte phenotype is thought to be regulated and maintained by adipogenic transcription factors such as the CCAAT enhancer binding proteins (C/EBP) α and peroxisome proliferator activator receptor (PPAR) isoforms. The cytoplasmic signals controlling the induction of these transcription factors in differentiating preadipocytes are initiated by extracellular differentiative factors. The nuclear signal is propagated by *trans*-acting modulators of transcription, such as c-*fos*, c-*jun*, c-*myc* and the C/EBPs β and γ, which are expressed transiently during the time-course of differentiation.

1.6.2) *Trans*-activation of Adipocyte-specific Genes

In addition to C/EBPα, C/EBP β and δ are suggested to play an important transient role in early adipocyte differentiation by relaying the effects of hormonal stimulants such as glucocorticoids, GH, insulin and stimulators of the cyclic AMP signalling pathways (Cao *et al*, 1991; Clarkson *et al*, 1995; Yeh *et al*, 1995b). In fact, it was recently demonstrated that C/EBPβ induces PPARγ expression in the preadipocyte, subsequently triggering differentiation (Wu *et al*, 1995). C/EBPβ and δ are also thought to be involved in the *trans*-activation of the C/EBPα gene (Lane *et al*, 1996).

A dramatic increase in C/EBPβ and δ early in preadipocyte differentiation is preceded by a transient increase in the expression of several immediate-early genes [eg c-*fos*, c-*jun* and c-*myc* (Stephens *et al*, 1992; Stone *et al*, 1990)]. Homo- and heterodimers of c-*jun* and c-*fos* were the first proposed regulators of adipocyte-specific gene expression (Spiegelman *et al*, 1988). These protein complexes were found to bind to AP-1 consensus sequences in the adipocyte-specific fatty acid binding protein (aP2) gene thereby reducing transcriptional activity (Spiegelman *et al*, 1988). Transcription of c-*fos* and c-*jun* is rapidly induced by GH in both 3T3-F442A (Gurland *et al*, 1990) and Ob1771 (Barcellini-Couget *et al*, 1993) preadipocytes which coincides with the GH-induced expression of C/EBPβ and δ (Clarkson *et al*, 1995). In 3T3-L1 preadipocytes both insulin and IGF-I induce expression of c-*fos* (Weiland *et al*, 1991). Furthermore, c-*fos* plays an intermediary role in the modulation of LPL gene expression (Barcellini-Couget *et al*, 1993). Therefore, it appears that c-*fos* and c-*jun* may relay specific "temporal cues" from extracellular stimuli to adipocyte-specific genes. However the complex role that c-*fos* and c-*jun* play in controlling gene expression during differentiation is, as yet, not completely understood.

C-*myc* is involved in the regulation of cellular proliferation (reviewed by Kato and Dang, 1992) and its induction during preadipocyte differentiation accompanies post-confluent mitoses (Christy *et al*, 1991). Following clonal expansion, c-*myc* expression decreases concomitant with transcriptional activation of the C/EBPα gene (Christy *et al*, 1991). The C/EBPα promoter contains a consensus c-*myc* binding site (Christy *et al*, 1991), that can bind c-*myc in vitro* (Legraverend *et al*, 1993), consequently the induction of C/EBPα towards the end of clonal expansion may involve the binding of c-*myc* to its cognate site in the C/EBPα promoter. The C/EBPα promoter also possesses a C/EBP binding site (Christy *et al*, 1991) that apparently mediates *trans*-activation by its own gene product and probably contributes to the maintenance of steady-state levels of C/EBP in the mature adipocyte (Legraverend *et al*, 1993; Lin *et al*, 1993a).

In addition to its role in the termination of clonal expansion, C/EBPα is known to be involved in the transcriptional activation of a number of adipocyte specific genes. Following the expression of C/EBPα transcriptional activation of a large number of adipocyte genes occur (Table 1.1). Many of these genes, including the *ob* gene-product leptin, are *trans*-activated by C/EBPα (Table 1.3). The exact number of genes regulated by C/EBPα during differentiation remains unknown but is estimated to be large. Similarly, PPARγ has been implicated in mediating the expression of fat-specific genes, including aP2 (Tontonoz *et al*, 1994a) and phosphoenolpyruvate carboxykinase (Tontonoz *et al*, 1995).

Table 1.3) Adipocyte-specific Genes *Trans*-activated by CCAAT/Enhancer Binding Protein α During Preadipocyte Differentiation

Gene Product	Reference
Angiotensinogen	Brasier *et al*, 1990
Fatty Acid binding Protein (aP2)	Christy *et al*, 1989
Steroyl CoA Desaturase	Christy *et al*, 1989
GLUT 4	Kaestner *et al*, 1990
Phosphoenolpyruvate Carboxykinase	Park *et al*, 1993
Insulin Receptor	McKeon and Pham, 1991
Leptin	Hwang *et al*, 1996

In summary, many of the mechanisms which regulate adipocyte differentiation are beginning to be elucidated. However, a great deal of work is still required to delineate the exact roles of individual DNA elements and transcription factors. The identification of adipocyte-specific elements and the factors which regulate them is clearly a fundamental requirement for a complete understanding of the process.

1.7) HORMONAL CONTROL OF DIFFERENTIATION

The process of cell differentiation is controlled by communication between individual cells or between cells and the extracellular microenvironment. Molecules which mediate this communication include the classic diffusible growth factors that act *via* specific external receptors to transduce external signals through a cascade of intracellular events (Johnson and Vaillancourt, 1994). The precise combination of hormones and growth factors are not known and likely to vary amongst different cell types. Most of the studies on the role of adipogenic factors (i.e. factors which provide a single differentiative stimulus) have employed preadipocyte cell lines (section 1.4). Identification of factors that affect adipocyte differentiation involves the addition of various hormones and pharmacological agents and determination of the extent of differentiation. This is judged by staining for accumulated lipid, measuring activation of lipogenic enzymes, such as α-glycero-3-phosphate dehydrogenase, or by measuring the expression of adipocyte specific genes. Early studies were performed on cells differentiated in the presence of serum which made interpretation of results difficult. Several laboratories have developed chemically defined media (Guller *et al*, 1988; Hauner 1990; Schmidt *et al*, 1990) and have implicated GH, glucocorticoids, triiodothyronine (T_3), cyclic AMP-elevating agents, insulin and IGF-I as possessing adipogenic activities.

Clearly a great deal of information is required to enable us to understand the molecular mechanisms which allow these factors to control differentiation and permit the cell to assume the metabolic functions of a mature adipocyte. Inevitably, no single signalling event or molecular change can alone account for the differentiation process, but rather many such events will control this complex process. What is apparent is that signal-transduction cascades control transcription of both immediate and delayed early-response genes which ultimately leads to coordinated changes in the transcription of differentiation associated gene networks (Johnson and Vaillancourt, 1994). The signal transduction cascades controlling these responses often utilise sequential protein-kinase reactions such as the protein kinase A cascade (section 1.7.3.3), or those initiated at the GH and insulin receptors (section 1.7.2.4). Alternatively, some hormones interact directly with nuclear receptors containing transcriptional modulatory domains (section 1.7.4).

1.7.1) Agents which Signal *via* Nuclear Receptors

1.7.1.1) Triiodothyronine (T_3)

It has been known for some time that blood levels of thyroid hormones can affect adipose tissue development; hyperthyroidism provokes an increase in the cellularity of adipose tissue during development, whereas hypothyroidism exerts the opposite effect (Levacher *et al*, 1984; Levacher *et al*, 1985; Picon and Levacher, 1979). In addition, it has been suggested that the hyperplasia in white adipose tissue, provoked by thyroid hormone, resulted from an earlier differentiation of preadipocytes and not from an increased preadipocyte multiplication (Levacher *et al*, 1984).

Little is known about thyroid hormone action on adipose cell lines. T_3 appears essential for the differentiation of Ob17 cells in both serum-supplemented and serum-free conditions (Grimaldi *et al*, 1982), where it provokes an increase in lipogenic enzyme activities (Gharbi-Chchi *et al*, 1983). Conversely, in 3T3-F442A cells, it appears that T_3 does not act as an adipogenic factor by itself, but may rather act in synergy with true adipogenic factors (Flores-Delgado *et al*, 1987). This is the case in cultured porcine preadipocytes where T_3 acts synergistically with insulin to promote differentiation (Hausman, 1989). Consistent with its regulatory role in lipid metabolism *in vivo* (Czech *et al*, 1980; Mariash *et al*, 1975), T_3 has been shown to act during preadipocyte differentiation to induce the expression of many proteins involved in the regulation of lipid metabolism, including fatty acid synthase, GPDH, malic enzyme and the β_3-adrenoceptor (Flores-Delgado *et al*, 1987; Hadri *et al*, 1996; Hausdorf *et al*, 1988; Moustaid and Sul, 1991). In this respect, T_3 is thought to exert its effects transiently, during the early stages of terminal differentiation, by stimulating *de novo* fatty acid synthesis and triglyceride accumulation (Ailhaud, 1982; Levacher and Picon, 1989;).

1.7.1.2) Glucocorticoids

Steroid hormones are able to influence body fat mass by their regulation of metabolism in adipocytes. In rats the triglyceride amounts in adipocytes is decreased by injection of the synthetic glucocorticoid dexamethasone (DEX; Steingrimsdottir *et al*, 1980) probably by directly regulating the lipolytic pathway (Lai *et al*, 1982). Paradoxically, glucocorticoids in chronic excess, such as in Cushing's syndrome associated with hyperinsulinemia, is associated with fat depot-specific increases in fat mass (Rebuffé-Scrive *et al*, 1985). The role of glucocorticoids in adipogenesis has received little attention, but a small number of studies on different preadipocyte cell types have also produced conflicting results. DEX addition after confluence and in the presence of

insulin has been shown to increase cellular differentiation of 3T3-L1 preadipocytes (Rubin *et al*, 1978; Schivek and Löffler, 1987) and primary cultured adipose precursors of rats (Xu and Björntorp; 1990) and humans (Hauner *et al*, 1989). Inhibitory effects of DEX on terminal differentiation have been reported on 3T3-F442A preadipocytes (Pairault and Lasnier, 1987) and insulin counteracted the DEX effects here. From studies such as these it is becoming increasingly apparent that the different results obtained *in vitro* are due to the varying conditions employed, namely the type of medium used, the time-point of DEX addition and the presence of insulin (Xu and Björntorp, 1990). Differential effects of glucocorticoids on cell-types from different anatomical locations, both *in vivo* and *in vitro*, may also explain discrepancies between various reports.

1.7.1.3) Fatty Acids and Retinoids

In recent years it has become clear that fatty acids and retinoids act through related nuclear receptors to directly regulate gene expression (see section 1.7.1.4). Adipose tissue represents the major storage organ for triglyceride in the body (Angel *et al*, 1974). Furthermore, studies on rat adipose depots have shown that serum retinol-binding protein and cellular retinol-binding protein are expressed at high levels, strongly suggesting that adipose tissue is also a major tissue for retinol storage (Okino *et al*, 1995). Given this abundant source of putative transcriptional regulators, it is likely that these factors should play a predominant modulatory role in the adipose conversion of preadipocytes *in vivo*. Indeed, fatty acids and retinoids at very low concentrations co-operate synergistically to promote the terminal differentiation of Ob1771 preadipose cells (Safonova *et al*, 1994a) and co-regulate 3T3-L1 preadipocyte proliferation, differentiation and survival (Chawla and Lasar, 1994). Fatty acid treatment of preadipocytes induces expression of several genes encoding proteins implicated in fatty acid metabolism. These include the adipocyte lipid-binding protein, aP2 (Distel *et al*, 1992), the acyl CoA synthase (Amri *et al*, 1991), the acetyl CoA synthase (Amri *et al*, 1991) and a recently cloned membrane protein involved in fatty acid binding and transport (Abumrad *et al*, 1993). Similarly, whereas retinoids are ineffective on the expression of early marker genes they are extremely effective in inducing late markers of preadipocyte differentiation (Safonova *et al*, 1994b).

1.7.1.4) Nuclear Hormone Receptors

Critical to the signalling of retinoids, fatty acids, thyroid and glucocorticoid hormones is a group of related nuclear hormone receptors (Corsa-Junica, 1990; Evans, 1988; Green and Chambon, 1988). Studies on the specific receptors for retinoids,

glucocorticoid and thyroid hormones have revealed that each receptor undergoes a structural alteration, or "transformation", upon hormone binding, which in turn enables DNA binding (Carlstedt-Duke *et al*, 1982; Wrang-Ökret, *et al*, 1984). Transcriptional regulation by active nuclear receptors is mediated by direct interaction with specific *cis*-acting DNA sequences, or DNA-protein complexes, in the promoter regions of target genes (Evans 1988; Karin *et al*, 1984).

These sequences, or hormone response elements (HREs), function in a position- or orientation-independent fashion and thus behave like transcriptional enhancers (Chandler *et al*, 1983). For example, a glucocorticoid response element sequence can be found in the aP2 gene of 3T3-L1 preadipocytes, which may be responsible for transcriptional induction by DEX during adipose conversion (Cook *et al*, 1988). Typically, HREs have an apparent dyad symmetry which suggests that they interact with receptor dimers. This is true of fatty acid-activated nuclear receptors, such as PPARγ2, which form heterodimeric complexes with retinoid receptors which can then bind corresponding HREs in adipocyte-specific genes (Amri *et al*, 1995; Tontonez *et al*, 1994). Despite our current understanding of nuclear hormone receptor action, we are still far from discerning how multiple receptor isoforms interact to coordinate gene network expression during such a complex biological phenomenon as adipogenesis.

1.7.2) Peptide Growth Factors and Hormones

1.7.2.1) Growth Hormone (GH)

Pituitary GH regulates the number of fat cells in both man and animals. The findings that GH-deficient children have less fat cells than normal children, and that hormonal replacement in these children shifts adipose tissue cellularity towards normal, indicate that GH is important for the development of the human fat cell complement (Wabitsh and Heinze, 1993). In addition, patients with hypopituitary dwarfism have a reduced number of adipose cells, even though fat cell mass is large, whereas hypophysectomised rats, when administered with GH, develop reduced fat cell mass but increased cellularity (Lindahl *et al*, 1991). Moreover, treatment of rats with antiserum to rat GH results in a reduction in adipocyte numbers in a depot specific manner (Flint and Gardner, 1993). A direct permissive role for GH in the differentiation process has been demonstrated in Ob17, $10T^1/_2$ and, under serum free conditions, in 3T3-F442A and 3T3-L1 preadipocytes (Grimaldi *et al*, 1984; Guller *et al*, 1988; Morikawa *et al*, 1982; Nixon and Green, 1983; Schmidt *et al*, 1990).

The exact mechanism by which GH exerts its adipogenic effects in preadipocytes is far from being understood. It has been shown that GH induces a rapid and transient stimulation of c-*fos* gene transcription in Ob1771 cells (Doglio *et al*, 1989) and c-*fos* and c-*jun* in 3T3-L1 and 3T3-F442A cells (Sumantran *et al*, 1992). This coincides with transcriptional activation of C/EBPδ and translational activation of C/EBPβ, which may contribute to the initiation of the differentiation program (Clarkson *et al*, 1995; section 1.6). However, part of the GH effect could be attributed to IGF-I, since GH has been shown to induce IGF-I gene expression in Ob1771 (Doglio *et al*, 1987) and rat preadipocytes (Wabitsch *et al*, 1996) and to increase sensitivity of 3T3-F442A cells to the action of IGF-I (Zezulak and Green, 1986). IGF-I, in turn has been reported to promote adipose conversion of clonal cell lines (Guller *et al*, 1989; Smith *et al*, 1988). In rat preadipocytes, GH-induced IGF-I production stimulates a round of cell division during differentiation (Wabitsch et al, 1996). This observation is in full agreement with the dual effector theory of Green *et al* (1985) which was originally devised to explain the differentiation of 3T3-F442A preadipocytes in culture. According to this theory, GH first directly stimulates adipocyte differentiation, then the number of young, differentiated cells is increased by limited multiplication (clonal expansion) mediated by the mitotic effects of IGF-I.

Quite often, the net effect of adding GH to cultures of differentiating preadipocytes is a reduction in the proportion of newly formed adipocytes and late markers of differentiation (Hausman and Martin, 1989; Wabitsch *et al*, 1996). The inhibitory effect of GH on preadipocyte differentiation is due to disturbance of glucose and lipid metabolism both during the differentiative phase and in the mature fat cell (Fain *et al*, 1985; Goodman *et al*, 1990; Schwartz and Carter-Su, 1988; Wabitsch *et al*, 1996). Therefore the effect of GH on preadipocyte differentiation appears to be differential. GH acts at an early stage to prime preadipocytes and contributes to their expansion by inducing local production and secretion of IGF-I. At later stages the actions of GH aim generally at reducing the lipid content of adipocytes, thereby limiting adipose tissue size. The biphasic action of GH on adipogenesis is certainly not unique. The accumulated evidence from a number of sources suggests that EGF (section 1.7.2.3), glucocorticoids (section 1.7.1.2) and cyclic AMP (section 1.7.3.1) may also exert stage-specific, differential effects.

1.7.2.2) Insulin

Developing adipocytes became highly responsive to insulin. The increase in insulin responsiveness has been attributed to an increase in insulin receptor number (Rubin *et al*, 1978) and to increased expression of proteins involved in metabolic control which

serve as an end-point for regulation by insulin eg GLUT 4 and ACC (Park and Kim, 1991). Insulin is required for the adipocyte differentiation of 3T3-F442A, 3T3-L1 and Ob17 cells (Doglio *et al*, 1986; Smith *et al*, 1988; Guller *et al*, 1988). It is thought not to be involved in commitment to differentiate (Steinberg and Brownstein, 1982), but as an enhancer of lipid synthesis in previously committed cells, thereby promoting the characteristic lipid filling of immature adipocytes (Smith *et al*, 1988). In this respect, insulin acts as a positive modulator in the expression of late enzyme markers involved in triglyceride metabolism, such as phosphoenolpyruvate carboxykinase and α-glycero-3-phosphate dehydrogenase (Dani *et al*, 1986). Furthermore, in 3T3-F442A adipocytes, insulin has been shown to increase the expression of PPARγ1 and γ2 and regulate the transcription of C/EBPs (MacDougald *et al*, 1995; Puig *et al*, 1996). This raises the intriguing possibility that insulin serves to modulate the activity of these critical adipogenic transcription factors during the time-course of preadipocyte differentiation.

To optimise adipose conversion in preadipocyte cell systems insulin is often used at supraphysiological concentrations where effects may be mediated via the IGF-I receptor (Guller *et al*, 1988). Furthermore, the addition of IGF-I to preadipocyte cell lines (Schmidt *et al*, 1990; Smith *et al*, 1988) and to preadipocytes in primary culture (Deslex *et al*, 1987; Hausman, 1989; Nougués *et al*, 1993) stimulates adipogenesis *in vitro*. It has recently been demonstrated that rat adipose tissue *in vivo* abundantly expresses IGF-I in addition to IGF-binding proteins (Peter *et al*, 1993), which are believed to control IGF bioavailability and thus modulate IGF action (McCusker and Clemmons, 1992; Shimasaki and Ling, 1992). Therefore, there may exist a subtle interplay between the insulin and IGF-I receptors to modulate the maximal expression of terminal differentiation.

1.7.2.3) Epidermal Growth Factor (EGF)

The peptide growth factor EGF has been reported as being one of the growth factors, such as PDGF (Fig 1.3; Ringold *et al*, 1988), to inhibit the entry of preadipocytes into a program of *in vitro* differentiation (Corin *et al*, 1990; Serraro, 1987). Moreover, administration of EGF to neonatal rats suppresses body weight gain and carcass fat deposition due to an increase in the number of mature adipocytes (Serrero and Mills, 1991). This is contrary to the finding that EGF is required for the maintenance of 3T3-F442A and 3T3-L1 preadipocyte differentiation (Guller *et al*, 1988; Schmidt *et al*, 1994) and that elevated EGF levels are a causative factor for adipocyte hypertrophy in obese mice (Kurachi *et al*, 1993). These apparent paradoxes can be explained by the fact that EGF shows sequential changes in its effects during preadipocyte differentiation

(Adachi *et al*, 1994). EGF has been shown to promote the insulin-supported accumulation of triglycerides in primed preadipocytes, however at an earlier stage EGF inhibits the adiposity of preadipocytes by enforcing cell-cycle re-entry (Fig 1.3; Adachi *et al*, 1994). A possible explanation for the potentiating action of EGF on lipid accumulation in differentiating preadipocytes is that EGF reduces catecholamine-mediated lipolysis (Taber *et al*, 1993).

1.7.2.4) Growth Hormone (GH) and Insulin Signalling Cascades

Given the critical roles of GH and insulin in promoting and maintaining adipogenesis (sections 1.7.2.1 and 1.7.2.2) it is apparent that elucidation of their respective signalling mechanisms will be critical to understanding the mechanisms of action of these hormones. Early transduction events appear to be common to both the GH and insulin receptors and involve tyrosine phosphorylation of several cellular proteins, including the so-called insulin-receptor substrate (IRS-I) (Anderson, 1992; Souza *et al*, 1994; Sun *et al*, 1991; White *et al*, 1985; White *et al*, 1987). Unlike the insulin receptor (Kasug *et al*, 1983), the GH receptor lacks intrinsic tyrosine kinase activity (Leung *et al*, 1987), but instead stimulates the activity of JAK-2, a tyrosine kinase that associates with the cytoplasmic domain of the activated GH receptor (Argetsinger *et al*, 1993). Putative substrates for JAK-2 in 3T3-F442A preadipocytes include 91 kDa (Stat 91) and 84 kDa components of the ISGF3 transcription factor complex that, when tyrosine phosphorylated, migrate to the nucleus and participate in the activation of gene transcription (Kilgour and Anderson, 1994; Sadowski *et al*, 1993).The precise consequences of ISGF3 activation by GH in terms of preadipocyte differentiation remain to be investigated.

A further point of divergence between the GH and insulin signalling pathways is at the level of protein kinase C (PKC; Clemens *et al*, 1992). Insulin is not thought to activate this pathway, however in Ob1771 preadipocytes GH has been reported to stimulate diacylglycerol (DAG) production, by means of phosphatidylcholine breakdown, leading to PKC activation (Catalioto *et al*, 1990; Catalioto *et al*, 1992). Activation of the PKC pathway in Ob1771 preadipocytes is thought to contribute to the adipogenic actions of GH in this cell type (Catalioto *et al*, 1992).The mechanism by which GH stimulates production of DAG from phosphatidylcholine is not known, but a pertussis-toxin sensitive phospholipase C activity, specific for glycerophospholipids other than phosphoinositol, has been implicated (Catalioto *et al*, 1990), Down-stream targets of GH-activated PKCs may include the mitogen activated protein (MAP) kinase cascade (Adams and Parker, 1991; Anderson 1992) and/or the phosphorylation of components of the transcriptional and translational machinery (Boyle *et al*, 1991; Morley *et al*,

1991). These effects are likely to result in changes in gene expression at both the transcriptional and translational levels.

Recently it has emerged that activation of the MAP kinase cascade (Fig 1.5), a signalling pathway used by mitogenic and differentiative agents, is absolutely required for the adipose conversion of 3T3-L1 preadipocytes (Sale *et al*, 1995). Here, tyrosine phosphorylation of the receptor, or receptor-associated molecule (eg JAK-2 or IRS-I), directs the association of transducing molecules *via* phosphotyrosyl-binding domains, termed SH2 and SH3 (Pawson and Gish, 1992). The association of adapter proteins, such as Grb2, ultimately leads to activation of the small GTP-binding protein Ras (Buddy and Downward, 1993). Ras then triggers a protein kinase cascade, involving the sequential activation of two kinases Raf and MEK (Kyriakis *et al*, 1992), leading to activation of MAP kinase by threonyl- and tyrosyl- phosphorylation (Ray and Sturgill, 1988). Activated MAP kinase can translocate into the nucleus where it targets a range of substrates including the nuclear transcription factor $p62^{TCF}$, having important implications for the regulation of gene expression (Hill and Treisman, 1995). Both GH and insulin have been shown to activate the MAP kinase pathway (Anderson, 1992; Ray and Sturgill, 1988) but the role of these events in the adipogenic actions of these hormones is not defined. Since MAP-kinases and other elements in this pathway exist in multiple isoforms, it will be necessary to clarify which isoforms are targeted by which hormone.

Other considerations, such as time of activation and subcellular localisation, are also likely to be important (Marshall, 1995). The duration of MAP kinase activation has been shown to be a determining factor in the decision of cells to differentiate or proliferate (Marshall, 1995); sustained activations provoke nuclear translocation of MAP kinases and cellular differentiation, whereas transient activations stimulate cell cycle re-entry. The duration of MAP kinase activation can be modulated, either positively or negatively, by regulatory "cross-talk" with other signalling pathways, such as the cyclic AMP cascade (Malarkey *et al*, 1995; Marshall,1995). Given the importance of cyclic AMP in transducing differentiative signals (section 1.7.3.1), it is plausible that interactions with crucial growth cascades, such as the GH- and insulin-stimulated MAP kinase pathways, could contribute to the effects of cyclic AMP on preadipocyte differentiation.

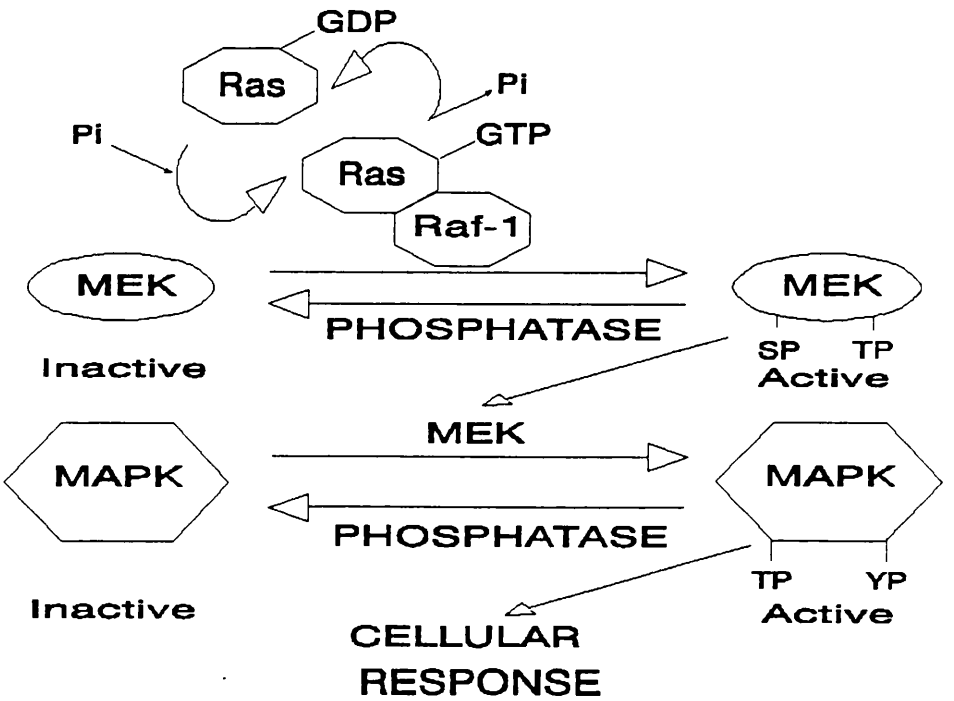


Fig 1.5) Immediate Regulation of the Mitogen Activated Protein (MAP) Kinase Cascade
The seryl/threoyl kinase Raf is activated by association with the G-protein Ras. Raf phosphorylates and activates the dual specificity threonyl/tyrosyl kinase MEK which phosphorylates and activates MAP kinases (MAPK). Potential sites for regulation by phosphatases are also shown.

1.7.3) Cyclic AMP

The actions of many hormones and neurotransmitters are mediated at the cellular level by cyclic AMP. The intracellular concentrations of cyclic AMP are regulated by adenylate cyclase and cyclic nucleotide phosphodiesterases, enzyme systems responsible for the formation and destruction of cyclic AMP (Fig 1.6; Gilman, 1987). Stimulatory and inhibitory receptors transmit extracellular stimuli *via* stimulatory (G_s) or inhibitory (G_i) guanine nucleotide binding proteins (G-proteins) to the catalytic unit of adenylate cyclase, which catalyses the formation of cyclic AMP from ATP (Vaughan and Moss, 1985). Several adenylate cyclase isoforms have been identified and characterised and it appears that all are membrane proteins (Bakalayer and Reed, 1990). Cyclic nucleotide phosphodiesterases (PDEs) convert cyclic nucleotides to their respective 5' mononucleotides and represent the only known pathways for the destruction and metabolism of cyclic nucleotides. The PDEs comprise a complex group of enzymes, multiple forms of which have been isolated from various tissues and cell-types (Beavo and Reifsnyder, 1990).

1.7.3.1) Cyclic AMP-elevating Agents

β-adrenoceptors are integral transmembrane receptors that mediate the effects of catecholamines secreted from the adrenal medulla and postganglionic sympathetic neurones (Lefkowitz, 1976). Receptor occupancy by catecholamines allows the β-adrenoceptor to couple to the G_s protein complex which promotes cyclic AMP production by adenylate cyclase (Fig 1.6). In turn, cyclic AMP exerts a key role in processes such as cellular growth, differentiation and adaptations to environmental conditions (Pastan *et al*, 1975). These processes are regulated in a tissue- and/or cell-specific manner (Pastan *et al*, 1975). Thus in white adipose tissue it is well documented that, essentially through the β-adrenoceptors, cyclic AMP has pleiotropic effects in the control of adipocyte differentiation and metabolism. In white adipose cell lines (Gaillard *et al*, 1989; Schmidt *et al*, 1990) or in the primary culture of adipose precursors (Björntorp *et al*, 1980; Wiederer and Löffler, 1987), β-agonists or cyclic AMP are well-known triggers of adipose conversion. *In vivo*, local denervation of white adipose lead to a marked hyperplasia of adipocyte precursors demonstrating that catecholamines exert an anti-mitogenic (ie pro-differentiative) influence on early-marker expressing preadipocytes (Cousin *et al*, 1993).

In addition to adrenergic regulation, adipose tissue may also influence its own adiposity through autocrine mechanisms by local production of cyclic AMP-elevating agents (Shilabeer *et al*, 1989). Angiotensin is a locally produced peptide secreted by

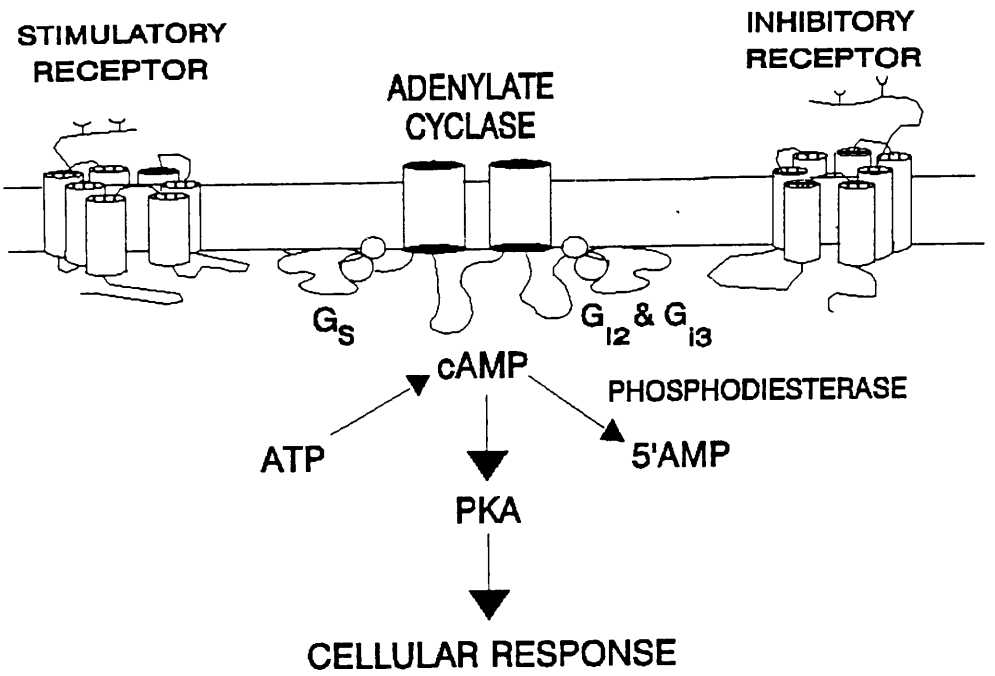


Fig 1.6) The Mammalian Adenylate Cyclase/Phosphodiesterase System. The actions of many hormones and neurotransmitters are mediated *via* cyclic AMP. The intracellular concentrations of cyclic AMP are regulated by adenylate cyclase and cyclic nucleotide phosphodiesterases, enzymes responsible for the formation and destruction of cyclic AMP, respectively. The adenylate cyclase system consists of a series of interacting proteins which lie predominantly in the plane of cellular membranes. Stimulatory and inhibitory effectors transmit their signals *via* stimulatory and inhibitory receptors coupled to heterotrimeric guanine nucleotide binding (G-) proteins. The activation status of adenylate cyclase, which catalyses the formation of cyclic AMP from ATP, is determined by the net actions of stimulatory- (G_s) and inhibitory- (G_{i2} and G_{i3} in preadipocytes) G-proteins. Cyclic AMP produces biological effects by activating cyclic AMP-dependent protein kinase (PKA) which phosphorylates specific substrates involved in the regulation of the biological response.

adipocytes whose expression is regulated nutritionally (Fredrich *et al*, 1992). Microdialysis of rat adipose tissue *in vivo* with angiotensin stimulates a specific increase in the production of the prostaglandin prostacyclin (Ailhaud *et al*, 1994). Prostacyclin has been shown to be a potent inducer of intracellular cyclic AMP and free intracellular Ca^{2+} in preadipocytes (Vassaux *et al*, 1992; Vassaux *et al*, 1993). These two signalling pathways synergise to play a cardinal role in triggering terminal differentiation of preadipose Ob17 cells, as well as modulating positively the differentiation of rat, human and 3T3-F442A preadipocytes (Négrel *et al*, 1989). Adenosine is another small molecule produced in adipose tissue which has been shown to potentiate the positive effect of prostacyclin on adipogenesis in Ob1771 and rat preadipocytes by *positively* coupling to adenylate cyclase through a type A_2 adenosine receptor (Vassaux *et al*, 1993). In mature adipocytes the adipogenic action of both prostacyclin and adenosine disappears, since adipocytes do not express prostacyclin receptors and the adenosine A_1 receptor emerges , coupled *negatively* to adenylate cyclase through G_i, and the A_2 receptor subtype disappears (Vassaux *et al*, 1992; Vassaux *et al*, 1993). These observations indicate that the differentiative effects of elevated intracellular cyclic AMP and Ca^{2+} mobilisation, promoted by prostacyclin and adenosine, are transient and restricted to the preadipose cell. Brief exposure of 3T3-L1 preadipocytes to cyclic AMP phosphodiesterase inhibitors [such as isobutylmethylxanthine (IBMX)] accelerates their adipose conversion in serum-containing or serum-free media (Elks and Manganiello, 1988; Schmidt *et al*, 1990). IBMX can be replaced by cyclic AMP analogues, or activators of adenylate cyclase, demonstrating that the potentiating action of intracellular cyclic AMP on preadipocyte differentiation can be dissociated from Ca^{2+} mobilisation.

1.7.3.2) Heterotrimeric Guanine Nucleotide-binding Proteins (G-proteins).

The hormonal stimulation of fatty acid and glycerol release from adipose tissue correlates with elevated intracellular cyclic AMP levels and an increase in the activity of hormone sensitive lipase (Kawamura *et al*, 1981). This characteristic and functionally important enzyme of adipose tissue controls rates of lipid mobilisation and is activated by phosphorylation on specific serine residues by cyclic AMP-dependent protein kinase (Kawamura *et al*, 1981). During differentiation, 3T3-L1 cells develop catecholamine-sensitive adenylate cyclase activity in parallel with increased numbers of β-receptors and increased expression of the stimulatory GTP-binding protein G_s (Cai and Rosen, 1981). The initial report on this topic demonstrated a dramatic increase in the levels of the 47 kDa and 42 kDa forms of $G_{s\alpha}$, as detected by cholera toxin-catalysed ADP-ribosylation (Cai and Rosen, 1981). This observation was confirmed by subsequent

studies on the same cells (Watkins *et al*, 1982; Watkins *et al*, 1987) and in 3T3-F442A cells (Kilgour and Anderson, 1993). Although the increased expression of the 42 kDa $G_{s\alpha}$ isoform is the most profound, the specific functions of the 42 kDa or 47 kDa isoform are not known and so the significance of this is unclear at present. Exposure of non-differentiating 3T3-C2 cells to the same differentiation protocol for 3T3-L1 cells, also results in the increased expression of 42 kDa and 47 kDa $G_{s\alpha}$ isoforms, however, without increased responsiveness of adenylate cyclase to lipolytic agents (Watkins *et al*, 1982). This indicates that changes in $G_{s\alpha}$ can not alone account for the magnitude of increased responsiveness of 3T3-L1 cells to β-adrenergic agonists. A reduction in cellular levels of the alpha subunit of the inhibitory GTP-binding protein, G_i may also contribute (Giershik *et al*, 1986). However, studies of Gi expression have yielded some conflicting results. Watkins *et al* (1987) reported that during differentiation of 3T3-L1 cells, an increase in the levels of 40 kDa α-subunits occurred, as detected by pertussis toxin-catalysed ADP-ribosylation, whereas Giershik *et al* (1986) reported a significant decrease in the levels of $G_{i\alpha}$ plus $G_{o\alpha}$ detected with an antiserum raised to these purified subunits.

The cellular levels of β-subunits have been reported to both decrease (Giershik *et al*, 1986) and increase (Watkins *et al*, 1982) during differentiation. These discrepancies may be explained by the fact that pertussis toxin catalyses the ribosylation of $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ and $G_{o\alpha}$ and is dependent on the dissociative state of the G-protein. In addition, the difficulty in obtaining homogeneous preparations, free of contamination by other G-protein subunits, for the raising of specific antisera rendered the interpretation of these early data very difficult. However, in a later report Watkins *et al* (1989), using anti-peptide antisera, specific for $G_{i2\alpha}$ and $G_{\beta 1}$ and $G_{\beta 2}$, demonstrated a decrease in expression of these proteins and corresponding mRNA levels during differentiation of 3T3-L1 cells.

Although it is clear that extracellular ligands can control adipogenesis by modulating adenylate cyclase activity through G_s- or G_i-coupled receptors, it has become apparent that these heterotrimeric G-proteins may exert additional regulatory influences on preadipocyte differentiation independent of adenylate cyclase (Wang and Malbon, 1996). Oligodeoxynucleotides in an antisense orientation to the α-subunit of G_s were shown to accelerate the rate of differentiation of 3T3-L1 cell differentiation induced by other agents, as well as acting as inducers themselves (Wang *et al*, 1992). Conversely, constitutive activation of $G_{s\alpha}$ with cholera toxin was found to provoke a blockade of differentiation, whereas cyclic AMP-elevating agents were reported, in this instance, to have no effect on differentiation (Wang *et al*, 1992). Increased expression of a constitutively active mutant form of G_i (Q205L-$G_{i2\alpha}$), whose activity counter-regulates

$G_{s\alpha}$, also promotes terminal differentiation of fibroblasts to adipocytes (Su *et al*, 1993). These data which parallel studies in mouse F9 teratocarcinoma stem cells (Watkins *et al*, 1992), and studies on the suppression of G_i *in vivo* in transgenic mice (Moxam *et al*, 1992), highlight a critical role of G-proteins in differentiation and development.

The domain responsible for the repression of adipogenesis in the $G_{s\alpha}$ protein (amino acids 146-235) was found to map to a region which was distinct from that which facilitated control of adenylate cyclase (Wang *et al*, 1996a). It is interesting to note that a mutant substituting valine for glycine at position 49 of $G_{s\alpha}$ was found to promote neuronal PC12 cell differentiation independently of adenylate cyclase activation (Kabir *et al*, 1993). In addition to adenylate cyclase, Mg^{2+}-transport (Birnbaumer *et al*, 1990; Gilman, 1987) and Ca^{2+} channels (Birnbaumer *et al*, 1990; Gilman, 1987) have been implicated as effector units regulated *via* $G_{s\alpha}$. Whether these, or other effectors which have yet to be discovered, mediated the negative regulatory action of $G_{s\alpha}$ on preadipocyte differentiation remains to be established. In addition, further investigation will be necessary to consolidate adenylate cyclase-independent actions of $G_{s\alpha}$ in light of overwhelming evidence supporting cyclic AMP as a regulator of adipogenesis (section 1.7.3.1).

1.7.3.3) The cyclic AMP-dependent Protein Kinase (PKA) Cascade

Cyclic AMP mediates its biological effects through a protein serine/threonine phosphorylation-dephosphorylation system, composed of a cyclic AMP-dependent protein kinase (PKA; Walsh *et al*, 1968) and a cognate protein phosphatase (Krebs and Beavo, 1979). The PKA holoenzyme consists of two identical cyclic AMP binding subunits and two protein-phosphorylating subunits (Rosen *et al*, 1975). The phosphorylating, or catalytic (C), subunits are active only when dissociated from the holoenzyme complex. Dissociation is promoted by the interaction of cyclic AMP with the nucleotide binding regulatory (R) subunits (Brostrom *et al*, 1970).The degree of phosphorylation of substrate proteins can therefore be related to the level of cyclic AMP within the cell. In mice there are four R genes (encoding RIα, RIβ, RIIα and RIIβ) and two C genes (encoding Cα and Cβ) which are expressed in a tissue specific manner (McKnight, 1991). The RIIβ isoform is abundant in adipose tissue and brain with limited expression elsewhere (McKnight, 1991).

PKA plays a fundamental role in mature adipocytes by mediating the regulation of lipolysis by catecholamines (Honnor *et al*, 1985a; Honnor *et al*, 1985b). In addition the regulation of fatty acid transport (Abumrad *et al*, 1986) and aromatase activity (Evans *et al*, 1970) by hormones is mediated, at least in part, by PKA. These facts, together with

observations that cyclic AMP positively regulates adipogenesis early in the differentiation program (section 1.7.3.1), indicate that induction of the relevant machinery for cyclic AMP signalling is essential to the expression of a differentiated adipose phenotype. During the adipocyte conversion of murine TA1 preadipocytes it has been shown that regulated increases in the expression of RIIβ, and to a lesser extent Cα and RIβ, precede morphological differentiation (Kurten *et al*, 1988). Moreover, transgenic ablation of the RIIβ subunit of protein kinase A leads to morphologically lean mice which display smaller adipocytes than their wild-type counterparts with substantially diminished triglyceride stores (Cummings *et al*, 1996). These observations indicate that regulated changes in PKA subunit expression, particularly RIIβ, can have a major impact on the developmental program of adipocyte maturation.

The subcellular localisation of PKA is directed through the R-subunit (Sarkar *et al*, 1984) and certain tissues contain up to 75% of either RII isoform in particulate form, associated with either the plasma membrane, cytoskeletal components, secretory granules or the nuclear membrane (Joachim and Schwoch, 1990; ; Nigg *et al*, 1985;; Rubin *et al*, 1972; Salvatori *et al*, 1972). Following their induction in TA1 preadipocytes, PKA subunits are predominantly associated with a reticular cytoplasmic structure abutting the nucleus (Kurten *et al*, 1988). This raises the exciting possibility that specific targeting of PKA to nuclear-associated RIIβ-anchoring proteins directs cyclic AMP-mediated signalling to adipocyte-specific substrates, perhaps modulators of adipogenesis *per se*.

1.7.3.4) Transcriptional Regulation by Cyclic AMP

The transcription of several adipocyte-specific genes, have been shown to be up-regulated by cyclic AMP during adipose conversion. These include α-glycero-3-phosphate dehydrogenase (Bhandari *et al*, 1991), acetyl-CoA dehydrogenase (ACC; Park and Kim, 1991) and fatty acid binding protein (aP2; Yang *et al*, 1989). The regions of the 5' flanking sequences of the ACC and aP2 genes which are necessary for regulation by cyclic AMP have been partially characterised.

ACC gene expression is induced during preadipocyte differentiation by insulin, and this requires prior action of cyclic AMP on the gene (Park and Kim, 1991). The DNA sequence responsible for cyclic AMP action on ACC induction has been identified as one which binds the activator protein (AP)-2 transcription factor (Park and Kim, 1993). AP-2 can be phosphorylated by PKA, but this does not affect its binding activity (Park and Kim, 1993), i.e. unphosphorylated AP-2 can bind the ACC promoter region as well as the phosphorylated form. This suggests that phosphorylation of transcription

factors by the cyclic AMP-PKA cascade may modulate differentiation-dependent transcriptional events through mechanisms other than DNA-binding.

The up regulation of aP2 gene expression only occurs in confluent, differentiation permissive preadipocytes and seems to depend on the presence of activator protein (AP) 1 and C/EBP consensus sequences in the promoter region (Yang *et al*, 1989). Intriguingly, part of the action of cyclic AMP on the triggering of preadipocyte differentiation may be the induction of the adipogenic transcription factor C/EBPβ (Yeh *et al*, 1995). Moreover, C/EBPβ has been shown in non-preadipocyte cell systems to be located in the cytoplasm where it is phosphorylated in response to elevations in intracellular cyclic AMP (Metz and Ziff, 1991; Yamamoto *et al*, 1988). This results in increased nuclear translocation and enhanced *trans*-activation of target genes (Metz and Ziff, 1991; Yamamoto *et al*, 1988). These observations, together with the fact that C/EBP family members form heterodimers with other transcription factors (Vallejo *et al*, 1993) including the CREB (cyclic AMP-response-element-binding protein) family, strongly suggest that functional cross-talk with C/EBPs may contribute to the effects of cyclic AMP on differentiation-associated transcription.

The transcription factor CREB has been shown in many cell systems to mediate transcriptional activation by cyclic AMP. Elevated intracellular cyclic AMP leads to phosphorylation of CREB by PKA at a key regulatory site (Ser 133; Gonzalez and Montminy, 1989; Lee *et al*, 1990; Sheng *et al*, 1991). This leads to nuclear translocation and activation of transcription through the binding of CREB proteins to cyclic AMP response elements (CREs) in target genes (Hagiwara *et al*, 1993). Phospho-CREB also interacts with a CREB-binding protein (CBP) which performs as a transcriptional co-activator which augments expression of CRE-directed genes and may be important for the activation AP-1 dependent promoters (Arias *et al*, 1994; Kwok *et al*, 1994). Curiously, many cyclic AMP-regulated adipocyte-specific genes do not contain CRE elements (Flores-Riveros *et al*, 1993) or contain CRE elements which do not contribute to cyclic AMP-responsiveness (Park and Kim, 1993). Therefore, it seems that gene-induction by cyclic AMP during preadipocyte differentiation may not be mediated solely by CREB. However, given that CREB can cooperate with other transcription factors, such as AP-1 and C/EBP, can be phosphorylated by a number of other protein kinases, including protein kinase C, CaM kinases II and IV, and p90RSK (Ginty *et al* , 1994; Gonzalez and Montminy, 1989; Sheng *et al*, 1991; Sun *et al*, 1994) suggests that CREB may serve to integrate and coordinate divergent signalling pathways at the transcriptional level. The consequences of such interactions in terms of preadipocyte differentiation remain to be investigated.

In contrast, to its effects on preadipocytes cyclic AMP appears to modulate terminal differentiation by repressing the expression of a number of genes coding for essential proteins of the adipocyte, especially lipogenic enzymes (Antras *et al*, 1991). Thus, in adipocytes cyclic AMP mRNA levels of adipose-specific markers which include lipoprotein lipase (Raynolds *et al*, 1990; Antras *et al*, 1991), fatty acid synthase (Paulauskis and Sul, 1988), α-glycero-3-phosphate dehydrogenase (Dobson *et al*, 1987; Antras *et al*, 1991; Bhandari et al, 1991), GLUT 4 (Kaestner *et al*, 1991), adipsin (Antras *et al*, 1991) and leptin (Gettys *et al*, 1996). The mechanisms involved in transcriptional repression by cyclic AMP are poorly understood, but may involve *trans*-acting nuclear factors which are distinct from CREB, C/EBPs, AP-1 and AP-2 (Rangan *et al*, 1996).

In conclusion, it appears that cyclic AMP exerts differential effects on adipocyte development, acting both at an early stage to promote preadipocyte differentiation, by inducing adipocyte-specific gene expression, and during the terminal stages, limiting fat cell hypertrophy by suppressing lipogenic gene expression and promoting lipolysis (section 1.2).

1.8) AIMS OF PROJECT

It is apparent that signal transduction pathways such as the cyclic AMP cascade, and those initiated at the GH and insulin receptors, can modulate preadipocyte differentiation depending on the stage of the developmental program at which they are activated (section 1.7). Among the consequences of initiating such adipogenic cascades will be changes in the expression of adipocyte-specific proteins and critical *trans*-acting transcription factors such as the C/EBP isoforms (section 1.6). Therefore, a major challenge in developmental cell biology is to elucidate the complex network of interactions which lead from cell-surface receptors to effect key transcriptional events in the nucleus. Furthermore, the discovery of pivotal "cross-talk" events between adipogenic signalling pathways will provide a better understanding of the mechanisms and consequences of intracellular hormone action.

Recently it has been demonstrated that G_s and G_i may play an important regulatory role during adipogenesis (section 1.7.3.2) and that changes in their expression contribute to the development of hormonal responsiveness in the mature adipocyte (section 1.7.3.2). Given the functional importance of these proteins it is likely that some of the phenotypic changes that occur during preadipocyte differentiation are reflections of alterations to the G-protein complement during development. Therefore, "permissive hormones" that can increase or decrease the expression of G_s and G_i, and thereby the stimulation of adenylate cyclase, can have a profound effect on the extent of adipose conversion. The present project aims to investigate perturbations to the cyclic AMP signalling system in the context of preadipose conversion and determine the potential consequences for terminal differentiation. The study will therefore cover three main areas:

1) Changes in the Expression of Guanine Nucleotide-binding Proteins During the Growth Hormone-dependent Differentiation of 3T3-F442A Cells.

The initial aim of this project is to determine whether changes in G-protein expression occur during the two stages of the differentiation program of 3T3-F442A preadipocytes (section 1.5); either during the GH-priming stage or the insulin-promoted maturation phase. G-protein levels will be assessed using a range of specific anti-peptide antisera. This approach will provide a better understanding of the hormonal influences which are responsible for the development of catecholamine-sensitive adenylate cyclase in mature adipocytes (section 1.5.3) and may provide an insight into stage-specific changes in G-protein expression which may underlie key regulatory events in the differentiation process. In addition, the use of specific antisera may help to clarify some of the

contradictory reports on differentiation-dependent changes in G-protein expression in other preadipocyte cell systems (section 1.5.3). Finally, the potential of the stimulatory- and inhibitory-G-proteins to modulate fat cell conversion will be assessed by cholera and pertussis toxin intoxication.

2) The Effects of Cyclic AMP on the Differentiation of 3T3-F442A Preadipocytes

That G_s and G_i have the potential to modulate adipogenesis (section 1.7.7.2) suggests that their major intracellular effector, adenylate cyclase, plays a supportive role in modulating differentiative processes. Indeed, there is much supportive evidence to suggest that cyclic AMP can influence preadipocyte differentiation *in vivo* and *in vitro* (sections 1.7.3.1 and 1.7.3.3). In light of recent reports that G_s and G_i may act through adenylate cyclase-independent mechanisms to effect fat cell conversion indicates that a critical re-evaluation of the adipogenic potential of cyclic AMP is required. The second aspect of this study will be to address this question by defining the roles of cyclic AMP in modulating preadipocyte differentiation. Cyclic AMP-elevating agents will be used to determine stage-specific aspects of the response of differentiating 3T3-F442A preadipocytes to cyclic AMP, in terms of the ultimate effect on terminal differentiation. The use of a serum-free, chemically defined differentiation medium will also reveal which permissive hormones cyclic AMP interacts with to determine cellular fate.

3) Interactions Between the Cyclic AMP and MAP Kinase Pathways: Effect on the Differentiation of 3T3-F442A Preadipocytes

One potential means of cyclic AMP to effect differentiation is to positively or negatively regulate obligatory differentiative signal transduction cascades, such as those initiated by insulin- and GH-receptor binding (section 1.7.2.4). The MAP kinase cascade has been implicated as a transducer of differentiative stimuli in 3T3-L1 preadipocytes (section 1.7.2.4). Further work will explore whether cyclic AMP can effect the activation of MAP kinases through regulatory cross-talk during the priming or maturation phases of 3T3-F442A adipogenesis. By correlating the effects of cyclic AMP on MAP kinase activation with stage-specific effects on terminal differentiation, it may be possible to assign a putative role for this ubiquitous signalling molecule in the control of fat cell development.

Chapter Two

Materials And Methods

2.1) MATERIALS

2.1.1) Chemicals

General laboratory chemicals were obtained from ICN Biomedicals (Oxfordshire, UK), Sigma Chemical Co. (Poole, UK) or BDH (Poole, UK). Other specialised laboratory reagents are listed in Table 2.1. Unless stated otherwise, all water was single distilled mains water.

Table 2.1) Specialised Laboratory Reagents

Chemical	Source	Catalogue Number
Acetonitrile (HPLC grade)	Sigma	27,071-7
Acrylamide/bis-Acrylamide (37.5:1 v/v solution)	Severn Biotech. Ltd. (Worcs., UK).	A 3423
Activated Charcoal (100-400 mesh)	Sigma	C 5260
Bisbenzimide (Hoechst H33258)	Fluka Biochemika (Dorset, UK)	14530
Bradford Reagent (dye reagent concentrate)	Bio-Rad Ltd (Herts., UK)	500-0006
BSA (improved standard grade)	Advanced Protein Products (West Midlands, UK).	PF-201-47
BSA (fraction V)	Sigma	A 2153
3', 5' Cyclic AMP-Dependent Protein Kinase (from bovine heart)	Sigma	P 5511
Cholera Toxin (from *Vibrio cholerae*)	Sigma	C 3012
Collagenase (type VIII, for adipocyte isolation)	Sigma	C 2139
CPT-cAMP	Boehringer Mannheim (East Sussex, UK)	405 647
DHAP (lithium salt)	Sigma	D 7137
1, 9-Dideoxyforskolin (from *Coleus forskohlii*)	Sigma	D 3658

DMEM (high glucose)	Life Technologies	41965-096
EGF (human, recombinant)	Life Technologies	13247-010
Fetuin (from foetal calf serum)	Sigma	F 2379
Forskolin (from Coleus forskohlii)	Sigma	F 6886
Freund's Adjuvant (complete)	Life Technologies	15721-012
Freund's Adjuvant (incomplete)	Life Technologies	15720-014
KLH	Pierce & Warriner, Chester UK	77100
Glutaraldehyde (grade I)	Sigma	G 7526
H-89 (dihydrochloride)	Calbiochem	371963-Q
IBMX (crystalline)	Sigma	I 5879
Insulin (cell culture tested)	Sigma	I 1882
(-)-Isoproterenol (hydrochloride form)	Sigma	I 6504
Lipofectin Transfection Reagent	Life Technologies	18292-037
MBP (from bovine brain)	Sigma	M 1891
Medium 199 (with Earle's Modified Salts and 1.25g/l sodium bicarbonate)	Life Technologies	31153-026
NADH (reduced form)	Sigma	N 8129
NP-40 (protein grade)	Calbiochem	492017
Mixture Ham's F12 Nutrient Mixture	Life Technologies	21765-029
Oil Red O (solvent red 27)	Sigma	O 9755
PBS	Life Technologies	20012-019
PD098059	Calbiochem (Nottingham, UK)	513000
Pertussis toxin (from *Bordetella pertussis*)	Life Technologies	13176-011
Phenyl-Sepharose Cl-4B	Pharmacia (Milton Keynes, UK)	17-0810-02

Rp-cAMPS	Biolog (Bremen, Germany)	B001 (B003-K)
RO-20-1724	Calbiochem	557502
Salmon Sperm DNA	Sigma	S 3245
T_3 (cell culture tested)	Sigma	T 5516
Thimerosal	Sigma	T 5125
Transferrin (cell culture tested)	Sigma	T 1428
Triethylamine Acetate (2.0M solution)	Applied Biosystems	400613
Trifluoroacetic Acid	Applied Biosystems	400445
Trypsin-EDTA Solution (containing 0.5g/l trypsin and 0.2g/l EDTA)	Life Technologies	45300-043
Urea (ultra pure)	Sigma	U 0631

2.1.2) Radiochemicals

Radiochemicals were obtained from Amershem International and are listed in table 2.2. ^{3}H- and ^{32}P- radioactive counts (cpm) were determined using a 1600TR liquid scintillation analyser with Opti-fluor scintillation fluid (both from Canberra Packard).

Table 2.2) Radiochemicals

Radiochemical	Source	Catalogue Number
[8-^{3}H] Adenosine 3', 5'-cyclic monophosphate	Amersham International (Slough, England)	TRK 304
[γ-^{32}P] Adenosine 5'-triphosphate	Amersham International	PB 10132

2.1.3) Peptides and Recombinant Proteins

The 7-residue synthetic peptide substrate, Kemptide (Kemp *et al*, 1977), and the 20-residue peptide inhibitor of cyclic AMP-dependent protein kinase (PKI; Cheng *et al*, 1986) were generously donated by Dr Roger Clegg (Hannah Research Institute, Ayr, UK). The amino-acid sequences were as follows:

PKI: Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp

Kemptide: Leu-Arg-Arg-Ala-Ser-Leu-Gly

Recombinant bovine GH (rbGH) and recombinant p42 MAP kinase, in which the lysine at position 52 is mutated to arginine (K52R), were gifts from Monsanto (St Louis, USA) and Professor Thomas W. Sturgill (University of Virginia, Charlottesville, USA) respectively. Recombinant insulin-like growth factor-1 (IGFI) and ^{125}I-rbGH were generously provided by Dr James Beattie (Hannah Research Institute, Ayr, U.K.); rbGH was iodinated to a specific activity of ca. 50μCi/μg as described previously (Beattie, 1992) using the Iodogen coated tube method (Fraker and Speck Jr, 1978).

2.1.4) Oligodeoxynucleotides

Phosphorothioate oligodeoxynucleotides were a generous gift of Drs Elizabeth and Graham Sale (University of Southampton) and were synthesised on a series 391 automated DNA synthesiser (Applied Biosystems). Antisense MAP kinase oligonucleotides were designed to complement a 17-base nucleotide sequence unique to p42 and p44 MAP kinase (Sale *et al*, 1995) cDNAs; 5'-ATG GCG GCG GCG GCG GC-3', where ATG is the initiation codon. In the mouse this corresponded to nucleotides 25-41 of p42 MAP kinase (Her *et al*, 1991) and 1-17 of p44 MAP kinase (Pagès *et al*, 1995). The corresponding 17mer antisense probe (antisense EAS 1) and control phosphorothioate oligonucleotides were synthesised with the following sequences:

EAS1	5'-GCC GCC GCC GCC GCC AT-3'
Sense	5'-ATG GCG GCG GCG GCG GC-3'
Scrambled	5'-CGC GCG CTC GCG CAC CC-3'

2.1.5) Antibodies

The monoclonal antibody to p42 MAP kinase (ERK 2), rabbit antiserum to rbGH and rabbit antiserum to the 35 and 36 kDa forms of G_β were generous gifts from Professor

Ailsa Campbell (I.B.L.S, Glasgow, UK), Dr David J. Flint (Hannah Research Institute, Ayr, UK) and Professor Graeme Milligan (I.B.L.S, Glasgow, UK) respectively. Rabbit polyclonal antibodies to p44 MAP kinase (ERK 1) were raised to a synthetic peptide from the rat sequence (residues 325-345) as previously described (Kilgour *et al*, 1996). Anti-mouse and anti-rabbit IgG horse radish peroxidase conjugates were obtained from Amersham International plc. (Buckinghamshire, UK) and Sigma, respectively. Donkey anti-serum to rabbit IgG (anti rabbit IgG) was from the Scottish Antibody Production Unit, (Carluke, UK).

2.1.6) Animals

Female Wistar rats were obtained from A.Tuck and Son, Essex, UK. New Zealand-breed rabbits were purchased from either Shrubacre Rabbits, Suffolk, UK or Hyline Rabbits, Cheshire, UK. Both diet and tap-water were available *ad libitum*.

2.1.7) Cell Lines

3T3-F442A and 3T3-L1 cells were provided by Dr Howard Green (Harvard Medical School) and Dr Steve Baldwin (University of Leeds) respectively.

2.2) METHODS

2.2.1) Standard Procedure for Cell Culture

Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) new born calf serum and glutamine (2.5mM). Stocks were seeded at a density of approximately $1x10^3$ cells/cm^2 in 75cm^2 plastic flasks (Costar Ltd., Bucks., UK) containing 0.2ml/cm^2 and grown at 37°C in a humidified atmosphere containing 5% CO_2.

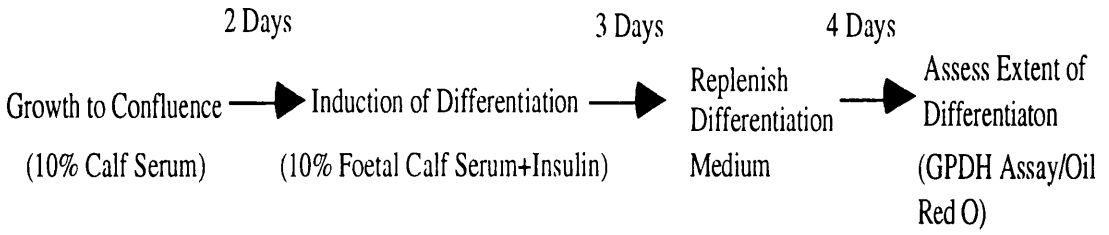
Cells were subcultured every 4-6 days when they had attained approximately 80% confluence. The growth medium of a 75cm^2 plastic flask of cells was decanted asceptically and the monolayer rinsed twice with 12.5ml PBS pre-warmed to 37°C. Trypsin/EDTA solution (1ml; trypsin 5g/l; 500µM EDTA; 145mM NaCl) was then added to the monolayer and incubated at room temperature for 1-2 minutes until cells detached. The resulting cell suspension was then diluted to seeding density with growth medium.

For experiments, 100mm culture dishes (Costar) were inoculated at a density of approximately $1x10^3$ cells/cm^2 in 0.12ml/cm^2 growth medium. Alternatively, for some experiments, cells were seeded at the same cell density onto 30mm, 6-well culture clusters (Costar) containing 0.5ml/cm^2 growth medium.

2.2.2) Induction of Differentiation with FCS and Insulin

The growth medium of two days post-confluent 3T3-F442A cells was removed and replaced by the same volume of DMEM containing 10% FCS and insulin (5µg/ml). Cultures were then incubated at 37°C in a humidified atmosphere containing 5% CO_2. Pharmacological agents under test were prepared aseptically and added directly to the medium at the initiation of differentiation. After three days the medium was replaced with fresh differentiation medium without agents. After a further four days the extent of differentiation was assessed. For schematic see Fig 2.1.

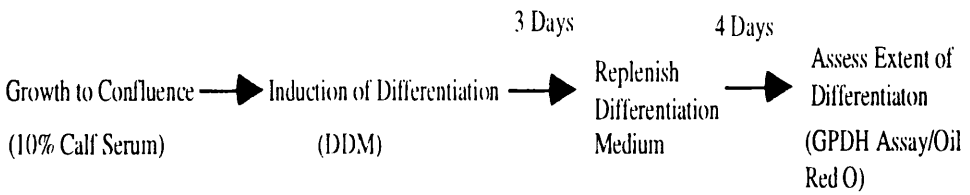
Fig 2.1) Induction of Differentiation with FCS and Insulin



2.2.3) Induction of Differentiation under Serum-Free Conditions

The growth medium of confluent cells was removed by aspiration and replaced by a defined differentiation medium (DDM; 0.12ml/cm^2; Guller *et al*, 1988) after washing monolayers three times with phosphate buffered saline (PBS). The DDM consisted of a basal medium of F12 Ham's Nutrient Mixture and DMEM (2:1 v/v, F12/DMEM) containing rbGH (2nM), insulin (1.8μM), T_3 (0.1ng/ml), EGF (50ng/ml), transferrin (10μg/ml), fetuin (50μg/ml), glutamine (2.5mM) and BSA (1mg/ml). Stock solutions of DDM components were added directly to a premeasured volume of F12/DMEM and sterilised through a disposable sterile filter (0.45μM, Fischer Scientific, Leicestershire, UK). As with differentiation with FCS/insulin, pharmacological agents were incorporated in the DDM for the first three days of differentiation, after which monolayers were washed once with PBS and the medium replaced with fresh DDM without agents (see Fig 2.2).

Fig 2.2) Induction of Differentiation with DDM



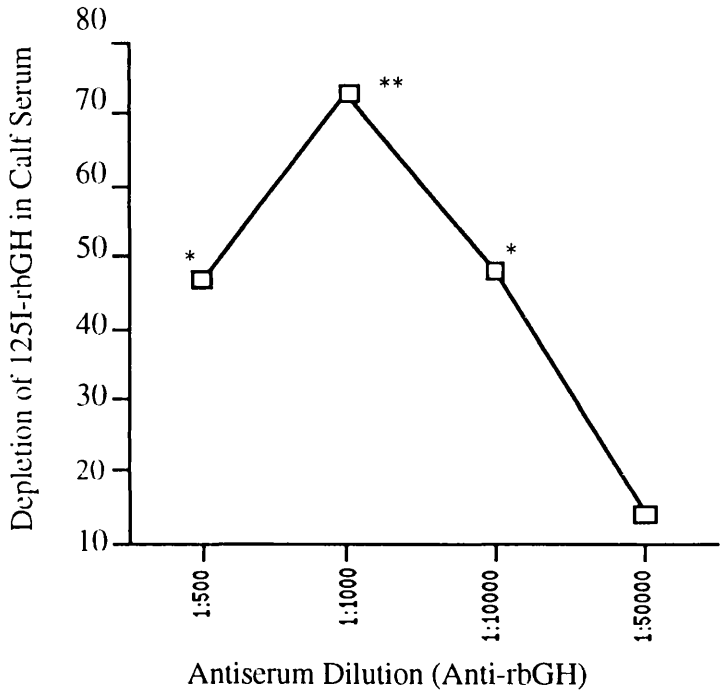


Fig 2.3) Immunoneutralisation of GH in Calf Serum.

Samples of calf serum were incubated with ^{125}I-rbGH (20000cpm) and various dilutions of anti-rbGH as indicated. Anti-rbGH was immunoprecipitated with anti-rabbit IgG (1:10) and the ^{125}I-activity of samples measured. Values are expressed as a percentage of the ^{125}I-activity depleted from samples relative to cells treated with non-immune serum (control) and are means ± standard error mean (S.E.M.) for 3 observations. Significant differences with respect to control values are indicated; *$p<0.01$, **$p<0.001$.

2.2.4) Two-Phase Protocol for Cellular Differentiation of 3T3-F442A Cells.

A serum free, two-phase differentiation protocol was developed to demonstrate the GH-dependency of 3T3-F442A preadipocyte differentiation (for further details refer to Section 3.2). For these experiments it was necessary to grow cells in the presence of calf serum depleted of GH.

2.2.4.1) *Preparation of calf serum depleted of GH*

Serum GH was neutralised with rabbit antiserum to recombinant bovine GH (anti-rbGH) and immunoprecipitated with anti-rabbit IgG. The generation and characterisation of anti-rbGH has been previously described (Beattie *et al*, 1992). The optimal neutralising concentration of anti-GH was determined by a radioligand-binding assay. Aliquots of calf serum were incubated overnight with ^{125}I-rbGH (20000 CPM) and either pre-immune rabbit serum or a range of anti-rbGH dilutions (Fig 2.3). Following incubation samples were treated with anti-rabbit IgG (1:10) for a further 4 hours at room temperature and then centrifuged in a 2.0RS Minifuge (Heraeus Sepateck, West Germany) at 2000g_{max} for 30min. The amount of ^{125}I-rbGH remaining in the supernatant was determined by gamma-radiation counting using a Cobra auto-gama counter (74% efficiency, LKB., Croydon, UK).

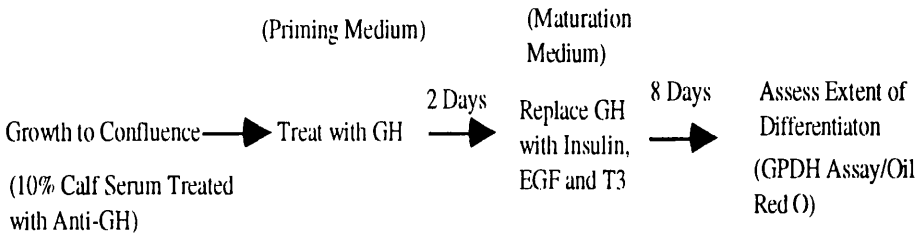
Control samples treated with anti-rabbit IgG alone were found to have no significant depletion of ^{125}I-rbGH. Fig 2.3 demonstrates that optimal depletion of ^{125}I-rbGH occurred with a 1:1000 dilution of anti-rbGH. The concentration of bovine GH in one batch of calf serum was estimated by radioimmunoassay to be 14.2ng/ml (D.J. Flint, personal communication). Assuming that anti-rbGH can bind 1mg of rbGH per ml (D.J. Flint, personal communication), then a 1000 fold dilution of anti-GH represents an approximate 70 fold excess over serum GH.

2.2.4.2) *Two-Phase Differentiation Protocol*

Cells were passaged at least twice, in DMEM containing 2.5mM glutamine and 10% GH-depleted calf serum, prior to use for differentiation studies. Confluent cells were washed three times in PBS then primed for 2 days in serum free medium [F12:DMEM (2:1)] containing transferrin (10µg/ml), fetuin (50µg/ml), glutamine (2.5mM) and BSA (1mg/ml)] and GH (2nM). Cultures were then washed, as before, and the medium replaced with maturation medium [serum free medium containing insulin (1.8µM), T_3

(0.1ng/ml) and EGF (50ng/ml)]. The extent of preadipocyte differentiation was assessed after a further 8 days (Fig 2.4).

Fig 2.4) Two-phase Differentiation Protocol



2.2.5) Oil Red O Staining

The extent of morphological differentiation in cell cultures was determined histochemically by staining accumulated lipids in terminally differentiated adipocytes. Cultures were washed twice with PBS and then fixed for 1 hour in formaldehyde (10% v/v in PBS). Fixed cultures were stained with the neutral lipid stain Oil Red O (5µg/ml in isopropanol) for 1 hour and then washed extensively with distilled water. Cells were then photographed under Phase Contrast Optics using an Olympus IMT3 inverted microscope.

2.2.6) Assay of α-Glycerophosphate Dehydrogenase Activity

Cells from one 100mm or 30mm culture dish were harvested by scraping into 500µl ice-cold extraction buffer (100mm triethanolamine, 1mM EDTA, 0.1mM β-mercaptoethanol, pH 7.5) and then lysed on ice with 8 passages through a $26^1/_2$g needle attached to a 1ml disposable syringe. Lysates were then centrifuged ($14000g_{max}$, 4°C) for 10 min in a microfuge and the resulting supernatant was removed and stored in small aliquots at -20°C until use.

Samples of supernatant were assayed for GPDH activity by the spectrophotometric method originally described by Wise and Green (1979). GPDH activity of cell extracts was measured at 25°C in a Cecil 5000 series double beam spectrophotometer in a 1ml reaction mixture. The basic reaction mixture (100mM triethanolamine, pH 7.5, 1mM

EDTA, 0.12mM NADH) was placed in a cuvette with a 1cm pathlength and set in the spectrophotometer. Samples of cell lysate were diluted with extraction buffer to 100µl (1-100µg protein) and added to the cuvette containing the reaction mixture. The reaction was then initiated with the addition of dihydroxyacetone phosphate (DHAP) to final concentration of 0.2mM. The biochemical nature of the reaction catalysed by GPDH is:

dihydroxyacetone phosphate+NADH <-> glycerol-3-phosphate+ NAD^+

Initiation of the reaction with DHAP results in a decrease in the absorbance at 340nM due to the oxidation of NADH to NAD^+. The absorbance at 340nM was recorded at 30s after initiation and at 30s intervals thereafter.

Calculation:

The enzyme activity per plate of cell in katals (number of moles of NADH oxidised/second) was calculated from the molar absorption coefficient for NADH at 340nM ($6.3x10^3$ $lmol^{-1}cm^{-1}$).

EQUATION 1

$$\frac{\Delta A_{340/sec}}{6.3x10^3} \times \frac{1.0}{1x10^3} \times \frac{0.5}{0.1}$$

A **B** **C**

A. The use of molar absorption coefficient for NADH to calculate the concentration change ($mol.l^{-1}sec^{-1}$)with the pathlength of the cuvette being 1cm.
B. Calculation of the amount of NADH oxidised in a 1ml reaction volume in 1 second.
C. Correction for sample volume used and estimation of GPDH activity for one 100mm plate.

In accordance with others (Lai *et al*, 1981) the amount of protein per cell was found to be 2.3 fold higher in adipocytes compared to fibroblasts. GPDH activities are therefore expressed relative to DNA content rather than the amount of cellular protein.

EQUATION 2

$$\frac{\text{(Solution from equation 1)}}{\text{µg of DNA/plate}} \times \frac{1x10^3}{1.0}$$

A **B**

A. Calculation of the number of katals of GPDH activity per µg of DNA.

B. Adjustment to katals per mg of DNA

2.2.7) Antisense Protocols

2.2.7.1) *Purification of Oligonucleotides*

Oligonucleotides were purified manually on oligonucleotide purification (OP) cartridges (Applied Biosystems) according to manufacturers instructions. Additions were made to columns with a 5ml syringe and washes and eluants were collected through a $26^1/_2$G needle at a rate of 1-2 drops per second. Cartridges were flushed sequentially with 5ml acetonitrile and then 5ml of 2.0M triethylamine acetate. Phosphorothioate oligonucleotides (approximately 20 OD units at 260nM in deionised water) were then loaded onto the cartridge. OP cartridges perform like affinity matrices which absorb the trityl group of the trityl-on oligonucleotide. Non-trityl bearing failure sequences, by- products and other impurities were washed through with 5ml of 1.5M ammonium hydroxide followed by two washes with 5ml deionised water. The trityl group was then removed by mild acid cleavage with 5ml 3% (v/v) trifluoroacetic acid in deionised water and the purified detritylated oligonucleotides eluted with 35% (v/v) acetonitrile in deionised water. Fractions contained approximately 10 OD units (at 260nM) of oligonucleotides and were dried overnight *in vacuo* on a Univap evaporator. Oligonucleotides were stored dried, in pellet form at -20°C.

2.2.7.2) *Oligonucleotide Treatment of Cells*

Cells were grown to confluence in 22mm dishes containing 10% calf serum in DMEM. Monolayers were washed three times with 2ml DMEM and appropriate dilutions of oligonucleotides in 100µl DMEM were preincubated at room temperature for 30 minutes with 100µl Lipofectin. This mixture was added to the cells together with a further 200µl of DMEM. The final concentration of oligonucleotides was 5µM. Cells were incubated for 8 hours at 37°C in the presence of 5% CO_2. After this time the medium containing Lipofectin was removed and the incubation continued for a further 40 hours using fresh medium containing 10% heat-treated (55°C for 30 minutes) calf serum in the presence or absence of 5µM oligonucleotide. After 40 hours the medium was removed and cells were washed and treated with FCS and exposed to differentiation medium in the presence or absence of oligonucleotide. The differentiation medium was replenished at two day intervals in the presence or absence of fresh oligonucleotide.

2.2.8) Preparation of Cell Membrane Samples

2.2.8.1) *Preparation of Membrane Fractions from 3T3-F442A Cells*

Cells were washed once in ice cold PBS then scraped into lysis buffer (50mM Hepes, pH 7.6; 0.25M sucrose, 5mM EDTA; 5mM EGTA; 1mM PMSF and leupeptin (1μg/ml). The volume of cell suspension was adjusted to 2ml and then cells were lysed in a 15ml tight-fitting Dounce homogeniser (Jencons Scientific Ltd., Leighton Buzzard, U.K.) followed by centrifugation in a microfuge (1000g, 10min, 4°C) to remove nuclei and unbroken cells. The resulting supernatant was centrifuged at 4°C for 45 min at $100000g_{max}$ in an Optima series TLX Tabletop Ultracentrifuge (Beckman-RIIC Ltd., Bucks., U.K.) fitted with a TLA-120.2 fixed angle rotor. Membrane pellets were resuspended in 50mM Hepes, pH 7.4/1mM EDTA at a concentration of 2-3mg protein/ml. Membrane suspensions were routinely snap-frozen in liquid nitrogen and stored at -80°C.

2.2.8.2) *Preparation of Crude Membrane Fractions from Rat Adipocytes*

Adipocytes were prepared by collagenase digestion of rat parametrial white adipose tissue according to the method of Rodbell (1964) with some modifications. Rats were killed by cervical dislocation and parametrial fat pads removed and washed in saline at 37°C. The fat pads from one rat were finely minced with scissors in 1ml of M199 medium prewarmed to 37°C then incubated in 10ml (final volume) of M199 containing 1mg/ml collagenase and 30mg/ml BSA (fraction V). Incubations were carried out for 1 hour at 37°C in stoppered flasks shaking at 180 oscillations per minute. The resulting cell suspension was then filtered through a nylon mesh (approx. 1mm in diameter) to remove undigested material and then washed successively at room temperature with 40ml of M199 and TES (20mM Tris/HCL, pH 7.4; 1mM EDTA; 0.255M sucrose). Adipocytes, which form a packed, buoyant layer on the wash solutions, were resuspended in 3.3ml TES and homogenised at room temperature with 10 strokes in a 15ml homogeniser fitted with a Teflon pestle (Jencons). Homogenates were then centrifuged at $100000g_{max}$ for 45 min in a pre-cooled (4°C) L7-65 Ultracentrifuge (Beckman-RIIC Ltd., Bucks., UK) fitted with a type 80Ti rotor. The resultant pellet was disaggregated with 8 strokes in a 10ml Uniform homogeniser (Jencons). Membrane suspensions were then made up to a final volume of 3.5ml in TES and subjected to further 45min centrifugation at $100000g_{max}$. Membrane pellets were resuspended in 20mM Tris/HCl, pH 7.4; 1mM EDTA and snap-frozen in liquid N_2 in small aliquots and stored at -80°C.

2.2.9) Peptide Synthesis, Conjugation and Immunisation

Immunogen decapeptides were synthesised by standard solid-phase fluorenylmethoxycarbonyl chemistry (Barany and Merrifield, 1979) on an Applied Biosystems 431A automated peptide synthesiser in the Hannah Research Institute core facility (Ayr, UK) and determined to be homogeneous by reverse-phase high-performance liquid chromatography as previously described (Beattie and Flint, 1992). The sequences were as described in Table 2.3.

Table 2.3 Amino-acid Sequences of Synthetic Peptides.

Protein Species	Amino Acid Sequence[1]
$G_{i1\alpha}$ (residues 159-168)	LDRIAQPNYI
$G_{i2\alpha}$ (residues 160-169)	LERIAQSDYI
$G_{i3\alpha}$ (residues 159-168)	LDRISQSNYI
$G_{i1+2\alpha}$ (C-terminal)	KNNLKDCGLF
$G_{s\alpha}$ (C-terminal)	RMHLRQYELL

[1] Single letter amino acid code.

Immunogen peptides were coupled to KLH, as a carrier protein to increase their immunogenicity and to prevent rapid degradation in the animal. KLH (3.3mg) and peptide (1mg) were dissolved in 0.1M phosphate buffer (0.1M NaH_2PO_4/Na_2HPO_4, pH 7.0). Glutaraldehyde was added dropwise over the course of 1 hour to a final concentration of 25mM. This bifunctional reagent couples primarily via amino groups (α-amino group of the peptide and ε-amino group of lysine residues). The coupling reaction was incubated for 24 hours at room temperature with gentle stirring.

Peptide-conjugate solutions were mixed with an equal volume of Freund's complete adjuvant by repeated expulsion through two luer lock syringes (Sherwood Medical, Sussex, UK) connected by a three-way plastic stopcock (Rocket of London, London, UK). The resulting emulsion was then injected in multiple subdermal sites in New Zealand White rabbits. Two weeks later each animal received a booster immunisation with material prepared identically except that one-half as much peptide and KLH were injected in Freund's incomplete adjuvant. Pre-immune sera were collected prior to the first injection, and subsequent bleeds were performed weekly beginning 2 weeks after the initial booster immunisation. The characterisations of G-protein antisera produced by this method are described in Chapter 3.

2.2.10) Gel Electrophoresis

2.2.10.1) *Standard Protocol for Gel Electrophoresis*

The protein mixture from cell extracts was boiled in Laemmli Sample buffer (0.06M Tris, pH 6.7; 1.25% (w/v) SDS; 12.5% (w/v) glycerol; 2.5% (v/v) β-mercaptoethanol; 0.01% (w/v) phenol red) and subjected to denaturing SDS polyacrylamide electrophoresis (SDS-PAGE) in a Protean II x i vertical electrophoresis system (Biorad) according to the method of Laemmli (1970). Samples were run on a 120 x 140 x 1.5mm or 160x 140 x 1.5mm gel slab containing 37.5mM Tris-HCl, pH 8.8, 10% (v/v) acrylamide, 0.26% (v/v) bisacrylamide, 0.1% (w/v) SDS. Gels were run at a constant current, 7mA, for 18 hours or 30mA for 4 hours. The composition of the running buffer was 49.5 mM Tris pH 8.3, 190mM glycine and 3.5mM SDS. All electrophoresis procedures were carried out at room temperature using water-fed cooling systems.

2.2.10.2) *Urea Gels*

Urea gels were used to obtain better resolution of the 40 kDa and 41 kDa isoforms of G_i. Proteins were resolved on 160 x 140 x 1.5 mm gels containing 6M urea, 37.5mM Tris, pH 8.8, 9% (v/v) acrylamide, 0.26% (v/v) bisacrylamide and 0.1% SDS. A constant current of 30mA was applied for 8-9 hours.

2.2.10.3) *Low-bisacrylamide Gels*

The phosphorylation and activation of MAP kinase is accompanied by a decrease in its electrophoretic mobility. Reliable separation of phosphorylated and unphosphorylated forms of the enzyme required the application of a constant current of 7mA for 18 hours to a 120 x140x 1.5 mm resolving gel containing 37.5mM Tris, pH 8.4, 10% (v/v) acrylamide, 0.11% (v/v) bisacrylamide and 0.1% (w/v) SDS.

2.2.11) Immunoblotting

Following electrophoresis proteins were transferred (Towbin *et al*, 1979), with water-cooling, to Hybond-C Super nitrocellulose membranes (Amersham International) in a Transphor electrophoresis-transfer unit with power lid (Hoeffer) for 2h at 400mA in ice-cold transfer buffer (25mM Tris, 192mM glycine, 20% methanol). To check for efficient transfer and even protein loading of lanes, the proteins adhered to the nitrocellulose membranes were visualised by the non-permanent stain Ponceau S

solution (0.5% w/v in 1% acetic acid). The stain was then removed by washing the membrane in distilled water several times. The membrane was then incubated for at least 16 hours in Tris- buffered saline (20mM Tris, pH 7.4, 154mM NaCl) containing 0.1% (v/v) Tween 20 (TBS/Tween) and 3% (w/v) BSA. This was to ensure that active sites on the membrane were blocked against further non-specific interactions. After incubation with blocking buffer the membrane was washed for 15min with Tween/TBS followed by three successive 5 min washes with Tween/TBS.

Primary antibodies were diluted in TBS/Tween containing 1% (w/v) BSA and 10µg/ml thimerosal and incubated with membranes for two hours at room temperature with gentle agitation. Antibody solutions were stored at 4°C. Following incubation, membranes were washed with TBS/Tween for 15 min, followed by three 5 min rinses, with vigorous shaking. HRP-conjugated secondary antibodies were incubated with the membranes for 1 hour at room temperature with gentle shaking. The dilutions of primary antibodies and corresponding secondary antibodies were as follows:

Table 2.4) Dilutions of Primary and Secondary Antisera.

Primary Antibody	Dilution	Secondary Antibody	Dilution
ERK1	1:1000	α-rabbit IgG	1:10000
ERK2	1:1000	α-mouse IgG	1:10000
$G_{i1+2\alpha}$	1:1000/(1:100)	α-rabbit IgG	1:10000/(1:1000)
$G_{i3\alpha}$	1:1000	α-rabbit IgG	1:10000
$G_{s\alpha}$	1:5000	α-rabbit IgG	1:10000
G_{β}	1:500	α-rabbit IgG	1:10000

Note: Numbers in parenthesis refer to the dilutions of antibodies when *o-dianisidine* was used to visualise bands (see text).

Following incubation with secondary antisera membranes were washed with vigorous shaking in TBS/Tween for 15 min, followed by three 5 min rinses. Immunoreactive bands were visualised by chemiluminescence using the ECL detection reagent. Washed membranes were allowed to drain and then the detection reagent was added to the protein-bound side of the membrane. Membranes were incubated with the detection reagent for one minute at room temperature. Following incubation the detection reagent was removed and excess reagent allowed to drain from the membrane. Membranes

were then wrapped in SaranWrap (Dow Chemical Co.) and placed, protein side up, in a film cassette. ECL-detected protein bands were visualised by exposing a sheet of autoradiography film (X-Omat AR, Kodak).

Alternatively, for some experiments, immunodetection of protein bands was achieved colourimetrically. Following exposure to primary and secondary antisera (Table 2.4) membranes were incubated with 10mg/ml o-*dianisidine* in 10mM Tris/HCL, pH 7.4. Addition of 0.1% (v/v) H_2O_2 initiated a HRP-catalysed reaction which stained protein bands brown.

2.2.12) Densitometry

ECL detected immunoblots were scanned with a type SI personal densitometer (Molecular Dynamics Ltd., Kensington, UK) and the optical density (OD) of protein bands quantified using ImageQuaNT^{TM} image-analysis software (Molecular Dynamics).

For ECL quantification to be accurate, it is important that the light produced is in the linear range of the film. Therefore, control experiments were carried out for each antiserum to establish the range of cellular protein concentrations which produced an intensity of labelling, following immunoblotting, which was proportional to the OD of the protein bands on the exposed film. Increasing concentrations of cellular protein (0.5-100μg) were applied to SDS/page (section 2.2.10) and analysed by immunoblotting (section 2.2.11). Quantitative data was obtained by densitometer analysis and plots of protein amount against OD were used to gauge the range of protein concentrations which fell within the linear range of the film (Table 2.5). When ECL detection systems were used care was also taken with the length of exposure so as to avoid underexposure or overexposure of the immunoblots.

Table 2.5) Concentrations of Cellular Protein which are within the Linear Range of the ECL Detection System.

Antiserum	Amount of Cellular Protein (µg)
ERK1	2.5-30
ERK2	2.5-40
$G_{i1+2\alpha}$	2.5-40
$G_{i3\alpha}$	5-40
$G_{s\alpha}$	10-40
G_{β}	5-30

2.2.13) Enzyme Assays

2.2.13.1) *Assay of Mitogen Activated Protein (MAP) Kinase Activity*

For assay of MAP kinase confluent cells from one 100mm plate were scraped into Buffer A (25mM Tris.HCl pH7.5, 25 mM NaCl, 40mM 4-nitrophenylphosphate, 10µM dithiothreitol, 10% (v/v) ethylene glycol, 1mM sodium orthovanadate, aprotinin (2 µg/ml), leupeptin (2µg/ml), pepstatin A (2µg/ml) and 100mM PMSF) and lysed by shearing through a $26^1/_2$G needle. The lysates were then centrifuged at $15000g_{max}$ for 5 min in a Sorvall centrifuge fitted with an SS34 rotor and supernatants retained. MAP kinase was assayed following partial purification of cell lysates by batch absorption to phenyl-Sepharose. Cell extracts (0.5ml) were mixed with 150µl (packed volume) of phenyl-Sepharose for 5 min on ice. The phenyl-Sepharose was then successively washed (0.5ml/wash) with Buffer A (twice) and Buffer A containing 35% (v/v) ethylene glycol (twice). MAP kinases, which bind tightly to this matrix (Ray and Sturgill, 1987), were eluted with 200µl Buffer A containing 60% (v/v) ethylene glycol for 5 minutes. After incubation the suspension was centrifuged in a Beckman microfuge (5 seconds) and the supernatant removed. Samples of supernatant were assayed immediately using MBP as a substrate (Anderson, 1992).

Components of the assay mixture were mixed in assay tubes at room temperature. Aliquots of eluant (5µl) from the phenyl Sepharose were added to 20µl of a solution of 1mg/ml MBP prepared in assay buffer (25mM HEPES, 1mM dithiothreitol, pH 7.5, 30ºC). To start the reaction, 15µl of a solution containing 133µM ATP, 26.7 mM $MgCl_2$ and 150µCi/ml [γ-^{32}P]-ATP was added to the reaction tube and mixed. The reaction was incubated for 10min at 30°C in a waterbath, and terminated by removing

30µl aliquots and spotting the aliquot onto 2x2cm squares of P81 phosphocellulose paper (Whatman, Kent, UK) which binds peptide substrates but not [γ-^{32}P]-ATP. After 10 seconds the square of phosphocellulose paper was dropped into a 500ml beaker containing of 180mM phosphoric acid (15ml/reaction). The papers were washed by stirring for 5min, after which the acid was discarded. Washes with phosphoric acid were repeated 5 more times. Finally, papers were rinsed once with 95% ethanol and allowed to dry. Dried papers were placed in a minivial with 4ml of scintillant and the radioactivity counted. Data were expressed as pmol of phosphate incorporated into MBP min per mg of cell lysate protein.

2.2.13.2) *Assay of MAP Kinase Kinase (MEK) Activity*

Cell lysates were prepared as described (section 2.2.13.1). MEK was assayed by measuring the incorporation of $^{32}P_i$ into recombinant, catalytically inactive K52R p42 MAP kinase (Wu *et al*, 1991a). Samples of cell extract (10µl) were incubated at 30°C for 25 min with 0.8µg of the K52R mutant of p42 MAP kinase in 40µl (total volume) of 10mM Hepes, pH 7.5; 40µM [γ-^{32}P]-ATP; 15mM $MgCl_2$; 1mM DTT. The reaction was stopped by the addition of one fourth volume of Laemmli sample buffer. After SDS/10% PAGE and staining the gel with Coomassie blue solution, incorporation of $^{32}P_i$ into K52R was visualised by autoradiography and the radioactivity of excised bands determined by liquid scintillation counting. Results were expressed as the amount of radioactivity incorporated into K52R (cpm).

2.2.13.3) *Assay of cyclic AMP-dependent Protein Kinase Activity (PKA)*

Cells from one 100mm plate were scraped in Buffer A containing 0.2% (v/v) NP-40 detergent, and lysed by shearing as described in the procedure for the assay of MAP kinase (section 2.2.13.1). The lysates were then centrifuged at 15000g for 5 min in a Sorvall centrifuge fitted with an SS34 rotor and supernatants retained. Samples of cell extract (10µl) were incubated at 30°C for 10 min with 0.26mM Kemptide (LRRASLG), a 7-amino acid substrate for PKA (Kemp *et al*, 1977), in a buffer containing 75mM Tris.HCl, pH 7.5; 15mM $MgCl_2$, 4mM DTT; 100µM ATP; 3µCi/ml [γ-^{32}P]-ATP. Assays were carried out in the presence or absence of an 18-residue peptide ligand containing the inhibitory domain sequence of a protein inhibitor of PKA (PKI) originally described by Cheng *et al* (1986). PKA activity was calculated as the amount of Kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the presence of PKI. Data were expressed as pmol of phosphate incorporated into Kemptide min^{-1} mg $protein^{-1}$.

2.2.14) DNA Assay

The DNA content of cellular homogenates was determined using the fluorescence enhancement of the fluorochrome bisbenzimide complexed with DNA (Brunk *et al*, 1979). The fluorescence increase produced by the DNA of cellular homogenates was compared directly with the fluorescence increase produced by a range of DNA standards. Reactions were prepared in assay buffer (10mM Tris, pH 7.5, 100mM NaCl, 10mM EDTA) and contained either a known amount of salmon sperm DNA standard (0.25-6.0μg/tube) or an aliquot of cellular homogenate (equivalent to approximately 1μg DNA). Bisbenzimide was added to each reaction to a final concentration of 1μg/ml and a final volume of 3ml. Reactions were then incubated for 1 hour at room temperature. The intensity of fluorescence of standards and samples were measured in arbitrary units using a model TKO 100 DNA fluorimeter (Hoefer Scientific Instruments Ltd., Newcastle-under-Lyme, UK). All measurements were performed in series using the same dye solution. A standard curve was constructed and the DNA content of test samples was determined by interpolation.

2.2.15) Protein Assay

The protein content of samples was assessed by the method of Bradford (1976). Reactions were prepared in a final volume of 100μl on microtitration plates (Greiner Labortechnik Ltd, Gloustershire, UK) and consisted of either a known amount of bovine serum albumin protein standards (0-10μg) or an aliquot of sample under test. Dye-binding was initiated with the addition of 240μl of fourfold diluted Bradford reagent. Coloured complex formation was measured at a wavelength of 620nM using a iEMS microplate reader (Labsytems Ltd., Hants., UK).

2.2.16) Assay of Intracellular Cyclic AMP Concentration

Samples were prepared according to the method described by Farndale *et al* (1992). Cell monolayers from 100mm plates were washed three times with ice cold PBS and intracellular cyclic AMP extracted with 1ml ethanol/0.5M HCl overnight at -20°C. Extracted samples were evaporated to dryness then reconstituted in 100μl cyclic AMP assay buffer (100mM Tris/HCl, pH 7.0, 8mM EDTA) and aliquots were assayed for cyclic AMP content.

Measurement of cellular cyclic AMP was carried out according to a modified method of Brown *et al* (1971); the assay was based on the competitive binding of [^{3}H]-cyclic

AMP and sample cyclic AMP to a binding protein (3', 5' cyclic AMP dependent protein kinase, Sigma). Reactions were prepared in assay buffer and contained either a known amount (0.1-10 pmol) of cyclic AMP standard, an aliquot of sample or assay buffer with no additions to a final volume of 300µl. In addition, each reaction contained [^{3}H]-cyclic AMP (25nCi/tube) and 8mg binding protein. Reactions were incubated for 4 hours at 4°C, following which a 100µl suspension of 26mg activated charcoal (Sigma) in assay buffer containing 2% (w/v) BSA was added to each tube. After centrifugation ($12000g_{max}$ for 2 min at 4°C) in a microfuge, a 200µl aliquot of the supernatant was taken for determination of radioactivity. Cyclic AMP concentration was determined by interpolation from a standard curve and was expressed as pmol per 10^6 cells.

2.2.17) Statistical Analysis

Statistical significance was determined by Student's *t*- test for unpooled samples (Samuels, 1989). Means were determined for test and control samples and used to calculate the *t* statistic (*ts*) using the following formula:

$$ts = \frac{y1-y2}{SE_{(y1-y2)}}$$

y1 = test sample means
y2 = control sample means
$SE_{(y1-y2)}$ = standard error of the difference

The standard error of the difference ($SE_{(y1-y2)}$) was calculated by the unpooled method (Samuels, 1989):

$$SE_{(y1-y2)} = \sqrt{((SE1)^2+(SE2)^2)}$$

SE1 = standard error of test sample
SE2 = standard error of control sample

P values were then determined from *ts* by cross-reference to a table of critical values of Student's *t*--distribution (Samuels, 1989). P-values were considered to be significant if they were less than a threshold significance level of 2% (p=0.02).

Chapter Three

Changes in the Expression of Guanine Nucleotide-binding Proteins During the Growth Hormone-dependent Differentiation of 3T3-F442A Preadipocytes

3.1) INTRODUCTION

Signal transduction processes involving heterotrimeric G-proteins play a major role in mediating the response of cells to external hormonal and growth factor stimuli and it is becoming increasingly clear that G-proteins transduce growth-regulatory signals that mediate changes in cellular proliferation, hypertrophy, differentiation and transformation (Post and Brown, 1996). Some of the most compelling evidence that G-proteins can serve as regulators of cell growth comes from studies of $G_{s\alpha}$ and $G_{i\alpha}$ in preadipocyte cell systems. Antisense oligodeoxynucleotides, which reduce the expression of the $G_{s\alpha}$ subunit, or overexpression of an inactivating mutant of $G_{s\alpha}$ (Gordeladze *et al*, 1997) have been shown to accelerate the rate of differentiation of 3T3-L1 preadipocytes into fat cells (Wang *et al*, 1992a). Conversely, activation of $G_{s\alpha}$ by cholera toxin intoxication blocks the ability of cells to differentiate (Wang *et al*, 1992). Conversely, over-expression of a constitutively active mutant form of $G_{i2\alpha}$ was shown to promote terminal differentiation of fibroblasts to adipocytes *in vitro* (Su *et al*, 1993; Gordeladze *et al*, 1997) and expression of inactivating $G_{i2\alpha}$ mutants abolish preadipocyte differentiation (Gordeladze *et al*, 1997), whereas *in vivo*, $G_{i2\alpha}$ has been shown to be crucial for development of fat tissues in neonatal mice (Moxham *et al*, 1993; Su *et al*, 1993). Together these observations highlight a critical role for G-proteins in adipocyte development.

Given the functional importance of G-proteins in cell morphogenesis it is likely that many of the phenotypic changes that occur on differentiation are reflections of alterations to the G-protein composition at different stages of development. In this respect changes in $G_{s\alpha}/G_{i\alpha}$ levels associated with preadipocyte differentiation have been reported (Lai *et al*, 1981; Watkins *et al*, 1982; Giershik *et al*, 1986; Watkins *et al*, 1987; Huppertz *et al*, 1993; McFarlane-Anderson *et al*, 1993) and these accompany the development of catecholamine-sensitive adenylate cyclase activity (Lai *et al*, 1981; see section 1.7.9.1). Adenylate cyclase activity can be regulated by both stimulatory (eg glucagon and β-adrenergic) and inhibitory (eg adenosine and prostaglandin) receptors which exert their actions through G_s and G_i respectively (Gilman, 1987). It is well documented that adipocyte differentiation is accompanied by increased expression of the 42 kDa and 47 kDa forms of $G_{s\alpha}$ which may contribute to the enhanced responsiveness of terminally differentiated adipocytes to lipolytic agents (Lai *et al*, 1981; Watkins *et al*, 1982; Watkins *et al*, 1987; Kilgour and Anderson, 1993; see section 1.7.9.1). However, studies on the expression of $G_{i\alpha}$ subtypes during preadipocyte differentiation have produced somewhat contradictory data, perhaps due to different detection methods used to assess G-protein expression (see section 3.1.1).

The traditional method used to identify and quantitate G-proteins, based on the ability of particular bacterial exotoxins to catalyse the transfer of [^{32}P]ADP-ribose of [^{32}P]NAD to the α-subunit of a specific G-protein has several drawbacks (McKenzie, 1992). For example pertussis toxin, isolated from *Bordetella Pertussis*, has been characterised as being able to catalyse the mono-ADP-ribosylation of $G_{i\alpha}$ (McKenzie, 1992). Although the substrate for pertussis toxin is defined as the α-subunit of "G_i", it has become apparent that a considerable number of pertussis-toxin-sensitive G-proteins exist, including G_{i1}, G_{i2}, G_{i3} and G_o (Jones and Reed, 1987; Lochrie and Simon, 1988). To compound matters, accurate G-protein quantitation may also be influenced by secondary factors such as β-subunit expression (Spiegel, 1992) and the expression of cellular ADP-ribosylation factors (Huppertz *et al*, 1993). Immunological probes provide a more specific means of detecting G-proteins (Milligan, 1990) and, in recent years, the isolation of complementary DNAs (cDNAs) for many proteins has facilitated the generation of antisera base on individual portions of the deduced amino-acid sequences (Milligan, 1990). Anti-peptide antisera have been used successfully to quantify changes in G-protein expression following the adipose conversion of 3T3-F442A preadipocytes (Kilgour and Anderson, 1993).

Until recently, the use of serum to differentiate preadipocytes has precluded the study of G-protein expression during individual stages of the adipogenic program, however the development of a serum-free medium capable of supporting adipocyte differentiation of 3T3-F442A preadipocytes has suggested that preadipocyte differentiation may be divided into two distinct stages (Guller *et al*, 1988; Schmidt *et al*, 1990; Darimont *et al*, 1994). The differentiation of 3T3-F442A preadipocytes is dependent upon the sequential actions of GH and insulin, with other factors exerting a modulatory influence (Guller *et al*, 1988). GH is thought to induce a primed state in the preadipocytes (Gp) in which cells acquire increased responsiveness to the growth promoting effects of insulin (Morikawa, 1986; Madon *et al*, 1986). The possibility therefore exists that regulated changes in G-protein expression may contribute critically to the cellular phenotype during both of these phases of preadipocyte differentiation. Initial studies from our laboratory have shown that terminal differentiation of 3T3-F442A cells under serum-free conditions has been reported to correspond with independent changes in the expression of $G_{s\alpha}$, $G_{i2\alpha}$ and $G_{i3\alpha}$ and increased responsiveness of adenylate cyclase to catecholamines (Kilgour and Anderson, 1993). The aim of the present work is to extend these studies to determine whether distinct changes occur in the G_s/G_i composition of 3T3-F442A cells during the different stages of their differentiation., therefore suggesting a role for the cyclic AMP signalling system in the control of these developmental stages.

Given the success of previous anti-peptide antisera (Anderson and Kilgour, 1993), peptides corresponding to various regions of the α-subunits of G_s, G_{i1}, G_{i2} and G_{i3} were utilised to generate sequence-specific antibodies. These tools subsequently permitted an assessment of the influence of differentiation on the levels of expression of the stimulatory- (G_s) and inhibitory- (G_{i1}, G_{i2} and G_{i3}) G-protein subunits in 3T3-F442A cell membranes during different stages of their development; either early in the differentiation process, during the GH priming stage, or later during the insulin-dependent maturation stage. In addition, recent evidence has suggested that βγ-dimers are involved in the transmission of growth signals (Faure *et al*, 1994; Crespo *et al*, 1994; Koch *et al*, 1994). Therefore, the effect of differentiative stimuli on the levels of β-subunit expression were also determined during the two phases of 3T3-F442A preadipocyte differentiation.

3.2) EXPERIMENTAL PROCEDURES

3.2.1) Standard Procedure for Cell Culture and Differentiation

3T3-F442A cells were cultured in DMEM containing 10% calf serum as described in Section 2.2.1). Confluent cultures (day 5 after seeding, subsequently referred to as JC cells) were washed three times in PBS and incubated for 10 days in a defined medium (DDM) containing GH (2nM), insulin (1.8μM), T_3 (0.1 ng/ml), EGF (50ng/ml) and other factors as described by Guller *et al* (1988). Cells cultured under these conditions, in which at least 80% of the cells exhibited adipocyte morphology as assessed by light microscopy and Oil Red O staining, are subsequently referred to as DDM cells. To assess the role of stimulatory and inhibitory G-proteins in modulating preadipocyte differentiation, cholera toxin, which activates G_s (McKenzie, 1992), and pertussis toxin, which inactivates G_i (McKenzie, 1992) were included in the DDM for the first three days of differentiation. After three days, the medium was replaced with fresh DDM in the absence of toxins and differentiation was assessed 7 days later.

3.2.2) Two-phase Protocol for Cellular Differentiation

For these studies cells were grown to confluence in the presence of 10% calf serum which had previously been depleted of GH (described in section 2.2.4.1). Cells were passaged at least twice, in DMEM containing 2.5mM glutamine and 10% GH-depleted calf serum (standard GH (-) medium), prior to use for differentiation studies. Confluent cells were washed three times in PBS then incubated for two days in serum free medium [F12:DMEM (2:1) containing GH (2nM), transferrin (10μg/ml), fetuin (50μg/ml), glutamine (2.5mM) and BSA (1mg/ml), subsequently referred to as priming medium]. Cultures were then washed as before and the medium replaced with maturation medium [serum free medium containing insulin (1.8μM), T_3 (0.1ng/ml) and EGF (50ng/ml)]. After a further 8 days the extent of differentiation was assessed by light microscopy and Oil Red O staining. As a control, parallel cultures were maintained at confluence for 10 days in standard GH (-) medium.

3.2.3) Peptide Synthesis, Conjugation and Immunisation

Decapeptides were provided by Dr James Beattie (Hannah Research Institute, Ayr, Scotland) and were assembled stepwise by the Merrifield solid-phase method (Barany and Merrifield, 1979) and determined to be homogeneous by HPLC as described in section 2.2.9. The amino acid sequences are listed in Table 3.1.

Following synthesis peptides were stored desiccated at -20°C until required. Polyclonal antibodies were raised in rabbits using immunogen-peptides coupled to KLH (see section 2.2.9). Pre-immune sera were collected just prior to the

commencement of the immunisation schedule which is detailed in section 2.2.9. Immune and pre-immune sera were stored in aliquots at -20°C until used for immunoblotting.

Table 3.1) Amino-acid Sequences of Immunogen Peptides

Protein Species	Sequence[1]
$G_{i1\alpha}$ (residues 159-168)	LDRIAQPNYI
$G_{i2\alpha}$ (residues 159-168)	LERIAQSDYI
$G_{i3\alpha}$ (residues 159-168)	LDRISQSNYI
$G_{i1+2\alpha}$ (C-terminal)	KNNLKDCGLF
$G_{s\alpha}$	RMHLRQYELL

[1]Single letter amino acid code

3.2.4) Membrane Preparations

Isolation of rat white adipocytes and preparation of membrane fractions was carried out according to the methods described in section 2.2.8.2. Membrane fractions were prepared from 3T3-F442A cells as described in section 2.2.8.1, during growth (pre-confluence) or at various time-points during their differentiation with the two-phase differentiation protocol (Section 3.2.2.2). Membranes were stored at -80°C until required for immunoblotting.

3.2.5) Immunoblotting

Membrane samples were separated by SDS/PAGE on 10%-acrylamide gels (15cm-length gels for the detection of $G_{i\alpha}$ and G_{β}, 20cm-length gels for $G_{s\alpha}$). Immunoblotting was performed essentially as described in section 2.2.11. Briefly, proteins were transferred to nitrocellulose and then blocked overnight in 3% BSA. Primary antiserum, diluted in 1% BSA, was then added for 2 hours. Primary antisera were used at the dilutions described in Table 3.2. After removal of primary antiserum, the blot was washed extensively and then incubated with anti-rabbit IgG conjugated to HRP (secondary antiserum) at the dilutions indicated in Table 3.2. Immunoreactive bands were visualised with the Enhanced ChemiLuminescence system or by colourimetric staining (see section 2.2.11). Where indicated the primary antiserum was pre-incubated with immunogen peptide (Table 3.1) prior to incubation with the immunoblot. This was to confirm the specificity of immunoreactivity.

Table 3.2) Dilutions of Primary and Secondary Antisera for G-protein Immunoblots.

Primary Antibody	Dilution	Secondary Antibody	Dilution
$G_{i1+2\alpha}$	1:1000/(1:100)	α-rabbit IgG	1:10000/(1:1000)
$G_{i3\alpha}$	1:1000	α-rabbit IgG	1:10000
$G_{s\alpha}$	1:5000	α-rabbit IgG	1:10000
G_{β}	1:500	α-rabbit IgG	1:10000

Numbers in parenthesis refer to antibody dilutions when the o-diansidine detection system was used

3.2.6) GPDH and DNA Assays and Expression of Results

GPDH activity was measured by the method of Wise and Green (1979). In accordance with others (Lai *et al*, 1981), adipocytes were found to contain approximately 2.3 times more protein per cell than in fibroblasts (Table 3.6). GPDH activities are therefore expressed relative to DNA content rather than amount of cellular protein. The DNA content of cellular homogenates was determined fluorimetrically as described in Chapter 2. The enzyme activity per plate of cells was expressed as the number of moles of NADH oxidised/second (katals)/mgDNA.

3.3) RESULTS

3.3.1) Detection of Gsα-subunits in 3T3-F442A Fibroblasts

There are two major isoforms of G_s α-subunits which have different relative mobilities on SDS/PAGE, displaying apparent molecular masses of 42 kDa and 47 kDa respectively (Mattera *et al*, 1986; Robishaw *et al*, 1986).These two species are produced by differential splicing of a common mRNA and differ by the presence (long-form) or absence (short form) of a fifteen amino-acid (residues 72-86) internal peptide (Mattera *et al*, 1986; Robishaw *et al*, 1986). Isoforms of $G_{s\alpha}$ also contain an arginine residue (Arg 187/188 or 201/202, depending on the splice variant) which is a target for ADP-ribosylation by cholera toxin (Gill and Meren, 1978). The use of specific anti-peptide antisera has revealed that both forms of $G_{s\alpha}$ are abundantly expressed in white adipose tissue of both rats and mice (Bégin-Heick, 1990; Granneman *et al*, 1990) and are localised to the plasma membrane, Golgi and endosomal fractions of fat cells (Bégin-Heick, 1990; Haraguchi and Rodbell, 1990; Strassheim *et al*, 1991). For this study antiserum (anti-$G_{s\alpha}$) was generated against the carboxy-terminal (RMHLRQYELL) amino-acid sequence of $G_{s\alpha}$ predicted from the cDNA sequences from several species, including rat and mouse (Bray *et al*, 1986; Mattera *et al*, 1986; Robishaw *et al*, 1986; Jones and Reed, 1987; Cerione *et al*, 1988). In agreement with others (Strassheim *et al*, 1991), rabbit immune-serum identified a doublet, composed of roughly equal quantities of a 42 kDa and a 47 kDa protein species, on immunoblots of membrane samples from rat adipocytes (Fig 3.1A). In contrast no reactivity was detectable with pre-immune serum (Fig 3.1A).

The abundance of G_s isoforms was next determined in membrane fractions of confluent murine 3T3-F442A preadipocytes. Anti-$G_{s\alpha}$ also revealed two forms of $G_{s\alpha}$ in immunoblots of 3T3-F442A cell membranes which migrated with the same apparent molecular mass as the long- and short-forms in rat adipocytes (Fig 3.1B). 3T3-F442A cell membranes were found to contain similar amounts of the long-form of $G_{s\alpha}$ that found in rat adipocyte membranes, whereas considerably less of the short form of $G_{s\alpha}$ (approximately 6-fold less) was detected in 3T3-F442A membranes when compared to rat adipocytes (Fig 3.1B).

3.3.2) Detection of $G_{i2\alpha}$ in Membranes from 3T3-F442A Cells.

$G_{i\alpha}$ exists as three closely related isoforms, $G_{i1\alpha}$ (41 kDa), $G_{i2\alpha}$ (40 kDa) and $G_{i3\alpha}$ (41kDa), which all contain a cysteine residue that is a putative ADP-ribosylation site and is the fourth residue from the carboxy-terminus (West *et al*, 1985). Each of the

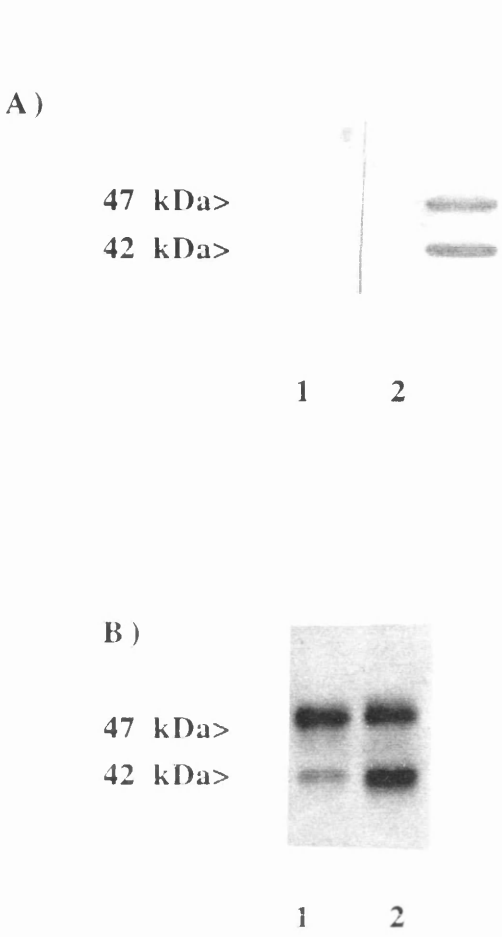


Fig 3.1) Detection of 42 kDa and 47 kDa Isoforms of $G_{s\alpha}$ in Rat Adipocyte and 3T3-F442A Cell Membranes

Equal quantities of membrane protein (50μg) from 12 day confluent 3T3-F442A cells or rat adipocytes were separated by SDS/PAGE and immunoblotted with pre-immune serum or antiserum raised against $G_{s\alpha}$ (anti-$G_{s\alpha}$) as described in Chapter 2. In panel A, rat adipocyte membranes were probed with pre-immune serum (*lane 1*) or with anti-$G_{s\alpha}$ (*lane 2*) at a dilution of 1:5000. In panel B, membranes from 3T3-F442A cells (*lane 1*) and rat adipocytes (*lane 2*) were probed with anti-$G_{s\alpha}$ (1:5000). The 42 kDa and 47 kDa isoforms of $G_{s\alpha}$ are indicated with arrow heads. Results are representative of experiments done on two separate occasions.

three $G_{i\alpha}$ subtypes is coded for by a distinct mRNA species which is the product of a single gene in a haploid genome (Bray *et al*, 1987; Jones and Reed, 1987). Anti-peptide antisera have proved capable of discriminating between three isoforms of Giα and have been used to demonstrate that white adipocytes of both rats and mice contain G_{i1}, G_{i2} and G_{i3} α-subunits (Mitchell *et al*, 1989; Bégin-Heick, 1990). To begin to identify the $G_{i\alpha}$–subtypes expressed in 3T3-F442A cells, rabbit antiserum was generated against a decapeptide corresponding to the C-terminal of $G_{i1\alpha}$ and $G_{i2\alpha}$ (termed $G_{i1+2\alpha}$; Table 3.3).

Table 3.3) Comparison of Amino Acid Sequences of Carboxy-terminal Decapeptides from G-protein α-subunits

α-subunits	Sequences[1]
G_{i1} and G_{i2}	KNNLKDCGLF
G_{i3}	KNNLKECGLY
Gs	RMHLRQYELL

[1]Single acid amino acid code

Anti-$G_{i1+2\alpha}$ recognised a single immunoreactive polypeptide in 3T3-F442A fibroblast membranes which migrated with an apparent molecular mass of 40 kDa on SDS/PAGE (Fig 3.2A), whereas no immunoreactivity was detected with pre-immune serum (Fig 3.2A). The immunoreactive species co-migrated with the major immunoreactive species detected in immunoblots of rat adipocyte membranes (Fig 3.2B). Rat adipocytes express both $G_{i1\alpha}$ and $G_{i2\alpha}$ and the major 40 kDa immunoreactive band detected with this antiserum represents a mixture of both of these isoforms (Mitchel *et al*, 1989). The weaker signal detected in 3T3-F442A membranes could be attributable to the presence of lower levels of $G_{i1\alpha}$ and/or $G_{i2\alpha}$ and possibly only one of these isoforms is present. To assess the relative levels of $G_{i1\alpha}$ and $G_{i2\alpha}$ present in 3T3-F442A membranes it was necessary to obtain better resolution of these subtypes than that afforded by standard SDS/PAGE conditions. Accordingly, G-proteins were resolved by electrophoresis in the presence of 6M urea as described in Chapter 2. Under these conditions anti-$G_{i1+2\alpha}$ identified two polypeptides in rat adipocyte membranes which migrated with apparent molecular masses of 41 and 40 kDa (Fig 3.3). These correspond to $G_{i1\alpha}$ and $G_{i2\alpha}$ respectively (Mitchel *et al*, 1989). In the same experiment only a single 40 kDa polypeptide ($G_{i2\alpha}$) was identified in membranes from 3T3-F442A fibroblasts or 3T3-F442A cells

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Anti-$G_{i1+2\alpha}$ recognised a single immunoreactive polypeptide in 3T3-F442A fibroblast membranes which migrated with an apparent molecular mass of 40 kDa on SDS/PAGE (Fig 3.2A), whereas no immunoreactivity was detected with pre-immune serum (Fig 3.2A). The immunoreactive species co-migrated with the major immunoreactive species detected in immunoblots of rat adipocyte membranes (Fig 3.2B). Rat adipocytes express both $G_{i1\alpha}$ and $G_{i2\alpha}$ and the major 40 kDa immunoreactive band detected with this antiserum represents a mixture of both of these isoforms (Mitchel *et al*, 1989). The weaker signal detected in 3T3-F442A membranes could be attributable to the presence of lower levels of $G_{i1\alpha}$ and/or $G_{i2\alpha}$ and possibly only one of these isoforms is present. To assess the relative levels of $G_{i1\alpha}$ and $G_{i2\alpha}$ present in 3T3-F442A membranes it was necessary to obtain better resolution of these subtypes than that afforded by standard SDS/PAGE conditions. Accordingly, G-proteins were resolved by electrophoresis in the presence of 6M urea as described in Chapter 2. Under these conditions anti-$G_{i1+2\alpha}$ identified two polypeptides in rat adipocyte membranes which migrated with apparent molecular masses of 41 and 40 kDa (Fig 3.3). These correspond to $G_{i1\alpha}$ and $G_{i2\alpha}$ respectively (Mitchel *et al*, 1989). In the same experiment only a single 40 kDa polypeptide ($G_{i2\alpha}$) was identified in membranes from 3T3-F442A fibroblasts or 3T3-F442A cells

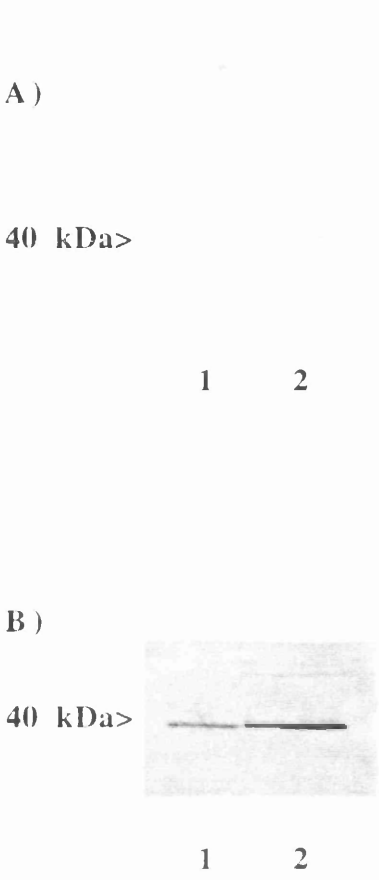


Fig 3.2) Immunoreactivity of Antiserum to $G_{i1\alpha}$ Plus $G_{i2\alpha}$ G-proteins in Rat Adipocyte and 3T3-F442A Cell Membranes

In panel A, 10µg of 3T3-F442A cell membrane protein was separated by SDS/PAGE and immunoblotted with pre-immune serum (*lane 1*) or antiserum which recognises both $G_{i\alpha1}$ and $G_{i\alpha2}$ (anti-$G_{i\alpha1+2}$, *lane 2*) at a dilution of 1:100 as described in Chapter 2. In *panel B*, 10µg of membrane protein from 3T3-F442A fibroblasts (*lane 1*) and rat adipocytes (*lane 2*) were probed with anti-$G_{i\alpha1+2}$ at a dilution of 1:100. The single immunoreactive species of 40 kDa is indicated with an arrow-head. Results are representative of an experiment repeated on two separate occasions.

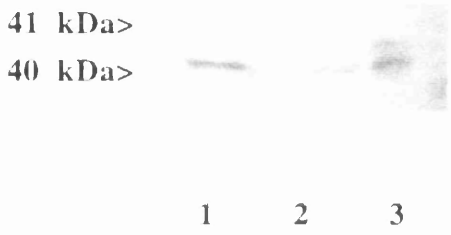


Fig 3.3) Abundance of $G_{i1\alpha}$ and $G_{i2\alpha}$ Membranes from 3T3-F442A Cells and Rat Adipocytes

Equal quantities of membrane protein (50μg) from confluent 3T3-F442A fibroblasts (*lane 1*), 3T3-F442A cells differentiated with FCS/insulin medium (*lane 2*) or rat adipocytes (*lane 3*) were resolved on 10% polyacrylamide gels containing 6M urea as described in Chapter 2. Gels were then immunoblotted with anti-$G_{i\alpha 1+2}$ at a dilution of 1:100. The 41 kDa ($G_{i1\alpha}$) and 40 kDa ($G_{i2\alpha}$) forms of $G_{i\alpha}$ are indicated with arrow heads. Results are representative of an experiment repeated on two separate occasions.

differentiated with FCS/insulin (Fig 3.3). As previously reported (Anderson and Kilgour, 1993), the abundance of $G_{i2\alpha}$ was lower in FCS cell membranes than in fibroblasts. Moreover, antisera which specifically recognises only $G_{i1\alpha}$ fails to detect an immunoreactive band in 3T3-F442A cell membranes (Anderson and Kilgour, 1993; Yarwood, Anderson and Kilgour, unpublished). Together, these observations demonstrate that 3T3-F442A cells express detectable levels of $G_{i2\alpha}$, but not $G_{i1\alpha}$. Hence anti-$G_{i1+2\alpha}$ antibodies can be used specifically as a probe for $G_{i2\alpha}$ in these cells.

3.3.3) Detection of $G_{i3\alpha}$ in Membranes from 3T3-F442A Fibroblasts.

In an attempt to raise antiserum specific for $G_{i3\alpha}$ (anti-$G_{i3\alpha}$) rabbits were immunised with a decapeptide corresponding to a distinct, internal region (residues 159-168, Table 3.4) of the deduced amino acid sequence of $G_{i3\alpha}$ (Goldsmith *et al*, 1987). Although the sequences in this region are very similar in all forms of $G_{i\alpha}$ (Table 3.4), antisera generated against these peptides have been shown to be highly specific for individual α_i-subtypes (Spiegel, 1990).

Table 3.4) Comparison of Deduced Amino Acid Sequences Corresponding to Residues 159-168 in $G_{i\alpha}$.

α-subunits	Sequences[1]
G_{i1}	LDRIAQPNYI
G_{i2}	LERIAQSDYI
G_{i3}	LDRISQSNYI

[1]Single acid amino acid code

Antisera from animals challenged with the $G_{i3\alpha}$ internal decapeptide revealed a 41 kDa immunoreactive polypeptide in membrane fractions from 3T3-F442A fibroblasts and rat adipocytes which was not apparent when pre-immune serum was used (Fig 3.4). To further test whether this protein species corresponded to $G_{i3\alpha}$ immunoblots were carried out in the presence of excess amounts of the synthetic peptide antigen (LDRISQSNYI) used for immunisations (see section 3.2.2). Addition of immunogen-peptide was found to block the reactivity of anti-$G_{i3\alpha}$ with the 41 kDa polypeptide on immunoblots of 3T3-F442A and rat adipocyte membranes. In contrast, a decapeptide conforming to the same internal sequence of $G_{i2\alpha}$ (Fig 3.5) was found to have a relatively small effect on anti-$G_{i3\alpha}$ reactivity. Similarly, a decapeptide corresponding to the equivalent region in $G_{i1\alpha}$ (Table 3.4) was unable to block the detection of the 41 kDa polypeptide (Fig 3.6). These results demonstrate the specificity of anti-$G_{i3\alpha}$ for

$G_{i3\alpha}$ and show that 3T3-F442A fibroblasts express this subtype of G_i in their cell membranes.

Although it is apparent that the anti-$G_{i3\alpha}$ antiserum specifically recognises $G_{i3\alpha}$ in 3T3-F442A cells and rat adipocytes this antiserum also cross-reacts with a 39 kDa protein species in these two cell types (Fig 3.4). The immuno-reactivity of this protein with anti-$G_{i3\alpha}$ is also blocked by $G_{i3\alpha}$ peptide antigen (Fig 3.4). While the identity of this protein remains to be determined it is unlikely to be $G_{i2\alpha}$, which shares a similar relative mobility (Fig 3.2), since the cross-reactivity of anti-$G_{i3\alpha}$ with this protein is unaffected by $G_{i2\alpha}$ peptide antigen (Fig 3.5).

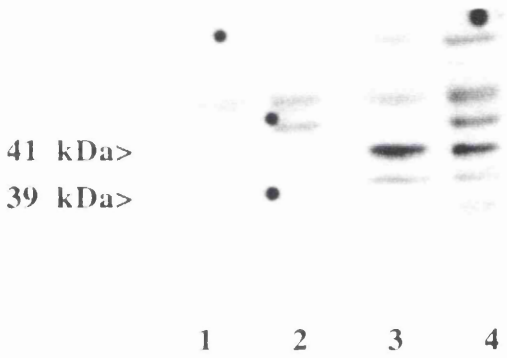
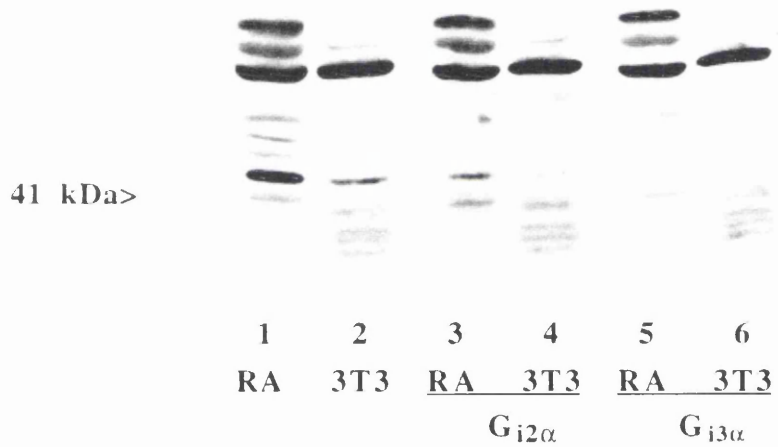


Fig 3.4) Immunoreactivity of Antiserum to $G_{i3\alpha}$ with Rat Adipocyte and 3T3-F442A Cell Membranes.

Equal quantities of membrane protein (50μg) from confluent 3T3-F442A fibroblasts (*lanes1 and 3*) or rat adipocytes (*lanes 2 and 4*) were separated by SDS/PAGE and immunoblotted as described in the legend to Fig 3.1. Antisera used were pre-immune (*lanes 1 and 2*) and anti-$G_{i3\alpha}$ (*lanes 3 and 4*) at a dilution of 1:1000 as described in Chapter 2. Major immunoreactive species of 39 kDa and 41 kDa which were specific to immunoblot strips probed with anti-$G_{i3\alpha}$ are indicated with arrow-heads. Results are representative of an experiment repeated on two separate occasions.



1 2 3 4 5 6

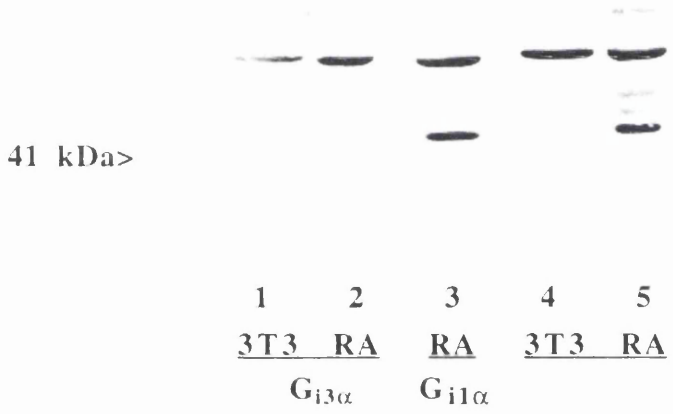
RA 3T3 RA 3T3 RA 3T3

$G_{i2\alpha}$ $G_{i3\alpha}$

Fig 3.5) Assessment of the Specificity of an Anti-$G_{i3\alpha}$ Antiserum by Competition with Synthetic Peptides: Cross-reactivity of Anti-$G_{i3\alpha}$ with $G_{i2\alpha}$.

Equal quantities of membrane protein (50µg) from confluent 3T3-F442A fibroblasts (*lanes 2, 4 and 6*) or rat adipocytes (*lanes 1,3 and 5*) were separated by SDS/PAGE and immunoblotted with anti-$G_{i3\alpha}$ (1:1000) as described n the legend to Fig 3.4.

Immunoblot strips in *lanes 3 and 4*, and in *lanes 5 and 6*, contained 50µg of synthetic peptide included with the first antibody during immunoblotting. Peptides used were LERIAQSDYI (*lanes 3 and 4*), which corresponds to residues 159-168 of $G_{i2\alpha}$, and LDRISQSNYI (*lanes 5 and 6*) corresponding to residues 159-168 of $G_{i3\alpha}$. The position of the 41 kDa immunoreactive species ($G_{i3\alpha}$) which was specifically depleted in the presence of LDRISQSNYI is indicated with an arrow-head. Results are representative of an experiment repeated on two separate occasions.



1	2	3	4	5
3T3	RA	RA	3T3	RA
	$G_{i3\alpha}$	$G_{i1\alpha}$		

Fig 3.6) Assessment of the Specificity of an Anti-$G_{i3\alpha}$ Antiserum by Competition with Synthetic Peptides: Cross-reactivity of Anti-$G_{i3\alpha}$ with $G_{i1\alpha}$.

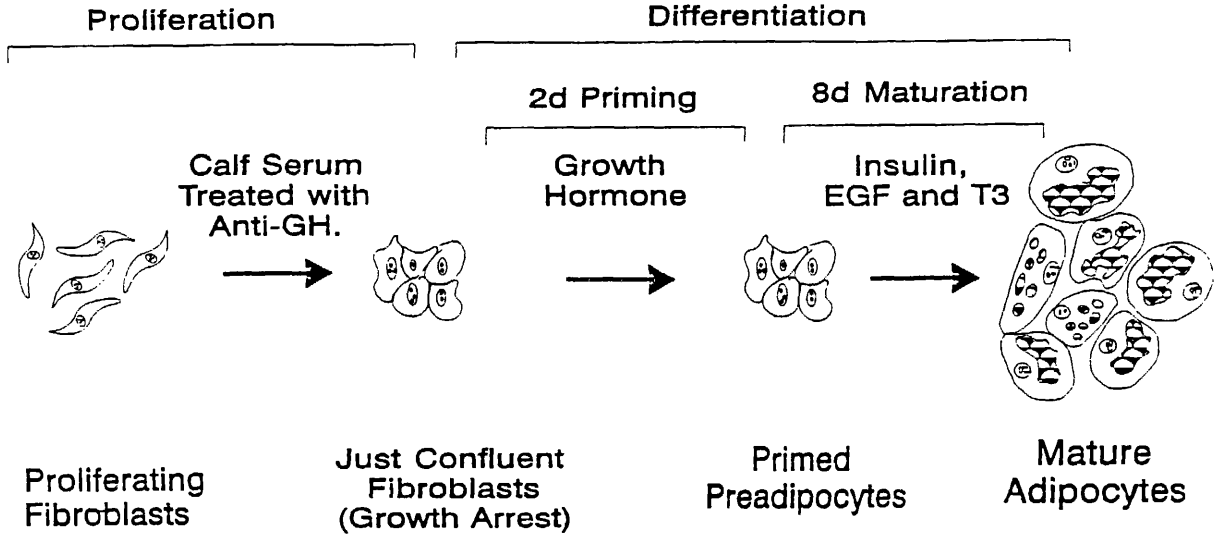
Equal quantities of membrane protein (50µg) from confluent 3T3-F442A fibroblasts (*lanes 1 and 4*) or rat adipocytes (*lanes 2,3 and 5*) were separated by SDS/PAGE and immunoblotted with anti-$G_{i3\alpha}$ (1:1000) as described n the legend to Fig 3.4.

Immunoblot strips in *lanes 1 and 2* contained 50µg of the synthetic peptide LDRISQSNYI (residues 159-168 of $G_{i3\alpha}$) along with the primary antibody as described in the legend to Fig 3.5. included with the first antibody during immunoblotting. The immunoblot strip in *lane 3* was incubate in the presence of the decapeptide LDRIAQPNYIwhich corresponds to residues 159-168 of $G_{i1\alpha}$, The position of the 41 kDa immunoreactive species ($G_{i3\alpha}$) which was specifically depleted in the presence of LDRISQSNYI is indicated with an arrow-head. Results are representative of an experiment repeated on two separate occasions.

3.3.4) Characterisation of the Two-Phase Protocol for 3T3-F442A Cell Differentiation.

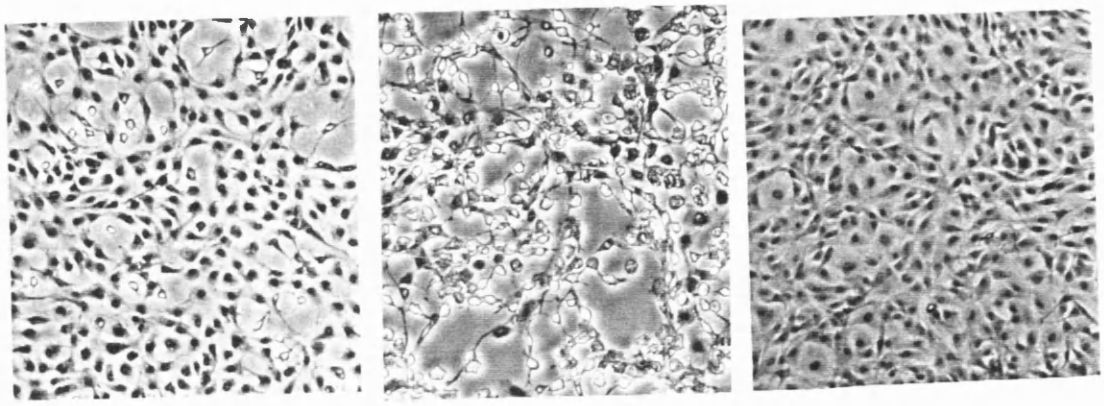
Preadipocyte differentiation involves a program of multiple developmental stages coordinated by external and genetic modulators (Charnels *et al*, 1994). The external, hormonal factors which control the adipose conversion of 3T3-F442A preadipocytes are GH, insulin, EGF and T_3 (Charnels *et al*, 1994; Guller *et al*, 1988). GH has been proposed to act at an early stage of preadipocyte differentiation by inducing the expression of early markers of the process (Clarkson *et al*, 1995; Barcellini-Couget *et al*, 1993; Ashcom *et al*, 1992) and sensitising cells to the actions of other differentiative factors which then induce terminal differentiation (Morikawa *et al*, 1982; Nixon and Green, 1984; Zezulak and Green, 1986; Guller *et al*, 1988). In order to study the effect of differentiative factors on the expression of heterotrimeric G-proteins during these two phases a two step differentiation protocol was developed in which the priming action of GH was clearly observed (Fig 3.7).

Cells were grown to confluence in calf-serum which had been depleted of GH (see section 2.2.4.1) and then primed for two days in serum-free medium in the presence or absence of GH. Following priming, the medium was replaced with fresh medium containing the maturation agents, insulin, EGF and T_3. The extent of differentiation was assessed eight days later. Cells primed with GH were found to adopt the morphological characteristics of adipocytes (Novikoff *et al*, 1980) in response to subsequent exposure to insulin, EGF and T_3, in that cells became rounded and accumulated triacylglycerol droplets in their cytoplasm (Fig 3.8A). In contrast, cells primed in the absence of GH retained a fibroblastic appearance after treatment with the maturation agents (Fig 3.8A). The efficiency of adipose conversion was also assessed biochemically by measuring cellular GPDH activity which is a sensitive marker of terminal adipocyte differentiation (Wise and Green, 1979). Following exposure to insulin, EGF and T_3 there was a substantial increase in GPDH activity expressed in cells which had been GH-primed whereas no significant increase in GPDH activity was detected in cells primed in the absence of GH (Fig 3.8B). Cells grown in normal serum which had not been depleted of GH and which were subsequently primed in the absence of GH differentiated normally (Fig 3.8B), indicating that depletion of calf-serum GH is necessary to observe the priming effect of GH during 3T3-F442A preadipocyte differentiation. Together, these observations demonstrate that in 3T3-F442A cells pre-exposure to GH is necessary for maturation agents to induce the changes in cell phenotype associated with fat-cell conversion.



3.7) Two-phase Protocol for the Differentiation of 3T3-F442A Preadipocytes

A)



Primed Minus GH Primed Plus GH 10DC

B)

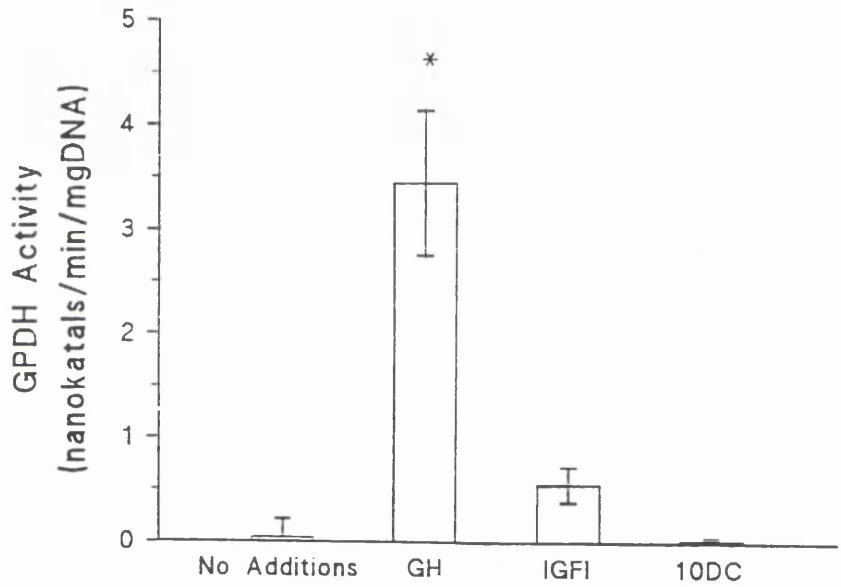


Fig 3.8) Effect of GH on the Priming of 3T3-F442A Preadipocytes.
Cells were grown to confluence in 10% calf serum which had been depleted of GH. At confluence cells were primed in serum-free medium in the presence or absence of GH or IGF-I as indicated. After 2 days the priming medium was replaced with maturation medium and 8 days later the extent of differentiation was assessed by either phase-contrast microscopy (*panel A*) or by measuring cellular GPDH activity (*panel B*). For further details see Section 2.2.4. Alternatively, cells were grown to confluence in CS which had not been depleted of GH (CS cells) and were primed in the absence of GH (*Panel B*). Parallel cultures were maintained at confluence for 10 days (10DC) as a control. GPDH activities are expressed as the number of moles of NADH oxidised/second (katals)/mgDNA and are means±S.E.M. for 3 observations. * indicates the value differs significantly from 10DC cells; $p<0.01$.

Although insulin-like growth factor I (IGF-I) is known to mediate many of the actions of GH (Ashcom *et al*, 1992), the priming action of GH on 3T3-F442A differentiation is not due to local production of IGF-I since IGF-I alone was unable to mimic the effect (results not shown). Furthermore it has been reported that GH does not promote IGF-I production in 3T3-F442A fibroblasts (Barcellini-Couget *et al*, 1993). These results show that differentiation of 3T3-F442A cells involves at least two distinct stages. Exposure to GH is necessary to prime cells to the actions of insulin, EGF and T_3 which act in the second, maturation phase to promote terminal differentiation.

3.3.5) Changes in G-protein Content in 3T3-F442A Cell Membranes During Confluence and Differentiation.

A number of studies have suggested that G-protein α-subunits play a crucial role in modulating fat cell development (Wang *et al*, 1992; Watkins *et al*, 1992; Moxham *et al*, 1993; Su *et al*, 1993) and that alterations in G-protein expression occur during preadipocyte differentiation (Lai *et al*, 1981; Watkins *et al*, 1982; Giershik *et al*, 1986; Watkins *et al*, 1987; Anderson and Kilgour, 1993; Huppertz *et al*, 1993). Having demonstrated that the differentiation of 3T3-F442A preadipocytes can be divided into a priming and maturation stage (section 3.3.3.1), the levels of $G_{s\alpha}$, $G_{i\alpha}$ and G_{β} were next determined in 3T3-F442A cell membranes, during these two phases of preadipocyte differentiation. A comparison was made between 3T3-F442A cells treated with the two-phase differentiation protocol and non-differentiating control cultures which were grown to confluence in 10% CS and maintained at confluence in 10% CS for a further 10 days. As noted in Fig 3.8, by day 10 approximately 90% of GH-primed 3T3-F442A cells have differentiated into adipocytes whereas virtually no 10-day confluent cells differentiate.

To determine whether changes in the expression of $G_{s\alpha}$ occur during the time-course of differentiation, a specific antiserum, which cross-reacts with the 42 kDa and 47 kDa forms of $G_{s\alpha}$ (section 3.1.3.1), was used to measure changes in the levels of these proteins in membranes of 3T3-F442A cells at different stages of their adipose conversion. Immunoblot analysis demonstrated that profound changes occurred in the levels of both the 42 kDa and the 47 kDa isoforms of $G_{s\alpha}$ ($G_{s\alpha 42}$ and $G_{s\alpha 47}$) during the two phase differentiation process (Fig 3.9A). No significant difference was detected in the levels of either $G_{s\alpha}$ subunit when membranes from sub-confluent, proliferating cells were compared to those from confluent cells. However, following confluence the levels of both $G_{s\alpha}$ subunits began to increase significantly. Maximal levels of $G_{s\alpha 47}$ were detected in cell membranes at the end of the 2 day priming stage and persisted for at least the first two days of the maturation stage then declined,

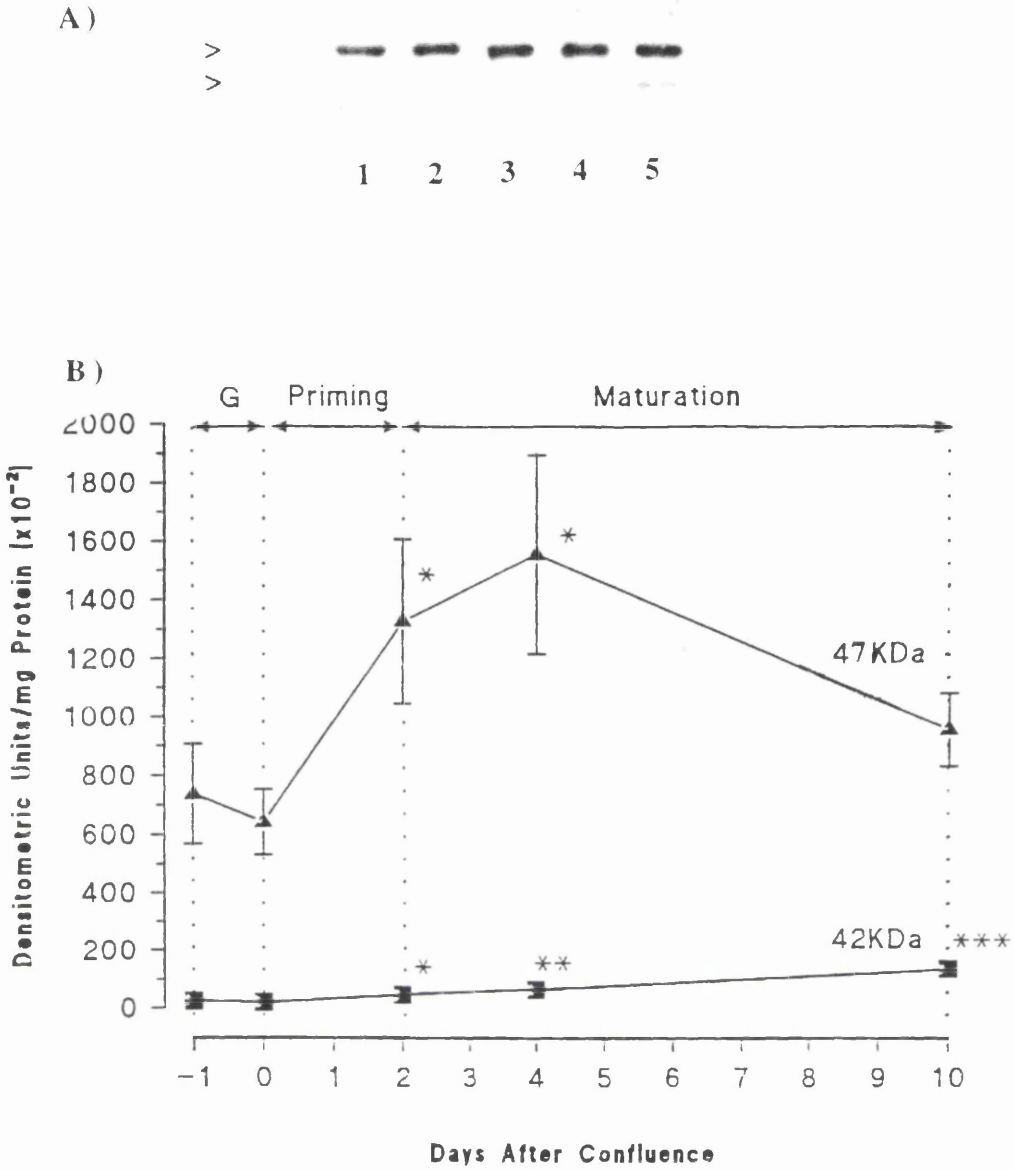


Fig 3.9) Expression of $G_{s\alpha 42}$ and $G_{s\alpha 47}$ in 3T3-F442A Membranes During Differentiation by the Two Phase Differentiation Protocol.

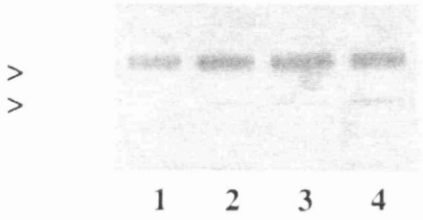
Equal quantities of membrane protein (10µg) extracted from 3T3-F442A cells during the various stages of their adipose conversion were subject to SDS/PAGE and immunoblotting with specific anti-$G_{s\alpha}$ antibodies (*panel A*). Membrane samples were prepared from pre-confluent, proliferating (*lane 1*), just confluent cells (*lane 2*), cells primed for 2 days in the presence of GH (*lane 3*), cells exposed to maturation medium for 2 days (*lane 4*) or from mature adipocytes (*lane 5*). The 42 kDa and 47 kDa forms of $G_{s\alpha}$ are indicated with arrow-heads. *Panel B* demonstrates the changes in densitometric values (arbitrary units) for $G_{s\alpha 42}$ and $G_{s\alpha 47}$ obtained from immunoblots. Just confluent cells are denoted day 0, whereas cells in the proliferative phase (G) and mature adipocytes are represented as being one day pre-confluent and ten day post-confluent respectively. Immunoblots were repeated on three separate occasions and densitometric units are expressed as means±S.E.M. for three separate observations. *,** and *** indicates that the value differs significantly from those obtained from just confluent (day 0) cells; $p<0.02$, $p<0.01$ and $p<0.001$, respectively.

reaching levels approaching those found in JC membranes by day 10 of differentiation (ie in mature adipocytes). In comparison to the levels detected in JC membranes, levels of G_{sa42} were significantly increased by the end of the 2 day priming stage (2.4±0.29 fold increase, $p<0.02$) and continued to rise, resulting in a final 7.18±0.08 fold, $p<0.01$) increase by the end of the differentiation process (day 10). However, similar changes in G_{sa42} and G_{sa47} levels were apparent in parallel cultures maintained in growth medium for 10 days following confluence (Fig 3.10), although these cultures displayed no increase in GPDH activity and none of the morphological changes associated with the differentiation process.

Immunoblots analysis of cell membranes with antiserum specific for $G_{i2\alpha}$ (section 3.3) showed that there was no significant change in the expression of this subunit when pre-confluence proliferating cells were compared to confluent cultures (day 0, Fig 3.11). There was, however, a significant 1.51±0.06 fold ($p<0.01$, n=3) increase in $G_{i2\alpha}$, relative to JC cells, following the two-day priming phase. These levels were sustained for at least the first 2 days of the maturation phase, after which the levels of $G_{i2\alpha}$ declined dramatically to fall below those found in JC cells by the end of the differentiation period (0.15±0.01 fold decrease, $p<0.001$, n=3). The amount of $G_{i3\alpha}$ in cell membranes did not fluctuate significantly during the two stages of differentiation (Fig 3.12). However, the amount of $G_{i3\alpha}$ in terminally differentiated adipocytes were found to be slightly lower than in JC fibroblasts (0.40±0.04, $p<0.05$, n=3). The changes in the levels of $G_{i2\alpha}$ and $G_{i3\alpha}$ which occurred at different stages of the differentiation process appear to be specifically regulated by the differentiative agents present (GH, insulin, EGF and T_3) as such changes were not observed in 10dc cells, in the absence of morphological change.(Fig 3.12).

G-protein α-subunits are believed to be anchored in the plasma membrane as a heterotrimer with β- and γ- subunits (Sternweiss, 1986). The γ-subunits of G-proteins in 3T3-F442A cells were not probed in this study. The steady-state levels of the β-subunit were, however, analysed using a pan-reactive antiserum which recognises the β_{35}- and β_{36}- isoforms, as previously described (Strassheim *et al*, 1991). This antiserum recognises the 35 kDa and 36 kDa forms of the β-subunit in membranes prepared from rat adipocytes (Fig 3.13A). However, only the β_{36}-subtype was detectable in membranes of 3T3-F442A adipocytes (Fig 3.13A). The levels of $G_{\beta 36}$ were not found to change significantly when membranes from pre-confluent cells were compared to confluent cells. However, levels of β_{36} were appreciably increased by a factor of 2.62±0.28 ($p<0.05$, n=3) by the end of the priming phase and by a further 1.60±0.11 fold ($p<0.05$, n=3) during the first two days of maturation. Levels declined during the final stages of maturation until the amount of $G_{\beta 36}$ detectable in

A)



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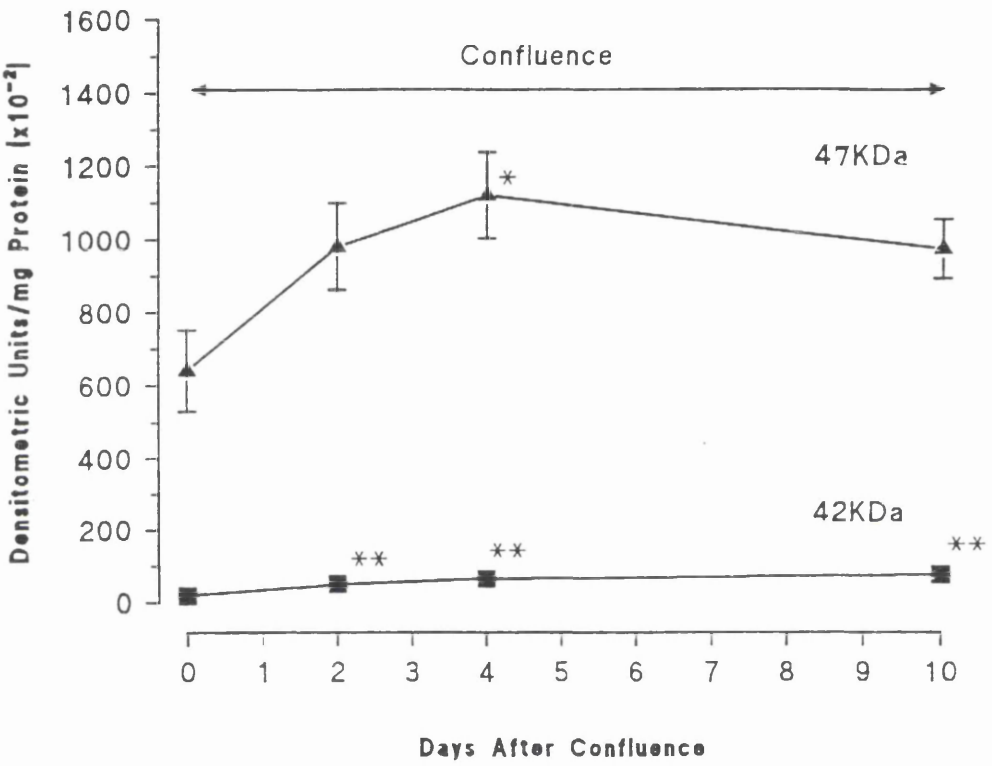
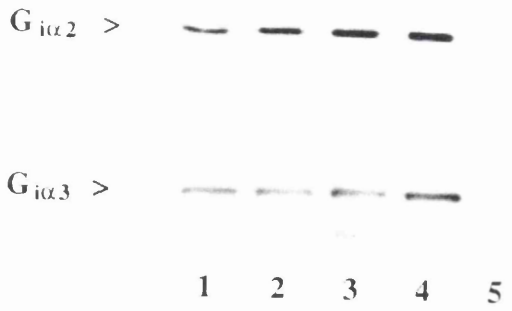


Fig 3.10) Expression of $G_{s\alpha 42}$ and $G_{s\alpha 47}$ in Membranes During the Maintenance of 3T3-F442A Preadipocytes at Confluence.

Equal quantities of membrane protein (10μg) extracted from 3T3-F442A cells maintained at confluence in growth medium were subject to SDS/PAGE and immunoblotting with specific anti-$G_{s\alpha}$ antibodies (*panel A*) as described in the legend to figure 3.10). Samples were taken from just confluent cells (*lane 1*) and at days 2 (*lane 2*), 4 (*lane 3*) and 10 of confluence (*lane 4*). The 42 kDa and 47 kDa forms of $G_{s\alpha}$ are indicated with arrow-heads. *Panel B* demonstrates the changes in densitometric values (arbitrary units) for $G_{s\alpha 42}$ and $G_{s\alpha 47}$ obtained from G-protein immunoblots. Just confluent cells are denoted day 0. Immunoblots were repeated on three separate occasions and densitometric units are expressed as means±S.E.M. * and ** indicates that the value differs significantly from those obtained from just confluent (day 0) cells; $p<0.01$ and $p<0.001$, respectively.

A)



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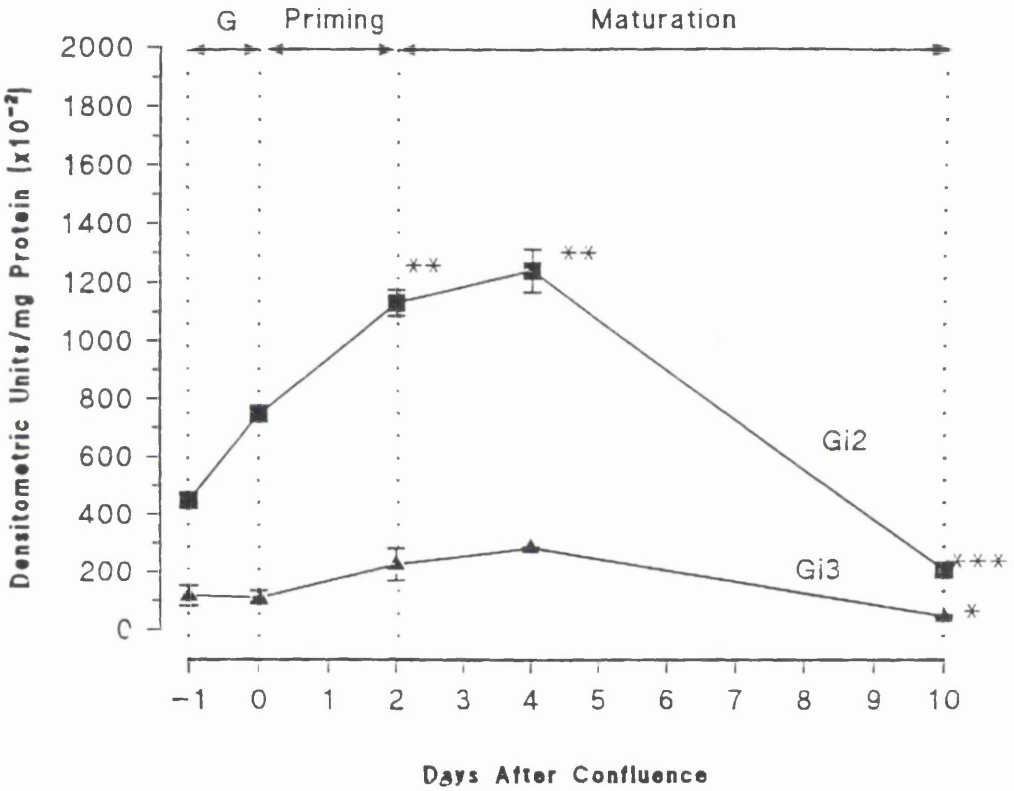
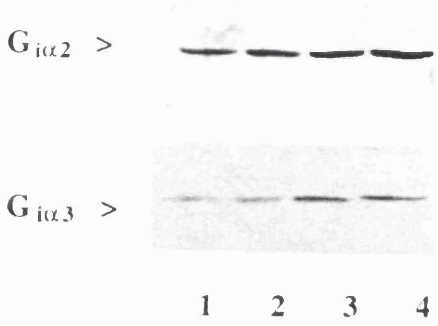


Fig 3.11) Expression of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ in 3T3-F442A Membranes During Differentiation.

Equal quantities of membrane protein (10μg) from 3T3-F442A cells during proliferation (*lane 1*), at confluence (*lane 2*), after priming for 2 days (*lane 3*), during maturation (*lane 4*) or from mature adipocytes (*lane 5*) were separated by SDS/PAGE and immunoblotted with specific antisera to $G_{i\alpha 2}$ and $G_{i\alpha 3}$ (*panel A*) as described in Section 2.2.11.The α-subunits of G_{i1} and G_{i2} are indicated with arrow-heads. Immunoblots were repeated on three separate occasions and densitometric values (arbitrary units) obtained at each stage of differentiation from immunoblots are shown in *panel B* and are expressed as means±S.E.M. for 3 observations. Significant differences with respect to just confluent (day 0) cells are indicated; *, ** and *** ($p<0.05$, $p<0.01$ and $p<0.001$ respectively).

A)



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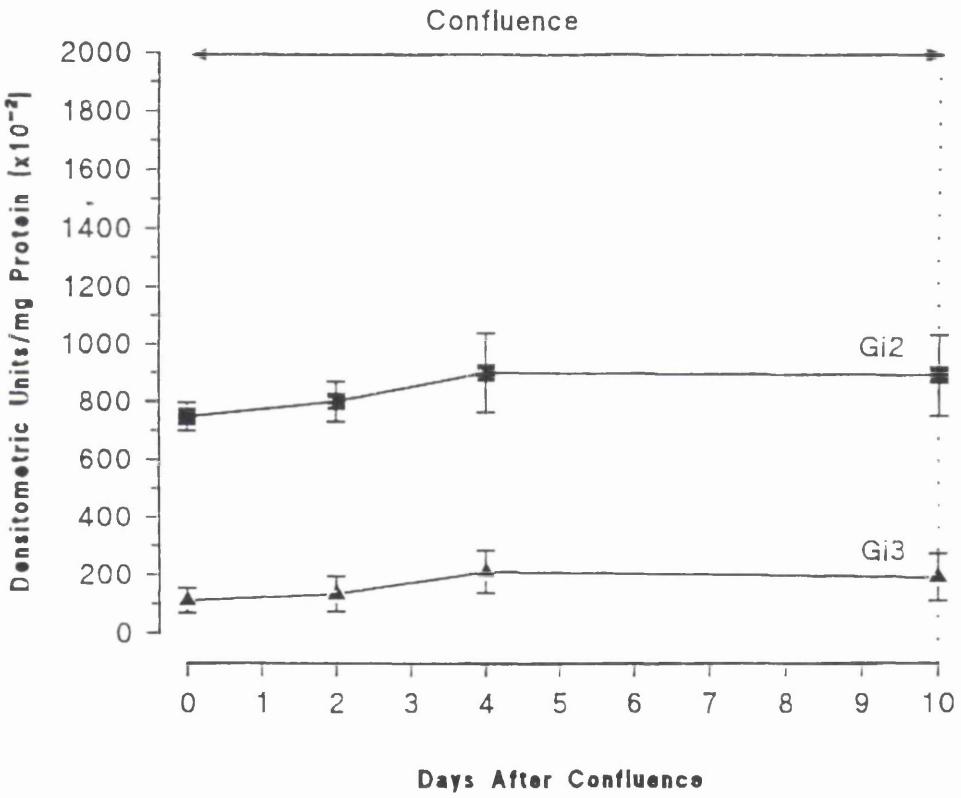
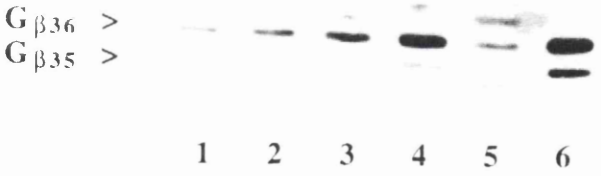


Fig 3.12) Expression of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ in Membranes from 3T3-F442A Preadipocytes During their Maintenance at Confluence.

Equal quantities of membrane protein (10µg) from just confluent 3T3-F442A cells (*lane 1*), and at days 2 (*lane 2*), 4 (*lane 3*) and 10 (*lane 4*) of confluence were separated by SDS/PAGE and immunoblotted with specific antisera to $G_{i\alpha 2}$ and $G_{i\alpha 3}$ (*panel A*) as described in the legend to Fig 3.11). The α-subunits of G_{i1} and G_{i2} are indicated with arrow-heads. Densitometric values (arbitrary units) were obtained from immunoblots of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ and are shown in *panel B*. Immunoblots were repeated on three separate occasions and densitometric units are expressed as means±S.E.M. for 3 observations.

A)



B)

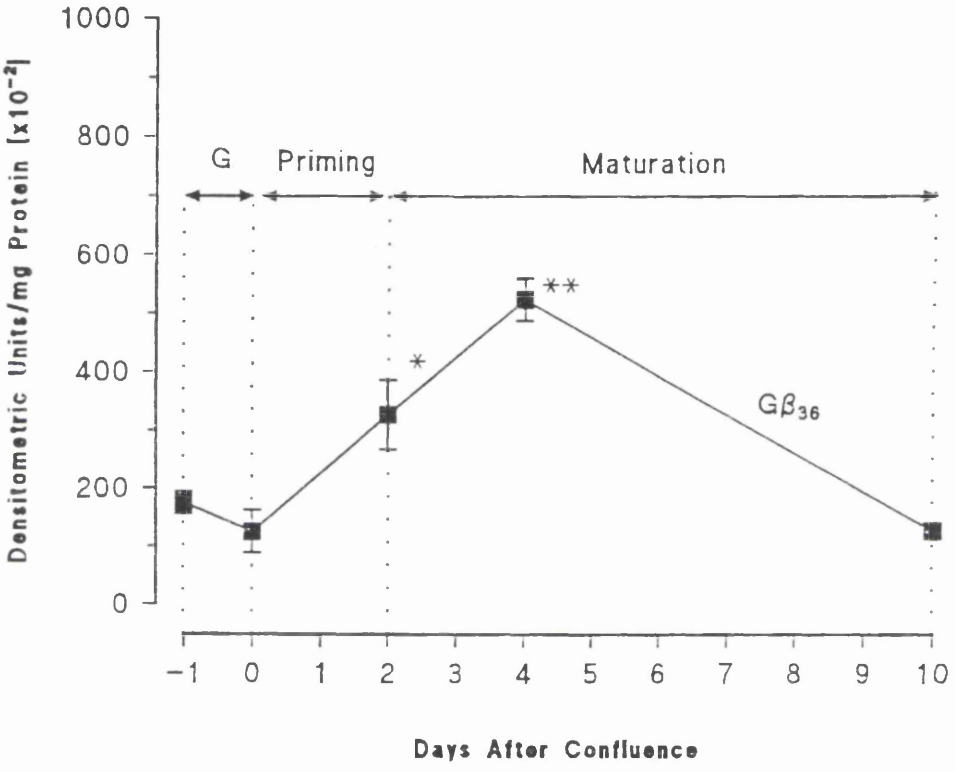


Fig 3.13) Expression of $G_{\beta 36}$ in 3T3-F442A Membranes During Differentiation.

Equal quantities of membrane protein (10μg) from 3T3-F442A cells during proliferation (*lane 1*), at confluence (*lane 2*), after priming for 2 days (*lane 3*), during maturation (*lane 4*) or from mature adipocytes (*lane 5*) or from rat adipocytes (*lane 6*) were separated by SDS/PAGE and immunoblotted with antisera which recognises the 35 kDa and 36 kDa forms of G_β (*panel A*). The two forms of G_β are indicated with arrow-heads. Immunoblots were repeated on three separate occasions and densitometric values (arbitrary units) obtained at each stage of differentiation from immunoblots are shown in *panel B* and are expressed as means±S.E.M. for 3 observations. Significant differences with respect to just confluent (day 0) cells are indicated; * and ** ($p<0.05$ and $p<0.01$ respectively).

3T3-F442A adipocyte membranes was similar to that found in JC membranes (Fig 3.13B). As with $G_{i2\alpha}$ and $G_{i3\alpha}$ the regulated changes in $G_{\beta 36}$ expression during differentiation were probably influenced by the differentiative factors present since levels of $G_{\beta 36}$ did not change in cells maintained at confluence in growth medium (10dc; Fig 3.14).

3.3.6) Changes in G-protein Subunit Expression Per Cell During Differentiation and Confluence.

During differentiation 3T3-F442A cells alter their size and, as a consequence, the yield of membrane protein/cell varies at different developmental stages (Table 3.5). Hence, the densitometric values obtained from G-protein immunoblots were transformed into values indicating expression per cell. Figure 3.15A demonstrates that on a per cell basis the expression of $G_{s\alpha 42}$ increased following priming and during maturation by 143.6±29.5% ($p<0.02$, n=3) and 242.3±29.5% ($p<0.01$, n=3) respectively, and was further elevated towards the end of the maturation phase by a substantial 1551±7.8% ($p<0.001$, n=3). In addition, the levels of $G_{s\alpha 47}$ also increased following priming (207±43.8% increase; $p<0.01$, n=3) and during maturation (242.5±52.9% increase; $p<0.01$, n=3) and were maintained at an elevated level in terminally differentiated cells (149.4±19.5; $p<0.01$, n=3). Similar changes in $G_{s\alpha 42}$ and $G_{s\alpha 47}$ expression were also observed in control cultures maintained at confluence (Fig. 3.15B). These results demonstrate that increases in $G_{s\alpha 42}$ and $G_{s\alpha 47}$ expression can occur independently of the morphological changes initiated by differentiative factors.

Significant increases in $G_{i2\alpha}$ expression per cell were also detected following priming and during the early stages of maturation (51.3±5.9%, $p<0.01$, n=3 and 65.9±9.8%, $p<0.01$, n=3 respectively; Fig 3.16). Elevations in $G_{i3\alpha}$ were also apparent at these time-points, however these values were not significant. Towards the end of maturation the amount of $G_{i2\alpha}$ and $G_{i3\alpha}$ per cell declined substantially to levels which were significantly below those found in JC fibroblasts (36.8±1.5%, $p<0.01$, n=3 and 27.5±4.0%, $p<0.05$, n=3 decrease, respectively). As there was no apparent change in the expression of either $G_{i2\alpha}$ or $G_{i3\alpha}$ during confluence (results not shown), it appears that $G_{i2\alpha}$ and $G_{i3\alpha}$ are subject to differential regulation during the different stages of 3T3-F442A cell differentiation. During GH-priming and maturation, cells receive signals to specifically increase levels of $G_{i2\alpha}$, whereas terminally differentiated cells concomitantly decrease expression of both $G_{i\alpha}$-subtypes.

When normalised to cell number, the pattern of $G_{\beta 36}$ expression during differentiation was similar to that obtained for $G_{i2\alpha}$, in that levels were significantly elevated following priming and during maturation (by 161.6±47.7%, $p<0.05$, n=3 and 319.4±29.0%, $p<0.01$, n=3). Unlike $G_{i2\alpha}$, the relative amount of $G_{\beta 36}$ in terminally differentiated cells was significantly greater than those found in JC cells (Fig 3.16). In contrast, there was no appreciable change in the expression of $G_{\beta 36}$ in cells maintained at confluence in growth medium (results not shown). These results suggest that the expression of $G_{\beta 36}$ is influenced by hormonal regulation during the two phases of 3T3-F442A cell differentiation. During GH-priming cells receive signals to increase the expression of $G_{\beta 36}$. Levels are further increased during maturation and then fall in terminally differentiated cells to those observed in GH-primed cells.

3.3.7) Effect of Pertussis and Cholera Intoxication on the Differentiation of 3T3-F442A Preadipocytes.

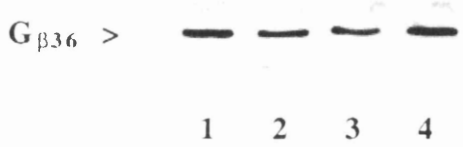
That G-protein levels are elevated in the crucial priming phase of 3T3-F442A cell differentiation suggests a potential regulatory role for these proteins in modulating fat-cell conversion. To determine whether G-proteins can influence preadipocyte differentiation the effects of bacterial exotoxins, namely cholera toxin (C.Tox) and pertussis toxin (P.Tox), on 3T3-F442A cell differentiation were investigated. C.Tox catalyzes the ADP-ribosylation of $G_{s\alpha}$ thereby reducing this protein's GTPase activity (Cassel and Selinger, 1977). In cell systems this results in constitutive activation of adenylate cyclase and a corresponding increase in intracellular cyclic AMP (McKenzie, 1992). Treatment of 3T3-F442A preadipocytes with C.Tox dramatically reduced the extent of cellular differentiation in the presence of DDM (by 67.8±1.1%, $p<0.001$, n=3) as assessed by GPDH activity (Fig 3.17). P.Tox catalyzes the ADP-ribosylation of $G_{i\alpha}$ with the result the $\alpha\beta\gamma$ heterotrimer is maintained in an inactive conformation (Buns *et al*, 1983; Kuruse *et al*, 1988). Treatment of cells with P.Tox lead to a much smaller 12.4±1.1% ($p<0.01$, n=3) but never the less significant inhibitory effect on differentiation (Fig 3.17).

Table 3.5) Parameters of Cell Growth During the Two Phases of 3T3-F442A Cell Differentiation.

3T3-F442A cells were grown to confluence in GH-deleted calf-serum as described in the legend to Fig 4.2). Upon attaining confluence cells were induced to differentiate with the two-phase differentiation protocol as described in Section 2.2.4. Alternatively, control cultures were maintained at confluence for the duration of the differentiation process. At the indicated stages of cell development the number of cells on a 100mm petri dish was assessed with a haemocytometer and the amount of protein in cell-homogenates was determined by the dye-binding assay of Bradford (1976). Results are representative of a single experiment repeated on three separate occasions.

Stage of Cell Growth	Cells/ Plate	Protein/ 10^6 Cells (mg)	Days at Confluence	Cells/ Plate	Protein/ 10^6 Cells (mg)
Proliferation	$8.1x10^5$	0.26	-	-	-
Just Confluent	$1.0x10^6$	0.26	Just Confluent	$1.0x10^6$	0.26
2d Priming	$1.0x10^6$	0.26	2d	$1.0x10^6$	0.26
2d Maturation	$1.8x10^6$	0.27	4d	$1.0x10^6$	0.26
Mature Adipocyte	$2.1x10^6$	0.60	10d	$1.2x10^6$	0.28

A)



B)

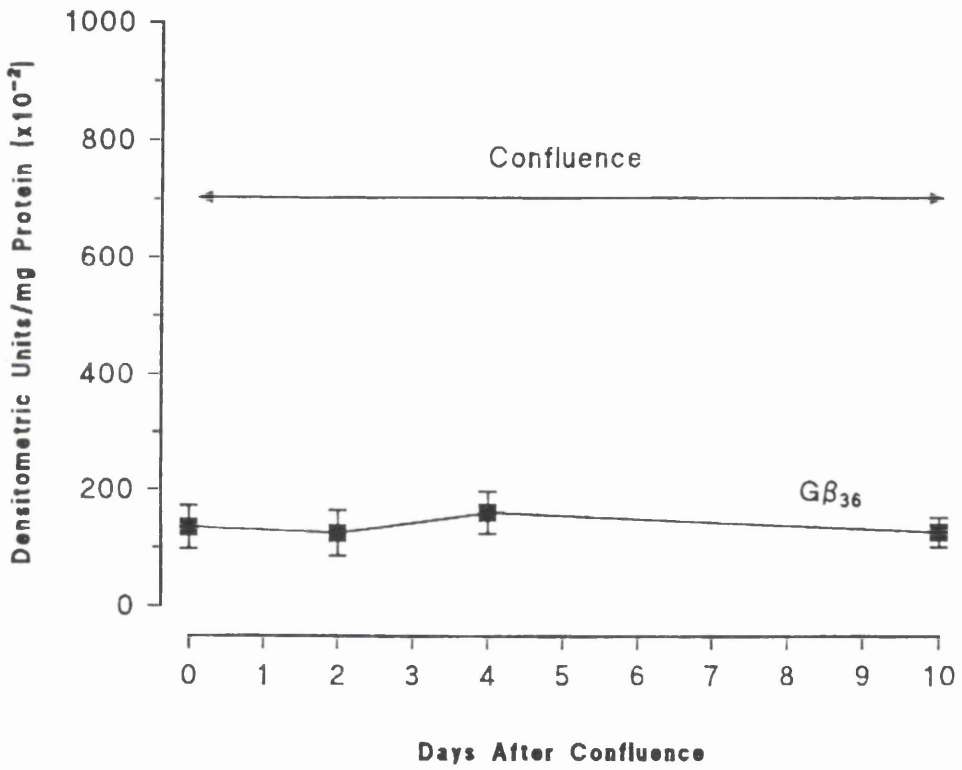


Fig 3.14) Expression of $G_{\beta 36}$ in Membranes from 3T3-F442A Preadipocytes During their Maintenance at Confluence.

Equal quantities of membrane protein (10μg) from just confluent 3T3-F442A cells (*lane 1*) and after 2 (*lane 2*), 4 (*lane 3*) and 10 (*lane 4*) days of confluence were separated by SDS/PAGE and immunoblotted with antisera which recognises the 36 kDa form of Gβ in 3T3-F442A cells (*panel A*). Densitometric values (arbitrary units) were obtained from immunoblots are shown in *panel B*. Immunoblots were repeated on three separate occasions and densitometric units are expressed as means±S.E.M. for 3 observations.

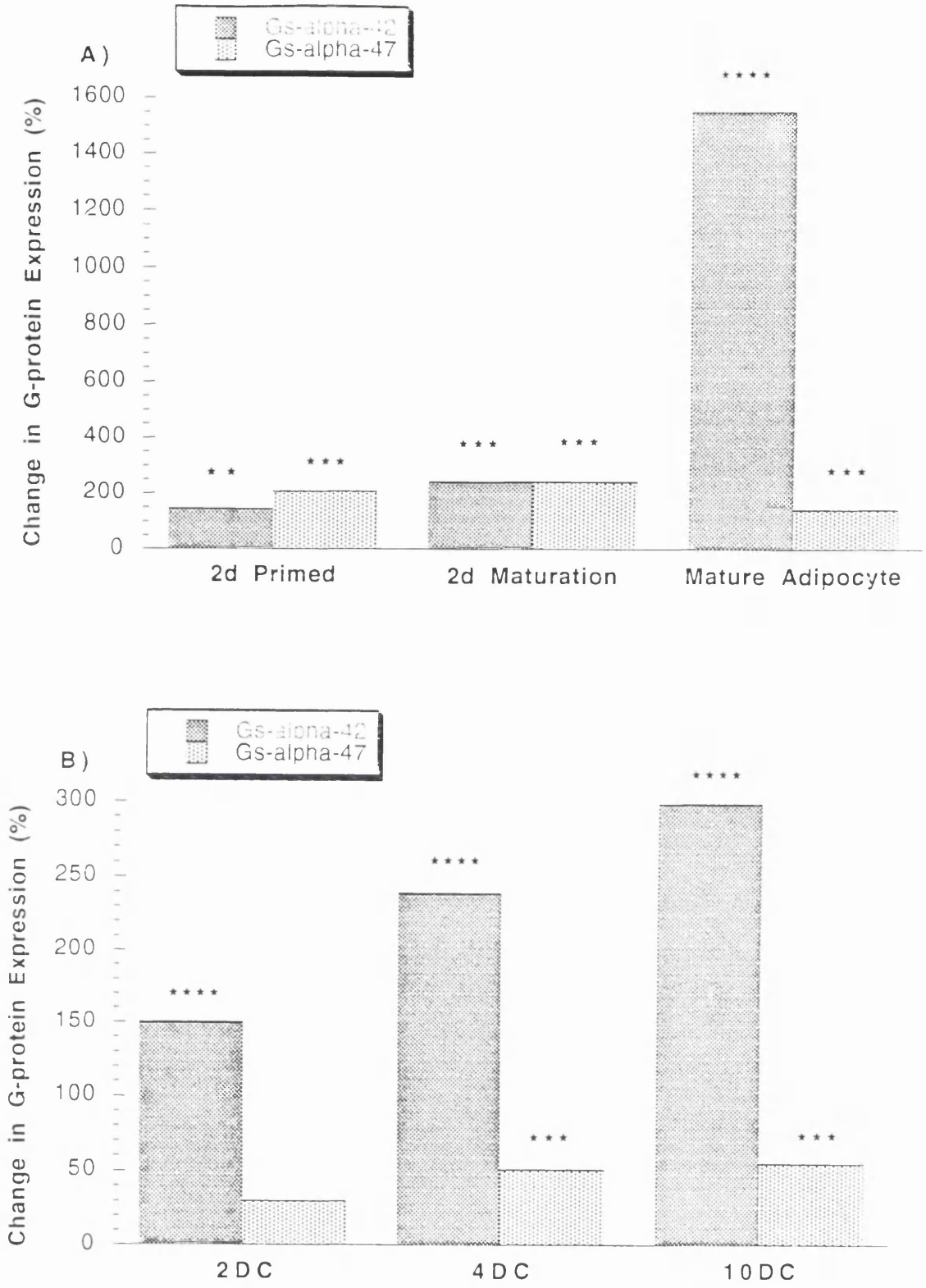


Fig 3.15) Changes in the Expression of $G_{s\alpha}$-subunits Per Cell in 3T3-F442A Cells During Differentiation or Confluence.

The change in expression of $G_{s\alpha}$-subunits during individual stages of 3T3-F442A cell differentiation (*panel A*) or the corresponding day of confluence (*panel B*) is shown relative to its expression in just confluent fibroblasts. Data represent average changes from 3 separate experiments. Data for G-protein expression were calculated on a 'per cell' basis as described in Section 3.2.2. Statistically significant changes in expression relative to just confluent fibroblasts are indicated as $^{**}P<0.02$, $^{***}P<0.01$ and $^{****}P<0.001$.

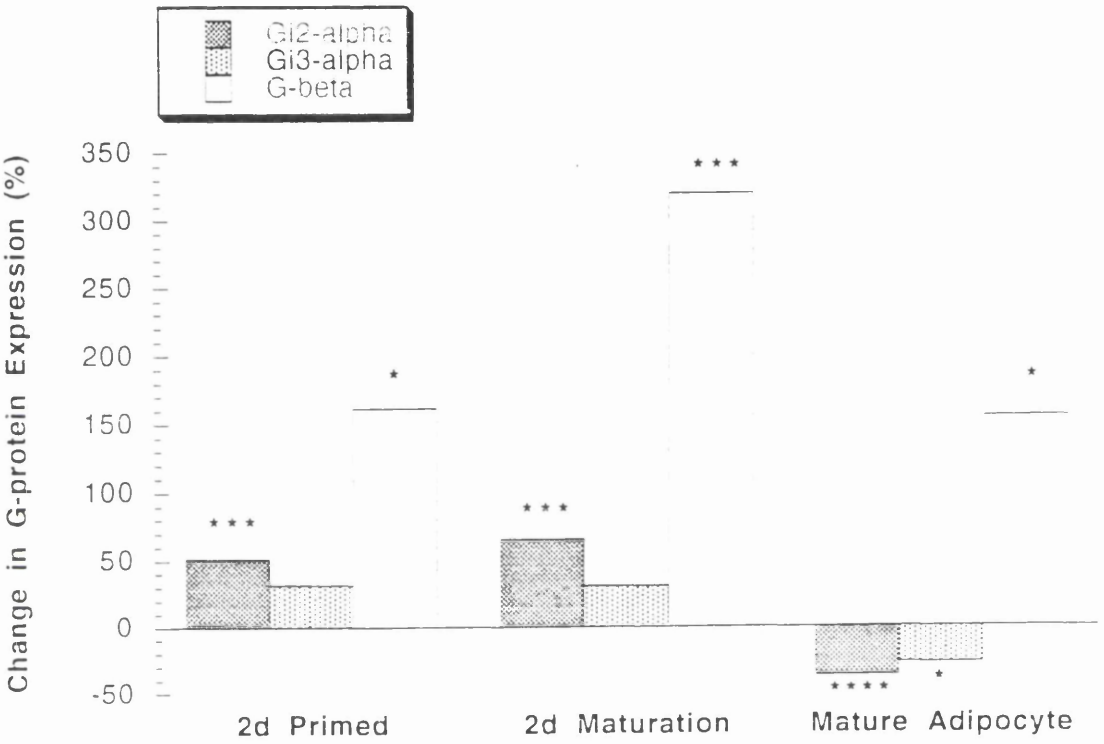


Fig 3.16) Changes in the Expression of $G_{i\alpha}$- and β-subunits Per Cell in 3T3-F442A Cells During Differentiation.

The change in expression of $G_{i\alpha}$- and β-subunits during individual stages of 3T3-F442A cell differentiation (*panel A*) is shown relative to its expression in just confluent fibroblasts. Data represent average changes from 3 separate experiments. Data for G-protein expression were calculated on a 'per cell' basis as described in Section 3.2.2. Statistically significant changes in expression relative to just confluent fibroblasts are indicated as $^{*}P<0.05$, $^{**}P<0.02$, $^{***}P<0.01$ and $^{****}P<0.001$.

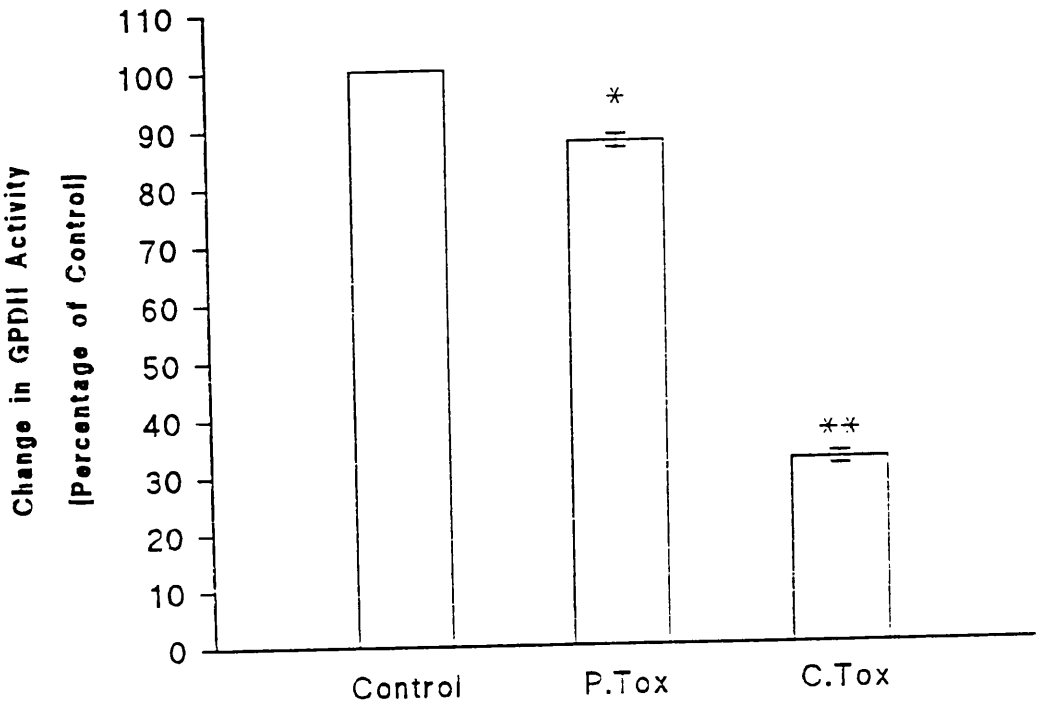


Fig 3.17) Effect of Cholera and Pertussis Intoxication on the Differentiation of 3T3-F442A Preadipocytes.

3T3-F442A cells were grown to confluence under standard conditions and induced to differentiate with a defined differentiation medium (DDM) as described in Section 2.2.3. For the first three days of differentiation cholera toxin (C.Tox, 10ng/ml) or pertussis toxin (P.Tox, 10ng/ml) were included in the DDM. After three days the DDM was removed and replaced with fresh medium without toxins. The extent of differentiation was assessed 7 days later by measuring the GPDH activity of cell extracts as described in the legend to Fig 3.6). GPDH values are expressed as a percentage of the values obtained in cells differentiated in the absence of toxins (control) and are means±S.E.M. for three observations. Significant differences with respect to control are indicated, *$p<0.01$, **$p<0.001$.

3.4) DISCUSSION

Specificity of signalling from seven-helix serpentine receptors is determined in part by the selectivity displayed by these receptors for coupling to specific members of the highly homologous heterotrimeric G-protein family. An added degree of specificity may be the result of differential localisation and regulated expression of particular G-protein subunits. Some phenotypic changes that occur on differentiation are likely to be reflections of changes in the expression of components of signal transduction pathways, including receptors, G-proteins and effectors. Therefore, levels of some of the G-protein constituents of the preadipocyte cell-line, 3T3-F442A, were measured before and after exposure to agents that induced phenotypic changes associated with differentiation.

The expression of G_s, G_{i2} and G_{i3} was demonstrated in membranes from 3T3-F442A fibroblasts by immunoblotting with specific anti-peptide antibodies generated against G-protein α-subunits. 3T3-F442A cells were found not to express detectable levels of $G_{i1\alpha}$. Consequently, anti-$G_{i1+2\alpha}$, an antiserum which recognises both $Gi_{1\alpha}$ and $G_{i2\alpha}$ can be used specifically as a probe for $G_{i2\alpha}$ in this cell type.

The 3T3-F442A cell system was shown here and in previous studies to display a strict requirement for GH as a differentiating signal (Fig 3.8; Morikawa *et al*, 1982; Nixon and Green, 1984; Guller *et al*, 1988). This is wholly consistent with a stimulatory effect of GH on the adipose conversion of several other clonal preadipocyte cell lines, such as 3T3-L1 (Hauner and Löffler, 1986), ob1771 and ob17UT (Doglio *et al*, 1986). GH acts on confluent, growth arrested cells to produce primed (Gp) cells. Gp-cells are in a differentiation permissive condition; i.e. they become sensitive to the differentiating activities of insulin, EGF and T_3 (Guller *et al*, 1988). These agents then promote maturation of Gp-cells by stimulating a limited increase in cell number (Table 3.5) and the induction of adipose-specific phenotypes such as cell rounding, fat accumulation and increased lipogenic enzyme activity (Fig 3.8).

Because of the selective reactivities of a range of antipeptide antisera (see Section 3.1) it was possible to distinguish between differentiation induced changes in the expression of $G_{s\alpha}$, the 36 kDa β-subunit and the closely related $G_{i2\alpha}$ and $G_{i3\alpha}$ subtypes during the two phases of 3T3-F442A cell differentiation (changes are summarised in table 3.6). Dramatic changes in the expression of the 42 kDa and 47 kDa forms of $G_{s\alpha}$ in cell membranes were also apparent during differentiation. However, since these changes were also observed in membranes from control cultures

Table 3.6) Changes in the Expression of G-protein Subunits During the Adipocyte Differentiation of 3T3-F442A Preadipocytes

The change in G-protein subunit expression during individual stages of 3T3-F442A cell differentiation is shown relative to its expression in undifferentiated, just confluent cells. The magnitude and direction of change is indicated, + (increase), ++ (large increase), - (decrease) and = (no change).

	Stage of Adipocyte Conversion		
G-protein Subunit	2d GH-primed	2d Maturation	Mature Adipocyte
$G_{s\alpha 42}$	+	+	++
$G_{s\alpha 47}$	+	+	+
$G_{i\alpha 2}$	+	+	-
$G_{i\alpha 3}$	=	=	-
$G_{\beta 36}$	++	++	++

in the absence of phenotypic change (Fig 3.10B), it was concluded that the expression of $G_{s\alpha}$ is not influenced by differentiative hormones in 3T3-F442A cells. In contrast, the relative abundance of $G_{i2\alpha}$,$G_{i3\alpha}$and G_{β} did appear to be subject to hormonal regulation either during the GH-priming phase or during maturation with insulin, EGF and T_3. Priming of 3T3-F442A cells with GH lead to a selective increase in the 36 kDa form of G_{β} and $G_{i2\alpha}$, whereas $G_{i3\alpha}$ levels were unchanged. Although there are many examples of agonist-stimulated alterations in G-protein expression (Milligan and Green, 1991), the results of this study demonstrate the first example of a GH-mediated increase in $G_{i2\alpha}$ and G_{β} expression. GH has been reported previously to specifically down-regulate $G_{i2\alpha}$ in rat adipocyte membranes, which may contribute to the chronic lipolytic effects of this hormone *in vivo* (Doris *et al*, 1994). This substantial difference between the two models provides evidence that the mechanisms involved in the differentiation-promoting actions of GH are not necessarily the same as those involved in the regulation of metabolism. In addition, 3T3-F442A cells have a distinct advantage as an experimental system in that they provide a convenient model for the study of such early events in the preadipocyte differentiation program.

The increases in $G_{i2\alpha}$ and G_{β} during priming were sustained into the maturation phase and then, together with $G_{i3\alpha}$, levels of these G-proteins declined in terminally differentiated adipocytes. This is consistent with previously reported decreases in $G_{i\alpha}$ protein (Giershik *et al*, 1986; Huppertz *et al*, 1993;) and mRNA (Uehara *et al*, 1994), and in G_{β} protein (Giershik *et al*, 1986) during the insulin-induced differentiation of 3T3-L1 preadipocytes. However, no initial increase in $G_{i2\alpha}$ and G_{β} was reported in 3T3-L1 cells. This is possibly attributable to differences between the two cell-types (3T3-L1 *versus* 3T3-F442A). Although GH is a potentiating factor for 3T3-L1 cell differentiation, it does not appear to be required for the terminal differentiation of these cells (Hauner and Löffler, 1986). As a consequence, the changes in G-protein expression which occur during the differentiation of 3T3-L1 cells are probably synonymous with changes during the insulin-responsive, maturation phase of 3T3-F442A differentiation. Due to the multi-component nature of the maturation media for 3T3-L1 (Schmidt *et al*, 1990) and 3T3-F442A (*vide infra*) preadipocytes, further investigation will be necessary to determine which factor(s) are specifically responsible for the changes in $G_{i2\alpha}$, $G_{i3\alpha}$ and G_{β} in the terminal stages of differentiation.

Further study will also be required to determine whether the changes in G-protein expression during differentiation are regulated at the level of transcription, translation or both. Despite the very large number of studies which have set out to document changes in G-protein mRNA or protein levels, there have been surprisingly few

investigations into the transcriptional regulation of G-protein expression. During the differentiation of polarised renal cells (Holtzman *et al*, 1993), increased $G_{i2\alpha}$ expression appears to require a member of the CCAAT box family of DNA binding proteins (Kinane *et al*, 1993), whereas CCAAT boxes do not contribute to the transcriptional activity of the $G_{i3\alpha}$ gene (Holtzman *et al*, 1993). Members of the CCAAT box family of transcription factors include the CCAAT enhancer binding proteins (C/EBPs; reviewed by Johnson and McKnight, 1989). In 3T3-F442A cells, conclusive evidence has shown that C/EBP family members can regulate preadipocyte differentiation (Umek *et al*, 1991; Freytag and Geddes, 1992; Freytag *et al*, 1994; Lin and Lane, 1994). It is therefore extremely tempting to speculate that hormonal regulation of one or more members of the C/EBP family could contribute to the selective changes in $G_{i2\alpha}$ protein expression during the differentiation of 3T3-F442A preadipocytes.

The stimulus for the specific increases in $G_{s\alpha42}$ and $G_{s\alpha47}$ observed in confluent cultures and differentiating cells is not known (Fig 3.9), but may be attributable to autocrine/paracrine mechanisms. An interesting aspect of the changes in the abundance of long- and short- forms of $G_{s\alpha}$ during differentiation and confluence is that they were not synchronous; i.e. dramatic increases in $G_{s\alpha42}$ occurred in terminally differentiated cells whereas the levels of $G_{s\alpha47}$ were not effected substantially (Fig 3.15). As these proteins are splice-variants of a single gene-product (Robishaw *et al*, 1986; Mattera *et al*, 1986) this implies that $G_{s\alpha42}$ protein levels may be subject to post-transcriptional regulation.

The importance of temporal G-protein α-subunit expression during differentiation has been convincingly demonstrated in lower eukaryotes (Firtel *et al*, 1989; Hardwiger and Firtel, 1992) and studies *in vitro* and in *vivo* have implicated α-subunits as being important for the development of fat in mice (Moxham *et al*, 1993; Su *et al*, 1993; Gordeladze *et al*, 1997). In support of this, in the present study P.Tox-induced inactivation of G_i (Ui, 1984) early in the differentiation of 3T3-F442A preadipocytes was found to exert an inhibitory influence on terminal differentiation. It has recently been demonstrated *in vivo* that $G_{i2\alpha}$ is a positive regulator of insulin receptor signalling and that loss of $G_{i2\alpha}$ in transgenic animals impairs the cardinal responses to insulin, namely glucose-transporter activity and recruitment, counterregulation of lipolysis and activation of glycogen synthase (Moxham and Malbon, 1996). Moreover, expression of a constitutively active form of $G_{i2\alpha}$ in transgenic mice was found to mimic insulin action in adipose tissue (Chen *et al*, 1997). It is interesting to note that before the fall in $G_{i\alpha}$-subunit expression in terminally differentiated cells that there is an initial rise in the expression of $G_{i2\alpha}$ during the insulin-dependent

maturation phase (Fig 3.13). This suggests that during this phase of the differentiation process that inhibitory G-proteins may play a prominent regulatory role, perhaps as positive-regulators of the insulin-stimulated differentiation signal. If this were the case then the anti-adipogenic action of chronic P.Tox treatment on the adipose conversion of 3T3-F442A preadipocytes could be partially explained by an inhibitory affect on insulin-promoted effects during the maturation phase of their differentiation.

In addition C.Tox, which constitutively activates $G_{s\alpha}$ (Gill and Meren, 1978), was found to severely retard differentiation of 3T3-F442A cells in DDM, which is consistent with previous reports of a negative modulatory effect of $G_{s\alpha}$ during the adipocyte differentiation of 3T3-L1 fibroblasts (Wang *et al*, 1992; Gordeladze *et al*, 1997). In addition to their effects on G-protein activities, long-term exposure of cellular systems to P.Tox and C.Tox can lead to selective down-regulation of target α-subunits (Chang and Bourne, 1989; Milligan *et al*, 1989; Klinz and Costa, 1990). Chronic P.Tox treatment has also been reported to induce down-regulation of β-subunits *in vivo* in 3T3-L1 cells (Watkins *et al*, 1989). Thus, while the results in this study highlight the functional importance of G-proteins in maintaining the differentiation of preadipocytes, the complex response of cells to chronic toxin treatment makes interpretation of results in terms of potential effector systems extremely difficult. This study does, however, demonstrate that G-proteins may be important for preadipocyte differentiation and that α- and β-subunits levels are altered in a stage-dependent manner. This implies that sequential alterations to G-protein levels may be important for efficient fat cell conversion. In light of this, caution should be observed in interpreting the results of transfection studies which have used activating- or inactivating-mutants of G-protein α-subunits (Wang *et al*, 1992; Gordeladze *et al*, 1997) since enforced expression of these mutant proteins will disrupt the temporal regulation of G-protein expression during fat cell development with unpredictable effects.

What role, if any, would temporal alterations in the abundance of cellular G-proteins play in determining the cellular phenotype of the differentiating preadipocyte? One potential consequence would be the altered coupling of extracellular stimuli to intracellular effector systems. $G_{i\alpha 1-3}$ were initially characterised for their ability to inhibit adenylate cyclase (Taussig *et al*, 1993) and have been implicated in Golgi function (Maltese and Robishaw, 1990) and shown to activate $p21^{ras}$ (Ras) and the MAP kinase and $p70^{S6K}$ growth-regulatory pathways (Winitz *et al*, 1993; Wilson *et al*, 1996) as well as K^+ channels (Yatani *et al*, 1988). $G_{s\alpha}$ is stimulatory to adenylate cyclase and can modulate a host of intracellular effectors by activating cyclic AMP-

dependent protein kinase (PKA), including the MAP kinase and $p70^{S6K}$ cascades (Withers *et al*, 1996, Yarwood *et al*, 1996; Graves and Lawrence, 1996). $G_{\beta\gamma}$-subunits can also modulate the activities of intracellular effector units, including some K^+ channels (Clapham and Neer, 1993; Logothetis *et al*, 1987), forms 2 and 3 of PLCβ (Rhee *et al*, 1989), certain adenylate cyclase isoforms (Tang and Gilman, 1991) and appear to be involved in the activation of MAP kinase by G_i-linked receptors (Alblas *et al*, 1993; Crespo *et al*, 1994; Faure *et al*, 1994; Koch *et al*, 1994; Thorburn and Thorburn, 1994). Since the ability to interact with the adenylate cyclase and MAP kinase pathways appears to be a feature common to G_s, G_i and $G_{\beta\gamma}$, it is likely that disregulation of these major signal transduction cascades may underlie the effects of P.Tox and C.Tox on the differentiation of 3T3-F442A preadipocytes.

Previous studies have suggested that cyclic AMP is a signalling molecule which contributes to the triggering of adipose conversion of adipogenic cell lines (Russel and Ho, 1976; Gaillard *et al* , 1989; Schmidt *et al,* 1990) as well as that of adipogenic primary cell ~~lines~~ cultures (Björntorp et al, 1980; Wierderer and Löffler, 1980). However cyclic AMP appears to play a dual role in modulating adipose differentiation since prolonged exposure of 3T3-F442A preadipocytes to cyclic AMP-elevating agents prevents terminal differentiation (Spiegelman and Green, 1981). This results in a down-regulation of mRNA expression for some adipocyte-specific products, including fatty-acid synthase (Paulausikis and Sul, 1988), α-glycero-3-phosphate dehydrogenase (Dobson *et al,* 1987) and lipoprotein lipase (Raynolds *et al*, 1990). This suggests the existence of a generic control mechanism which switches lipogenic and lipolytic states in an orderly biochemical fashion. Indeed, it is well documented that adipocyte differentiation is accompanied by a coincident increase in the expression of the 42kDa and 47kDa forms of $G_{s\alpha}$ and decreased expression of $G_{i\alpha}$ isoforms, which may contribute to increased intracellular concentrations of cyclic AMP and enhanced responsiveness of terminally differentiated adipocytes to lipolytic agents (Kilgour and Anderson, 1993). It appears, therefore, that large increases in intracellular cyclic AMP induced by chronic C.Tox exposure in the present study would exert an inhibitory effect on this late stage of differentiation. It is also possible that the elevations in $G_{i\alpha}$-subunit expression which were observed during the maturation phase of 3T3-F442A preadipocyte differentiation could, by limiting the activation of adenylate cyclase, prevent the action lipolytic agents thereby allowing lipid accumulation and terminal adipocyte differentiation. In addition to the dynamic changes in heterotrimeric G-protein expression during the late stages of preadipocyte differentiation, the present study demonstrates an "on-dependent" increase in $G_{i2\alpha}$ and $G_{\beta 36}$ during the essential GH-priming phase and a significant increase in $G_{s\alpha 42}$ which also occurred, in the absence of differentiative stimuli, in cultures which were maintained at confluence (Fig

3.13). This suggest that regulation of adenylate cyclase activity early in the differentiation program could also be critical for determining cellular fate. It is therefore apparent that the role of heterotrimeric G-proteins in controlling preadipocyte differentiation is complex and requires further clarification in terms of the cyclic AMP signalling system.

3.5) CONCLUSIONS

In this study, specific anti-peptide antisera were used to identify differentiation-induced changes in the concentrations of G-protein α- and β-subunits in 3T3-F442A cells. It is clear that not all G-proteins respond equally to the induction of differentiation and unique responses occur even amongst members of the same family. The crucial GH-priming stage of 3T3-F442A differentiation witnessed specific increases in $G_{s\alpha 42}$, $G_{i2\alpha}$ and $G_{\beta 36}$. Further increases were observed during the initial stages of maturation culminating in dramatic increases in $G_{s\alpha 42}$ and a more modest increase in $G_{s\alpha 47}$ expression occurring in terminally differentiated cells. In contrast, levels of $G_{i2\alpha}$, $G_{i3\alpha}$ declined in mature adipocytes. The importance of G-proteins in the maintenance of the differentiated state was demonstrated with bacterial toxins. This suggests that the regulated changes in G-protein expression occurring during 3T3-F442A cell differentiation may contribute to changes in cellular phenotype. Further studies will be needed to clarify the functional properties that specific changes in G-protein levels impart to the differentiating cell.

Chapter Four

The Effects of Cyclic AMP on the Differentiation of 3T3-F442A Preadipocytes

4.1) INTRODUCTION

The results from Chapter 3 demonstrate that changes occur in the cellular levels of individual G-protein isoforms at various stages of 3T3-F442A fat-cell conversion. Specific increases in the expression of $G_{s\alpha}$, $G_{i2\alpha}$ and G_{β}, during the essential GH-priming phase of differentiation and increases in $G_{s\alpha}$ in confluent cultures, where morphological conversion was negligible, provide indications as to the importance of G-proteins in modulating adipogenesis. Further evidence for a regulatory role for G-proteins in this process was obtained by using cholera toxin (C.Tox), which constitutively activates G_s (Gill and Meren, 1978), and pertussis toxin (P.Tox), which inactivates certain G-proteins, including members of the G_i family (Ui, 1984). Treatment with P.Tox proved to be slightly inhibitory to adipose conversion, whereas C.Tox provoked a dramatic blockade of differentiation. It is clear that treatment of cells with these toxins will lead to changes in adenylate cyclase activity (Gill and Meren, 1978; Ui, 1984) resulting in perturbations to intracellular cyclic AMP concentrations. In addition, changes in the level of G-protein expression probably contribute to the changes in acute hormonal regulation of adenylate cyclase which are characteristic of adipocyte development (Rubin *et al*, 1978; Lai *et al*, 1981; Kilgour and Anderson, 1993). With these considerations in mind it appears that modulation of intracellular cyclic AMP concentrations may play a functional role in determining the cellular fate of differentiating preadipocytes.

A large number of studies have demonstrated the functional significance of cyclic AMP in the modulation of cell growth (Table 4.1). Several hormones which are known to activate adenylate cyclase through G_s-coupled receptors (eg thyrotropin) are mitogenic in cell types in which cyclic AMP production appears to be a physiological mediator of cell proliferation (Dumont *et al*, 1989). Moreover, constitutively active mutants of $G_{s\alpha}$ have been identified in pituitary adenomas (Landis *et al*, 1989) and in autonomously functioning thyroid tumours (Lyons *et al*, 1990), the oncogenicity of which is thought to be mediated by continuous cyclic AMP formation (Valler *et al*, 1987; Gaiddon *et al*, 1994). However, a positive mitogenic effect of cyclic AMP is not evident in certain cell types and anti-mitogenic effects are observed in others (Table 4.1). Indeed, cyclic AMP can even promote reversion to the normal phenotype of certain transformed cells (Pastan *et al*, 1975). Therefore, it appears that the effects of cyclic AMP on proliferation are complex and depend very much on the cellular context.

In addition to its role as a mitogenic effector, cyclic AMP has been shown to promote the differentiation of certain cell types. Most notably, cyclic AMP stimulates the formation of specialised functions in thyroid cells (Lamny *et al*, 1987), melanocytes

Table 4.1) Mitogenic and Anti-mitogenic Effects of Cyclic AMP-elevating Agents.

Cell System	Stimulant	Effect On Proliferation
Rat Parotid Acinar Cells	β-adrenergic	+
Rat Hepatocytes	Glucagon/β-adrenergic	+
Rat Brown Adipocytes	β-adrenergic	+
Rat Pancreatic β-cells	Glucose	+
Murine Mammary Epithelial Cells	PGE[b]	+
Immature Rat Sertoli Cells	FSH[c]	+
Swiss 3T3 Fibroblasts	Insulin+Cholera toxin	+
Dog Thyroid Epithelial	TSH	+
Rat Atrial Smooth Muscle Cells	PGE[b]	-
Human Foreskin Fibroblasts	Forskolin	-
Neoplastic Human B Cells	Forskolin	-
Transformed Fibroblasts	8Br-cAMP[a]	-
Transformed NIH-3T3 Fibroblasts	Constitutively-active $G_{s\alpha}$	-

Adapted from Blomhoff *et al* (1987), Boynton and Whitfield (1983), Cannon *et al* (1996), Chen and Iyengar (1994), Heldin *et al* (1989), Hordijk *et al* (1994), Nilsson and Olsson (1984), Rabinovitch *et al* (1980), Roger *et al* (1983) and Rozengurt *et al* (1983).

[a]8Br-cAMP refers to the cell permeable analogues of cyclic AMP 8-Bromo-cyclic AMP.

[b]PGE refers to protaglandin E1.

[c] FSH refers to follicle stimulating hormone.

(Angler *et al*, 1995), neuronal cell lines (Frödin *et al*, 1994; Hoffman *et al*, 1994) and cultured brown adipocytes (Cannon *et al*, 1996). The effects of cyclic AMP on the differentiation of white preadipocytes are somewhat controversial (Table 4.2). The cellular model in which the effects of cyclic AMP have been most extensively studied is the differentiation of the murine 3T3-L1 preadipocyte cell line (Russell and Ho, 1976; Elks and Manganiello, 1985; Schmidt *et al*, 1990; Wang *et al*, 1992). Rather confusingly, in this cell type positive (Elks and Manganiello, 1985; Russell and Ho, 1976; Schmidt *et al*, 1990) and neutral (Wang *et al*, 1992) effects of cyclic AMP-raising agents have been reported. Indeed, in different studies differential effects of the same agent (10μM forskolin) on 3T3-L1 differentiation have even been reported (Schmidt *et al*, 1990; Wang *et al*, 1992). Further inconsistencies have arisen from studies on primary cultures of rat preadipocytes (Table 4.2). One potential reason for inconsistencies such as these is that different culture conditions were used in different studies. For example, in one study forskolin was shown, under serum-free conditions, to potentiate the differentiation of 3T3-L1 preadipocytes (Schmidt *et al*, 1990), whereas another study performed in the presence of serum forskolin had very little effect on 3T3-L1 differentiation (Wang *et al*, 1992).

Only two studies have been carried out on the effects of cyclic AMP on 3T3-F442A adipogenesis and these have also produced conflicting observations (Spiegelman and Green, 1981; Nègrel *et al*, 1989). Spiegelman and Green (1981) reported that long-term incubations with high concentrations of a cyclic AMP analogue, or a cyclic AMP analogue in combination with a phosphodiesterase inhibitor, severely attenuated foetal calf serum-induced 3T3-F442A differentiation by compromising production of mRNA for lipogenic enzymes. These effects appeared to be specific to the later stages of adipose conversion as similar results were obtained when cyclic AMP levels were raised in cells which had already undergone terminal differentiation (Spiegelman and Green, 1981). Moreover, the reduction in late-stage lipogenic enzyme synthesis induced by cyclic AMP was independent of differentiation-dependent morphological changes, again indicating a stage-specific effect of cyclic AMP (Spiegelman and Green, 1981). In contrast, in a different study short-term exposure at the onset of differentiation to the cyclic AMP-elevating agent prostacyclin (prostaglandin I_2) was found to promote the terminal differentiation of 3T3-F442A cells maintained under serum-free conditions, however the effect of prostacyclin on cyclic AMP accumulation in 3T3-F442A cells was not investigated in this study (Nègrel *et al*, 1989). Even so, prostacyclin has been shown to elevate intracellular cyclic AMP levels in Ob1771 preadipocytes as well as dramatically enhancing their differentiation (Nègrel *et al*, 1989). Together these observations suggest that long-term exposure to cyclic AMP inhibits differentiation of 3T3-F442A preadipocytes by effecting late-stage events,

whereas short-term exposure, at the beginning of differentiation, may potentiate differentiation.

In order to clarify the influence of cyclic AMP on preadipocyte differentiation a detailed study was carried out using 3T3-F442A preadipocytes. Multiple agents which influence intracellular cyclic AMP levels were used in an attempt to obtain information regarding the effects of differing magnitude and duration of elevations in intracellular cyclic AMP. Furthermore, the ability to divide differentiation of the cells into two discrete stages, using a two-phase differentiation protocol which employs defined media, as described in Chapter 3, has permitted an investigation of the effects of cyclic AMP at these different stages.

Table 4.2) Effects of Agents which Raise Intracellular Cyclic AMP on Preadipocyte Differentiation.

Cell Type	Agent	Effect on Differentiation	Culture Conditions	Assessment of Differentiation	References
Rat Preadipocyte	Pertussis toxin (P.Tox) (0.1ng/ml	+	Agents present in differentiation medium for 9 days.	GPDH Activity	Shinohara *et al*, (1991)
	Cholera toxin (C.Tox) (0.1ng/ml)	+			
	P.Tox (10ng/ml)+ dbcAMP (5mM)	-			
	Forskolin (0.5μM)+P.Tox (10ng/ml)	-			
Rat Preadipocyte	IBMX (500μM)	+	Agents present in the differentiation medium for 12 days.	Lipid Accumulation & Lipoprotein Lipase Activity	Björntorp *et al*, (1980)
	PGE_1 (2μg/ml)	+			
	C.Tox (10ng/ml)	+			
Rat Preadipocyte	PGI_2 (200nM)	+	Agents present in the differentiation medium 3 days out of 12.	GPDH Activity	Negrel *et al*, (1989)
Human Preadipocyte	PGI_2 (200nM)	+			
3T3-L1	PGI_2 (100mg/ml)	NE	Agents present in differentiation medium 4 days out of 6.	Lipid Accumulation	Russell and Ho, (1976)
	dbcAMP (1.0mM)	NE			
	IBMX (500μM)	+			
3T3-L1	C.Tox (10ng/ml)	-	Agents present in the differentiation medium 2 days out of 7.	Lipid Accumulation	Wang *et al*, (1992)
	Forskolin (10μM)	NE			
	P.Tox (10ng/ml)	NE			
	dbcAMP (1mM)	NE			
3T3-L1	IBMX (100μM)	+	Agents present in differentiation medium 3 days out of 10.	GPDH Activity Lipid Accumulation	Elks and Manganiello, (1985)
	RO 20-1724 (10μM)	+			
	Cilostamide (0.3μM)	NE			
3T3-L1	IBMX (500μM)	+	Agents present in the differentiation medium 4 days out of 8.	GPDH Activity	Schmidt *et al*, (1990)
	Forskolin (1μM)	+			
	Forskolin (10μM)	+			
	dbcAMP (1mM)	+			

3T3-F442A	dbcAMP (1mM) dbcAMP (0.5mM)+ Theophylline (0.5mM) Isoproterenol (1μM)+ Theophylline (0.5mM)	- - -	Agents present in the differentiation medium for 7 days.	Lipid Accumulation	Spiegel-man and Green, (1981)
3T3-F442A	PGI_2 (200nM)	+	Agent present in the differentiation medium 3 days out of 12.	GPDH Activity	Negrel *et al*, (1989)
TA1	Forskolin (5μM)	+	Agent present in the differentiation medium for 4 days.	Lipid Accumulation	Kurten *et al*, (1988)
TA1	Forskolin (0.5μM)+dbcAMP (5mM) P. Tox (0.1ng/ml)	- +	Agents present in the differentiation medium for 2 days out of 6.	GPDH Activity	Shinohara and Murata, (1992)
Ob1771	PGI_2 (200nM) Isoproterenol (1μM) Forskolin (1μM) 8Br-cAMP (0.5mM)	+ + + +	Agents present in the differentiation medium 3 days out of 12.	GPDH Activity	Vassaux *et al*, (1992)
ST13	Theophylline (10mM) Epinephrine (10μM)	+ -	Agent present in the differentiation medium for 14 days.	Lipid Accumulation	Hiragun *et al*, (1980)

Abbreviations: PGE_2 and PGI_2, prostaglandins E_2 and I_2 respectively. +, - or NE, increase, decrease or no effect.

4.2) EXPERIMENTAL PROCEDURES

4.2.1) Standard Procedure for Cell Culture and Differentiation

3T3-F442A fibroblasts were grown to confluence in DMEM containing 10% calf serum as described in section 2.2.1. Confluent cultures were induced to differentiate by replacing the growth medium with either DMEM containing 10% foetal calf serum and 5μg/ml insulin (FCS/insulin; section 2.2.2) or with a defined differentiation medium (DDM) consisting of 2:1 (v/v) F12/DMEM containing GH (2nM), insulin (1.8μM), T_3 (0.1ng/ml), EGF (50ng/ml) and other factors as described in section 2.2.3 and by Guller *et al* (1988). The cyclic AMP-elevating agents under test were included in the FCS/insulin or DDM media for the first three days of differentiation, following which cells were treated with fresh media, without agents, and the extent of differentiation was assessed after a further 3 days (FCS/insulin) or 7 days (DDM). The extent of differentiation was assessed both qualitatively, by Oil Red O staining, and quantitatively by measuring cellular GPDH activity. For further details see section 2.2.5 and 2.2.6.

4.2.2) Two-phase Protocol for Cellular Differentiation

For these studies cells were grown to confluence in the presence of 10% calf serum depleted of GH (described in section 2.2.4.1). Briefly, this was prepared by incubating calf serum with anti-rbGH (1:1000 dilution) for 24 hours at room temperature. Antiserum to rabbit IgG was then added to a final dilution of 1:10. After 4 hours the precipitate was pelleted by centrifugation at 3000g for 30 minutes and the supernatant (GH depleted calf-serum) carefully removed. Cells were passaged at least twice, in DMEM containing 2.5mM glutamine and 10% GH-depleted calf serum, prior to use for differentiation studies. Confluent cells were washed three times in PBS then incubated for 2 days in serum free medium [F12:DMEM (2:1) containing transferrin (10μ/ml), fetuin (50μg/ml), glutamine (2.5mM) and BSA (1mg/ml)] along with the other additions indicated in legends. Cultures were then washed, as before, and the medium replaced with maturation medium [serum free medium containing insulin (1.8μM), T_3 (0.1ng/ml) and EGF (50ng/ml)] along with the agents under test. After 2 days the drugs were removed and the extent of differentiation was measured after a further 6 days.

4.2.3) Oil Red O Staining, GPDH, DNA and Cyclic AMP Assays and Expression of Results

Qualitative assessment of differentiation was determined by staining neutral lipids accumulated in terminally differentiated cells with Oil Red O (see section 2.2.5). Cells were then photographed under Phase Contrast Optics (x24 magnification) using an

inverted microscope. GPDH activity was measured spectrophotometrically (section 2.2.5). GPDH activities were expressed relative to cellular DNA content to correct for differentiation-associated increases in cellular protein (Lai *et al*, 1981; Kilgour and Anderson, 1993). The DNA content of cellular homogenates was determined fluorometrically as described section 2.2.14. The enzyme activity per plate of cells was expressed as the number of moles of NADH oxidised/second (katals)/mg DNA.

Measurement of cellular cyclic AMP concentration was carried out essentially according to the competitive binding protein method of Brown *et al* (1971) and is described fully in section 2.2.16.

4.3) RESULTS

4.3.1) Effects of Agents which Elevate Intracellular Cyclic AMP Levels on Lipid Accumulation During Differentiation of 3T3-F442A Cells with Foetal Calf Serum and Insulin (FCS/Insulin).

In order to study the effects of cyclic AMP on 3T3-F442A preadipocyte differentiation, multiple agents were used which share the common ability to elevate intracellular cyclic AMP levels (Fig 4.1).The effects of agents were assessed initially on differentiation induced by FCS/insulin differentiation medium and the extent of cellular differentiation was determined qualitatively, by Oil Red O staining, and quantitatively, by measuring the cellular activity of the lipogenic enzyme GPDH which is a sensitive marker of the adipocyte phenotype (Smas and Sul, 1995). To assess the ability of cyclic AMP to modulate the differentiation of 3T3-F442A cells a range of agents were used which raise intracellular cyclic AMP by different mechanisms (Fig 4.1). These were forskolin, which directly activates adenylate cyclase, cholera toxin, which constitutively activates $G_{s\alpha}$, a cell permeable cyclic AMP analogue, CPT-cAMP, and IBMX, which inhibits cyclic AMP phosphodiesterases (Fig 4.1).

Confluent cell cultures were induced to differentiate in the presence of FCS/insulin and the cyclic AMP-elevating agents included for the first three days. The extent of differentiation was qualitatively assessed by light microscopy six days after the initiation of differentiation. Following exposure to the FCS/insulin medium cells acquired a larger and more rounded morphology (Fig 4.2A) characteristic of the adipocyte phenotype (Slavin, 1972). Differentiated cells also developed numerous, large cytosolic lipid spheres as revealed by Oil red O staining (Fig 4.2A). Addition of forskolin (50μM) severely attenuated triacylglyceride accumulation (Fig 4.2C) whereas dideoxyforskolin (50μM), a homologue of forskolin which does not activate adenylate cyclase, had no effect on the extent of lipid accumulation (Fig 4.2D). In contrast, inclusion of IBMX (500μM) in the FCS/insulin medium visibly enhanced the extent of lipid filling (Fig 4.2B). GPDH activity was undetectable in undifferentiated fibroblasts and rose to 7.80±0.52 nanokatals/mg DNA ($p<0.001$, n=5) after exposure to FCS/insulin for seven days. Inclusion of IBMX in the FCS/insulin medium (Fig 4.3) clearly increased GPDH enzyme activity relative to control cells by 99±17% ($p<0.001$, n=5). Interestingly the compound forskolin exerted different modulatory influences upon the differentiation process depending on the concentration to which the preadipocytes were exposed. Hence 50μM forskolin markedly inhibited GPDH activity by 90.90±3.20% ($p<0.001$, n=4) while 10nM forskolin significantly increased this activity by 94±13% ($p<0.001$, n=5). Dideoxyforskolin had no significant effect on

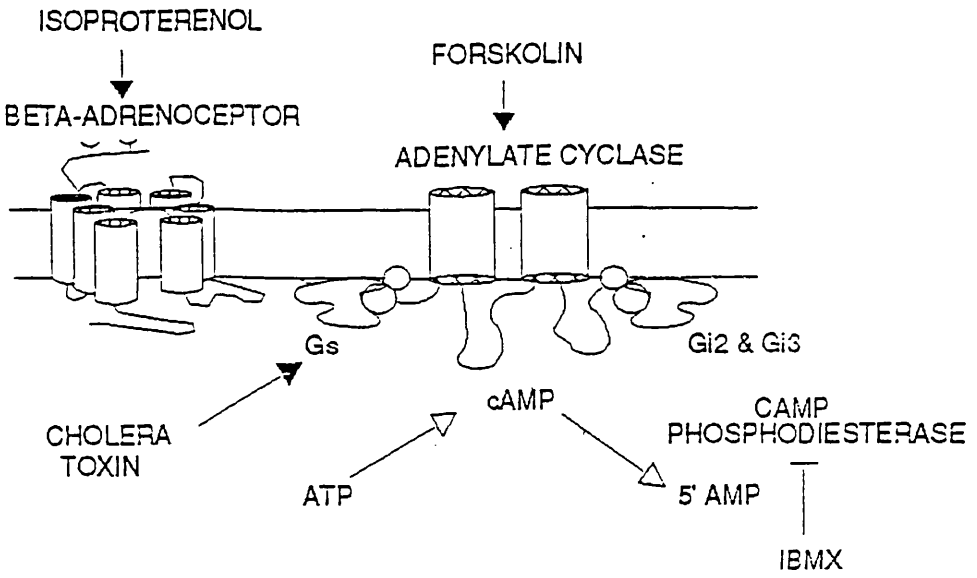


Fig 4.1) Sites of Action of Cyclic AMP-elevating Agents

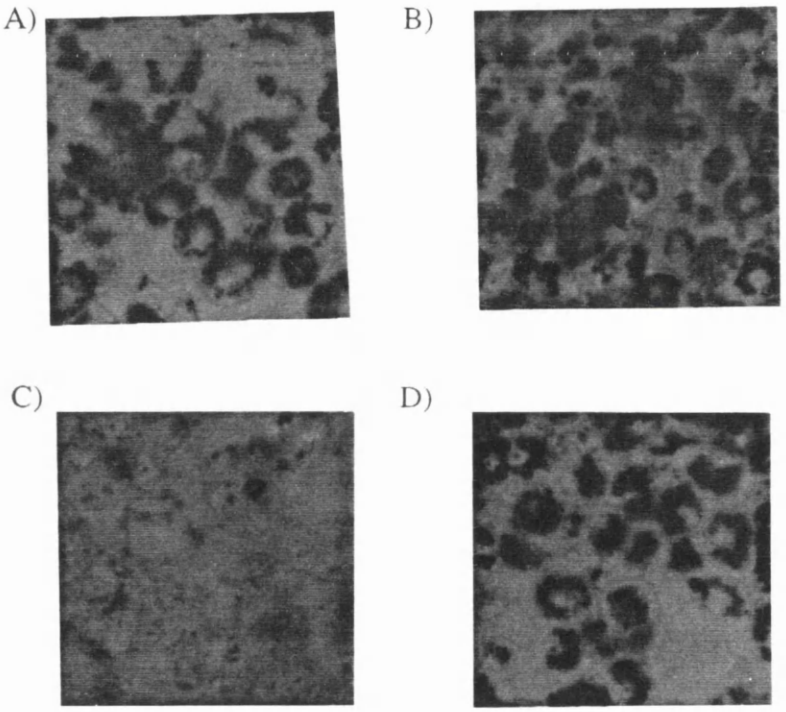


Fig 4.2) Effect of Agents which Elevate Cyclic AMP on Lipid Accumulation During Differentiation of 3T3-F442A Cells with Foetal Calf Serum and Insulin (FCS/Insulin).

Confluent cultures were induced to differentiate by replacing the growth medium with FCS/insulin medium in the presence of (A) diluent, B) 500μM IBMX, (C) 50μM forskolin or (D) 50μM dideoxyforskolin. After 3 days these agents were removed from the differentiation medium and after a further 7 days cells were stained with Oil Red O and photographed.

GPDH activity. To confirm that the effects of IBMX and forskolin on differentiation were attributable to elevated intracellular cyclic AMP levels, the effects of two other cyclic AMP-elevating agents, cholera toxin and CPT-cAMP, were investigated. Both cholera toxin and CPT-cAMP reduced the GPDH activity of differentiated cells by 91.03±1.41% ($p<0.001$, n=5) and 92±1% ($p<0.001$, n=5) respectively, when included for the first three days of differentiation (Fig 4.3). A possible explanation for these results is that small increases in intracellular cyclic AMP levels, as would be expected to occur in the presence of IBMX or 10nM forskolin, promote differentiation, while the larger and more sustained increases in intracellular cyclic AMP levels, which are achieved with 50μM forskolin CPT-cAMP and cholera toxin, inhibit the differentiation process.

To investigate further the effects of different intracellular cyclic AMP concentrations, cells were incubated with the FCS/insulin differentiation medium in the presence of various concentrations of forskolin or CPT-cAMP. As shown in Fig 4.4), both of these compounds exhibited striking differential dose-dependent effects upon differentiation. Low concentrations of forskolin and CPT-cAMP promoted differentiation, as assessed by GPDH activity, with the maximal effect occurring at around 1nM forskolin (122±11% increase, $p<0.001$, n=7) and 10nM CPT-cAMP (78±19% increase, $p<0.01$, n=7). Exposure of cells to increasing concentrations of these compounds resulted in a loss of effectiveness upon differentiation which occurred at between 100nM and 200nM forskolin and 100nM and 1μM CPT-cAMP. Subsequently, higher concentrations of these two agents markedly inhibited the differentiation process.

5.3.2) Effects of Agents which Elevate Cyclic AMP on Differentiation of Cells with a Defined Differentiation Medium (DDM)

The effects of elevated intracellular cyclic AMP levels on the cellular differentiation of 3T3-F442A preadipocytes with FCS/insulin appears to be two-fold, with low concentrations of cyclic AMP promoting the process and high concentrations enforcing an inhibitory influence. However, the complex nature of serum impedes a systematic analysis of interactions occurring between cyclic AMP and individual adipogenic factors. Further studies were therefore carried out to assess the effects of cyclic AMP under defined conditions, in the absence of serum. Confluent cultures were treated with DDM for 10 days with cyclic AMP-elevating agents present for the first three days of differentiation. As with FCS/insulin, the DDM promoted the formation of clusters of rounded, enlarged cells from the fibroblast monolayer (Fig 4.5A). As shown in figure 4.5, cholera toxin (10ng/ml), forskolin (50μM) and CPT-cAMP (0.25mM) inhibited the formation of fat-cell clusters, whereas IBMX (500μM) and a lower concentration of

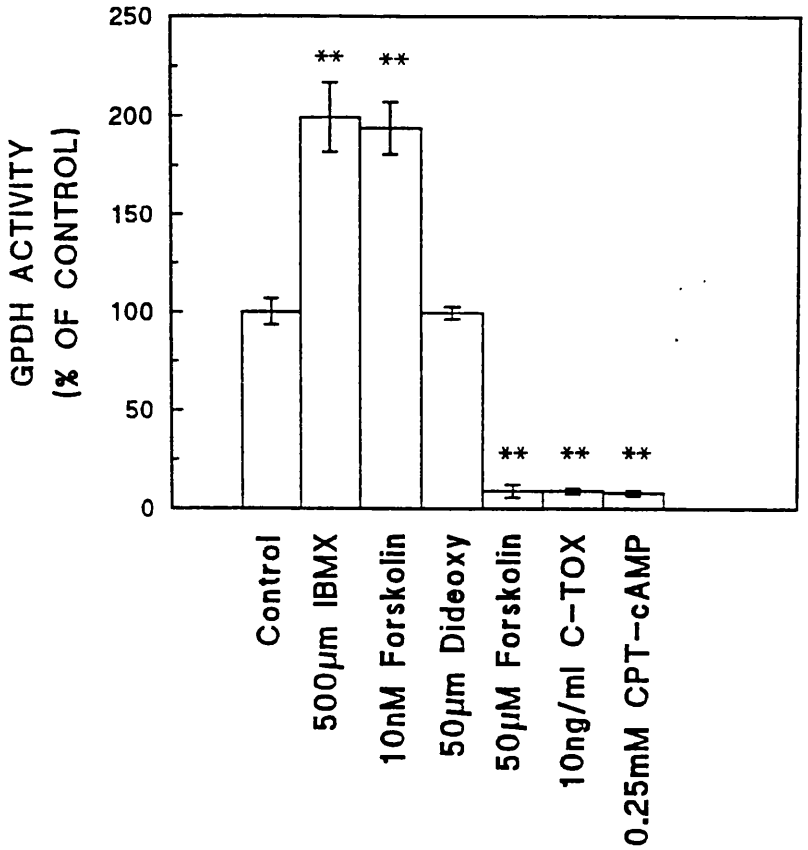


Fig 4.3) Effect of Agents which Elevate Cyclic AMP on α-Glycerophosphate Dehydrogenase (GPDH) Activity of 3T3-F442A Cells Differentiated with FCS/insulin.

Cells were induced to differentiate in FCS/insulin medium in the presence of diluent (control), 500μM IBMX, 10nM forskolin, 10ng/ml cholera toxin (C-TOX), 0.25M CPT-cAMP, 50μM forskolin or 50μM dideoxyforskolin (Dideoxy). After 3 days the test agents were removed from the differentiation medium and after a further 7 days the extent of differentiation was assessed by measuring the GPDH activity of cell extracts as described in *Materials and Methods*. The GPDH activity obtained in differentiated cultures which were exposed to diluent alone was 7.80±0.52 nanokatals/mg DNA (n=5) and all values are expressed as a percentage of this activity. Results are means ± S.E.M. for 4 to 5 observations. *,** indicates that the value differs significantly from control values; $p<0.01$, $p<0.001$ respectively.

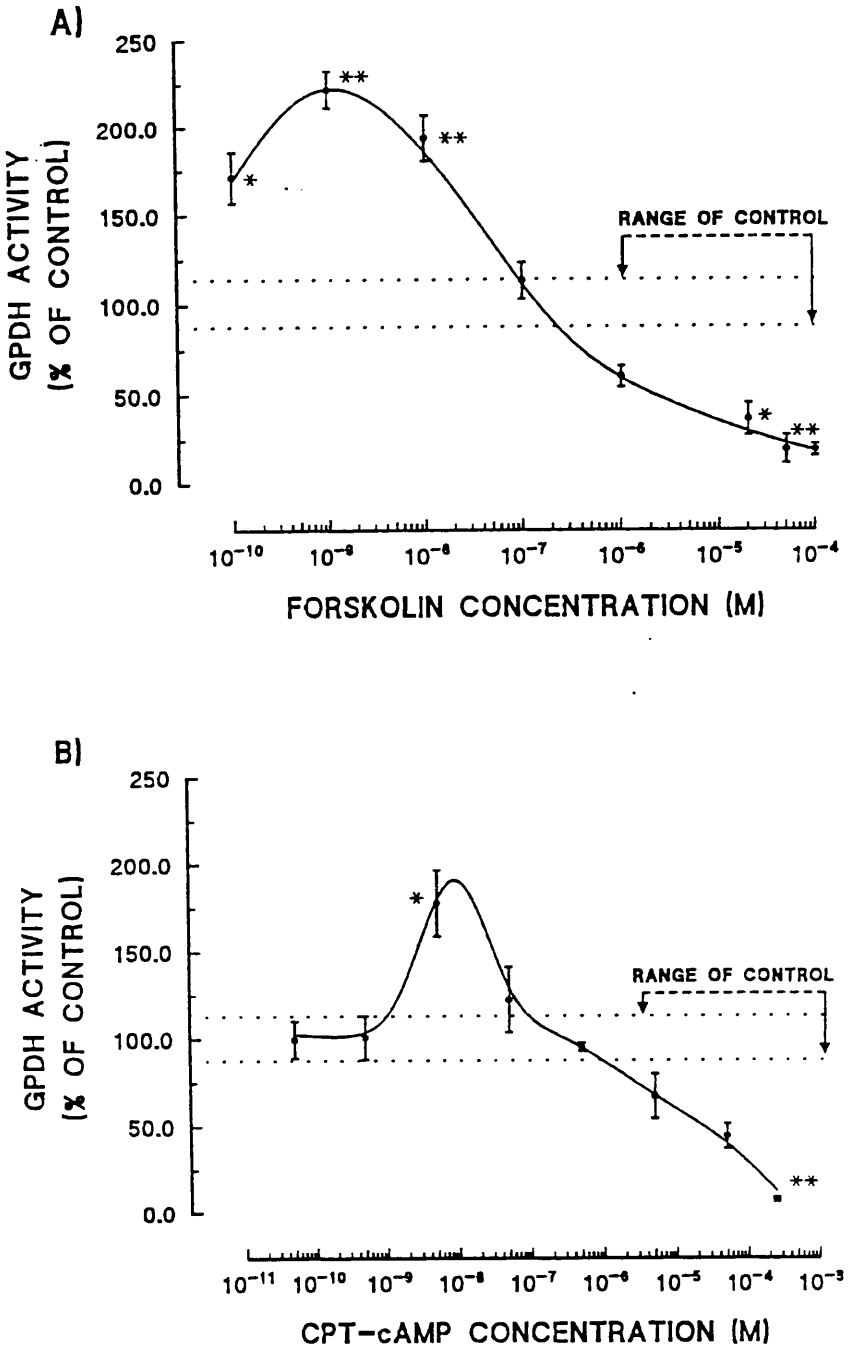


Fig 4.4) Effects of Various Concentrations of Forskolin and CPT-cAMP on the Differentiation of 3T3-F442A Cells with FCS/Insulin.
Confluent cultures were induced to differentiate with FCS/insulin in the presence of the indicated concentrations of A) forskolin or B) CPT-cAMP. After 3 days the drugs were removed from the differentiation medium and 7 days later GPDH activities were measured. Activities are expressed as a percentage of those obtained from differentiated cultures which were exposed to diluent alone (7.80±0.52 nanokatals/mgDNA, n=5). Results are means ± S.E.M. for 7 observations. *, ** indicates the value is significantly different from that for diluent treated cells (control cells); $p<0.01$, $p<0.001$. Broken lines denoted the range of GPDH values obtained from control cells.

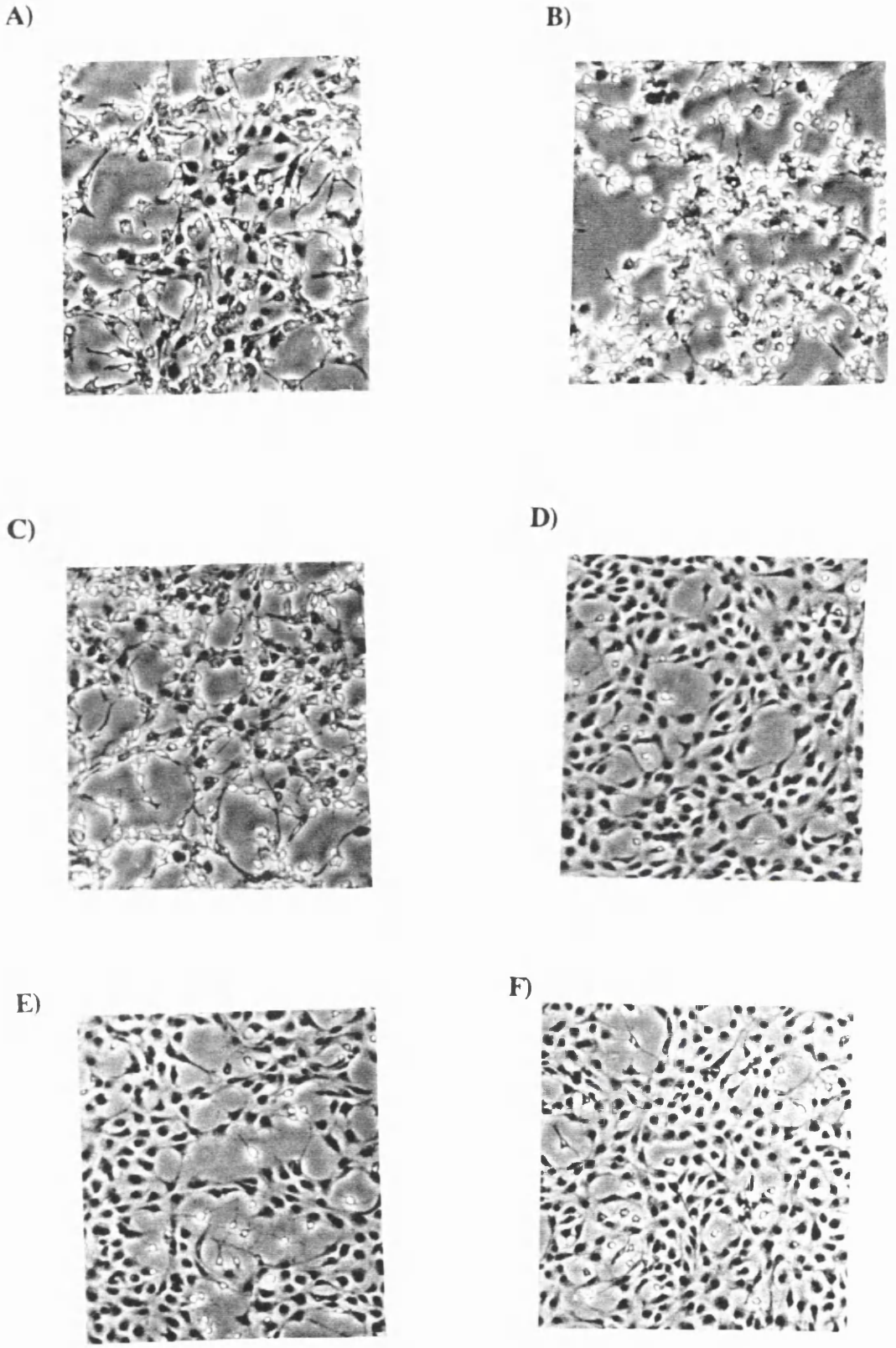


Fig 4.5) Effect of Agents which Elevate Cyclic AMP on the Differentiation of 3T3-F442A Cells Under Defined Conditions.
Confluent 3T3-F442A cells were incubated in DDM (defined differentiation medium) along with (A) diluent, (B) 500μM IBMX, (C) 50μM dideoxyforskolin, (D) 50μM forskolin, (E) 10ng/ml cholera toxin and (F) 0.25mM CPT-cAMP. After 3 days the agents were removed from the DDM and 7 days later cells were stained with Oil Red O and photographed as described in section 2.2.5.

forskolin (10nM) enhanced the number of fat cells formed. Hence qualitatively similar effects of these agents are observed when cells are differentiated in either FCS/insulin (Fig 4.2) or under defined conditions in DDM.

Inclusion of cyclic AMP elevating agents in the DDM induced changes in expression of GPDH activity which paralleled their effects on the morphology of differentiated cells (Fig 4.6). The GPDH activity of cells differentiated with DDM was 3.66±0.41% ($p<0.001$, n=9) nanokatals/mg DNA. Cholera toxin (10ng/ml) and CPT-cAMP (0.25mM) decreased the GPDH activity of differentiated cells by 64±1% ($p<0.001$, n=8) and 73±4% ($p<0.001$, n=7) respectively. While 500μM IBMX increased GPDH activity by 37±12% ($p<0.01$, n=9). As before (Fig 4.4) different concentrations of forskolin exerted differential effects upon preadipocyte differentiation. 10nM Forskolin was found to significantly increase GPDH activity, relative to control cells, by 47.±11% ($p<0.01$, n=9), whereas 50μM forskolin attenuated the GPDH activity in terminally differentiated cells by 67.76±1.11% ($p<0.001$, n=8).

The dose effects of forskolin and CPT-cAMP when cells were differentiated in DDM (Fig 4.7) were similar to those observed when differentiation was induced with FCS/insulin (Fig 4.4). Under DDM conditions the maximal increases in GPDH activity were observed in the presence of around 10nM forskolin (42±12% increase, $p<0.01$, n=7) and 10nM CPT-cAMP (31±9% increase, $p<0.01$, n=7). Maximal inhibition of GPDH activity occurred at around 50μM forskolin (95±1.5% inhibition, $p<0.001$, n=5) and 200μM CPT-cAMP (52±20% inhibition, $p<0.01$, n=7). For both of these agents there existed a range of concentrations where no effect on differentiation was observed. Thus, concentrations of forskolin between 100nM and 500nM, and CPT-cAMP between 4μM and 30μM, had no discernible impact on GPDH activity. This presumably reflects the net effect of stimulatory and inhibitory influences evoked by raising intracellular cyclic AMP levels. If these results are taken together, it appears that the differentiation of 3T3-F442A cells in the presence of serum, or under serum-free, chemically defined conditions, displays a strict inverse dose-responsive relationship to elevations in intracellular cyclic AMP concentrations. Low concentrations of intracellular cyclic AMP appear to be able to enhance stimulatory influences on preadipocyte differentiation, whereas high concentrations induce negative regulatory constraints. Therefore, in order to aid interpretation of the observed effects it is important to consider the magnitude of intracellular cyclic AMP production induced in response to exposure to the test agents. A further consideration is the duration of cyclic AMP production which will have consequences in terms of the stage of the differentiation process which is being influenced.

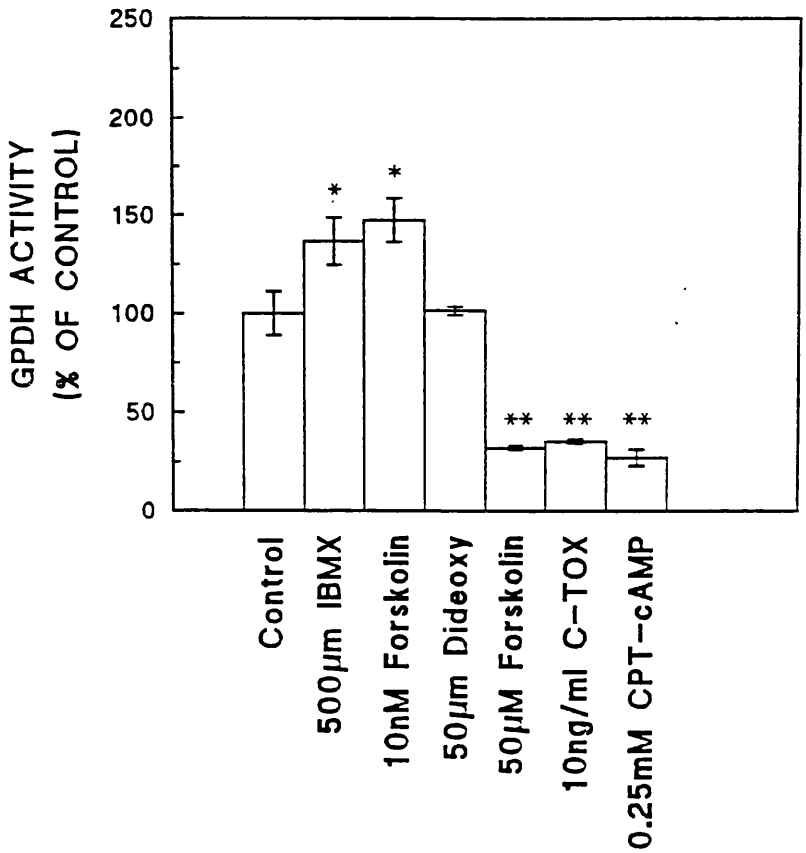


Fig 4.6) Effects of Agents which Elevate Cyclic AMP on GPDH Activity of Cells Differentiated with DDM.
Confluent cultures were induced to differentiate by replacing the growth medium with DDM medium along with the agents indicated (as described in the legend to Fig 5.3) or with diluent (control). After 3 days the test agents were removed and after a further 3 days GPDH activity was measured. Results are means ± S.E.M. for 5 to 9 observations. The GPDH activity obtained in cultures induced to differentiate with DDM alone was 3.66±0.41 nanokatals/mg DNA (n=9) and all values are expressed as a percentage of this activity. *, ** indicate the value differs significantly from that for cells differentiated in the absence of test agents. $p<0.01$, $p<0.001$.

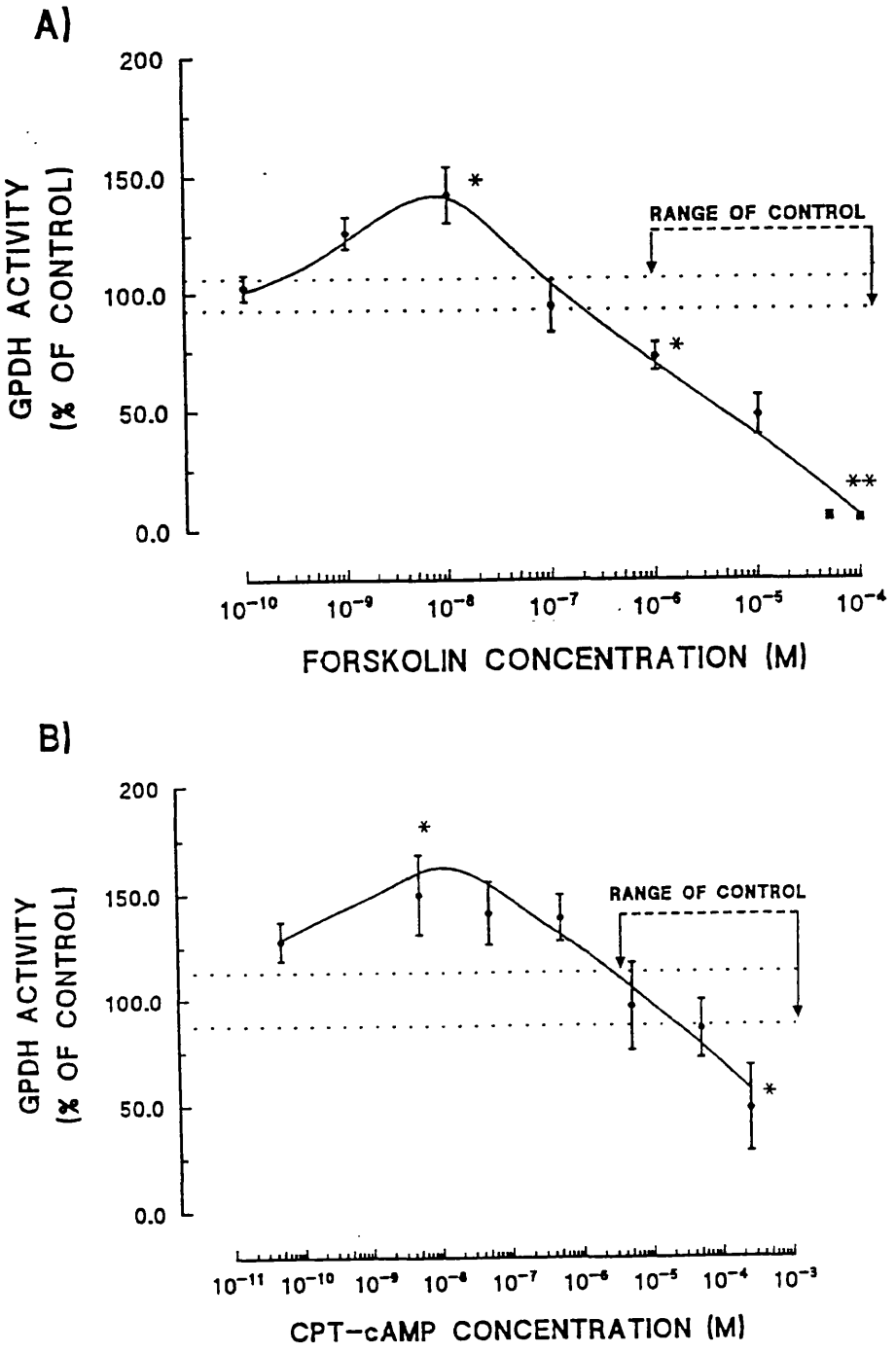


Fig 4.7) Dose Effects of Forskolin and CPT-cAMP on Differentiation of 3T3-F442A Cells in a Defined Differentiation Medium.
Confluent cultures were induced to differentiate with DDM medium in the presence of the indicated concentrations of A) forskolin or B) CPT-cAMP. After 3 days the drugs were removed and after a further three days GPDH activities were assessed. The GPDH activity obtained in cultures induced to differentiate with DDM medium alone was 3.9±1.1 nanokatals/mg DNA (n=7) and all values are expressed as a percentage of this activity. Results are means ± S.E.M. for 4-7 observations. *, ** indicates the value is significantly different from that for cells differentiated in DDM medium alone; $p<0.01$, $p<0.001$.

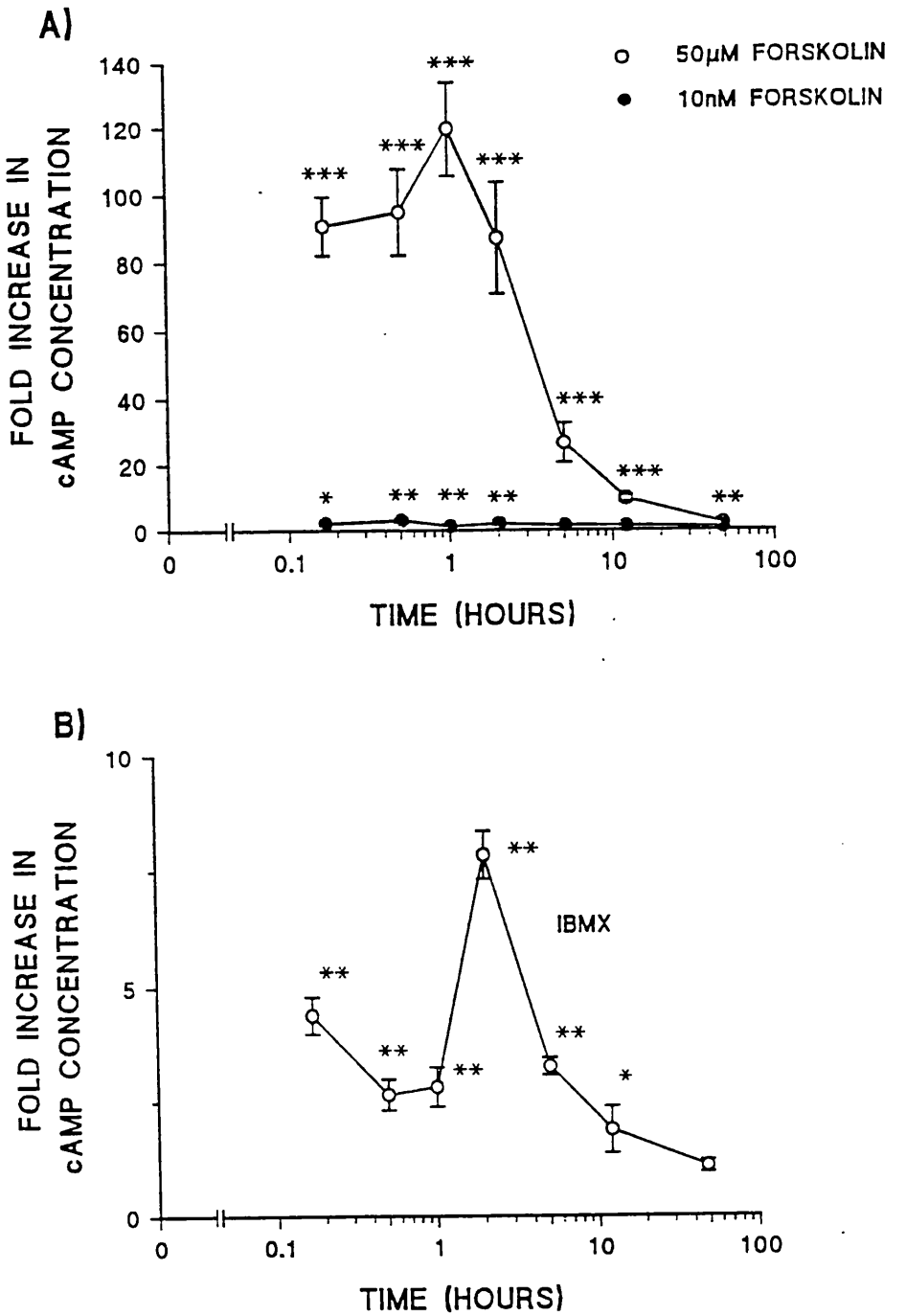


Fig 4.8) Effect of Forskolin and IBMX on the Intracellular Concentration of Cyclic AMP.
Confluent 3T3-F442A cells were incubated for the indicated times in culture medium containing A) forskolin (10nM or 50μM) or B) IBMX (500μM). Intracellular cyclic AMP was then extracted and the concentration measured as described in section 2.2.16. Data are presented as means ± S.E.M. for 4 to 8 observations and are expressed as the fold increase in intracellular cyclic AMP relative to cells incubated with diluent alone. Significant differences with respect to control are indicated; * ($p<0.02$), ** ($p<0.01$) and *** ($p<0.001$).

4.3.3) Effect of Forskolin and IBMX on the Intracellular Concentration of Cyclic AMP

In order to investigate these possibilities further the effect of the various compounds on intracellular cyclic AMP levels was addressed. IBMX (500μM) induced a relatively transient increase in intracellular cyclic AMP (Fig 4.8B), with maximal levels achieved after 2 hours incubation with the agent (approximately 7.8±0.5 fold increase relative to control, $p<0.001$, n=12). Levels returned to those detected in control cells after 12 hours exposure to IBMX. Similarly, 10nM forskolin induced a small and comparatively transient increase in intracellular cyclic AMP (Fig 4.8A) with a maximal 3.0±0.7 fold increase ($p<0.01$, n=6) achieved after 30 minutes incubation and a return to basal levels by 5 hours. In contrast, 50μM forskolin provoked a strikingly large and sustained increase in intracellular cyclic AMP (Fig 4.8A), with a maximal 119.6±14.2 fold increase ($p<0.001$, n=8) achieved after 1 hour. Elevated cyclic AMP concentrations were detectable even after 48 hours stimulation with 50μM forskolin (2.1±0.2 fold increase, $p<0.01$, n=5). Hence the large and sustained increases in intracellular cyclic AMP concentrations achieved with 50μM forskolin are capable of inhibiting preadipocyte differentiation whereas the smaller and more transient increases induced by 10nM forskolin and IBMX promote this process.

4.3.4) Effect of Elevating Intracellular Cyclic AMP Levels During the Priming and Maturation Stages of 3T3-F442A Preadipocyte Differentiation

The effects of transient increases in cyclic AMP will be confined to a discrete stage of the differentiation process, whereas larger, sustained elevations have the potential to effect multiple stages of development. In previous experiments cyclic AMP-elevating agents which potentiated differentiation (10nM forskolin and 500μM IBMX) were included in the differentiation media at the onset of the process (Figs 4.3 and 4.6). Therefore, the transient increase in intracellular cyclic AMP elicited by these agents must have been exerting its effects at an early stage of the differentiation program. As demonstrated in Chapter 4 the differentiation of 3T3-F442A preadipocytes can be divided into at least two stages. GH acts at an early stage of the differentiation process and primes 3T3-F442A cells to the actions of insulin, EGF and T_3 (maturation medium), which then induce terminal differentiation. In order to study the effects of cyclic AMP on these two distinct stages, the two-phase protocol for cellular differentiation (described in section 2.2.4.2) was employed. The effects of the β-adrenoreceptor agonist isoproterenol (10μM) were also examined during the two phases of differentiation. As discussed in the previous chapter, alterations in heterotrimeric G-

protein and adrenoreceptor expression during priming and maturation will contribute to cellular responsiveness to adrenergic stimulation. Treatment of cells with isoproterenol at these distinct stages will determine to what extent β-adrenergic stimulation contributes to the regulation of preadipocyte differentiation. To assess the effects of cyclic AMP and β-adrenergic stimulation on the priming stage 10nM forskolin, IBMX or isoproterenol were included in the priming medium for two-days, following which cells were washed and the medium was replaced with maturation medium in the absence of agents. Differentiation was then assessed 8 days later. For studies on the second phase of differentiation, cells were primed for two days with GH and then the medium was replaced with maturation medium containing 10nM forskolin, IBMX or isoproterenol. After a further two days this medium was removed and replaced with fresh maturation medium in the absence of agents. The extent of differentiation was assessed 6 days later. During the two day priming phase if included alone, in the absence of GH, 10nM forskolin, IBMX or isoproterenol, were observed to induce a very small, but not significant, increase in terminal differentiation as assessed by GPDH activity (Table 4.3). Significantly, each of these agents strongly enhanced the ability of GH to promote differentiation by 91.0±21.1% ($p<0.01$, n=9), 133.9±37.3% ($p<0.01$, n=6) and 76.3±7.2% ($p<0.001$, n=3) respectively (Table 4.3). In contrast to this effect the same agents, namely 10nM forskolin, 500μM IBMX and isoproterenol, significantly inhibited terminal differentiation when they were included during the initial two days of the maturation phase (Table 4.4). In this circumstance, GPDH activity was inhibited by 59.8±12.1% ($p<0.01$, n=6), 71.9±9.5% ($p<0.001$, n=6) and 78.9±8.0% ($p<0.001$, n=3), respectively. Inclusion of 50μM forskolin also inhibited differentiation by 73.7±6.2% ($p<0.001$, n=6) when added to cells at the maturation phase. Assessment of the effects of 50μM forskolin specifically on the priming stage was not practical as this high concentration produced an elevation in cyclic AMP levels which persisted into the maturation stage (Fig 4.8A). From Fig 4.8) it is clear that the addition of 10nM forskolin or IBMX to preadipocytes at the beginning of the priming phase, and the continued presence throughout this phase, would result in relatively transient increases in cyclic AMP levels which would be lost by commencement of the maturation phase (48 hours after initial addition of the agents). In contrast the addition of 50μM forskolin to cells at commencement of the priming phase would result in around a 2 fold elevation in intracellular cyclic AMP levels 48 hours later, at the start of the maturation phase. Hence, the effects of 50μM forskolin on the priming phase cannot be dissociated from effects on the subsequent maturation phase.

Intriguingly, isoproterenol did not induce significant elevations in intracellular cyclic AMP during priming or maturation (results not shown). This is consistent with previous observations in 3T3-L1 (Rubin *et al*, 1977) and 3T3-F442A (Kilgour and

Table 4.3) Effect of Forskolin, IBMX and Isoproterenol on the Priming of 3T3-F442A Preadipocytes.

Cells were grown in 10% calf serum which had been depleted of GH. At confluence the growth medium was removed and cells were primed for two days in serum free medium in the presence or absence of 2nM GH along with the agents indicated. This was then replaced with maturation medium and 8 days later GPDH activity was measured. For further details see section 2.2.4.2. The GPDH activity obtained in cultures primed in the absence of GH was 0.04±0.28 nanokatals/mgDNA (n=9) and all values are expressed as the relative increase (fold) in this activity. Results are means ± S.E.M. with the number of observations in parenthesis. *, ** indicate the value differs significantly from that for cells primed with GH alone; $p<0.01$, $p<0.001$ respectively.

Fold increase in GPDH activity

Stage	Additions	GH-	GH+
Priming	-	1	86.2±7.5 (n=5)
	10nM Forskolin	9.8±5.4 (n= 9)**	164.7±18.2* (n=9)
	10μM Isoproterenol	12.7±7.7 (n=3)**	152.0±6.2**(n=3)
	500μM IBMX	13.0±5.2 (n=3)**	201.7±32.2* (n=6)

Table 4.4) Effect of Forskolin, IBMX and Isoproterenol on the Maturation of 3T3-F442A Preadipocytes.
Cells were grown to confluence as described in the legend to Table 4.3) then primed for 2 days in serum free medium containing 2nM GH. After 2 days this was replaced with maturation medium and the agents indicated. Drugs were removed from the maturation medium after 2 days and 6 days later GPDH activity was measured. The GPDH activity obtained in cultures induced to differentiate with this protocol in the absence of drugs was 3.88±1.06 nanokatals/mgDNA (n=6) and all values are expressed as the relative change (fold) in this activity. Results are means ± S.E.M. for the number of observations in parenthesis. *, ** indicate the value differs from that for cells differentiated in the absence of additions, $p<0.01$, $p<0.001$ respectively.

Addition	Fold Change in GPDH Activity
10nM Forskolin	$0.40\pm0.12^{*}$ (6)
50μM Forskolin	$0.26\pm0.06^{**}$ (6)
10μM Isoproterenol	$0.21\pm0.08^{**}$ (6)
500μM IBMX	$0.28\pm0.09^{**}$ (6)

Table 4.5) Effect of Elevating Cyclic AMP with Forskolin, IBMX and Isoproterenol During Priming and Maturation on the DNA Content of 3T3-F442A Preadipocytes.

Cells were grown to confluence as described in the legend to Table 4.3). At confluence the growth medium was removed and cells were primed for 2 days in serum free medium in the presence of 2nM GH. After 2 days this was replaced with maturation medium and the amount of cellular DNA was measured fluorometrically 8 days later as described in section 2.2.14. Drugs were included either during the 2 day priming stage, along with GH, or alternatively for the first 2 days of the maturation stage, as indicated. DNA values are expressed as the amount (μg) of DNA extracted from one 100mm petri-dish. Results are means±S.E.M. for 6 observations.

DNA Content (μg)

	Drugs Included During...	
Addition	Priming	Maturation
Diluent	15.3±3.5	17.5±2.2
500μM IBMX	13.3±1.8	18.2±3.2
10nM Forskolin	16.0±2.1	16.3±3.3
50μM Forskolin	12.4±2.1	15.2±4.0

Anderson, 1993) preadipocytes and implies that the effects of isoproterenol in this study are attributable to mechanisms distinct from those utilised by cyclic AMP.

One of the effects of cyclic AMP on cell growth is to act as a positive- or negative-regulator of cellular proliferation (Table 4.1). Given that the number of cells increases during the maturation phase of 3T3-F442A differentiation (Table 3.2) part of the actions of cyclic AMP on preadipocyte differentiation may be to enhance or inhibit this expansion phase. To clarify this, cyclic AMP levels were elevated during either the priming and maturation phases and DNA content, a direct measurement of cell number, was measured in terminally differentiated cultures. The results presented in Table 4.5) demonstrate that elevation of cyclic AMP with IBMX or with 10nM or 50μM forskolin, during priming or maturation, had no significant effect on the number of terminally differentiated cells. This suggests that the positive and negative effects of cyclic AMP on the differentiation of 3T3-F442A preadipocytes are achieved through modulation of phenotypic differentiation and not by increasing the number of terminally differentiated adipocyte cells.

4.4) DISCUSSION

It is well established that cyclic AMP exerts a range of effects upon cell growth depending on the cell type being studied (Rozengurt *et al*, 1986; Dumont *et al*, 1989; Frödin *et al*, 1994; Englaro *et al*, 1995).To study the effects of cyclic AMP on the differentiation of 3T3-F442A preadipocytes multiple agents were employed which raise intracellular cyclic AMP levels by different mechanisms (Fig 4.1). In 3T3-L1 preadipocytes an accelerating effect of cyclic AMP on adipose conversion was suggested originally by Russel and Ho (1976), and later by Elks and Manganiello (1985), on the basis of studies using synthetic phosphodiesterase inhibitors such as IBMX. In rat preadipocytes Björntorp *et al* (1980) described a small, but significant, effect of IBMX on differentiation, whereas Gaillard *et al* (1989) reported a more substantial enhancement of Ob17 cell differentiation by this inhibitor. In the present study, IBMX very effectively promoted the expression of a late marker of adipose conversion of 3T3-F442A cells, namely GPDH. This effect was observed when cells were induced to differentiate with foetal calf serum and insulin (Figs 4.2 and 4.3) or under defined serum free conditions (Figs 4.5 and 4.6).

Two major pharmacological effects of IBMX are inhibition of cyclic nucleotide phosphodiesterases and inhibition of interaction of adenosine with its receptor (Elks and Manganiello, 1985). In this study the effects of IBMX were mimicked with specific activation of adenylate cyclase by low concentrations of the diterpene forskolin (10nM) and by a lipophilic cyclic AMP analogue, namely CPT-cAMP (Fig 4.4 and 4.7). This suggests that the effect of IBMX is due to its ability to elevate cellular cyclic AMP concentrations, by inhibition of cyclic AMP phosphodiesterase activity.

In contrast to the stimulatory effects of 10nM forskolin and IBMX, high concentrations of forskolin (50μM) potently inhibited the differentiation of 3T3-F442A cells induced by FCS/insulin (Figs 4.2 and 4.3) or DDM (Figs 4.5 and 4.6). Although the physiological consequences of forskolin treatment are, in general, thought to be mediated by increases in intracellular cyclic AMP, a number of cyclic AMP-independent events have been reported (Laurenza *et al*, 1989). For example, forskolin has been found to completely inhibit glucose transport in adipocyte plasma membranes (EC_{50}=240nM) by direct interaction with a transporter (Joost *et al*, 1988). Consequently, the anti-differentiation effects of 50μM forskolin could be due to inhibition of glucose-uptake for oxidative phosphorylation or preventing deposition of glucose into lipid. However, a number of observations indicate that the principal effects of 50μM forskolin on 3T3-F442A differentiation are primarily attributable to elevation of intracellular cyclic AMP. Firstly, the inability of an inactive analogue of forskolin,

1,9-dideoxyforskolin, to reproduce a forskolin like effect (Figs 4.3 and 4.5). This analogue has also been shown to completely inhibit glucose transport in rat adipocytes, but does not stimulate adenylate cyclase even at 100μM (Joost *et al*, 1988). Secondly, the effect of 50μM forskolin was mimicked by a high concentration of CPT-cAMP and by cholera toxin (Figs 4.3 and 4.5).

The inhibitory effect of cholera toxin on 3T3-F442A differentiation is in complete accordance with the work of Wang *et al* (1992) who also demonstrated that cholera toxin exerted a blockade on 3T3-L1 preadipocyte differentiation. In addition, antisense oligodeoxynucleotides to $G_{s\alpha}$ were shown to accelerate 3T3-L1 differentiation (Wang *et al*, 1992). These authors conclude that these effects were independent of an adenylate cyclase-mediated increase in cyclic AMP levels. This was based on the observation that 10μM forskolin had no effect on differentiation as assessed by qualitative Oil Red O staining. This is perhaps somewhat of a paradox since elevated cyclic AMP levels, induced by cyclic AMP phosphodiesterase inhibitors, have been shown to potently enhance 3T3-L1 differentiation as assessed quantitatively by GPDH activity (Elks and Manganiello, 1985; Schmidt *et al* 1990). Furthermore, the results in the present study are consistent with at least part of the inhibitory actions of cholera toxin on 3T3-F442A cell differentiation being mediated by cyclic AMP. This, together with the observations of Elks and Manganiello (1985) and Schmidt *et al* (1990), suggests that the conclusions made by Wang *et al* on the functional role of $G_{s\alpha}$ in preadipocyte differentiation should be carefully reevaluated in terms of a more sensitive marker of terminal differentiation, such as GPDH activity.

Interestingly, the present results indicated that small, transient increases in intracellular cyclic AMP, as induced by IBMX and 10nM forskolin (Fig 4.8), early in the 3T3-F442A differentiation process, promote terminal differentiation, while larger, more sustained increases, as induced by 50μM forskolin (Fig 4.8), inhibit the process. Thus, it seems that a discrete cyclic AMP signal, temporally localised to the initial stages of differentiation, exerts a potentiating influence, but a continued and prolonged cyclic AMP signal interferes with full expression of the fatty phenotype by exerting effects at a later stage.

Interestingly, elevations in intracellular cyclic AMP have also been shown to potentiate terminal differentiation of Ob1771 preadipocytes (Gaillard et al, 1989). In the present study transient elevation of cyclic AMP, with 10nM forskolin and IBMX, during the priming phase of 3T3-F442A cellular differentiation synergistically enhanced the ability of GH to promote terminal differentiation (Table 4.3). Since GH is also a critical requirement for the differentiation of Ob1771 cells (Doglio *et al*, 1987), it is tempting to

speculate that part of the adipogenic actions of cyclic AMP in this cell type are attributable to a similar interaction.

The molecular mechanisms used by GH to promote differentiation are not known, but are likely to involve the induction of genes which are early markers of this process. GH has been shown to induce the expression of lipoprotein lipase (Bacellini-Couget *et al*, 1993) and the transcription factors c-fos, c-jun (Sumantran *et al*, 1992), and C/EBP β and δ (Clarkson *et al*, 1995). Cyclic AMP has also been shown to be a direct inducer of C/EBP β and δ in mouse cortical astrocytes (Cardinaeux and Magistretti, 1996) and of C/EBPβ gene expression in 3T3-L1 preadipocytes (Yeh *et al*, 1995). C/EBPβ and C/EBPδ have been shown to play an essential, early pivotal role in the adipose conversion of 3T3-L1 cells (Yeh *et al*, 1995), it would therefore be extremely interesting to investigate to what extent GH and cyclic AMP cooperate in the induction of these "adipogenic" transcription factors.

A potential mechanism by which GH could induce changes in protein expression is through activation of growth regulatory signalling pathways such as the MAP kinase (Marshall, 1995) and $p70^{S6K}/p85^{S6K}$ (Kozma and Thomas, 1994) cascades. Studies using the pheochromocytoma cell line, PC12, have shown that the transmission of signals to MAP kinase, mediated by sequential activation of Ras, Raf and MEK , may serve to transduce differentiative signals (Noda *et al*, 1985; Wood *et al*, 1993; Cowley *et al*, 1994; Marshall, 1995; Pang *et al*, 1995). Recent studies have suggested that activation of this pathway may also be a requirement for preadipocyte differentiation. Transfection of 3T3-L1 preadipocytes with Raf or Ras (Porras *et al*, 1994) induces partial adipogenesis and a complete blockade is induced when cellular levels of MAP kinase are specifically depleted with antisense oligodeoxynucleotides (Sale *et al*, 1995). Since GH has been shown to activate MAP kinases in 3T3-F442A preadipocytes (Anderson, 1993) this could be a potential pathway underlying the adipogenic actions GH. In addition, a number of studies have suggested that part of the effects of cyclic AMP on cell growth may be mediated by regulatory "cross-talk" with the MAP kinase cascade (Graves and Lawrence, 1996). Consequently, it is possible that the potentiating action of cyclic AMP on the GH-priming of 3T3-F442A adipogenesis may be due to interactions between the GH and cyclic AMP pathways, perhaps at the level of MAP kinase.

The synthetic β-adrenoceptor agonist isoproterenol (10μM) mimicked the effects of forskolin (10nM) and IBMX on GH-priming. This is consistent with the findings of Vassaux *et al* (1992) who demonstrated that isoproterenol enhanced Ob1771 differentiation by 2 fold and rat preadipocyte differentiation by approximately 1.5 fold;

this was accompanied by an approximate 20 fold increase in intracellular cyclic AMP. However, isoproterenol does not elevate cyclic AMP levels in 3T3-F442A fibroblasts (Anderson and Kilgour, 1993) or activate PKA (this author, unpublished observations). Accordingly isoproterenol should induce some additional intracellular event(s), independent of activation of adenylate cyclase, which are able to act in concert with the GH signalling pathway to potentiate differentiation. The effects of isoproterenol on differentiation are likely be mediated by β_1-adrenergic receptors as this is the predominant subtype in 3T3-F442A fibroblasts (Fève *et al*, 1991) whereas the predominant isoform in differentiated cells is the β_3-subtype (Fève *et al*, 1994). Although β-adrenergic receptors are typically thought to activate G_s, these receptors have also been shown to interact with G_i in a variety of cells and reconstituted systems (Assano *et al*, 1984; Rubenstein *et al*, 1991).

In contrast to their priming actions during the initiation of differentiation, cyclic AMP and β-adrenergic stimulation were found to be inhibitory during the terminal stages of the process (Table 4.4). Spiegelman and Green (1981) reported that agents which increase intracellular cyclic AMP levels inhibit lipid accumulation and the expression of several lipogenic enzymes in 3T3-F442A cells. In their study the cyclic AMP-elevating agents were either present throughout the entire differentiation period or added 6 days after the induction of differentiation. Antras *et al* (1991) also demonstrated that isoproterenol and cyclic AMP-elevating agents evoked transcriptional down-regulation of genes encoding lipogenic enzymes in mature 3T3-F442A adipocytes. Hence these inhibitory effects are consistent with the inhibitory effect of cyclic AMP observed during the maturation period in the present study.

Insulin and EGF are essential components of the medium used to promote the second phase of differentiation whereas T_3 exerts only a modulatory influence (Guller *et al*, 1988). The molecular mechanisms underlying the effects of EGF and insulin are not known but each induce strong activations of MAP kinases in 3T3-F442A cells (chapter 5) which are essential for preadipocyte differentiation (Sale *et al*, 1995). In many cell types the biological effects of cyclic AMP appear to be mediated in part by changes in the activities of intermediate steps of transduction pathways utilised by insulin and growth factors (Graves and Lawrence Jr., 1996). For example, in certain fibroblastic cell lines (Burgering *et al*, 1993; Hordijk *et al*, 1994) and rat adipocytes (Sevetson *et al*, 1993) cyclic AMP inhibits the MAP kinase cascade and this correlates with antagonistic effects of cyclic AMP on growth factor-induced mitogenesis and insulin-mediated effects on metabolism respectively. Therefore, in defining mechanisms by which cyclic

AMP attenuates the transduction of differentiative signals by insulin and EGF, the effect of cyclic AMP on the activation of MAP kinases should be assessed.

4.5) CONCLUSIONS

This study demonstrates that increases in cyclic AMP levels exert differential effects upon the differentiation of 3T3-F442A preadipocytes which depend upon the stage of the differentiation process at which the levels are raised. During the early stages of the process cyclic AMP synergistically promotes differentiation in the presence of GH. Subsequently cyclic AMP inhibits differentiation at the maturation stage. These observations may go some way in helping to explain some the apparently contradictory findings presented in Table 4.2). The molecular mechanisms involved remains to be elucidated but are likely to involve either the direct modulation of gene expression by the cyclic nucleotide or modulation of the signal transduction pathways utilised by GH and by insulin and EGF.

Chapter Five

Interactions Between The Cyclic AMP and MAP Kinase Pathways: Effect On The Differentiation Of 3T3-F442A Preadipocytes.

5.1) INTRODUCTION

The results from Chapter 4 demonstrate that cyclic AMP can exert differential effects on the cellular differentiation of preadipocyte 3T3-F442A cells. These reciprocal actions appear to be dependent on the stage of differentiation at which levels are raised. During the initial priming stage of differentiation cyclic AMP interacts synergistically with GH to potentiate differentiation, whereas in the later, insulin-induced maturation phase cyclic AMP behaves as a potent inhibitor of terminal differentiation. How cyclic AMP can elicit such radically different responses in differentiating preadipocytes is not known. However, because the cellular-response to cyclic AMP is stage-specific, this suggests that the composition of the extracellular hormonal milieu may be a determining factor. It is therefore conceivable that cyclic AMP could be modulating the cellular response to particular adipogenic hormones by opposing or enhancing specific intracellular signals generated at their receptors. A complete understanding of the mechanisms of adipogenic hormone-receptor binding will be necessary to determine the consequences of such regulatory cross-talk. Nevertheless, in recent years our appreciation of the intracellular events involved in growth and differentiation factor signalling has improved substantially.

Commitment to progress through the cell cycle or to arrest in G_1 followed by differentiation is thought to involve changes in gene expression induced by growth and differentiation factors (MacDougald and Lane, 1995). Stimulation of cell growth, through G-protein-coupled serpentine receptors or through receptor-coupled tyrosine kinases, involves activation of at least two distinct protein kinase cascades. The first to be discovered involves the *erk* gene products p42 and p44 MAP kinase (Her *et al*, 1991; Pagès *et al*, 1995) which are activated simultaneously in response to signals transmitted sequentially through $p21^{ras}$, $p74^{raf}$ and $p45^{mek}$ (Marshall, 1995). Recently, members of the cytokine receptor superfamily, such as the GH receptor, have also been shown to feed into the MAP kinase pathway (Winston and Hunter, 1995). Once activated p42/p44 MAP kinase can be translocated to the nucleus (Chen *et al*, 1992; Lenormont *et al*, 1993) and directly phosphorylates transcription factors, including $p62^{TCF}$ and ATF-2 (Davis, 1993). In addition, a number of cytosolic substrates for MAP kinases have been identified, including MAPKAP kinase 1, phospholipase A_2, and Stat 1 (Sturgill *et al*, 1988; Lin *et al*, 1993b; Lin *et al*, 1994; Wen *et al*, 1995).

Although it is clear that the MAP kinases play a pivotal role in mitogenesis (Pagès *et al*, 1993), accumulated data from several sources has suggested that the MAP kinase cascade is also a transducer of differentiative signals. Different members of the cascade have been implicated in the cellular differentiation of a diverse range of cell

types; including thymocytes (Alberola-Ila *et al*, 1995; Crompton *et al*, 1996), pheochromocytoma (PC12) cells (Qui and Green, 1992; Pang *et al*, 1995) and *Drosophilla* photoreceptors (O'Neil *et al*, 1994). The most compelling evidence for the involvement of the MAP kinase pathway in cellular differentiation has come from the use of dominant negative interfering mutations of MEK which inhibit the differentiation of T-cells (Alberola-Ila *et al*, 1995) and PC12 cells (Cowley *et al*, 1994). A possible involvement of the MAP kinase pathway in the control of adipocyte development was initially suggested by the observation that dominant inhibitory mutants of Ras block differentiation of 3T3-L1 fibroblasts to adipocytes (Benito *et al*, 1991). However, evidence suggests that Ras is an upstream activator multiple pathways (Lowry and Willumsen, 1993). A more direct, anti-sense approach has been used recently to demonstrate an obligatory requirement for p42 and p44 MAP kinases for the terminal differentiation of 3T3-L1 preadipocytes (Sale *et al*, 1995).

Although cyclic AMP, induces an activation of MAP kinase in neuronal PC12 cells this is not sufficient to differentiate these cells (Frodin *et al*, 1994). This appears to be due to the transient nature of the cyclic AMP-stimulation which is not sufficient to induce translocation of MAP kinase to the nucleus (Yao *et al*, 1995). In contrast, differentiation induced by nerve growth factor (NGF) is characterised by prolonged activation and nuclear translocation of MAP kinase (Traverse *et al*, 1992; Nguyen *et al*, 1993). Still, cyclic AMP acts synergistically with NGF to potentiate PC12 differentiation (Yao *et al*, 1995). Therefore, it appears that there exists a threshold duration and magnitude of MAP kinase activation which must be achieved before a cell is committed to a pathway of differentiation. Indeed, EGF, which induces a transient activation of MAP kinase and proliferation in PC12 cells, can be converted into a differentiation agent by synergistic interaction with cyclic AMP (Yao *et al*, 1995). This differentiation was associated with sustained activation and nuclear localisation of MAP kinase (Yao *et al*, 1995). Thus, cyclic AMP may serve to augment cellular differentiation by enhancing activation of signalling pathways by differentiative agents.

To try and understand the molecular mechanisms underlying the effects of cyclic AMP on differentiation of 3T3-F442A cells presented in Chapter 4 a study was undertaken to determine if the differential effects of cyclic AMP in preadipocytes were attributable to functional interplay with the MAP kinase cascade. This required that the requirement for MAP kinases in transducing stage-specific differentiative signals be assessed. This involved cellular depletion of p42 and p44 MAP kinases with antisense oligodeoxynucleotides and the use of a recently discovered chemical inhibitor of MAP kinase activation. The ability of cyclic AMP to activate MAP kinases, or enhance the

signal propagated by other adipogenic agents, was assessed immunologically and by a specific phospho-transferase assay.

5.2) EXPERIMENTAL PROCEDURES

5.2.1) Standard Procedure for Cell Culture and Differentiation

3T3-F442A cells were grown to confluence in DMEM containing 10% calf serum. Confluent cultures were induced to differentiate with a chemically defined differentiation medium (DDM) or with foetal calf serum and insulin (FCS/insulin) as described in sections 2.2.2 and 2.2.3.

5.2.2) Two-phase Differentiation Protocol

For certain experiments cells were grown to confluence in the presence of 10% calf serum depleted of GH. This was prepared as described in Chapter 2. Cells were passaged at least twice in DMEM containing 2.5mM glutamine and 10% GH-depleted calf serum, prior to use for differentiation studies. Confluent cells were washed three times in PBS then incubated for 2 days in serum free medium [F12:DMEM (2:1)] containing transferrin (10µg/ml), fetuin (50µg/ml), glutamine (2.5mM) and BSA (1mg/ml)] along with the other additions indicated in figure legends. Cultures were then washed, as before, and the medium replaced with maturation medium [serum free medium containing insulin (1.8µM), T_3 (0.1ng/ml) and EGF (50ng/ml)]. The extent of differentiation was measured after a further 8 days.

5.2.3) Oligodeoxynucleotide Treatment of Cells

The design, synthesis and purification of phosphorothioate oligodeoxynucleotides has been described previously (Sale *et al*, 1995). Cells were grown in 22mm dishes and were treated with oligodeoxynucleotides having just attained confluence. Monolayers were washed three times with 2ml DMEM and appropriate dilutions of oligodeoxynucleotides in 100µl DMEM were preincubated at room temperature for 30 minutes with 100µl Lipofectin (Life Technologies). This mixture was added to the cells with a further 200µl DMEM. The final concentration of oligodeoxynucleotides was 5µM. Cells were incubated for 8 hours at 37°C in the presence of 5% CO_2. After this time the medium containing Lipofectin was removed and the incubation continued for a further 40 hours using fresh medium containing 10% heat-treated calf serum containing 5µM oligonucleotide. After 40 hours the medium was removed and cells were washed and treated with DDM or FCS/insulin differentiation media as described in section 5.2.1). The differentiation medium, containing fresh oligodeoxynucleotide, was replenished at two day intervals. For further details refer to figure 5.1.

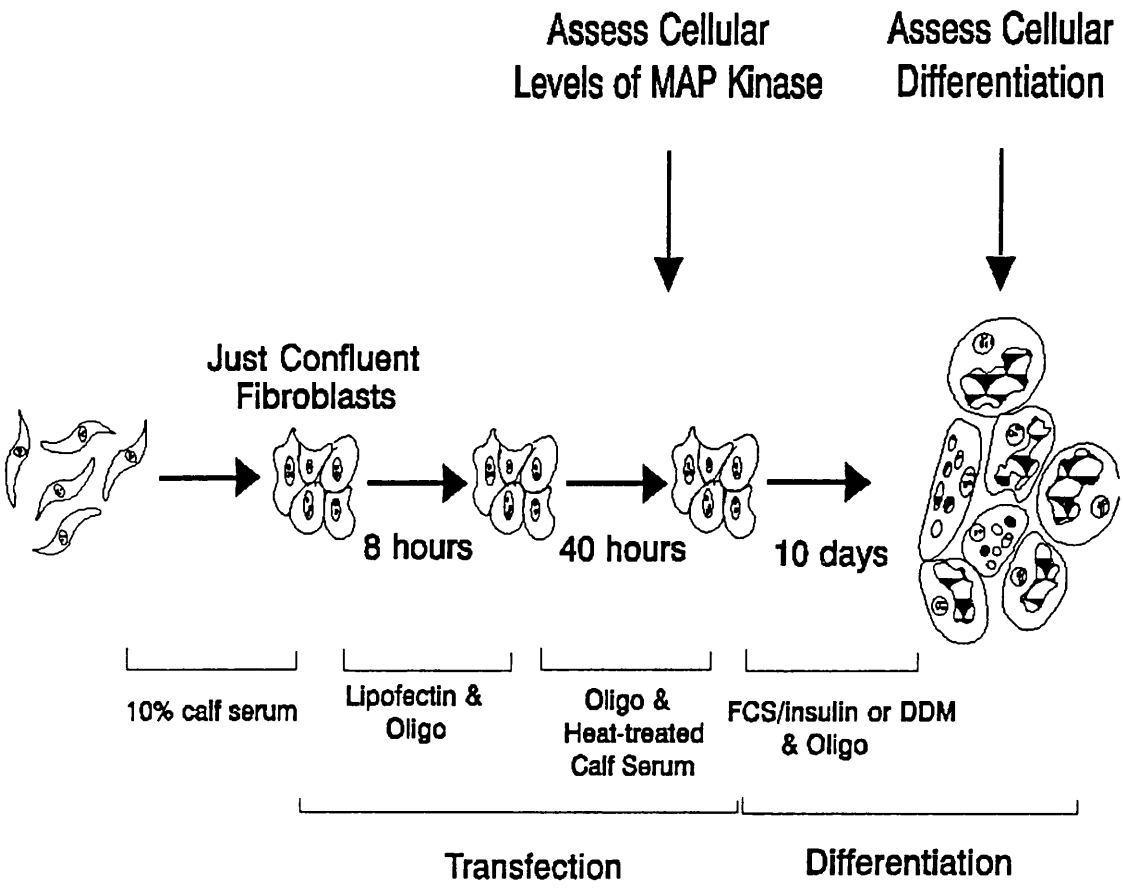


Fig 5.1) Protocol for Oligodeoxynucleotide Treatment and Subsequent Differentiation of 3T3-F442A Cells.
Confluent 3T3-F442A cells were treated for 8 hours with a combination of oligodeoxynucleotide (oligo) and the transfection reagent Lipofectin in DMEM. Following this the medium was replaced with medium containing fresh oligo and 10% heat inactivated calf serum. This second incubation was continued for 40 hours and then the levels of cellular MAP kinase were determined by immunoblotting as described in section 2.2.11. Parallel cultures were challenged with a defined differentiation medium (DDM) or medium containing foetal calf serum and insulin (FCS/insulin) and the extent of differentiation was assessed 10 days later. During the differentiation period the medium and oligo under test were replenished at two day intervals.

5.2.4) MAP Kinase Assay

Cells were scraped in Buffer A [25mM Tris.HCl pH 7.5, 25mM NaCl, 40mM p-nitrophenylphosphate, 10μM dithiothreitol, 10% (v/v) ethylene glycol, 1mM sodium orthovanadate, 100μM PMSF, leupeptin (2μg/ml), pepstatin A (2μg/ml) and aprotinin (2μg/ml)] and lysed by shearing through a $26^1/_2$G needle. The lysates were then centrifuged ($14000g_{max}$, 5min). MAP kinase was assayed following partial purification of cell lysates by batch absorption to phenyl-Sepharose, as described previously (Anderson *et al*, 1991). Briefly, cell extracts were mixed with 150μl phenyl-Sepharose for 5 min. The Sepharose was then washed, in a step-wise fashion, with buffers containing increasing concentrations of ethylene glycol. MAP kinases, which bind tightly to this matrix (Ray and Sturgill, 1991), were eluted with buffer containing 60% ethylene glycol and assayed using myelin basic protein as a substrate (Anderson, 1992).

5.2.5) Assay of Cyclic AMP-dependent Protein Kinase

PKA activity of cell lysates was assayed by measuring the phosphorylation of kemptide (0.26mM) at 30°C for 10 minutes in a buffer containing 75mM Tris, pH 7.5; 15mM $MgCl_2$; 4mM DTT; 100μM ATP; 3μCi/ml [γ-^{32}P]ATP in the presence or absence of PKI (5μM). PKA activity was calculated as the amount of kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the presence of PKI. For further details see section 2.2.13.3.

5.2.6) Immunoblotting of $p42^{MAPK}$/$p44^{MAPK}$

Cells were lysed in Buffer B (25mM Hepes pH7.5, 2.5mM EDTA, 0.2% NP-40, 50mM sodium chloride, 50mM sodium fluoride, 30mM sodium pyrophosphate, 10% glycerol, 1mM sodium orthovanadate, 400μM PMSF and 2μg/ml each of leupeptin, pepstatin A and aprotinin), clarified by centrifugation ($14000g_{max}$, 10min) and denatured by adding 0.25 volumes of 5X concentrated sample buffer. Equal quantities of lysate protein (50μg) were run on 10% polyacrylamide gels (30:0.32 acrylamide:bis-acrylamide) overnight with a constant current of 7mA. Following transfer to nitrocellulose (2 hours at 400mA), immunoblots were blocked with 3% BSA and probed with anti-MAP kinase antibodies (1:1000; see section 2.2.11) for 3 hours followed by horse radish peroxidase-conjugated anti IgG (1:10000) for 45 minutes. Immunoreactive bands were detected using the ECL (Amersham) system. For some experiments the activation status of MAP kinase was determined using this immunoblotting system. In common with many proteins, the phosphorylation and activation of MAP kinase is accompanied by a decrease in its electrophoretic mobility

on SDS-polyacrylamide gels (Leevers and Marshall, 1992). Activation of MAP kinases was characterised by the appearance of slower migrating forms on immunoblots.

5.2.7) GPDH and DNA Assays

GPDH activity was measured by the method of Wise and Green (1979) and activities were expressed relative to DNA content. The DNA content of cellular homogenates was determined fluorimetrically (Brunk *et al*, 1979). The GPDH enzyme activity per plate of cells was expressed as the number of moles of NADH oxidised/second (katals)/mgDNA.

5.3) RESULTS

In an attempt to understand the molecular basis for the effects of cyclic AMP on the differentiation of 3T3-F442A cells (Chapter 4) antisense depletion of cellular p42 and p44 MAP kinase was used to determine whether the MAP kinase pathway plays a functional role in modulating fat cell differentiation. The design of MAP kinase antisense oligodeoxynucleotides (antisense EAS1) and control oligodeoxynucleotides, and the development of the protocol for their usage, was first described by Sale *et al*, 1995. Antisense EAS1 was designed to complement a 17-base nucleotide sequence unique to p42 and p44 MAP kinases. In the mouse this corresponds to nucleotides 25-41 of the p42 MAP kinase cDNA (Her *et al*, 1991) and 1-17 of the p44 MAP kinase cDNA (Pagès *et al*, 1995). These sequences encompass the first 17-bases of the 5'-flanking regions of the MAP kinase genes and includes the ATG initiation codon (Fig 5.2). Control oligodeoxynucleotides were also synthesised in either a sense or random (scrambled) orientation.

Cultures of 3T3-F442A cells that had just attained confluence were treated for 48 hours with 5μM antisense phosphorothioate oligodeoxynucleotide EAS1 in the presence of 10% heat inactivated calf serum. The transfection agent Lipofectin was present during the first 8 hours of the incubation. Following treatment, the levels of cellular MAP kinase were determined using two different specific antibodies to the 42 and 44 kDa forms of MAP kinase (termed $p42^{MAPK}$ and $p44^{MAPK}$, respectively). Antisense EAS1 caused a dramatic depletion of cellular $p44^{MAPK}$ (approximately 90%) when compared to cells treated with the transfection agent alone (Fig 5.3A). Preliminary studies showed that Lipofectin treatment alone did not affect cellular levels of MAP kinases. The p42 isoform of MAP kinase exhibited exquisite sensitivity to depletion with antisense EAS1 as levels of the protein were undetectable even with long exposures of the immunoblot. Incubation of cells with 5μM oligodeoxynucleotide in a scrambled or sense orientation did not have a significant effect on the expression of $p42^{MAPK}$ or $p44^{MAPK}$ thereby indicating that the antisense depletion induced by EAS1 was specific. The effect of antisense EAS 1 on other major cellular proteins was assessed qualitatively by staining polyacrylamide gels with Coomassie blue. The general banding pattern of proteins extracted from cells treated with antisense EAS1 was very similar to that observed in cells treated with control oligodeoxynucleotides of with transfection agent alone. However, a small increase in the expression of a protein species of around 85kDa was consistently observed in EAS1-treated cells (Fig 5.3B).

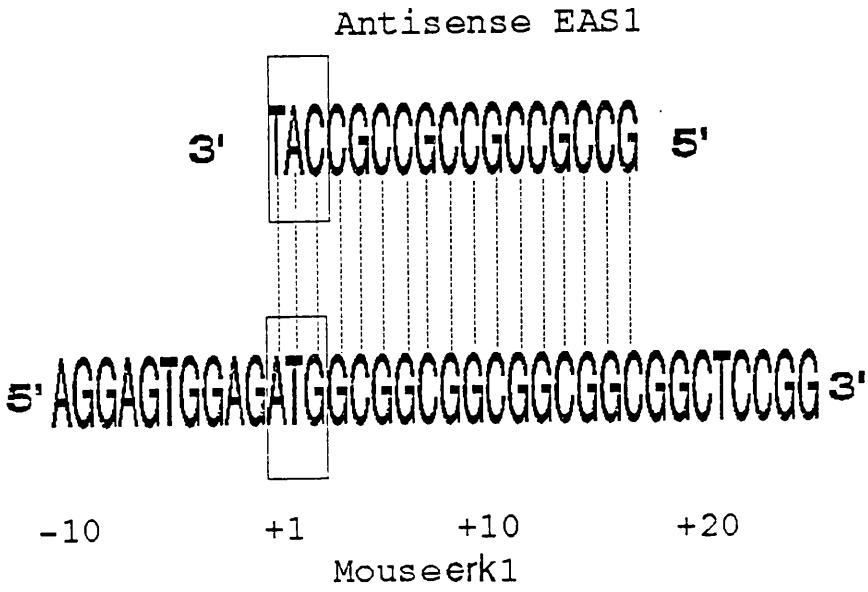


Fig 5.2) Design of Antisense EAS1 Phosphorothioate Oligodeoxynucleotides
Antisense EAS1 was designed to complement, in an antisense orientation, the first 17 bases of the mouse p44 and p42 MAP kinase genes (erks 1 and 2, respectively). This region is identical and unique to both erk genes and encompasses the ATG initiation codon (indicated as a boxed form in the diagram). Regions of complementary are indicated with broken lines.

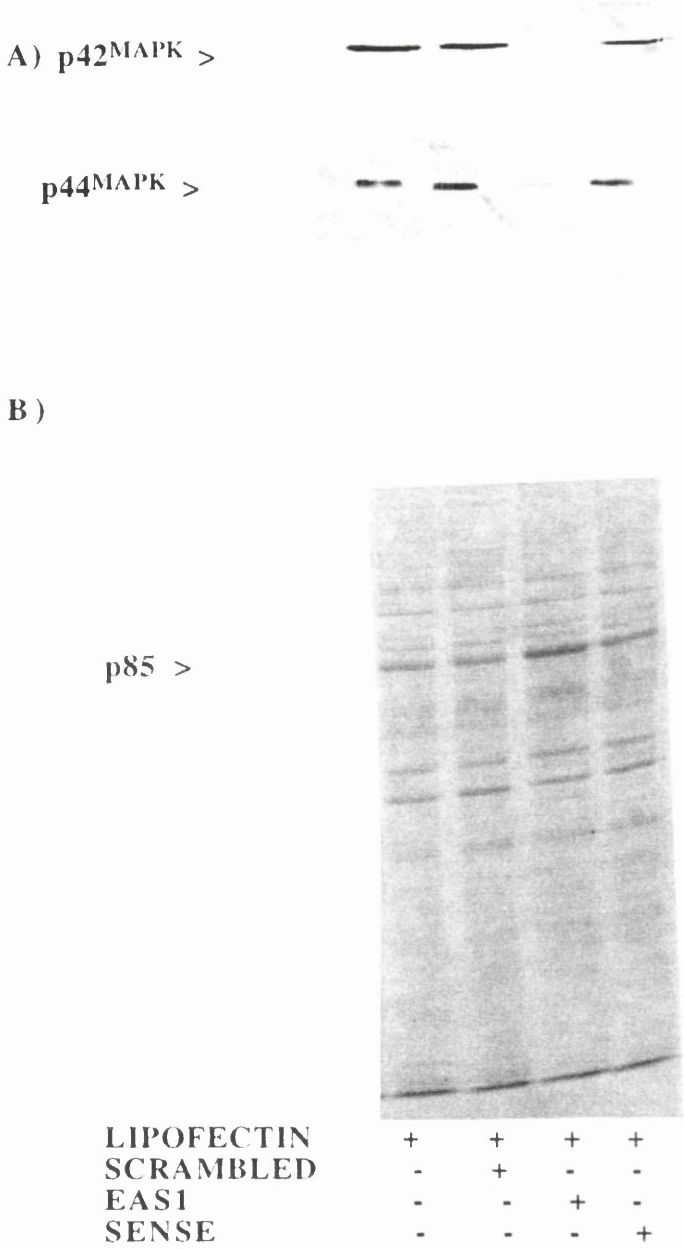


Fig 5.3) Effect of Antisense EAS1 on the Expression of Cellular Mitogen Activated Protein Kinases (MAP Kinases).
Just confluent cultures of 3T3-F442A cells were treated with antisense EAS1 (5μM), scrambled (5μM) or sense (5μM) oligodeoxynucleotides for 48 hours with Lipofectin present for the first 8 hours. The level of cellular MAP kinase was then assessed by immunoblotting equal quantities of protein (10μg) with specific antibodies to the 42 and 44 kDa MAP kinases ($p42^{MAPK}$ and $p44^{MAPK}$; Fig 5.3A) as described in section 5.2.5). Alternatively, samples of cell extract were subjected to SDS-PAGE and stained with Coomassie brilliant blue (Fig 5.3B). Results are representative of experiments carried out on at least three separate occasions.

Having determined that antisense EAS1 selectively and thoroughly depletes the expression of $p42^{MAPK}$ and $p44^{MAPK}$ proteins the role of these proteins in transducing differentiative signals by FCS/insulin was evaluated. Approximately 90% of the cells treated with transfection agent alone underwent adipose conversion under the influence of FCS/insulin as estimated by Oil red O staining (Fig 5.4). The GPDH activity in these cells was determined to be 7.6±0.3 (n=5) nanokatals/mgDNA. Incubation of cells with 5μM antisense EAS1 caused a dramatic reduction in the extent of differentiation as judged by lipid accumulation (Fig 5.4) and a corresponding reduction in GPDH activity (approximately 95% reduction; Table 5.1). Incubation with 5μM sense or scrambled oligodeoxynucleotides did not significantly effect lipid accumulation or GPDH activity of differentiated cells (Fig 5.4; Table 5.1). Therefore, antisense oligodeoxynucleotides to MAP kinase act specifically to inhibit the transmission of differentiative stimuli induced by FCS/insulin in 3T3-F442A cells.

To further confirm that the MAP kinase proteins are involved in the transduction of signals by adipogenic factors the effect of antisense EAS1 on the differentiation of 3T3-F442A cells under serum-free conditions was investigated. Cells were pretreated with oligodeoxynucleotides for 48 hours as described (Fig 5.2), following which differentiation was induced with the hormonally defined differentiation medium (DDM) in the continued presence of oligodeoxynucleotides. Under these conditions the GPDH activity of differentiated cultures, which were treated with Lipofectin in the absence of oligodeoxynucleotide, rose to 3.0±0.4 nanokatals/mgDNA (n=3). As observed for the FCS/insulin differentiation protocol antisense EAS1 markedly inhibited differentiation induced by the DDM producing a qualitatively similar reduction in expressed GPDH activity (approximately 99%; Table 5.1) whereas scrambled and sense oligodeoxynucleotides were without significant effect (Table 5.1). Together these observations demonstrate that p42 and p44 MAP kinases are required for the adipose conversion of 3T3-F442A preadipocytes.

Having shown an absolute requirement for MAP kinases in the differentiation process further studies were undertaken to define the role of these kinases in the modulation of differentiation by cyclic AMP. The results from Chapter 3 demonstrate that the differentiation of 3T3-F442A cells can be divided into two stages, the first phase requires GH and the later, maturation stage requires insulin, EGF and T_3. It was demonstrated in Chapter 4 that cyclic AMP acts synergistically with GH to promote priming of 3T3-F442A cells to differentiate. In contrast, during the maturation phase cyclic AMP is inhibitory. As previously reported (Anderson, 1992) GH activates MAP kinases approximately 5 fold in these cells (Table 5.2). It is well established in the literature that insulin and EGF, *via* their tyrosine kinase receptors, activate MAP

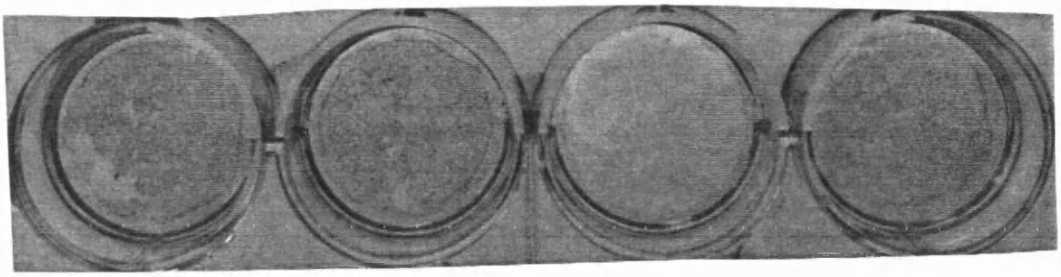


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Fig 5.4) Effect of Antisense Depletion of Cellular MAP Kinase on the Differentiation of 3T3-F442A Cells with Foetal Calf Serum and Insulin (FCS/insulin) or Defined Differentiation Medium (DDM).
Cells were grown to confluence and treated with antisense EAS1, sense or scrambled oligodeoxynucleotides as described in the legend to Fig 5.3). Alternatively, control cultures were treated with Lipofectin but no oligodeoxynucleotides. Cells were then transferred to FCS/insulin in the continued presence of oligonucleotides as described in section 5.2.3. After 10 days cells were stained with Oil red O (see section 2.2.5).

Table 5.1) Effect of Antisense Depletion of Cellular MAP Kinase on the Differentiation of 3T3-F442A Cells with Foetal Calf Serum and Insulin (FCS/insulin) or Defined Differentiation Medium (DDM)
Cells were grown to confluence and treated with antisense EAS1, sense or scrambled oligodeoxynucleotides as described in section 5.2. Alternatively, control cultures were treated with Lipofectin but no oligodeoxynucleotides. Cells were then transferred to FCS/insulin or DDM in the continued presence oligodeoxynucleotides (section 5.2). After 10 days cells were harvested and processed for the assay of GPDH activity. The GPDH activities when cultures were exposed to FCS/insulin or DDM medium alone were 7.6±0.3 (n=5) and 3.0±0.4 (n=3) nanokatals/mgDNA respectively. All values are expressed as a percentage of these activities. Results are means±S.E.M. for 3 (DDM) or 5 (FCS/insulin) observations. ** indicates the values are significantly different from those for cells differentiated in the absence of oligodeoxynucleotides; $p<0.001$.

	GPDH Activity (Percentage of Control)	
Treatment	**FCS/insulin**	**DDM**
5μM Scrambled	97.63±3.55% (n=5)	108.33±12.0% (n=3)
5μM Antisense	4.74±0.92% ** (n=5)	0.05±0.06% ** (n=3)
5μM Sense	98.55±2.76% (n=5)	105±11.3% (n=3)

Table 5.2) Effect of GH, Insulin, EGF and T_3 on the Activation of MAP kinase in 3T3-F442A Cells.

Serum starved cells were incubated for 10 minutes with GH (2nM), insulin (1.8μM), T3 (100pg/ml) or EGF (1ng/ml). Following stimulation cells were lysed and cytosolic extracts were processed for the assay of MAP kinase as described in section 2.2.13.1. Data are expressed as the relative increase (fold) in MAP kinase activity induced by each agent above that measured in unstimulated cells and are means±S.E.M. with the number of observations in parenthesis. ** indicates the values which differ significantly from values obtained when cells were incubated in the absence of agents; $p<0.001$.

Treatment	Fold Increase in MAP kinase Activity
-	1.0±0.1 (n=5)
GH	5.4±0.5 ** (n=4)
Insulin	5.0±0.9 ** (n=4)
EGF	21.2±2.5 ** (n=3)
T3	1.0±0.1 (n=5)

kinases (reviewed by Fantl *et al*, 1993). In 3T3-F442A preadipocytes there is a small activation by insulin (Table 5.2) and, consistent with the literature (Anderson, 1992), a potent activation by EGF (Table 5.2).

Recently a number of studies have demonstrated that the effect of cyclic AMP on MAP kinase activation varies, depending on the cell system studied (Angler *et al*, 1996; Graves and Lawrence, 1996; McKenzie and Pousségur, 1996; Saswati *et al*, 1996). Therefore, in 3T3-F442A cells it was necessary to establish the effect of cyclic AMP alone and in combination with other agents to examine if it correlated with the effects on differentiation. Initially, the diterpene forskolin was used to elevate intracellular cyclic AMP and MAP kinase activation status was determined by immunoblotting cell extracts with an antibody which specifically recognises $p42^{MAPK}$ and $p44^{MAPK}$. In common with a number of proteins which are activated by phosphorylation, activation of MAP kinase results in a retardation of its mobility in SDS-PAGE gels (Leevers and Marshall, 1992). The appearance of the more slowly migrating forms of phospho-MAP kinase is taken to represent activation of the enzyme. Fig 5.6 shows that after 5 minutes stimulation 50μM forskolin, which caused a maximal 90±9 (n=5) fold elevation of intracellular cyclic AMP levels (Fig 4.7A), induced an activation of $p42^{MAPK}$ (Fig 5.5A), as assessed by mobility shift. However, the degree to which MAP kinase is activated by forskolin appears to be relatively small. Anderson (1992) reported that the level to which MAP kinase can be activated by GH is sensitive to the incubation period of cells in serum-free medium. To test whether this is the case with forskolin, cells were incubated in serum-free medium for various times and the ability of forskolin to activate MAP kinase was determined. Little or no stimulation of $p42^{MAPK}$ phosphorylation was observed in cells serum-starved for only 5 hours (Fig 5.6A). Optimal responses were observed when cells were serum-starved between 18 and 48 hours (Fig 5.6A). Assessment of $p44^{MAPK}$ activation by forskolin after an overnight (18 hours) incubation in serum-free medium revealed a relative degree of activation similar to $p42^{MAPK}$ (Fig 5.6B). The resolution of the hyper-phosphorylated form of $p44^{MAPK}$ was not as distinct as with $p42^{MAPK}$, therefore further studies were carried out on the p42 isoform on cells which had been subjected to at least 18 hours serum deprivation. The activation of $p42^{MAPK}$ with forskolin was found to be transient, with the maximal effect occurring at around 5 minutes after the addition of forskolin (Fig 5.6). The transient nature of MAP kinase activation by forskolin is very similar to that observed with other factors which are adipogenic in 3T3-F442A cells (Anderson, 1992).

Although forskolin elevates cyclic AMP levels by direct interaction with adenylate cyclase a number of non-specific effects of this drug have been reported (Laurenza *et*

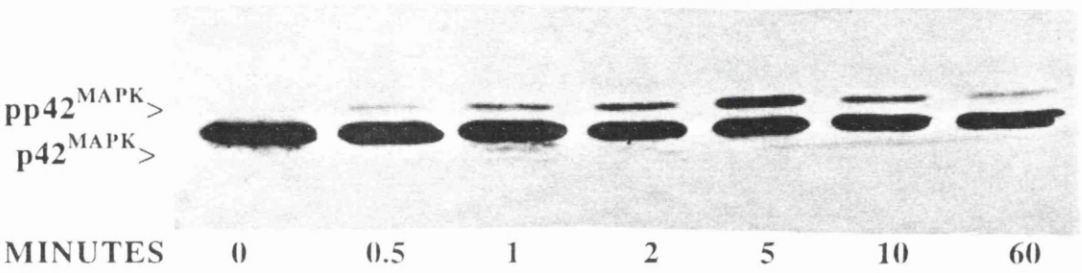

Fig 5.5) Time-course of MAP Kinase Activation by 50μM Forskolin in 3T3-F442A Cells.

3T3-F442A cells were serum starved for 18 hours then incubated with 50μM forskolin for the indicated times. Cells were then lysed and equal quantities of protein (50μg) were immunoblotted with a specific monoclonal antibody to $p42^{MAPK}$ as described in section 2.2.11. The non-phosphorylated (lower band) and phosphorylated (upper band) forms of the enzyme are indicated by arrows. Results are representative of an experiment carried out on two separate occasions.

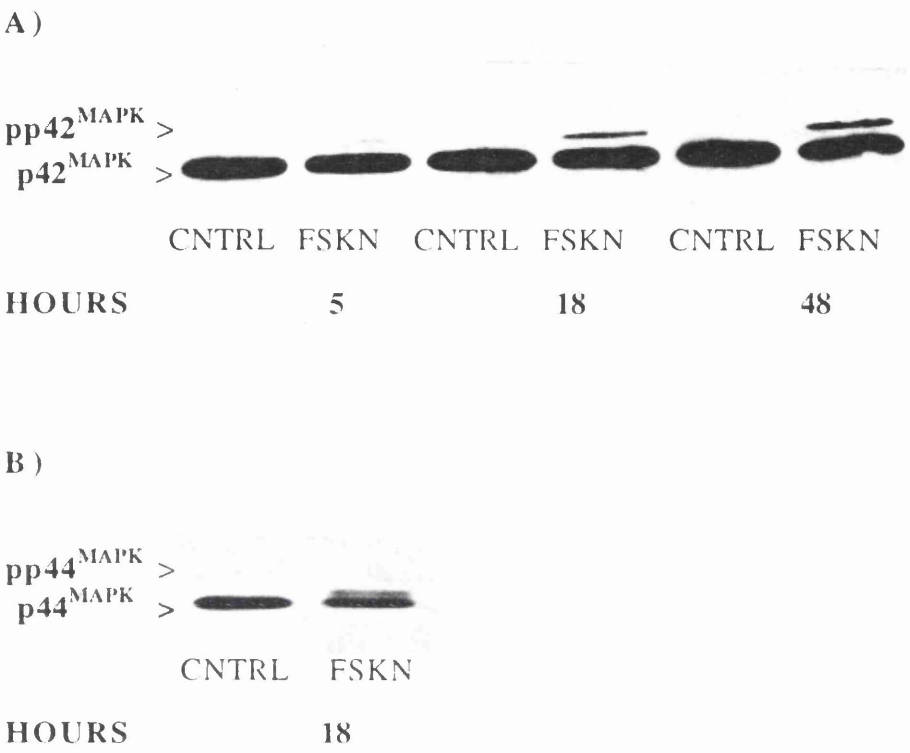

Fig 5.6) Comparison of Short- and Long-term Serum Deprivation on Subsequent Stimulation of MAP Kinase by 50μM forskolin.
Cells were incubated in the absence of serum for the indicated times prior to the addition of 50μM forskolin (FSKN) or diluent (CNTRL) for 5 minutes. Cytosolic extracts were then prepared and 50μg of protein immunoblotted with specific antisera to A) $p42^{MAPK}$ or B) $p44^{MAPK}$ as described in section 2.2.11. The non-phosphorylated (lower band) and phosphorylated (upper band) forms of the enzyme are indicated by arrows.

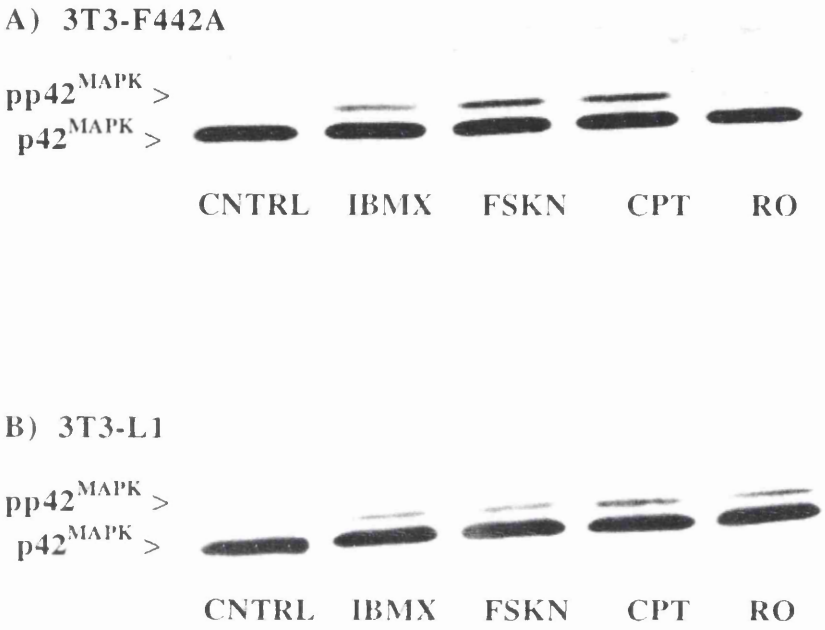

Fig 5.7) Effect of Agents which Elevate Intracellular cyclic AMP on the Activation of MAP Kinase in 3T3-F442A Cells and 3T3-L1 Cells.
Serum starved 3T3-F442A cells (A) or 3T3-L1 cells (B) were incubated for 5 minutes in the presence of diluent (CNTRL), 500μM IBMX, 50μM forskolin (FSKN), 1mM CPT-cyclic AMP (CPT) or 10μM RO-201724 (RO). Activation of $p42^{MAPK}$ was detected by immunoblotting as described in the legend to Fig 5.3).

al, 1989). To confirm that the effects of forskolin on MAP kinase were mediated *via* cyclic AMP a range of agents were tested which elevate intracellular cyclic AMP by different mechanisms. The cell permeable analogue CPT-cAMP and the cyclic AMP phosphodiesterase inhibitors IBMX and Ro-201724 each induced the shift in $p42^{MAPK}$ mobility indicative of MAP kinase activation (Fig 5.7). Further experiments were carried out to examine whether cyclic AMP is a positive effector of MAP kinase activation in the related 3T3-L1 preadipocyte cell line. All agents tested were found to provoke a comparable degree of activation of $p42^{MAPK}$ in 3T3-L1 cells to that induced in 3T3-F442A cells (Fig 5.7). The relative potencies of agents at elevating MAP kinase activity were as follows:

3T3-F442A: CPT-cAMP>Forskolin>IBMX>Ro-201724
3T3-L1: CPT-cAMP>Ro201724>Forskolin=IBMX

Thus, it appears that the phosphodiesterase inhibitor Ro-201724 is comparatively more effective at activating MAP kinase in 3T3-L1 cells than in 3T3-F442A cells.

Full enzymatic activation of MAP kinases requires dual phosphorylation on Thr-183 and Thr-185 (Anderson *et al*, 1990). To confirm that the observed increases in MAP kinase phosphorylation induced by cyclic AMP reflected an increase in MAP kinase enzyme activity, the phosphotransferase activity was measured in phenyl Sepharose-purified cell extracts using MBP, a selective substrate of MAP kinase. MAP kinases bind tightly to phenyl Sepharose and represent the major kinase activity eluted from this matrix with 60% ethylene glycol (Ray and Sturgill, 1987). Table 5.3) shows that after 5 minutes stimulation IBMX, forskolin, CPT-cAMP and Ro-201724 all increased cellular MAP kinase activity in both 3T3-F442A and 3T3-L1 preadipocytes, above the level observed in unstimulated cells. The relative degree of activation induced by each agent was comparable in each cell type and correlated well with the relative amount of phospho-MAP kinase detected by gel shift (Fig 5.7). Inclusion of PKI in the assay at a concentration (5μM) which completely inhibits forskolin-stimulated PKA activity measured in cell lysates, did not cause a significant reduction in forskolin-stimulated MBP-kinase activity (Table 5.3), indicating that PKA is not responsible for the phosphorylation of MBP observed in response to elevation of intracellular cyclic AMP levels.

Although the vast majority of cellular responses to cyclic AMP are mediated *via* activation of PKA, there are a few notable exceptions (Piper *et al*, 1993; DiFrancesco and Tortora, 1991). To determine whether a relationship exists between the activation of PKA and MAP kinase by cyclic AMP in 3T3-F442A cells, PKA and MAP kinase

Table 5.3) Effect of Agents which Elevate Intracellular cyclic AMP on MAP Kinase Activity in 3T3-F442A Cells and 3T3-L1 Cells.
Serum starved 3T3-F442A cells and 3T3-L1 cells were incubated for 5 minutes with various cyclic AMP-elevating agents as indicated. Following incubation, cells were lysed and cytosolic extracts were processed for the assay of MAP kinase as described in section 2.2.13.1. For some experiments a specific inhibitor of PKA (indicated PKI) was included during the assay of MAP kinase. Data are expressed as the relative increase (fold) in MAP kinase activity induced by each agent above that measured in unstimulated cells and are means±S.E.M. with the number of observations in parenthesis. * indicates the values which differ significantly from control values; $p<0.01$.

Fold increase in MAP kinase Activity

Treatment	3T3-F442A	3T3-L1
500μM IBMX	1.40±0.15 (n=3)*	1.40±0.02 (n=3)*
50μM Forskolin	1.94±0.09 (n=3)*	1.40±0.05 (n=3)*
50μM Forskolin+5μM PKI	1.87±0.05 (n=3)*	-
1mM CPT-cAMP	2.36±0.11 (n=3)*	2.80±0.23 (n=3)*
10μM RO-201724	1.16±0.06 (n=3)*	1.91±0.07 (n=3)*

activities were measured following stimulation of cells with a range of forskolin concentrations. Results demonstrated broadly overlapping dose-dependent activations of both PKA and MAP kinase (Fig 5.8). Half maximal activation (EC_{50}) of both PKA and MAP kinase was observed at around 50μM and maximal stimulation of both kinases occurred at around 100μM forskolin. However, PKA appeared to be more sensitive than MAP kinase to forskolin; the lowest dose of forskolin observed to elevate PKA activity significantly was 1μM, whereas the lowest dose of forskolin observed to activate MAP kinase significantly was 10μM.

Further evidence that the effects of forskolin on MAP kinase were mediated via the activation of PKA was obtained by the use of two chemical inhibitors. H89 is a protein kinase inhibitor which shows a high degree of selectivity for PKA over other protein kinases (Chijiwa, 1990). (*R*p)-cAMPS inhibits the activation of PKA by binding to the regulatory subunit and preventing dissociation of the catalytic subunit (Dostmann, 1995; Gjertsen *et al*, 1995). Fig 5.9 shows that preincubation with both H89 and (*R*p)-cAMPS blocked the retardation in $p42^{MAPK}$ mobility induced by forskolin. Together, the results presented in Figs 5.8 and 5.9 strongly suggest that the activation of MAP kinase by cyclic AMP is mediated by PKA.

The only known mechanism for the activation MAP kinase is *via* direct phosphorylation on the regulatory threonine and tyrosine residues by the dual-specificity kinase MEK. To test whether cyclic AMP induced activation of MEK in 3T3-F442A cells, the kinase activity of MEK was measured following treatment of cells with forskolin. A catalytically inactive recombinant version of p42 MAP kinase (K52R), in which Lys 52 is mutated to Arg, was used as a specific substrate for MEK. Fig 5.10A) shows that treatment of cells with forskolin for 5 min resulted in MEK activation. After 10 min of forskolin treatment the activity of MEK had returned to basal levels. Thus the activation of MEK by forskolin is temporally compatible with the activation of MAP kinase (Fig 5.6) suggesting that MEK is responsible for the activation of MAP kinase by forskolin. To test this further a recently discovered specific inhibitor of MEK activation, PD098059 (Alessi *et al*, 1995), was employed. Preincubation of cells with PD098059 completely blocked the activation of MAP kinase by forskolin (Fig 5.10B). The inhibitor also blocked activation of MAP kinase by GH. In accordance with others (Alessi *et al*, 1995), the activation of MAP kinase by EGF was largely unaffected by PD098059. These results confirm that the activation of MAP kinase by cyclic AMP requires the activation of MEK. Moreover, since PD098059 is known to preferentially inhibit the activation of the MEK isoform, MEK1 (Alessi *et al*, 1995) these results suggest that the activation of MAP kinase by cyclic AMP in 3T3-F442A preadipocytes involves prior activation of MEK1.

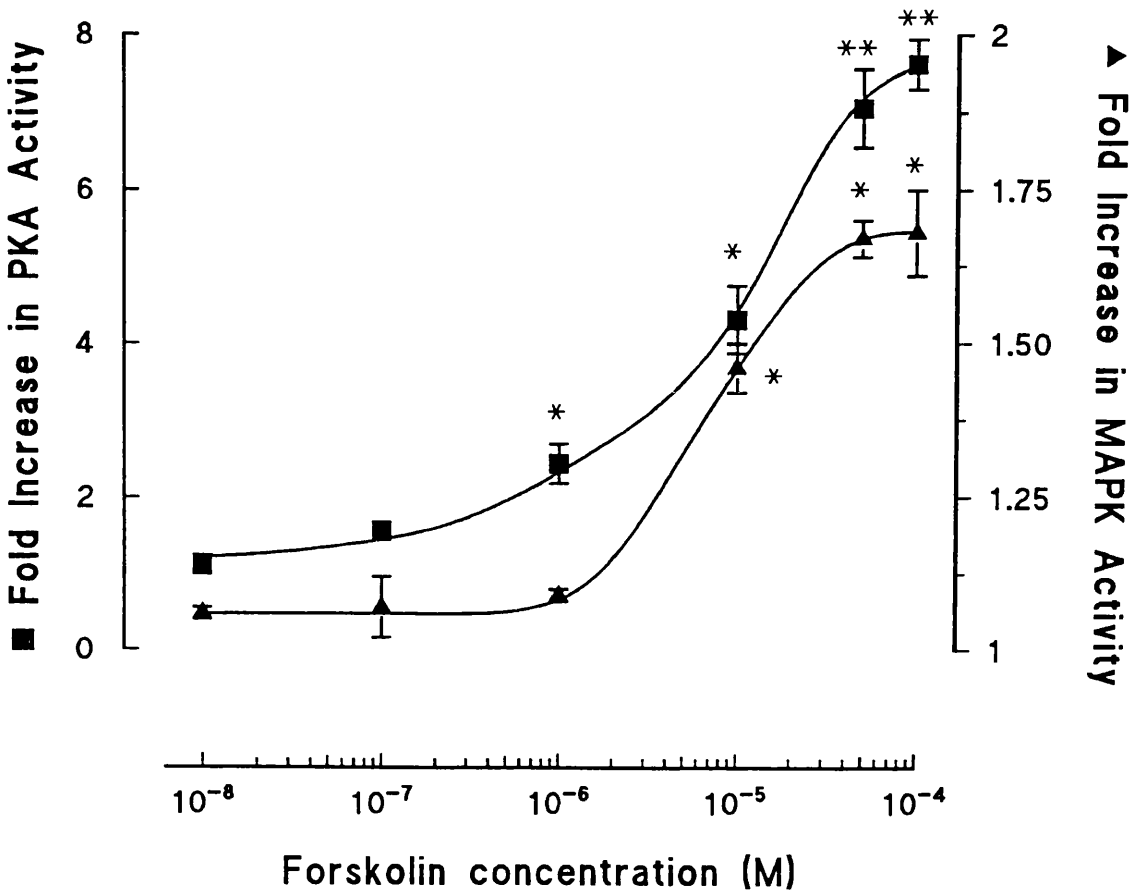

Fig 5.8) Dose-dependent Stimulation of MAP Kinase and Protein Kinase A (PKA) by Forskolin.
3T3-F442A cells were serum starved and then incubated with the indicated concentrations of forskolin for 5 min. Cell extracts were prepared and assayed for MAP kinase and PKA activities as described in section 2.2.13.3. Data are expressed as the fold increase in enzyme activity relative to unstimulated cells and are expressed as mean±S.E.M. for triplicate samples. *, ** indicate the value differs significantly from control cells; $p<0.01$, $p<0.001$.

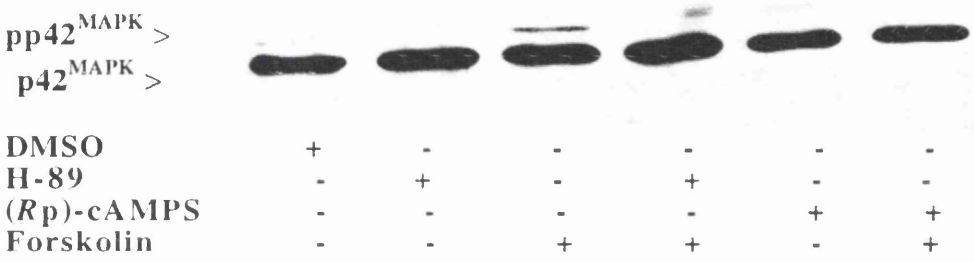

Fig 5.9) Effect of H-89 and (*R*p)-cAMPS on the Activation MAP kinase by Cyclic AMP.
Serum starved cells were preincubated for 10 minutes with either H89 (30μM) or (*R*p)-cAMPS (1mM) and then stimulated for 5 min with 50μM forskolin or diluent (DMSO). Cell extracts were prepared and the activation of $p42^{MAPK}$ was assessed by electrophoretic mobility shift as described in the legend to Fig 5.3.

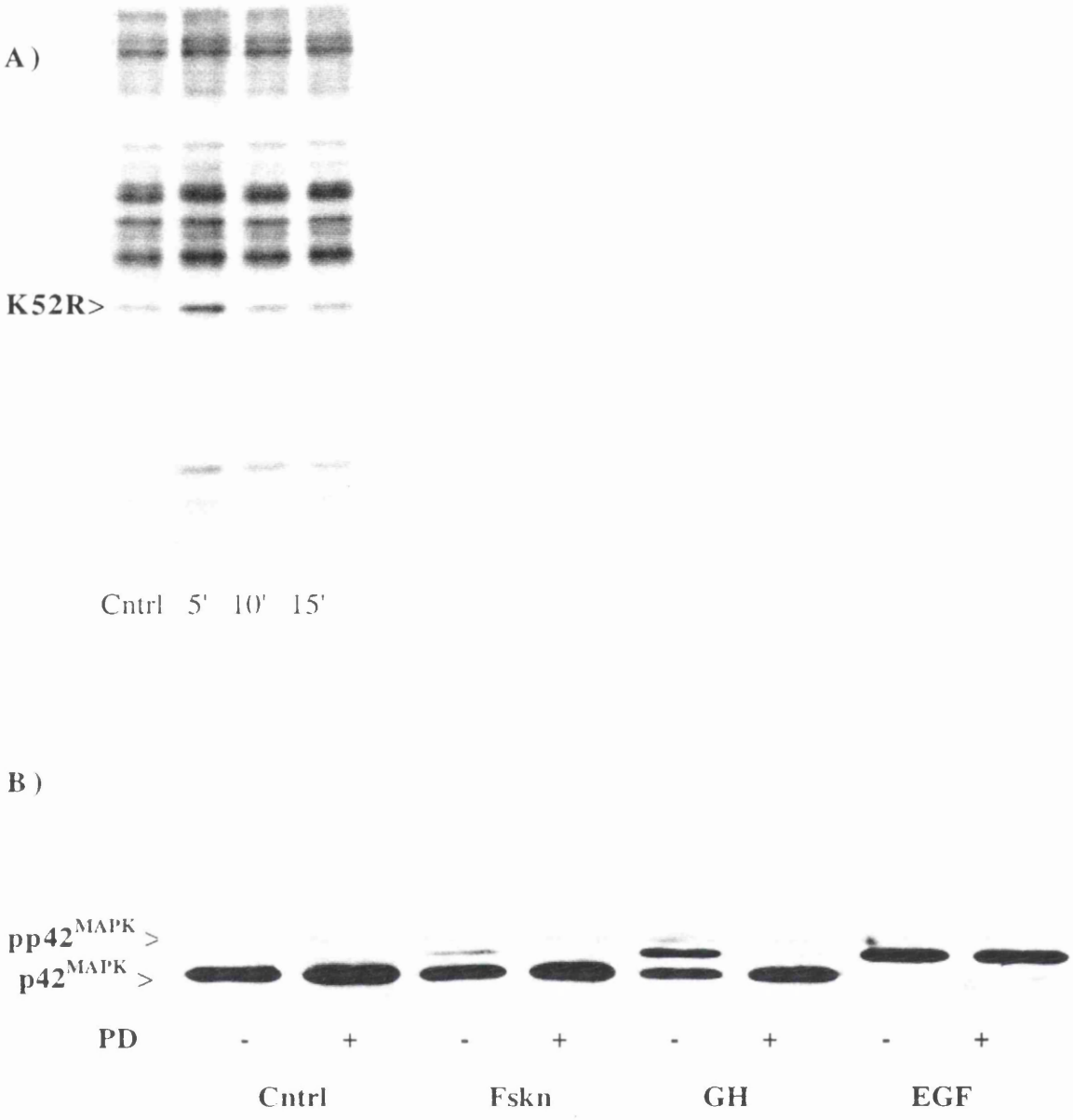

Fig 5.10) Cyclic AMP Induces MEK Activation in 3T3-F442A cells. Serum starved cells were stimulated with 50μM forskolin for the indicated times. Cell extracts were then prepared and assayed for MEK activity as described in section 2.2.13.2. The position of the phosphorylated recombinant catalytically inactive $p42^{MAPK}$ used as the substrate is indicated and the radioactivity (cpm) incorporated was 183 (control), 305 (5 min), 202 (10 min) and 218 (15 min). Serum starved cells were incubated with 50μM PD098059 (PD) for 90 minutes followed by stimulation with 50μM forskolin (5 min), 10nM EGF (5 min) or 10nM GH (10 min). Activation of $p42^{MAPK}$ was determined by electrophoretic mobility shift as described in the legend to Fig 5.3.

Work in our laboratory has shown that PKC mediates the activation of MAP kinases by GH, but not by EGF in 3T3-F442A cells (MacKenzie *et al*, 1997). Given that interactions can occur between the cyclic AMP and PKC signalling systems it may be interesting to assess the role of PKC in the activation of MAP kinases by cyclic AMP. To investigate whether cyclic AMP might feed into the MAP kinase cascade by activating PKC the effect of overnight incubation of 3T3-F442A cells with the phorbol ester 12-myristate 13-acetate (PMA) was examined. Pretreatment with PMA abolished the ability of PMA to induce an activation-dependent gel shift in $p42^{MAPK}$ confirming the efficiency of PKC down-regulation (Fig 5.11). In contrast, down-regulation of PKC did not inhibit the ability of forskolin to induce phosphorylation of $p42^{MAPK}$ (Fig 5.11). These findings suggest that cyclic AMP activates MAP kinase independently of stimulation of a PMA-sensitive PKC in 3T3-F442A cells.

Results so far have demonstrated that cyclic AMP evokes a PKA-mediated activation of MAP kinase in 3T3-F442A preadipocytes. The activation is transient and much smaller than those elicited by other differentiative agents (Table 5.2). Therefore, it seems unlikely that such a small and transient activation of MAP kinase could alone account for the potentiating action of cyclic AMP during the priming stage of 3T3-F442A preadipocyte differentiation (Chapter 4). Given that many of the biological effects of cyclic AMP on cell growth correlate with modulation of growth or differentiative factor-induced MAP kinase activity (Table 5.2), it was necessary to study the interactive effect of cyclic AMP with other differentiative hormones.

As previously shown in Table 5.2, 50μM forskolin induced a small but significant increase in MAP kinase activity in phenyl Sepharose-purified cell extracts. GH evoked a more substantial activation of MAP kinase (Table 5.3). Preincubation with forskolin was found to potentiate the GH-activated MAP kinase (Table 5.4). T_3 did not stimulate a significant activation of MAP kinase, which is consistent with the current understanding that the signalling mechanism for T_3 is independent of intracellular phosphorylation events (see the *Signalling* section in Chapter 1). Forskolin had very little effect on the activation of MAP kinase by insulin, EGF or T_3 in isolation or the three agents in combination. Individually insulin and EGF evoked a significant activation of MAP kinase (Table 5.4). When cells were stimulated with a combination of insulin, EGF and T_3 this also elicited an activation of MAP kinase which was comparable to that when cells were stimulated with EGF alone (Table 5.4). This observation is in accordance with the data presented in Fig 5.10 in which EGF alone could induce phosphorylation of the total cellular pool of MAP kinase. Interestingly, although cyclic AMP does not inhibit the activation of MAP kinases by EGF or insulin, it does inhibit their activation by PMA (29.9±2.6% reduction; $p<0.01$; $n=3$).

Table 5.4) Effect of Cyclic AMP on the Ability of Insulin, EGF and T_3 to Activate MAP kinase in 3T3-F442A Cells.
Serum starved cells were incubated for 10 minutes with forskolin (50μM) and then stimulated with either GH (2nM), insulin (1.8μM), T_3 (100pg/ml) or EGF (1ng/ml) for 10 minutes. Alternatively, cells were incubated for 10 minutes with a combination of insulin (1.8μM),T_3 (100pg/ml) and EGF (1ng/ml). Following stimulation cells were lysed and cytosolic extracts were processed for the assay of MAP kinase as described in section 2.2.13.1. Data are expressed as the relative increase (fold) in MAP kinase activity induced by each agent above that measured in unstimulated cells and are means±S.E.M. with the number of observations in parenthesis. * indicates the values which differ significantly from values obtained when cells were incubated in the absence of forskolin; $p<0.01$.

Fold increase in MAP kinase Activity

Treatment	-Forskolin	+Forskolin
-	1.0±0.1 (5)	1.9±0.1*(3)
GH	5.4±0.5 (4)	9.6±0.8*(3)
Insulin	5.0±0.9 (4)	7.4±1.4 (5)
EGF	21.2±2.5 (3)	23.3±3.5(3)
T_3	1.0±0.1 (5)	1.6±0.4 (3)
EGF/Insulin/T_3	20.9±1.9 (3)	20.7± 1.3 (3)

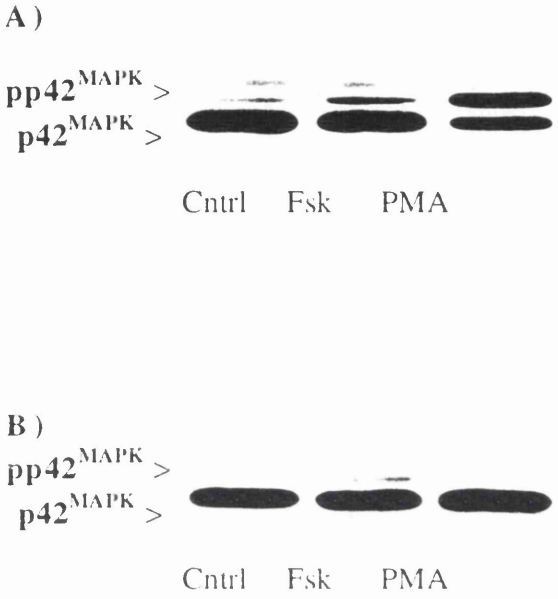

Fig 5.11) Effect of Down-regulation of PKC on the Activation of MAP Kinase by Cyclic AMP.
Cells were depleted of cellular PKC by pretreatment with 16μM PMA for 16 hours (Anderson *et al*, 1991). One hour before stimulations the PMA was removed by two washes with PBS. Cells were then exposed to 50μM forskolin for 5 minutes or 10nM PMA for 10 minutes. Activation of $p42^{MAPK}$ was determined by electrophoretic mobility shift as described in the legend to Fig 5.1). Panel A) are representative of cells which have been incubated with diluent (DMSO) alone for 16 hours, whereas panel B) represent cells which have been subjected to chronic PMA treatment.

As yet the significance of this is unknown. These data demonstrate that the interactive effects of cyclic AMP on MAP kinase activation do not account for its effects on the maturation stage of 3T3-F442A cellular differentiation. However, cyclic AMP does potentiate GH-activated MAP kinase and this does correlate with a potentiating effect of cyclic AMP during the priming stage of 3T3-F442A cellular differentiation (Chapter 4). To investigate this further it was necessary to determine at which stage of differentiation MAP kinases are required. These crucial experiments were not carried out earlier due to the unavailability of an agent which could act specifically on the priming stage of differentiation to inhibit MAP kinase activation. However, at a later stage a specific inhibitor of MAP kinase activation, PD098059 (Alessi *et al*, 1995), became available and this was then employed.

The difficulty of restricting antisense depletion of MAP kinase specifically to the priming phase precludes the use of antisense EAS1 during this stage of differentiation. The MEK-inhibitor PD098059 can act specifically to inhibit GH-activated MAP kinase during priming and will not exert an effect in the maturation phase as this compound is unable to inhibit the degree of MAP kinase-activation induced by the combined effect of agents in the maturation medium (Figs 5.10; Alessi *et al* 1995). To validate this study a control experiment was carried out to confirm the continuing efficiency of the PD098059 inhibitor during the 48 hour incubation period of GH priming. Incubation of cells in the presence of PD098059 during the two-day priming period completely blocked the acute activation of MAP kinase by GH and by GH and 10nM forskolin (results not shown). This demonstrates that PD098059 is still effective after a 48 hour period and will therefore be continually effective in inhibiting the activation of MAP kinase by GH and cyclic AMP during the whole priming phase. Inclusion of PD098059 in the priming medium failed to inhibit the ability of GH to prime cells and, moreover, failed to inhibit potentiation of GH-priming by 10nM forskolin (Fig 5.12).

These results suggest that although MAP kinases are an essential requirement for differentiation (Table 5.1), they appear not to be essential for the transduction of a priming signal by GH. In addition, the site of synergy between cyclic AMP and GH which underlies the potentiation of 3T3-F442A differentiation is not at the level of MAP kinase. Given that they are not involved during the early stage of the process, MAP kinase must be required for the positive coupling of maturation signals initiated by insulin/EGF and T_3.

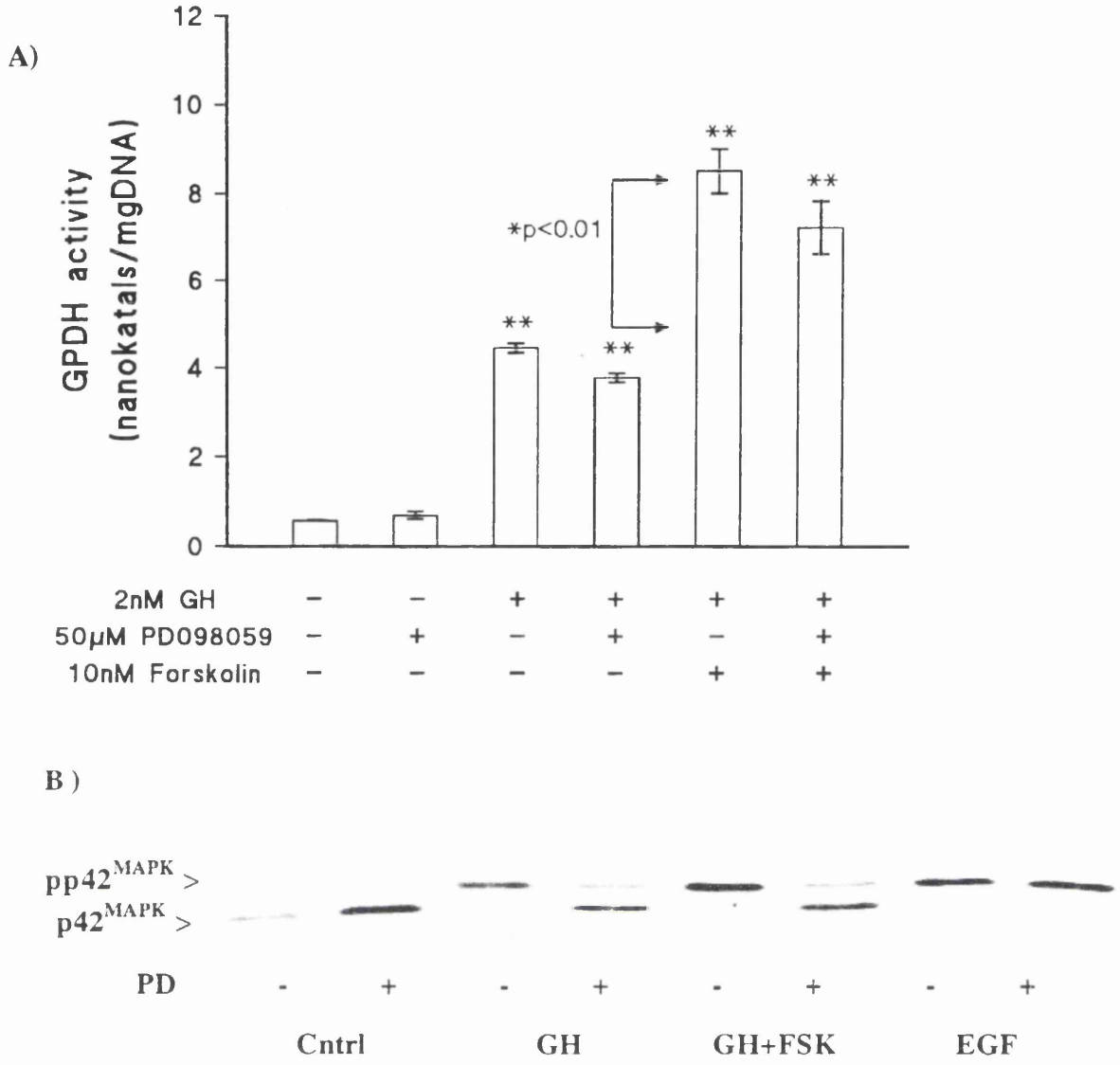

Fig 5.12) Effect of PD098059 on the Priming of 3T3-F442A Cells to Differentiate.

A) Cells were grown to confluence in GH-depleted calf serum as described in section 2.2.4. At confluence the medium was removed and cells were then primed for 2 days in serum-free medium along with the additions indicated. This was then replaced with maturation medium and 8 days later GPDH activity was measured. Results are means±S.E.M. for 3 observations. *, ** indicates the value is significantly different from that obtained in cells primed with no additions; $p<0.01$, $p<0.001$. The potentiation of GH-evoked GPDH activity by 10nM forskolin was also significant ($^{*}p<0.01$).

B) Cells were incubated in serum-free medium either with or without PD098059 as indicated. After 48 hours cells were acutely stimulated with 2nM GH (10min), 10nM EGF (10min) or a combination of 2nM GH and 10nM forskolin (10min). Activation of $p42^{MAPK}$ was then determined by electrophoretic mobility shift as described in the legend to Fig 5.3.

5.4) DISCUSSION

The aim of the initial part of this study was to determine to what extent 3T3-F442A adipocyte differentiation was dependent on MAP kinases. To this end, expression of cellular $p42^{MAPK}$ and $p44^{MAPK}$ was specifically suppressed by treatment of cells for 48 hours with a phosphorothioate oligodeoxynucleotide probe, antisense EAS1. This antisense probe was originally designed for use in 3T3-L1 cells where it was found to efficiently and effectively reduce expression of $p44^{MAPK}$ and, to a lesser degree, $p42^{MAPK}$ presumably by inhibiting synthesis of new MAP kinases, thus preventing protein-turnover (Sale *et al*, 1995). Treatment of 3T3-F442A cells with 5μM antisense EAS1 caused a dramatic reduction in the expression of both $p42^{MAPK}$ and $p44^{MAPK}$ to near undetectable levels in immunoblots (Fig 5.3A). Cells subjected to this treatment were viable and exhibited apparently normal phenotypes. There are several mechanisms proposed by which antisense oligonucleotide may inhibit gene expression. These include prevention of new protein synthesis by translational arrest, promotion of RNA degradation by an RNase H-dependent mechanism, inhibition of mRNA maturation by masking sequences required for the formation of a spliceosome, inhibition of RNA transport out of the nucleus, inhibition of gene transcription by forming a triple helix structure, or other unidentified mechanisms (Stein and Cohen, 1988; Zon, 1988; Dolnick, 1990). Since antisense oligonucleotides bind to the target mRNA or pre-mRNA through normal Watson-Crick base pairing, there is the potential for interaction with other, non-specific, gene-targets which contain complementary, or semi-complementary, sequences to the antisense probe.

Two points of evidence were obtained that the action of depleting EAS was specific. Firstly, the expression of MAP kinase was not significantly effected by sense or scrambled phosphorothioate oligodeoxynucleotides (Fig 5.3A). Secondly, the banding pattern of Coomassie blue-stained proteins was generally unaffected by antisense EAS1 treatment (Fig 5.3B). A small increase in the expression of an ≈85kDa species was observed in antisense EAS1 treated cells, whereas no change was observed in cells treated with control oligodeoxynucleotides. That this is an effect specific to cells in which MAP kinases have been depleted suggests that these kinase may regulate the expression of the 85kDa protein. Whether or not this protein is involved in modulation of 3T3-F442A preadipocyte differentiation remains to be determined.

Treatment of 3T3-F442A fibroblasts with 5μM of antisense EAS1 completely inhibited differentiation induced by FCS and insulin as assessed by two markers of terminal differentiation (Table 5.1; Fig 5.4). This agrees with Sale *et al* (1995) who found that EAS1 blocked the differentiation of 3T3-L1 preadipocytes with FCS. Since

FCS contains a multitude of growth factors, each with the potential to influence differentiation, these studies fail to address which agents are primarily responsible for eliciting the necessary activation of MAP kinase. The development of a hormonally defined medium capable of supporting differentiation allowed an examination of the role individual factors played in the process. The results in Table 5.1 indicate that the differentiation of cells induced with a combination of GH, insulin, EGF and T_3 displays an absolute requirement for MAP kinase, since antisense depletion of MAP kinases severely impaired differentiation. This effect was specific as incubation with control oligodeoxynucleotides had no significant effect on the extent of differentiation (Table 5.1). Although the precise mechanism involved is unknown, it is concluded that MAP kinase is required for the transduction of differentiative signals induced by serum components.

MAP kinases do not appear to be necessary for the transmission of a priming signal by GH. This is based on the observation that PD098059, a specific inhibitor of the MAP kinase cascade (Alessi *et al*, 1995), does not inhibit the priming effect of GH in 3T3-F442A cells (Fig 5.12). Therefore, the combination of antisense and PD098059 results concludes that MAP kinases are required for maturation of 3T3-F442A preadipocytes. Although MAP kinases do not appear to be required for GH priming under the conditions used, they may be required when lower concentrations of GH are used. This is based on the premise that high concentrations of GH will trigger multiple pathways to initiate the priming response in cells by recruiting many signalling molecules to JAK2 (see the *Signalling* section in Chapter 1). At submaximal concentrations of GH, when fewer signalling molecules associate with JAK2, then the MAP kinase cascade may be required to produce a significant priming signal.

That MAP kinases are not involved in GH-priming, suggests that the potentiating action of cyclic AMP during the priming phase of 3T3-F442A preadipocyte differentiation does not involve the MAP kinase pathway. This is contrary to a number of other studies which have suggested that the Ras-MAP kinase pathway may control differentiation in cells which use cyclic AMP as a differentiative signal (Johnson and Vailancourt, 1994). In these cell systems, however, components of the MAP kinase pathway appear to be essential requirements for the transduction of an initiating differentiative signal. For example, studies using the pheochromocytoma cell line, PC12, have shown that oncogenic Ras and Raf can trigger neuronal differentiation (Noda *et al*, 1985; Wood *et al*, 1993) Additionally, transfection of PC12 cells with dominant negative MEK, or preincubation with the MEK-inhibitor PD098059, blocks neurite outgrowth triggered by nerve growth factor (NGF; Cowley *et al*, 1994;Pang *et al*, 1995), whereas constitutively active mutants accelerate the process (Cowley *et al*,

1994). In the PC12 cell system cyclic AMP activates MAP kinase and potentiates MAP kinase activation by differentiative agents such as NGF (Frödin *et al*, 1994; Young *et al*, 1994; Yao *et al*, 1996). This is consistent with the synergistic interaction between NGF and cyclic AMP on the promotion of PC12 differentiation (Frödin *et al*, 1994; Young *et al*, 1994; Yao *et al*, 1996). Since the synergistic effect of cyclic AMP and GH in the potentiation of 3T3-F442A cells does not appear to require interaction at the level of MAP kinase, then other molecular interaction/s must be involved.

As discussed in chapter 4, the molecular mechanisms used by GH to promote differentiation are likely to involve the induction of early markers of the process, such as C/EBP β and δ (Clarkson *et al*, 1995), which have themselves been shown to enhance adipogenesis (Yeh *et al*, 1995). The activation of MAP kinase by GH is thought to involve Ras, Raf and the intracellular tyrosine kinase JAK2 (Sotiropolous *et al*, 1994; Winston and Hunter, 1995). JAK2 can also phosphorylate and activate a family of transcription factors called Stats (signal transducers and activators of transcription) 1, 3 and 5 which can bind to specific sequences in GH-inducible genes (Campbell *et al*, 1995; Smit *et al*, 1995; Han *et al*, 1996; Silva *et al*, 1996). One such gene is c-*fos* which contains *sis*-inducible elements which interact with Stats and are thought to contribute to the rapid activation of the gene by GH (Campbell *et al*, 1995;. Smit *et al*, 1995). A number of observations implicate c-*fos* as playing a regulatory role in the adipogenic program. Firstly, GH activates c-*fos* transcription rapidly (Gurland *et al*, 1990) prior to the induction of C/EBPs β and δ (Clarkson *et al*, 1995). Secondly, the c-*fos* gene-product forms a nucleoprotein complex in the regulatory region of the differentiation-specific aP2 gene (Distel *et al*, 1988). Given that c-*fos* is also induced in many cell types, including preadipocytes, by cyclic AMP (Gaskins *et al*, 1989) it is possible that cooperativity between cyclic AMP and GH at the level of c-*fos* transcription may have a potentiating effect on preadipocyte differentiation. Since transcriptional regulation by cyclic AMP is often governed by the CREB transcription factor (see section 1.7.3.4), it would be extremely interesting to investigate whether Stat-CREB interactions contribute to the potentiating action of cyclic AMP on GH-promoted preadipocyte differentiation.

One of the actions of GH in promotion of preadipocyte differentiation is thought to be the production of an anti-mitogenic state in proliferating cells which primes them to the actions of other hormones (Tang *et al*, 1995). An obligate step for the mitogen-stimulated passage of cells through the G1-S phase of the cell cycle is the induction of the nuclear protein cyclin D (Baldin *et al*, 1993). Correspondingly, the antimitogenic effects of GH in preadipocytes is thought to be mediated partially by the suppression of cyclin D expression (Tang *et al*, 1995). Cyclin D expression is also blocked

following exposure to cyclic AMP of certain fibroblastic cell-lines in which cyclic AMP is anti-mitogenic (McKenzie and Pousségur, 1996). Perhaps the effects of cyclic AMP on GH-mediated priming could be attributable to cooperativity at the level of cyclin D expression. In a similar vein, another mechanism by which GH could cause growth arrest in proliferating preadipocytes is by increasing the expression inhibitors of cell proliferation, such as the cyclin-dependent kinase inhibitor $p21^{WAF1}$ which is induced in a Stat1 dependent manner (Chin *et al*, 1996; Timchenko *et al*, 1996). This area requires further investigation but could provide another potential site of synergy between the GH and cyclic AMP pathways.

In sharp contrast to its effects in the priming phase cyclic AMP potently inhibited terminal differentiation of 3T3-F442A preadipocytes. EGF and insulin are essential components of the medium used to promote this second phase whereas T_3 exerts only a modulatory influence (Guller *et al*, 1988). The molecular mechanisms underlying the effects of EGF and insulin are not known but each induces MAP kinase activation, though to differing degrees (Table 5.2). MAP kinases must be essential for the maturation phase of differentiation, since they are not a requirement for priming (Fig 5.12) but are required for differentiation *per se* (Table 5.1). However, the inhibitory effect of cyclic AMP on maturation was not mirrored by an inhibition of MAP kinase activity as stimulated by insulin, EGF and T_3 (Table 5.3). There are a number of examples where the effects of cyclic AMP on cell growth are dissociated from the activation or inhibition of the MAP kinase pathway (Withers *et al*, 1995; McKenzie and Pouysségur, 1996). For example, MAP kinases display neutral responsiveness in both Swiss 3T3 fibroblasts, where cyclic AMP is mitogenic (Withers *et al*, 1995), and in CCL39 fibroblasts, in which cyclic AMP is anti-mitogenic (McKenzie and Pouysségur, 1996). The intracellular targets which allow cyclic AMP to modulate cell growth in these cell-types are not known. However, in Swiss 3T3 cells cyclic AMP-stimulated cell division was shown to correlate with the activation $p70^{S6K}$, an important transducer of proliferative signals (Withers *et al*, 1995). A specific inhibitor of $p70^{S6K}/p85^{S6K}$, rapamycin, was found to inhibit the differentiation of 3T3-F442A cells (Yarwood, Kilgour and Anderson, unpublished observations), suggesting that the $p70^{S6K}/p85^{S6K}$ is also important for the transmission of the differentiative signal by insulin, EGF and T_3. Therefore, interaction with the $p70^{S6K}/p85^{S6K}$ cascade may contribute to the inhibitory effects of cyclic AMP during the maturation phase of 3T3-F442A cellular differentiation. Whether or not such interactions occur remains to be determined.

Although the effects of cyclic AMP on 3T3-F442A adipose conversion do not involve the MAP kinase cascade, a range of pharmacological agents which share the

common ability to elevate intracellular cyclic AMP were found to evoke a small activation of MAP kinase in both 3T3-L1 and 3T3-F442A preadipocytes in a PKA-dependent manner (Fig 5.9; Table 5.2).Therefore, these cell systems could provide a convenient system for the study of the molecular basis of MAP kinase/PKA interactions.

Although PKA is known to mediate many of the biological responses of cells to cyclic AMP, PKA-independent events have been reported (DiFrancesco and Tortora, 1991; Piper *et al,* 1993). However, in 3T3-F442A cells the EC_{50} and maximal doses for the activation of MAP kinase and PKA by forskolin are equivalent (Fig 5.8). In addition, two selective inhibitors of PKA, H-89 and (*R*p)-cAMPS, which act via different mechanisms, were found to block the ability of forskolin to activate MAP kinase (Fig 5.8. Taken together, these results indicate that PKA mediates the activation of MAP kinase by cyclic AMP.

Figure 5.8 shows that low doses of forskolin (around 1μM) activate PKA but not MAP kinase suggesting that there exists a threshold level of PKA activation that must be achieved before activation of MAP kinase can occur. Additionally, the degree to which MAP kinase is activated in response to cyclic AMP is transient and relatively small when compared to growth/differentiation factors such as EGF and GH and the phorbol ester PMA (Table 5.4; unpublished observations). This is not the case in other cyclic AMP responsive cell types. In PC12 cells (Frödin *et al,* 1994; Young *et al,* 1994) and ovarian granulosa cells (Saswati *et al,* 1996) cyclic AMP was shown to produce an activation of MAP kinase which was equivalent in magnitude to that evoked by TPA. These observations indicate that the cyclic AMP-stimulated MAP kinase pathway in 3T3-F442A cells is under stringent negative-regulatory influences.

An involvement of protein kinase C in the activation of MAP kinases by certain agonists has been postulated (Rossomando *et al,* 1992; Burgering and Bos, 1995; Mitchell *et al,* 1994; Sim *et al,* 1995). However, phorbol ester-sensitive isoforms of PKC are unlikely to mediate the activation of MAP kinase by cyclic AMP since their cellular depletion by chronic PMA treatment had no effect on MAP kinase activation by forskolin (Fig 5.11).

The activation of MEK occurs through direct phosphorylation by the serine/threonine protein kinases Raf-1 (Huang *et al,*1993; Avruch *et al,* 1994) and B-Raf (Jaiswal *et al,* 1994). However, Raf-1 is unlikely to mediate the positive effects of cyclic AMP on MEK and MAP kinase reported in Fig 5.10, since phosphorylation by PKA at Ser 43 inhibits Raf-1 function and impairs MAP kinase activity in fibroblasts (Wu *et al,* 1993;

Cook and McCormick, 1993). However, in PC12 cells where cyclic AMP has been demonstrated to be an activator of MAP kinase (Frödin *et al*, 1994; Young *et al*, 1994), MEK is activated by B-Raf and not Raf-1 (Jaiswal *et al*, 1994; Moodie *et al*, 1994). Intriguingly, B-Raf activity is not inhibited by cyclic AMP in PC12 cells maintained in serum-containing medium, (Erhardt *et al*, 1995). In light of this, it will be important to determine the expression pattern of Raf isoforms in 3T3-F442A cells and determine whether PKA can phosphorylate and/or inhibit Raf activation. Other putative MEK activators, other than the Raf isoforms, have also been partially characterised (Blank *et al*, 1996), but their responsiveness to cyclic AMP remains to be determined.

As well as exerting positive effects on components upstream of MEK, cyclic AMP could also relieve negative constraint on the MAP kinase pathway at one or more points. For example, although phosphatases which regulate the dephosphorylation and hence inactivation of kinases such as MEK and Raf are incompletely characterised, inhibition of a protein phosphatase by PKA-mediated phosphorylation could result in MAP kinase activation. Indeed, inhibition of PP2A with the tumour promoter okadaic acid, or with the small T antigen of simian virus 40, can lead to MAP kinase activation (Haystead *et al*, 1990; Sontag *et al*, 1993). Although initial studies indicate that PP2A contributes to the inactivation of cyclic AMP-stimulated MAP kinase in 3T3-F442A cells (Yarwood, Anderson and Kilgour, unpublished observations), these further considerations remain to be addressed. In addition, this study did not address the contribution of protein tyrosine kinases to PKA mediated signal to MAP kinases. This may be an important area of investigation as tyrosine phosphorylation of Raf, by kinases such as $pp60^{src}$, is critical for activation (Dent *et al*, 1995).

5.5) CONCLUSIONS

Transient increases in cyclic AMP levels exert differential effects upon the differentiation of 3T3-F442A preadipocytes which depend upon the stage of the differentiation process at which levels are raised (Chapter 4). The results from the present study demonstrate an overall requirement for MAP kinase in serum-free differentiation conditions. Cyclic AMP was found to potentiate GH-, but not EGF/Ins/T_3-activated MAP kinase. In addition, cyclic AMP alone activates MAP kinase. This suggested that the cooperativity between cyclic AMP and GH could be at the level of MAP kinase but MAP kinase was not the site of action of cyclic AMP in inhibiting maturation. However, the availability of the MEK inhibitor, PD098059, enabled the testing of the MAP kinase requirement during priming and this showed a lack of MAP kinase requirement at this stage.

General Discussion and Future Investigations

GENERAL DISCUSSION

The results from the present study demonstrate that stage-specific alterations in the expression of heterotrimeric G-protein subunits accompany the adipose conversion of 3T3-F442A preadipocytes (chapter 2). The crucial GH-priming stage of 3T3-F442A differentiation witnessed specific increases in $G_{s\alpha 42}$, $G_{i2\alpha}$ and $G_{\beta 36}$. Further increases were observed during the initial stages of insulin-supported maturation culminating in dramatic increases in $G_{s\alpha 42}$ occurring in terminally differentiated cells. In contrast, levels of $G_{i2\alpha}$, $G_{i3\alpha}$ declined in mature adipocytes. Two conclusions can be drawn from these observations. First, it is clear that not all G-proteins respond equally to the induction of differentiation, and unique responses occur even among members of the same family. Second, it is apparent that the onset of individual stages of preadipocyte differentiation can lead to varied ratios of G-protein α- and β-subunits which will contribute to the selectivity of receptor coupling to specific signal transduction processes. Hence some of the phenotypic changes which occur on differentiation are likely to be reflections of these changes in G-protein expression.

There is increasing evidence that G-proteins contribute to the control of complex biological phenomena such as cellular differentiation. For example, the development of nerve growth cones and primitive endoderm in mice have been reported to be under the control of pertussis toxin-sensitive G-proteins (Strittmater *et al*, 1990; Watkins *et al*, 1990) and $G_{i2\alpha}$ has been implicated in the control of preadipocyte differentiation (Su *et al*, 1993; Gordeladze *et al*, 1997) and fat development in mice (Moxham et al, 1993). In support of this the present study demonstrates that pertussis toxin-induced inactivation of G_i in 3T3-F442A preadipocytes inhibited their adipocyte differentiation in hormonally defined differentiation medium (Fig 3.16). Although the exact mechanisms underlying the potentiating action of G_i on preadipocyte differentiation have not been addressed in this or other studies, it is possible that interaction with the $p70^{S6K}/p85^{S6K}$ and p42/p44 MAP kinase signalling pathways will be critical to its action. This is based on observations that $G_{i\alpha}$ has been shown to activate these pathways (Winitz *et al*, 1993; Wilson *et al*, 1996) in, what appears to be, a $G_{\beta\gamma}$-dependent manner (Alblas *et al*, 1993; Crespo *et al*, 1994; Faure *et al*, 1994; Koch *et al*, 1994; Thorburn and Thorburn, 1994). Intriguingly, a link between differentiation-dependent alterations in G_i expression and activation of MAP kinases has recently been proposed to contribute to the control of morphogen induced teratocarcinoma stem-cell progression (Gao and Malbon, 1996). In this respect the present study demonstrates that the relative expression of $G_{i2\alpha}$, $G_{i3\alpha}$ and $G\beta$ appear to be maximal during the insulin-supported maturation stage of 3T3-F442A preadipocyte differentiation (Fig 3.15) when the MAP kinase (see Chapter 5) and $p70^{S6K}/p85^{S6K}$ (Yarwood, Anderson and Kilgour,

unpublished observations) pathways are required for the transmission of differentiative stimuli. It is likely that during this stage of preadipocyte differentiation $G_{i2\alpha}$ contributes to the promotion of differentiation by facilitating signal propagation from the insulin receptor, since this G-protein subunit is known to be a positive regulator of insulin action *in vivo* (Moxham and Malbon, 1996; Chen *et al*, 1997).

In addition to the positive-acting role of G_i-isoforms in differentiation, this study also shows that cyclic AMP and the β-adrenergic agonist isoproterenol act synergistically with GH early in the differentiation program to potentiate preadipocyte differentiation (see chapter 4). This demonstrates that $G_{s\alpha}$, by activating adenylate cyclase, can serve as a positive regulator of fat cell formation. During the later insulin-promoted maturation phase cyclic AMP is inhibitory to the process. While it has been postulated for some time that cyclic AMP plays a dual role in modulating the adipose differentiative potential of preadipocytes (Antras *et al*, 1991) this is the first occasion that the temporal aspects of this bifunctional role of cyclic AMP has been demonstrated in the same preadipocyte cell system. The importance of $G_{s\alpha}$ in controlling fat cell development has been demonstrated clearly in the 3T3-L1 preadipocyte cell system in which antisense depletion of $G_{s\alpha}$ has been shown to potentiate differentiation (Wang *et al*, 1992). Moreover, an inactivating mutant of $G_{s\alpha}$ has also been shown to enhance 3T3-L1 adipose conversion (Gordeladze et al ,1997). These observations demonstrate that $G_{s\alpha}$ exerts an inhibitory influence on 3T3-L1 cellular differentiation, which is in agreement with the inhibitory role of cyclic AMP on the later stages of 3T3-F442A preadipocytes reported in this study. Whereas it is generally accepted that prolonged exposure to cyclic AMP prevents terminal adipocyte differentiation by causing a decrease in the expression of lipogenic enzymes (Spiegelman and Green, 1981; Bhandari and Miller, 1985) the authors concluded that the inhibitory role of $G_{s\alpha}$ in 3T3-L1 differentiation is independent of adenylate cyclase activation despite the fact that under the differentiation conditions used in their study cyclic AMP has been shown to be a powerful positive regulator of 3T3-L1 adipogenesis (Schmidt *et al*, 1990). Therefore, care should be taken in interpreting the results of gain-of- and loss-of-function studies on the role of G-proteins in controlling preadipocyte differentiation. The results from the present study indicate both a positive and negative regulatory role for $G_{s\alpha}$ in the adipose conversion of adipogenic 3T3-F442A preadipocytes in what is clearly a cyclic AMP-mediated process. There is, however, a suggestion that $G_{s\alpha}$ may play an inhibitory role during preadipocyte differentiation in a cyclic AMP-independent manner based on observations using chimeric constructs of $G_{s\alpha}$ which were expressed stably in 3T3-L1 cells to define the region controlling adipogenesis (Wang *et al*, 1996). This study revealed that the $G_{s\alpha}$ protein contains a adipogenic-repression region (amino acids 146-220) which is spatially distinct from those regions which map for control of

adenylate cyclase (Wang *et al*, 1996). Clearly further work should address the mechanistic aspects of the obviously complex role of $G_{s\alpha}$ in modulating preadipocyte differentiation.

The current investigation examined whether interaction with the MAP kinase growth-regulatory cascade contributed to the biphasic action of cyclic AMP on preadipocyte differentiation. Results demonstrated an overall requirement for MAP kinase in serum-free differentiation conditions. Cyclic AMP was found to potentiate GH-activated MAP kinase, but did not affect the degree of MAP kinase activation induced by the agents which promote maturation of 3T3-F442A preadipocytes (insulin, EGF and T_3). In addition, cyclic AMP alone activates MAP kinase. This suggested that the cooperativity between cyclic AMP and GH could be at the level of MAP kinase but MAP kinase was not the site of action of cyclic AMP in inhibiting maturation. However, the availability of the MEK inhibitor, PD098059, enabled the testing of the MAP kinase requirement during priming and this showed a lack of MAP kinase requirement at this stage. This suggests that MAP kinases are required during the later maturation stage of differentiation. If MAP kinases are required for the insulin-promoted maturation phase of preadipocyte differentiation, and not during GH priming, this raises the question as to what is the down-stream target of MAP kinase action at this stage. Once activated p42/p44 MAP kinase can be translocated to the nucleus (Chen *et al*, 1992; Lenormont *et al*, 1993) and directly phosphorylates transcription factors, including $p62^{TCF}$ and ATF-2 (Davis, 1993). In addition, a number of cytosolic substrates for MAP kinases have been identified, including members of the $p90^{RSK}$ family, phospholipase A_2, and Stat 1 (Sturgill *et al*, 1988; Lin *et al*, 1993b; Lin *et al*, 1994; Wen *et al*, 1995; Xing *et al*, 1996). It is worth noting that GH induces a transient activation of MAP kinase, whereas that induced by the maturation agents is much larger and sustained (Yarwood, Kilgour and Anderson, unpublished observations). Prolonged activation of MAP kinase is associated with nuclear translocation (Traverse *et al*, 1992; Nguyen *et al*, 1993), therefore it is likely that MAP kinases activated during the maturation phase undergo nuclear translocation and activate transcription factors by phosphorylation. Intriguingly, it has recently been demonstrated that the adipogenic transcription factor PPARγ undergoes MAP kinase-mediated serine phosphorylation (ser 122) in response to insulin (Hu *et al*, 1996). Given that MAP kinase activation and PPARγ are required for the completion of the adipogenic program, it will be interesting assess whether this insulin-induced serine phosphorylation is important for modulating preadipocyte differentiation.

Activation of the MAP kinase cascade (see chapter 5) and the $p70^{S6K}$ cascade (Yarwood, Kilgour and Anderson, unpublished observations) do not appear to be

necessary for GH to prime 3T3-F442A preadipocytes for a program of differentiation. However, antisense oligodeoxynucleotides which specifically deplete the GH-receptor-associated tyrosine kinase JAK2 are inhibitory to 3T3-F442A differentiation (N.G. Anderson, unpublished observation). This suggests that other JAK2-mediated signalling events are responsible for the priming action of GH. Following binding of GH, receptor dimerisation leads to the activation of JAK2 which tyrosine phosphorylates and activates latent cytoplasmic transcription factors termed Stats (signal transducers and activators of transcription) 1, 3 and 5 (Han *et al*, 1996; Smit *et al*, 1996). Tyrosyl-phosphorylated Stats bind to the *sis*-inducible element of the c-*fos* promoter and other genes. Current evidence suggest that these response elements bind Stat1 and Stat3 homodimers as well as Stat1/Stat3 heterodimers, all of which appear to be formed in response to GH (Granowski *et al*, 1994; Meyer *et al*, 1994; Campbell *et al*, 1995; Granowski *et al*, 1995). Following GH-treatment of 3T3-F442A cells c-*fos* induction precedes the induction of C/EBPs β and δ and it has been postulated that c-*fos* contributes to the *trans*-activation of these important adipogenic transcription factors (Clarkson *et al*, 1995). It appears, therefore, that activation of the JAK/Stat signalling pathway by GH has the potential to directly initiate a program of preadipocyte differentiation by inducing the "tertiary messenger" c-*fos* and early markers of adipose conversion.

Interaction between the cyclic AMP/PKA pathways and the JAK/Stat cascade may underlie the potentiating action of cyclic AMP on GH-priming reported in the present study (see chapter 4). Maximal activation of Stats 1 and 3 requires both tyrosine and serine phosphorylation (Wen *et al*, 1995). Therefore, it could be envisaged that increased serine kinase activity triggered through cyclic AMP elevation, Ras-MAP kinase activation or protein kinase C activation has the potential to affect transcriptional activation of adipogenic transcription factors by increasing the transcriptional-activating abilities of Stats. In this respect GH stimulates phosphorylation of Stats 1, 3 and 5 on serine, threonine and tyrosine residues *in vivo* in a manner that either enhances (Stats 1 and 3) or substantially alters (Stat 5) the binding of each Stat to its cognate DNA element (Ran *et al*, 1996). The serine phosphorylation of GH-activated Stats could result from the activation of the MAP kinase cascade, the $p70^{S6K}$ cascade and/or the PKC cascade which are all known to be activated by GH (McKenzie *et al*, 1997; *vide infra*). The synergistic potentiation of 3T3-F442A preadipocyte differentiation by cyclic AMP which occurs during GH-priming could therefore be contributed to by potentiation of GH-activated MAP kinase (see chapter 5), resulting in increased Stat activation. It is unlikely that increased Stat activation results from a cyclic AMP-induced potentiation of GH-activated PKC and/or $p70^{S6K}$ as cyclic AMP was not found to potentiate GH-signalling through these pathways in 3T3-F442A cells (Yarwood,

Kilgour and Anderson, unpublished observations). Therefore, future study should be directed towards determining which GH-activated Stats are important for 3T3-F442A preadipocyte differentiation and to determine whether the cyclic AMP potentiation of GH-activated MAP kinase increases Stat-mediated transcriptional activation.

Another potential site of synergy between the cyclic AMP and GH signalling cascades is at the level of C/EBPβ which is required and sufficient to initiate the adipogenic program (Yeh *et al*, 1995). Increases in intracellular cyclic AMP levels stimulated by IBMX treatment have been shown to be a direct inducer of C/EBPβ expression in 3T3-L1 preadipocytes (Yeh *et al*, 1995). In addition, GH-treatment of 3T3-F442A preadipocytes has also been shown to increase expression of C/EBPβ (Clarkson *et al*, 1995). It is therefore likely that the combined effect of GH-treatment and elevated cyclic AMP levels leads to optimal C/EBPβ expression during the priming phase of 3T3-F442A differentiation, thereby potentiating adipose conversion. Interestingly, it has been demonstrated in hepatocytes and neuroblastoma cells that optimal transcriptional activation of certain cytokine-responsive genes requires the binding of both Stats and C/EBPs to the gene promoter region (Symes *et al*, 1995; Kardula and Travis, 1996). Such cooperativity may underlie the synergistic action of GH and cyclic AMP on preadipocyte differentiation by optimising expression of differentiation-dependent genes.

Recent observations in a rat insulinoma cell line have demonstrated that impaired cyclic AMP-dependent phosphorylation renders CREB (cyclic AMP response-element-binding-protein) a repressor of C/EBPβ-induced transcription (Vallejo *et al*, 1995). This suggests that phosphorylation and activation of CREB is important for correct functioning of C/EBPβ and implies that GH-induced C/EBPβ activation in 3T3-F442A preadipocytes may be optimised by cyclic AMP-mediated CREB phosphorylation. Given the critical role of C/EBPβ in modulating preadipocyte differentiation (Yeh *et al*, 1996), increases in CREB activation therefore have the potential to modulate the extent of terminal differentiation. A signalling pathway has been elucidated whereby growth and differentiation factors activate CREB (Xing *et al*, 1996; Sato *et al*, 1997). Growth factor-stimulated CREB phosphorylation at serine 133 is mediated by the Ras-MAP kinase pathway (Xing et al, 1996). MAP kinase activates a member of the $p90^{RSK}$ family which, in turn, phosphorylates and activates CREB (Xing *et al*, 1996). In the present study cyclic AMP potentiated GH-activated MAP kinase and terminal differentiation of 3T3-F442A preadipocytes (see chapters 4 and 5). This may lead to increased CREB activation through MAP kinase-mediated activation of $p90^{RSK}$, thereby leading to increased activation of adipocyte-specific genes by cooperativity with C/EBPβ.

FUTURE INVESTIGATIONS

Future study aimed at elucidating the potentiating and inhibitory actions of cyclic AMP on the adipose conversion of 3T3-F442A preadipocytes should focus on the synergistic interactions between the cyclic AMP and GH signalling cascades and inhibitory interactions occurring between the cyclic AMP and insulin pathways. Particular attention should be paid to determining whether cooperativity between the cyclic AMP and these adipogenic pathways occurs at the level of transcription factor activation or induction, particularly those transcription factors which are known to be activated or induced by cyclic AMP (CREB and C/EBPs), insulin (C/EBPs) and GH (Stats and C/EBPs). It is already well established that the C/EBP family of transcription factors are extremely important for controlling adipogenesis, however the importance of Stats and CREB in the modulation of preadipocyte differentiation remains to be determined. The requirement for these transcription factors can be determined by specific depletion with antisense oligodeoxynucleotides which can be designed so as to deplete individual members of the same transcription factor family. The combined effect of cyclic AMP and GH on the expression of CREB, C/EBP and Stat proteins can be determined immunologically, by Western blotting, or, indirectly, by measuring the cellular abundance of mRNAs for each transcription factor. The activation status of individual transcription factors can be determined by electrophoretic mobility shift assay (EMSA; Ausubel, 1993).

A further measure of the activation status of transcription factors is the degree of nuclear translocation induced by extracellular stimulation. In this respect the degree of nuclear accumulation of adipogenic transcription factors in response to GH, cyclic AMP and/or insulin stimulation should be assessed by subcellular fractionation and confocal imaging (see for example Metz and Ziff, 1991; Kilgour and Anderson, 1994; King and Delaney, 1994).

As the activation status of transcription factors can be modulated directly by phosphorylation (Hunter and Karin, 1992), the level of phosphate incorporation into C/EBPs, Stats and CREB should be assessed in response to insulin and GH in the presence or absence of cyclic AMP. This can be achieved immunologically, in the case of CREB, since a commercial antiserum which specifically recognises phospho-CREB has recently been made available. The phosphorylation status of C/EBPs has been determined by gel retardation assay in a manner similar to that used to detect phospho-MAP kinases (see Chapter 5), whereas phosphorylation of Stats has been detected by

phosphotyrosine-specific antisera and by phospho-peptide mapping (Wen *et al*, 1995; Smit *et al*, 1996).

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