EFFECTS OF THE N-RAS ONCOGENE ON DIFFERENTIATION OF CO25 MYOBLAST CELLS

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By

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This thesis is dedicated to my father and my mother who supported me throughout my life.

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ABSTRACT

Effects of a mutant N-*ras* oncogene (under transcriptional control of the glucocorticoid- inducible MMTV-LTR promotor) on the differentiation of a CO25 myoblast cell line have been investigated using various techniques.

Scanning electron microscopy demonstrated that CO25 cells were more rounded in shape with numerous microvilli on their surface after induction of the N-*ras* oncogene by dexamethasone. After 10 days, they lost their anchorage dependency and formed foci. In contrast, the control cells formed long branched myotubes with a few microvilli on their surface in the absence of dexamethasone.

Distribution of F-actin was investigated by fluoresence microscopy using rhodamine-phalloidin. There was a dramatic change in actin organization during transformation of the cells. Stress fibers were replaced by a diffuse bright fluorescence in *ras*-induced cells.

Transcription levels of the cellular oncogenes, c-*myc*, *p53*, c-Ki-*ras* and a control β-actin gene, were examined by Northern blotting. During differentiation of CO25 cells, mRNA levels of these genes declined up to 2-3 days and returned to the initial levels in 4 days. Following induction of the N-*ras* oncogene, the level of c-*myc* mRNA first decreased in 2 days and then increased in 3 and 4 days. The level of β-actin mRNA was lower during transformation, and there were no changes in the mRNA levels of *p53* and c-Ki-*ras* genes.

Phosphorylation of tyrosine in cellular proteins has been analysed following stimulation of cells with PDGF-BB by the Western blotting techniques. In the *ras*-induced cells, PDGF-BB stimulation

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CHAPTER. 1.

GENERAL INTRODUCTION

1.1. CELL GROWTH AND DIFFERENTIATION

Cell growth and differentiation are complex processes, regulated by interdependent mechanisms. Generally, the proliferative potential is inversely related to the degree of differentiation, but this relationship varies in different cell systems. The development of muscle cells provides an excellent model system for experimental analysis of the processes of commitment, proliferation and differentiation. Myoblasts are proliferating, mononucleated stem cells which, upon withdrawal from the cell cycle, differentiate into myotubes. Muscle cell differentiation is characterized morphologically by the fusion of myoblasts into multinucleated myotubes, and the coordinated activation of a family of muscle-specific myofibrillar genes including myosins, α-actin and desmin (Whalen et al., 1976; Merlie et al., 1977; Endo and Nadal-Ginard, 1986; Schneider and Olson, 1988; Olson and Capetanaki, 1989; Capetanaki et al., 1990; Florini et al., 1991), and biochemically by the activation of muscle- specific enzymes such as muscle isoenzyme of creatine kinase, myokinase, the nicotinic acetylcholine receptor, acetylcholinesterase and glycogen phosphorylase (Merlie et al., 1977; Shani et al., 1981; Olson et al., 1983; Alemâ and Tato, 1987; Nadal-Ginard, 1978). The only phase of the cell cycle in which muscle gene activation and fusion into myotubes can occur is the G_1 (Li and Olson, 1992).

Recent studies have demonstrated that the transition along this differentiation pathway may be determined by a family of trans-acting transcription factors; the MyoD1 gene (Davis et al., 1987), the myogenin gene (Wright et al., 1989), the myd gene (Pinney et al., 1988), and four myf genes (Braun et al., 1989, 1990) (reviewed in Florini et al., 1991). In addition each gene can convert mouse

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fibroblasts into cells with myogenic characteristics (Olson, 1990; Weintraub, 1991; Li and Olson, 1992).

Myoblast cells in culture can proliferate in the presence of growth factors and do not express muscle - specific genes. When the concentration of growth factors is reduced or these growth factors are removed, the cells cease DNA synthesis, withdraw from the cell cycle, and fuse to form myotubes. But the exact signals that initiate myogenesis *in vivo* remain unknown (Yaffe and Saxel, 1977; Nadal-Ginard, 1978; Li and Olson; 1992). Thus many factors that influence cell proliferation, in particular mitogens such as serum component, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF–β), can affect myoblast differentiation by inhibition of activation of muscle-specific genes. TGF–β and in some cases FGF can arrest myoblasts in the differentiation pathway without stimulating cell proliferation (Linkhart *et al.*, 1981; Nguyen, *et al.* 1983; Olson, *et al.*, 1986; Spizz *et al.*, 1986; Clegg *et al.*, 1987; Li and Olson, 1992). PDGF exerts a mitogenic effect on C2 myoblast cells by promoting cell proliferation and depressing the frequency of differentiated myoblasts (Yablonka- Reuvei *et al.*, 1990). By contrast, insulin and IGFs stimulate rather than inhibit myogenic differentiation (Florini *et al.*, 1991).

Growth factors appear to act at multiple levels to inhibit myogenesis. Growth factor signals not only inhibit transcription of the genes encoding muscle-specific transcription factors myogenin and *MyoD*, but can also suppress the activity of the myogenin proteins (Li and Olson, 1992). Increased expression of a cellular oncogene such as c-*fos*, *junB*, c-*jun* or c-*myc* in response to growth factors may be involved in the inhibition of myogenesis by acting as the final mediators for diverse signals that are known to inhibit myogenesis

(Florini *et al.*,1991; Li and Olson,1992). Other agents can inhibit myogenic differentiation, including a variety of viral and cellular or proto-oncogenes (Alema and Tato,1987). More detail will be given in the next section.

1.2. ONCOGENES

Proto-oncogenes (c-oncogenes) are the normal cellular counter-parts of the transforming oncogenes carried by retroviruses termed as viral-oncogenes (v-oncogenes). Since it is known that viral oncogenes disrupt the control of cell proliferation and differentiation, it is thought that products of proto-oncogenes are functional proteins with various roles in cellular regulatory systems that control growth and differentiation (Varmus, 1984; Bishop,1983; Zelenka,1990).

1.2.1. Function of oncogenes

To date more than 60 different oncogenes are known which encode proteins localized in the nucleus or cytoplasm, or associated with the plasma membrane or even secreted (Bishop, 1991). These oncogenes can be classified into six groups according to the function of their product:

1) Growth factors: These oncogenes encode secreted proteins which act via autocrine loops to stimulate cell growth. For instance, the v-*sis* product which is almost identical to the β-chain of PDGF, and *int*-2 and *k-fgf-hst* oncogenes that are related to the FGF.

2) Cell surface receptor and nonreceptor protein - tyrosine kinases: A number of oncogenes encode either receptors for known growth factors (e.g., c-*erb B* and c-*neu-erB-2* are homologs of the receptor for EGF, ros for the insulin receptor, and v-*fms* is the colony-stimulating factor-1-receptor) or receptor-like proteins with

unknown ligands (eg., *kit*). These oncoproteins transform cells by delivering a continuous ligand-independent mitogenic signal.

Nonreceptor oncogenic protein-tyrosine kinases, such as the *src* family are membrane associated with the inner side of cytoplasm and activated upon ligand binding to cell surface molecules. Some other nonreceptor oncogenic protein-tyrosine kinases, such as *abl* are found in the nucleus. However *abl* protein has to act at the membrane for transformation.

3) GTP binding proteins: (e.g., *ras, gsp, gip*). The *ras* family of proteins are GTP binding GTPases, and they transduce the signal from cell surface growth factor receptors to an effector molecule.

4) Protein-serine / threonine kinases: (e.g. *raf/mil, mos*). Phosphorylation of c-*raf* protein is elevated when cells are stimulated by mitogens, leading to an increase in c-*raf* protein kinase activity.

5) Cytoplasmic regulators : V-*crk* oncogene product binds to protein tyrosine kinases and causes an increase in tyrosine phosphorylation;

6) Transcription factors or DNA binding proteins: This class of oncogenes encode nuclear proteins and many of them act as transcription factors. *Jun, fos* and *myb* oncogenes carry mutations that lead to loss of negative regulatory elements. The stimulation of resting cells by mitogens, rapidly induces the expression of *myc, jun, fos* and *rel* oncogenes, suggesting that these genes encode proteins that are required to initate a cascade of gene expression events that lead a cell through G1 into S phase.

In this class, three oncogenes, *p53*, RB (retinoblastoma) and WAGR (Wilms' tumor/aniridia/genitourinary/mental retardation), are known variously as tumor suppressor genes, growth suppressor genes, recessive oncogenes or anti-oncogenes. Since, these genes contribute to oncogenicity through their loss rather than through their

activation, it has been inferred that the genes serve as repressors of biochemical function and cellular proliferation (Nishimura and Sekiya,1987; Travali *et al.*,1990; Bishop, 1991; Hunter,1991; Solomon *et al.*,1991).

It has become clear that proto-oncogene products are involved in biochemical processes connected with either the proliferation or differentiation of cells. The first mechanism in the activation of proto-oncogenes is phosphorylation of proteins on serine, threonine and tyrosine (e.g. growth factors, growth factor receptors), followed by transmission of signals by GTPases (e.g. *ras* proteins), and finally by control of transcription from DNA by transcription factors which are encoded by proto-oncogenes (e.g. *fos* and *myc*) (Bishop,1991). How the biochemical functions of these proto-oncogene products are connected with their transforming potentials are still poorly understood. Genetic alterations have been found in one or other proto-oncogene with some consistency in a variety of human malignancies, causing changes either in gene expression or in the gene product itself (Bishop,1991). Cells overexpressing the growth factor related gene (c-*sis*) can grow in the absence of the related growth factor (PDGF) in the medium. A point mutation in a receptor gene (*Her2*) causes the receptor to become oncogenic. Oncogenes can replace growth factors, receptors for growth factors or some cellular genes that are induced by a growth factor. For example, over-expression of c-*myc* oncogene abrogates the requirement for PDGF (Travali *et al.*,1990). Analysis of the oncogene product then is important for understanding the mechanism of cell transformation, cell proliferation and differentiation.

1.2.2. Activation of oncogenes

Cancer is the result of the accumulation of multiple genetic changes. Two classes of genes are implicated in cancer including some cellular genes (the proto-oncogenes) which are activated by several mechanisms, and some other cellular genes, often referred to as tumor suppressor genes which are inactivated rather than through their activation (Solomon *et al.*, 1991).

Oncogenes have been identified as one of the essential elements in the process of malignant transformation. By modifiying or increasing the expression of a proto-oncogene, the regulation of cell growth is affected. In several systems there is evidence that the cells have to withdraw from cell cycle in G1 to differentiate, and by promoting continuous growth, oncogenes could block differentiation by preventing cells from leaving the cycle (Hunter, 1991). A proto-oncogene can become oncogenic by various mechanisms :

a) Point mutations which cause increased specific activity by alteration of the protein structure or by exchanging the amino acids that are targets for post-transcriptional modifications (Varmus, 1984; Nishimura and Sekiya, 1987). For example, the point-mutated cellular Harvey *ras* (H-*ras*) gene was the first transforming gene to be isolated from a human tumor, and was homologous to a retroviral oncogene that encodes a protein (p21) with a single amino acid substitution in codon 12 or 61. These mutations resulted in the alteration of the conformation of *ras* p21 and the specifity in the nucleotide binding site residing in this domain of the protein (Duesberg, 1985).

b) Gene rearrangement or translocation causing deregulated synthesis of gene products (Schönthal, 1990; Solomon *et al.*, 1991). For example, in chronic myelogenous leukaemia (CML), a reciprocal translocation between chromosome 9 and chromosome 22 was found

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with 95% frequency (Nishimura and Sekiya, 1987). The c-*abl* gene located on chromosome 9 was shown to join to the *bcr* (breakpoint cluster region) gene of chromosome 22 (Heisterkamp *et al.*, 1985), resulting in the formation of a fused gene that encodes a larger c-*abl* protein and unmasking the tyrosine kinase activity of *abl* gene (Konopka *et al.*, 1984).

c) Gene amplification causing overproduction of a gene product affecting the activity of individual effector genes (Schönthal, 1990; Solomon *et al.*, 1991). For example, amplification of N-*myc* gene was frequently observed in surgical specimens and cell cultures of neuroblastoma and retinoblastoma, resulting in the overproduction of the protein products (Emanuel *et al.*, 1985; Shiloh *et al.*, 1985).

One or more of these abnormalties may be responsible for the transforming activity of a proto-oncogene. Molecular analysis of human cancers and tumor cell lines commonly show multiple genetic lesions of these types in a single cancer (Nishimura and Sekiya, 1987). For example, in a human leukaemia cell line and in pancreatic cancer, N-*ras* and K-*ras* mutations respectively, coexist with an amplification of the c-*myc* gene (Murray *et al.*, 1983; Yamada *et al.*, 1986).

Several genetic alterations were observed in a human malignant carcinoma. At the early stages of colon carcinoma (hyperplastic adenoma), an allelic loss is observed in chromosome 5 (Vogelstein *et al.*, 1989). This results in inactivation of the suppressor genes *MCC* (mutated in colorectal carcinoma) and *APC* (adenomatous polyposis coli) (Kinzler *et al.*, 1991a, 1991b). In the earliest stages of polyp formation, the c-*myc* gene is also hypomethylated (Sharrard *et al.*, 1992). Two cytogenetic abnormalities associated with later stages are the 17p and 18q chromosome deletions (Vogelstein *et al.*, 1989; Fearon and Vogelstein, 1990). 75-80% colon carcinomas show a "loss"

of *p53* activity, one through deletion, the other through a point mutation affecting 17p (Vogelstein, 1990), and the *DCC* gene (deleted in colorectal carcinoma) on chromosome 18 is also lost. Mutations in the K-*ras* gene are also associated with colon cancer (Vogelstein, 1990). Thus, both types of oncogenes, the suppressor and activated classes are involved in the event.

One attractive possibility is that oncogenes may function by stimulating or repressing the expression of other regulatory proto-oncogenes or cellular genes. This mechanism is especially connected with oncogenes whose protein products are located in the nucleus, such as *myc*, *fos*, *myb*, *jun* and *p53* (Leibovitch *et al.*, 1987; Owen and Ostrowski, 1987). For example, transformation by the v-*abl* oncogene may cause the amplification of c-*myc* oncogene (Colledge *et al.*, 1989) or overexpression of the cellular *p53* protein (Rotter *et al.*, 1980).

1.3. EFFECTS OF ONCOGENE EXPRESSION ON
DIFFERENTIATION

Oncogenes have been shown to affect the differentiation of various cell types. For instance, v-*src*, v-*fps*, v-*erbB*, v-*myc* and v-*ski* transform myoblasts preventing the formation of myotubes (Holtzer *et al.*, 1975; Alemâ and Tato, 1987). Recently, it has been reported that c-*myc* inhibited the differentiation of NIH 3T3 cells rendered myogenic by transfection with the *MyoD* gene (Miner and Wold, 1991), and also the differentiation of L6 rat myoblasts (Denis *et al.*, 1987). V-*jun* transfection of quail myoblasts and myoblasts from chicken skeletal muscle, blocked myogenic differentiation by preventing expression of some muscle specific genes (Grossi *et al.*, 1991; Su *et al.*, 1991). In contrast, the *ski* oncogene has been shown to induce muscle development in quail embryo fibroblastoid cells (Colmenares

and Stavnezer, 1989). *Ras* and *fos* can block myogenesis by preventing the expression of the transcription factors *MyoD1* and *myogenin* which are involved in myogenic differentiation (Lassar *et al.*, 1989). It was reported that the loss of myogenic capacity by transformation with the v-*fos* oncogene, correlates with the inactivation of certain oncogenes c-*fos*, c-*myc*, *erbB*, *erbA*, *src* and *sis* in two neoplastic cell lines M4 and RMS4 derived from rat myogenic cells (Leibovitch *et al.*, 1987).

However, there are also circumstances in which oncogene expression is elevated in non-dividing or differentiated cells, suggesting that certain cellular oncogene products may have specific functions during cell differentiation. For example, expression of c-*fms* (Sariban *et al.*, 1985) and c-*fos* (Zelenka, 1990) is increased during the differentiation of monomyelocytes to form macrophages; c-*src* expression is also increased at particular stages of neural differentiation (Adinolfi, 1988); c-*myc* oncogene expression is generally associated with cell proliferation, but there are also several instances in which c-*myc* mRNA levels increase in the differentiation process of lens epithelial cells, PC12 cells, HL60 cells and chronic lymphocytic leukemia cells (Zelenka, 1990).

1.5. THE *RAS* FAMILY AND DIFFERENTIATION

The *ras* oncogene has been shown to affect differentiation in various cell types in different ways. It induces resistance to retinoic acid, which is a potent effector of epithelial cell growth and differentiation (Tainsky *et al.*, 1991), and neurogenic differentiation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Muroya *et al.*, 1992). Expression of transforming *ras* genes in different myoblast cell types inhibited myogenic differentiation by blocking or downregulating the

expression of transcription factors *MyoD1* and *myogenin* (Konieczny *et al.*, 1989; Lassar *et al.*, 1989). N-*ras* and H-*ras* expression in C2 and CO25 cells blocked the induction of muscle specific proteins (α-actin, desmin, Myosin Heavy Chain) , and led to expression of nonmuscle proteins β- and γ- actin and vimentin (Olson *et al.*, 1987; Gossett *et al.*, 1988; Olson and Capetanaki, 1989). In a mouse myogenic cells 23A2, the activated H-*ras* gene prevented muscle differentiation, and caused two- to threefold more PKC activity than wild-type myofibres (Vaidya et al., 1991)

1.6. MYOBLAST CELL SYSTEM

The progression of tumorigenesis has been studied in various systems, but the investigation of colorectal cancer has received most attention at the genetic level (Hunter, 1991). The difficulty of obtaining samples at different stages, and in controlling the possible interaction with other tissues has hindered systematic progress. Studies on cell cultures have therefore been more popular. In a cell culture system, the effects of an oncogene can be studied following its transfection.

CO25 myoblast cells have been chosen in this work as an attractive model system since it allows the identification of transformed cells which can be clearly distinguished from their differentiated counterparts which form multinucleated myotubes. There is also a considerable background of biological information available about the system.

A C2 cell line was established from primary cultures prepared from injured mouse thigh muscle (Yaffe and Saxel, 1977). As shown in Diagram.1, the CO25 cells were derived by transfection of the C2 cell with a plasmid containing a mutationally activated human N-*ras* gene

(in codon 61) under transcriptional control of the steroid-sensitive promotor of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Gossett *et al.*,1988). Dexamethasone (DEX) treatment of the cells leads to the induction of the *N-ras* gene and the development of the transformed phenotype. Differentiation is inhibited in mitogen deficient medium, horse serum (10% HS). But both C2 and CO25 cells can proliferate in 20% foetal calf serum (FCS). As the cells are transferred to 10% HS, with low mitogenic activity, they start to fuse and form multinucleated myotubes. Changes in the morphology of the cells either during differentiation or the transformation process enable the resercher to monitor the stages in either process.

In CO25 cells, the expression of the N-*ras* gene has been shown to mimic the inhibitory effects of FGF and TGF–β in myogenic differentiation (Olson *et al.*,1987). Inhibition of differentiation of CO25 myoblasts by the induction of N-*ras* oncogene do not require the proliferation of the cells. The mechanism of inhibition was transient and reversible, and *ras* interfered with the muscle- specific activation at an early stage of the process by preventing the activity of CK and ACh receptors (Gossett *et al.*,1988). CO25 cells continued to express vimentin, β- and γ- actin at elevated levels which normally declined during differentiation, following *ras* stimulation, and failed to upregulate either α- sarcomeric actin or desmin. This suggested that *ras* was responsible for downregulation of myoblast- specific genes as well as upregulation of myotube- specific genes (Olson *et al.*,1987; Olson and Capetanaki,1989).

mouse
muscle

C2 myoblast cell

Mutated
N-ras gene

CO25 cell

MYOBLASTS
growth medium
(20% foetal calf serum)

MYOTUBES
fusion promoting medium
(10% horse serum)

FOCI
transformation medium
(10% horse serum + dexamethasone
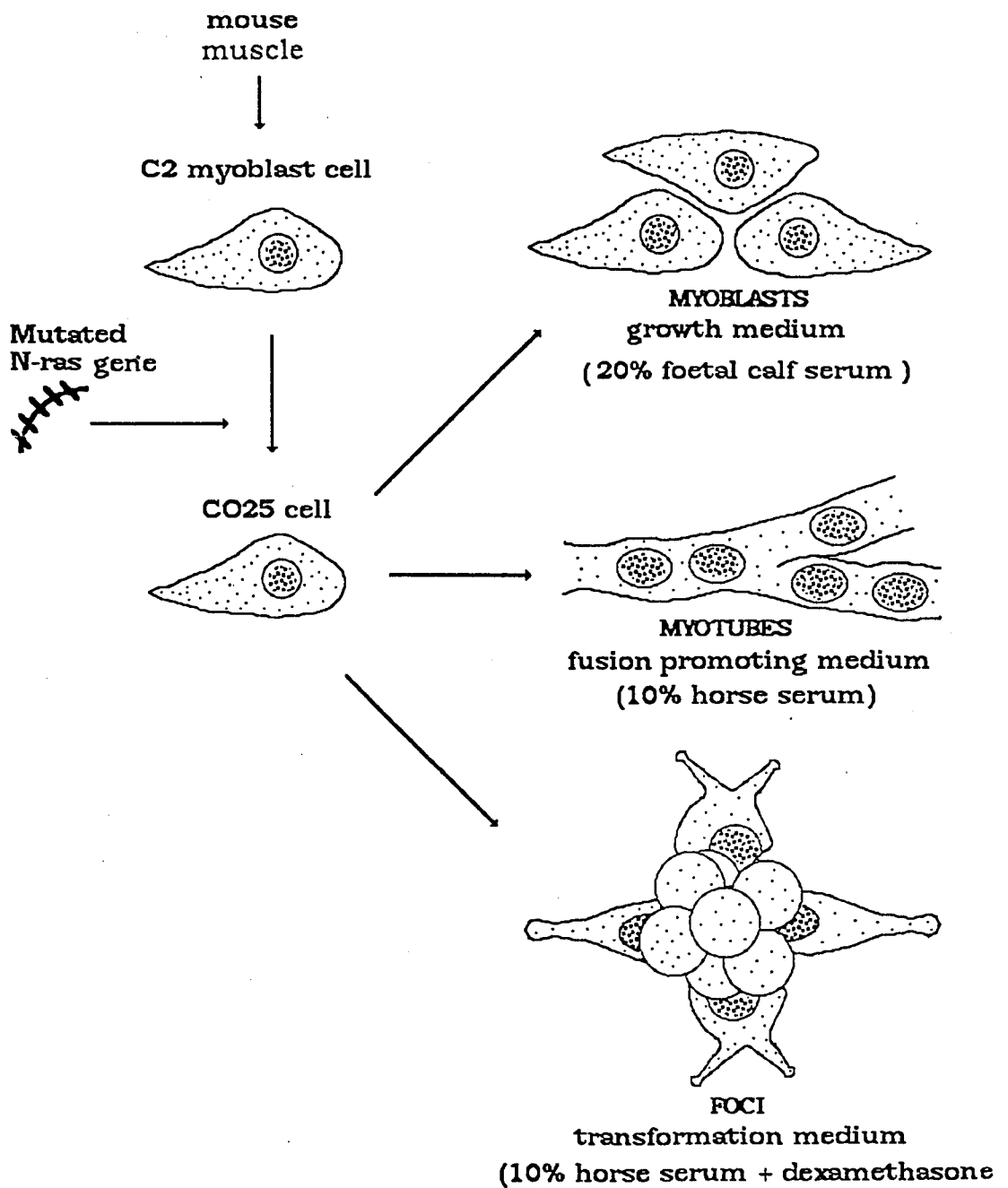
Diagram. 1. The system of CO25 myoblast cells used in this thesis. CO25 cells derived from C2 myoblasts by the transfection of a human N-*ras* oncogene (mutated at codon 61), were cultured as described in section 2.4.1 and 2.4.2. 20% foetal calf serum was used as agrowth medium. In 10% horse serum, they formed myotubes. Addition of dexamethasone into the horse serum induced the expression of the N-*ras* oncogene, and cells formed foci.

C2 myoblast fusion is also accompanied by a dramatic decline in levels of c-*myc* mRNA. In contrast, C2 cells bearing activated N-*ras* or H-*ras* oncogenes failed to down regulate, and exhibited an increase in levels of c-*myc* under fusion-promoting medium (Olson *et al.*,1987). In a recent report, Yablonka-Reuveni *et al.* (1990) demonstrated that C2 cells have high binding sites for the PDGF-BB growth factor which promoted cell proliferation and depressed the frequency of differentiated cells. The role of *ras* in interfering with the signal pathway stimulated by these receptors in myoblast cell lines is unknown.

1.7. THE AIM IN THIS STUDY

The inital part of this investigation comprised a study of the effects of N-*ras* gene expression on the growth rate, morphology, actin distrubition and transcriptional levels of some cellular oncogenes, c-*myc, p53* and K-*ras*. These cellular oncogenes have been shown to have a role in the differentiation and proliferation processes (Bishop, 1983; Adinolfi,1988; Zelenka,1990), and are also affected by the expression of *ras* oncogene in various cell systems (Owen and Ostrowski,1987; Sistonen *et al.*,1989; Godwin and Lieberman,1990).

During this study, the role of the N-*ras* product, p21 in signal transduction was reported (Wakelam *et al.*,1986; Downward *et al.*, 1990; Smith *et al.*,1990; Burgering *et al.*,1991). I therefore investigated effects of *ras* p21 on various stages in the signal transduction pathway stimulated by PDGF. For example, phosphorylation of cellular proteins at tyrosine residues following PDGF stimulation of the cells. The phosphorylation or quantitative variation in PDGF receptors, GAP and/or PLC proteins (which are involved in the early stages of the signal transduction) could be

investigated in normal, differentiated or *ras*-induced CO25 myoblast cells. The effects of *ras* p21 on the second messenger systems stimulated by other growth factors or hormones could also be studied. I also looked at the changes in the intracellular free calcium as an indicator of second messenger effects following the addition of PDGF, BK or FCS to these cells. This model system involved a study of the role of growth factors in cell proliferation and differentiation and an in depth study of the mechanism by which genes like *ras* control such processes at the biochemical level.

CHAPTER.2.

EXPRESSION OF THE *N-RAS* ONCOGENE AND THE INDUCTION OF THE TRANSFORMED PHENOTYPE IN CO25 MYOBLAST CELLS

2.1. INTRODUCTION TO THE *RAS* GENE

2.1.1. THE *RAS* GENE FAMILY

The *ras* oncogene family has an important role in transformation and tumorigenesis. Point mutations in ras genes are found at different frequencies in different human tumors types analysed with the most common point mutations occurring at codons 12 and 13 (Barbacid,1987; Bos,1988; Downward,1992). For example, K-*ras* mutations were found in 90% of pancreatic carcinomas, in 50% of colon carcinomas, in 40% of seminomas and in 30% of lung adenocarcinomas. N-*ras* mutations were found in 30% of acute myeloid leukemias, in 40% of seminomas and in 20% of melanomas (Bos *et al.*,1985,1989).

Ras protein (*ras* p21) is located on the inner side of the plasma membrane, and appears to act as a molecular switch in signal transduction from growth factor receptors on the cell surface to effector molecules (Grand and Owen,1991; Haubruck and McCormick,1991; Lowy *et al.*,1991). The attachment of *ras* p21 to the membrane is essential for biological function, and it is facilitated by a number of post-translational modifications at the carboxy terminus, called the CAAX motif (in which C is cysteine, A is any aliphatic amino acid, X is a variable residue) (Santos and Nebreda,1989; Gibbs,1991). *Ras* proteins are first isoprenylated at the C-terminal cysteine by a thioester linkage and the last three residues are cleaved (Goodman *et al.*,1990; Schaber *et al.*,1990; Sinensky and Lutz,1992), followed by methylation (Casey *et al.*,1989; Hancock *et al.*,1989) and a cysteine[186] is acylated through a thioester linkage with palmitic acid (Marshall,1986; Hancock *et al.*,1991; Haubruck and McCormick,1991; Grand and Owen,1991).

There are four members of the *ras* gene family, H-*ras* (Harvey *ras*), K-*ras* (Kirsten *ras*) and N-*ras* (Neuroblastoma *ras*) which is an oncogene with no viral equivalent. The primary structures of all *ras* proteins are very similar and highly conserved between species at the N-terminal end (Barbacid,1987; Grand and Owen,1991; Lowy *et al.*,1991). The carboxy-terminal region shows diversity between *ras* proteins. Several residues in this area undergo post-translational modification (Downward,1990).

Each *ras* gene encodes a 188-189 amino acid protein of 21 kD molecular weight derived from four coding exons and which is attached at its C-terminal end to the inner side of the cell membrane (Marshall,1986; Lowy *et al.*,1991). Various regions in p21 have been shown as interactive sites for guanine nucleotide and other proteins (positive or negative regulators and effector molecules, see Fig.1.) by mutational and crystallographic studies. Two areas of the protein comprising amino acids 116-119 and 145-147 are highly conserved in all *ras* proteins, and involved in binding to the purine base of GDP (Pai *et al.*,1990). The phosphate groups bind to amino acids 12-20, 32-35 and 60. The GAP (which is a negative regulator for p21) binds to the " effector region " at amino acids 32-34 (Adari *et al.*,1988; Grand and Owen,1991).

Ras genes are members of a supergene family, and these for code over 50 small proteins (20-30 kD). These proteins are usually grouped into four subfamilies; termed true *ras*, *ras*-like, *rho* and *rab/YPT*. All of them are involved in guanine nucleotide binding and they share amino acid sequence motifs. *Ras*-like proteins have about 50% homology, *rho* about 35% homology and *rab/YPT* about 30% homology with " true *ras* " proteins (Downward,1990; Bourne *et al.*,1991; Drivas *et al.*,1991; Grand and Owen,1991).

2.1.2. ACTIVATION AND INACTIVATION OF p21

The *ras* proteins represent a subset of 20 small GTP binding proteins that include the heterotrimeric G proteins (Landis *et al.*,1989; Birnbaumer,1990; Haubruck and McCormick, 1991). Like other guanine nucleotide-binding proteins, the activity of p21 is regulated by the binding of guanine nucleotides GDP (inactive state) and GTP (active state) with high affinity and possess intrinsic GTPase activity. Normally only a small proportion of cellular p21 is GTP bound (Gibbs *et al.*,1990; Satoh *et al.*,1990). However, mutationally activated *ras* genes encode proteins with reduced hydrolysis of GTP and therefore accumulate much higher levels of the GTP-bound form (Grand and Owen,1991; Haubruck and McCormick,1991; Lowy *et al.*,1991; Marshall,1991).

As shown in Fig.1., inactive p21.GDP is activated following the release of GDP by a group of membrane associated proteins termed Guanine Nucleotide Releasing Proteins (GNRP) of molecular mass between 35-160 kD, followed by the binding of GTP to the p21 molecule by inhibiting GTPase stimulating proteins (GAP, NF1). GNRP stimulates GDP release from other products of the *ras*-related superfamily and is thought to work by relaxing hydrogen bonding in

Fig.1. (next page) Model of p21^{ras} regulation showing the activation and inactivation states of the protein. Signals are transmitted through activated growth factor receptors, resulting in the activation of p21 by stimulating the guanine nucleotide releasing protein (GNRP), or inhibiting GTPase stimulators (GAP, NF1). Activated p21.GTP can be recognized by both GAP, NF1 and target effector molecule which transmits the biological signal from p21.GTP in to the cell. The hydrolysis of GTP to GDP by GAP or NF1 returns p21 to the inactive state (modified from Lowy et al.,1991 and from Downward,1992).
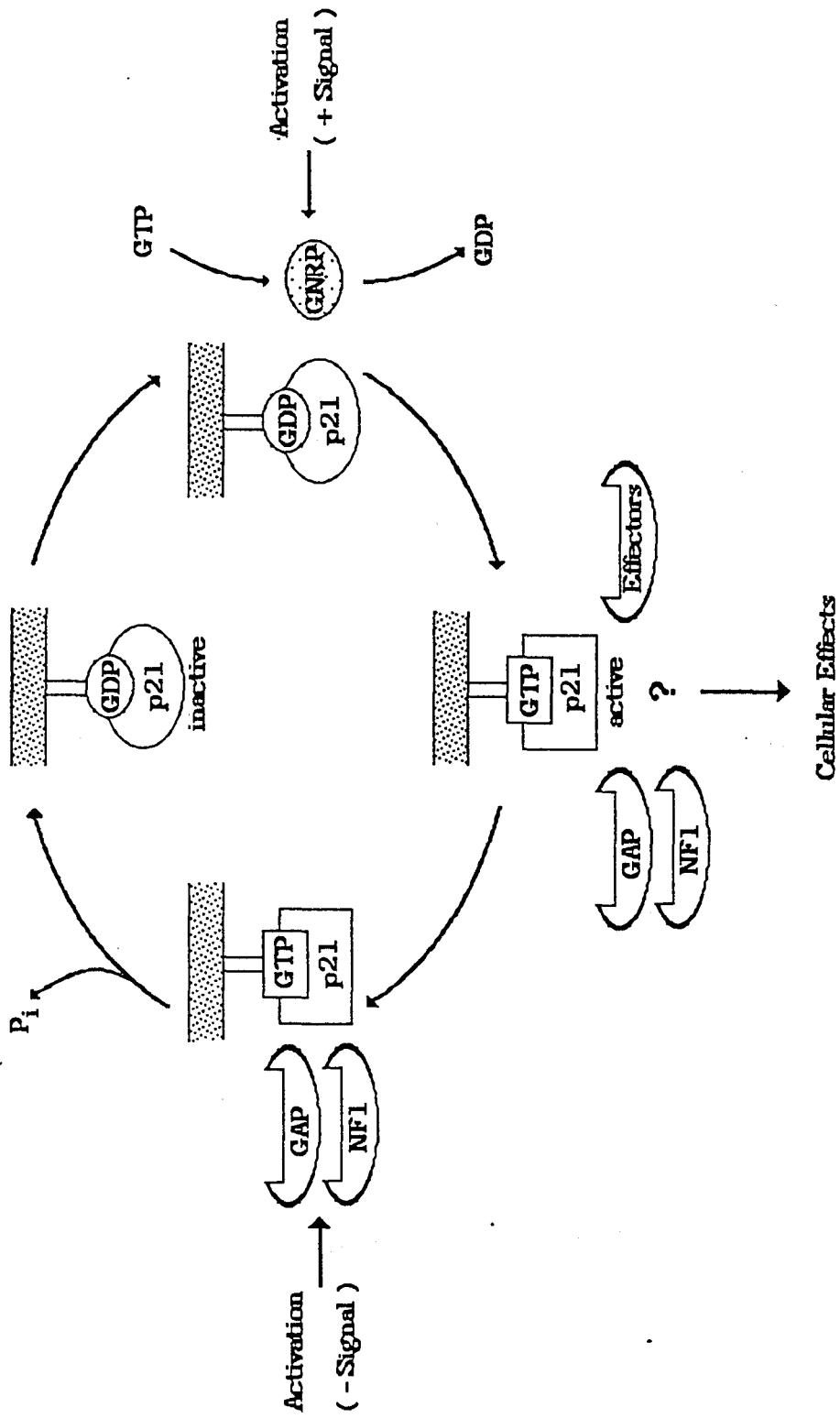
p21.GTP complex has an effector domain which can be recognized by both GTPase-stimulating proteins (GAP, NFl) and target effector molecules. GAP serves as a negative regulator of p21, potently stimulating the GTPase activity intrinsic to p21, and catalysing the conversion of p21.GTP to the inactive GDP bound form. There is a family of GAP molecules which interact with their own target within the *ras* superfamily. Thus *ras* GAP binds to *ras* and increase its GTPase activity. GAP is a 120 kD cytoplasmic protein and the normal proteins encoded by all mammalian *ras* genes are fully sensitive to GAP (Adari *et al.*,1988; Calés *et al.*,1988; Garrett *et al.*,1989; Santos and Nebreda,1989; Grand and Owen,1991; Lowy *et al.*,1991).

The second mammalian protein that may have GTPase stimulating activity towards p21 is encoded by the neurofibromatosis type 1 *(NF1)* gene. *NF1* is the gene responsible for which is characterised by a high frequency of benign tumors (neurofibromas) and malignant tumors (neurofibrosarcomas) of neural crest origin, along with a number of development abnormalities, probably caused by germ line mutations in one allele of the *NF1* gene (Grand and Owen,1991; Lowy *et al.*,1991). *NF1* gene encodes a protein, p280^{NF1}, designated as neurofibromin (DeClue *et al.*,1991a), with a large region of homology with the products of the yeast *IRA* genes which are the negative regulators for *RAS1* and *RAS2* in *S. cerevisiae* (Xu *et al.*,1990); and a small region of homology with the GAP (Trahey and McCormick, 1987; Grand and Owen,1991; Lowy *et al.*,1991). Recently, DeClue *et al.* (1992) reported that neurofibromin may be a tumor suppressor gene product that negatively regulates p21, since they found reduced neurofibromin,

decreased cellular GAP-like activity and increased GTP binding by nonmutant *ras* p21 in the tumor cell lines.

2.1.3. ONCOGENIC ACTIVATION OF *RAS* GENES BY MUTATIONS OR OVEREXPRESSION

Transformation by p21 can occur either by overexpression of normal p21 or through mutational activation of p21. In natural or induced malignancies, analysis of *ras* gene showed that oncogenic point mutations occured at amino acids 12, 13, 61 and 63, with residue 59 implicated from studies with viral *ras* analogues. All these residues lie near the sites of interaction of the p21 with the β and γ phosphate residues of the bound GTP. In these mutants, the p21 was found to be resistant to regulation by the activity of GAP and NF1 (see above), remaining bound to GTP. In additional studies, mutations *in vitro* of cloned *ras* genes have revealed many other sites where mutations give rise to transforming ability, at amino acids 116, 117, 119 and 146, sites of interaction of p21 with the guanine base of GTP. These mutants showed an increased rate of nucleotide dissociation (Taparowsky *et al.*,1983; Levinson,1986; Barbacid, 1987; Pai *et al.*,1989; Santos and Nebrada,1989; Downward,1990; Grand and Owen,1991; Lowy *et al.*,1991; Downward,1992). In all cases those activating mutations cause a significant decrease of the GTPase activity and therefore more of the GTP bound active p21 form than the inactive GDP form. Hydrolysis of p21-GTP is normally accelerated over 100 fold by GAP , but it has no affect on the GTPase activity of oncogenic *ras* proteins.

The analysis of mutated *ras* genes in the human tumor, adenocarcinoma of the lung, have revealed the possible involment of chemical mutagens, such as tobacco smoke, in the mutational event

(Bos, 1989). Similar activation of *ras* oncogenes have been observed in tumors induced experimentally by chemical carcinogens (Balmain, 1986). A chemical initiator dimethylbenzanthracene (DMBA), and a promotor 12-O-tetrade canoyl-phorbol-13acetate (TPA) induced specific A-T mutations at codon 61 in tumors (Quintanilla *et al.*, 1986). Nitrosomethylurea (NMU) induced mammary carcinomas in rats and produced the same G-A transitions at the second base of codon 12 in the H-*ras* gene (Zarbl *et al.*, 1985; Hamm, 1990).

In some cases, overexpression of the normal *ras* oncogene can induce transformation by the integration of a low number of copies of long terminal repeat (LTR)-linked *ras* genes in many cell systems. For example, in NIH-3T3 cells, over expression of normal N-*ras* gene bearing MMV-LTR, inducible by dexamethasone, fully transformed the cells, and induced DNA synthesis (McKay *et al.*, 1986; Santos and Nebreda, 1989).

2.1.4. *RAS* EFFECTS

Several lines of evidence have suggested that the normal *ras* gene acts as a transducer through a signal network following stimulation of cell proliferation by growth factors. The activity of growth factors is blocked when *ras*-specific neutralizing antibodies are injected into cells (Mulcahy *et al.*, 1985). Transformation of cells by *ras* oncogenes reduced growth factor requirement for their proliferation and induced the proliferation of quiescent cells (Weisman and Aaronson, 1983; Stacy and Kung, 1984). Overexpression of *ras* gene increased the rate of abnormal mitosis mostly during metaphase, induced disorganization of actin (Hagag *et al.*, 1990), cells also lost their anchorage dependency by growing on

top of each other, and developed membrane ruffling with extensive microvilli on their plasma membranes (Feramisco *et al.*,1984; Bar-Sagi and Feramisco,1986; Linstead *et al.*,1988; Hagag *et al.*,1990).

Recent studies have demonstrated that a certain class of growth factors stimulate the formation of active p21-GTP complexes. Satoh *et al.* (1991) reported that interleukin 2 (IL-2), IL-3 and granulocyte /macrophage colony-stimulating factor (GM-CSF) enhance the formation of p21-GTP complexes. Downward *et al.* (1990) reported that stimulation of the antigen receptor of T-lymphocytes causes activation of p21 by a mechanism which involves a decrease in the activity of GAP.

In fibroblasts, several studies have indicated that the expression level of p21 is increased by insulin and the process involves a rapid increase in p21-GTP levels (Burgering *et al.*,1991). Purified insulin receptor can phosphorylate p21 at a tyrosine residue and regulate its GTPase activity, thereby affecting its interaction with phospholipase C (PLC) (Korn *et al.*,1987). In contrast, O'Brien *et al.* (1987) reported that both normal and mutant p21 inhibited the autophosphorylation of the purified human insulin receptor tyrosyl kinase. Sjölander *et al.* (1991) recently demonstrated that phosphatidylinositol 3-kinase (PI-3K) activity is increased in a H-*ras* transformed epithelial cells which had been stimulated with insulin or insulin like growth factor (IGF).

Several results suggest that *ras* p21 play a key role in secondary messenger systems.The expression of normal N-*ras* p21 in fibroblasts leads to the coupling of certain growth factor receptors - bradykinin (BK), bombesin, vasopressin, PDGF, EGF and FCS - to the production of inositol phosphate via activation of PLC (Wakelam *et al.*,1986). Smith *et al.* (1990) indicated that *ras* protein is an

upstream effector of PLC activity in phosphoinositide- specific signal transduction, and PLC-gamma activity is necessary for *ras*-mediated induction of DNA synthesis. Regulation of PLC activity involves inositol phosphate hydrolysis and was observed in exponentially growing *ras* transformed cells (Korn *et al.*,1987; Bishop and Bell, 1988).

In different cell systems, studies have shown that the levels of inositol phosphates, diacylglycerol (DAG) and protein kinase C (PKC) are elevated after *ras* transformation (Fleischman *et al.*,1986; Parries *et al.*,1987; Wolfman and Macara,1987; Alonso,*et al.*,1988; Wakelam,1989). A major source of DAG is the agonist-stimulated hydrolysis of phosphatidyl-inositol 4,5-biphosphate (PIP$_2$) by a polyphospho-inositide specific PLC; this is accompanied by the formation of the Ca^{2+} mobilizing second messenger inositol 1,4,5-triphosphate (IP$_3$) DAG is known to activate PKC, a serine/threonine kinase which has implications in the *ras* pathway.

Zhang *et al.* (1990) reported that transformation of fibroblasts by normal H-*ras* was suppressed by GAP as a negative regulator, but transformation by activated H-*ras* (v-H-*ras*) was not suppressed. Transforming *ras* oncogene products have been found to be resistant to inactivation by GAP according to McCormick (1989). Molloy *et al.* (1989) also showed that GAP fails to effect hydrolysis of oncogenic p21-GTP complexes, allowing them to remain in their active states.

Several studies demonstrated that PDGF receptors in *ras* transformed cells are present in levels comparable to untransformed counterparts, but they are lost or diminished in their ability to stimulate PLC activities, and in their ability to phosphorylate the tyrosine residue of GAP (Benjamin *et al.*,1987; Parries *et al.*,1987;

Kaplan *et al.*,1990), production of IP_3 and Ca^{2+} release from intracellular stores (Parries *et al.*,1987; Benjamin *et al.*,1988; Polverino *et al.*,1990), and to induce c-*myc* and c-*fos* expression (Lin *et al.*,1988; Zullo and Faller,1988). In a recent report, Rake *et al.* (1991) demonstrated that in v-*ras* transformed cells, the autophos-phorylation of PDGF receptor is inhibited, without causing the loss of PDGF receptor expression, suggesting the effects of *ras* in the earliest steps of the signal transduction.

Furthermore, bradykinin receptor number has been found to be elevated (Downward *et al.*,1988; Roberts and Gullick,1989), and cells increased the production of cellular IP_3 by treatment of bradykinin following the expression of the *ras* oncogene (Parries *et al.*,1987; Downward *et al.*,1988; Hall *et al.*,1988). In *ras* transformation of rat kidney, EGF also failed to bind to its receptor (Chua and Ladda,1986). Activated *ras* has some negative regulatory effect in the control of adenylate cyclase and cyclic adenosine monophosphate (cAMP) levels after hormone stimulation in fibroblasts (Tarpley *et al.*,1986).

Following the transmission of the *ras* induced signal, changes in the expression of cellular genes have been reported. The expression of c-*myc*, TGF-α, transin, glucose transporter genes (Godwin and Lieberman,1990), transcription factor genes *jun B* and c-*jun* (Sistonen *et al.*,1989), v-*sis*-related cellular mRNA (Owen and Ostrowski,1987), and *fos* were increased in *ras* transfected cells after activation of the *ras* gene. In contrast, a decrease was found in the expression of PDGF receptor and fibronectin mRNA level (Godwin and Lieberman,1990).

Beside the role of p21 in cell proliferation, Spandidos and Wilkie (1988) showed that expression of the normal H-*ras* gene can

supress the transformed and tumorigenic phenotypes of cells containing mutant *ras* genes, suggesting its role as a tumor-supressor gene. Also several studies indicated the involment of *ras* gene in the cell differentiation rather than cell proliferation (see section 1.5).

These observations suggest that the role of normal *ras* protein in cellular regulation may be to couple cell surface receptor activation to a variety of intracellular effectors, such as enzymes, ion channels, DNA synthesis all of which modulate cellular activity. Activated *ras* can uncouple all these cellular responses at the level of the growth factor receptor and at many other stages. Despite of much work on p21 protein, it still remains a mystery how oncogenic p21 induces the transformed phenotype. It would seem that it must involve a complex series of biochemical events involving activation and inactivation of proteins.

2.2. THE TRANSFORMED PHENOTYPE OF THE CO25 CELLS

As explained in Chapter 1, a cell model system has been developed where differentiation can be suppressed or reversed by an activated *ras* oncogene. The action of the latter causes cells transformation. Characteristics of the transformed phenotype of the CO25 cells has been examined:

1) **Morphology:** As has been mentioned in section 2.1.4. chapter, morphological changes were observed in different cells including CO25. In CO25 cells, inhibition of myotube formation by the N-*ras* gene gives an excellent morphological data to the transformed phenotype. Electron microscopy was used to characterize the observed morphological changes in greater detail.

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2) Cytoskeletal changes: Cytoskeletal alterations are closely linked to changes in morphology and the particular rearrangement of F-actin stress fibres is a commonly reported phenomenon in transformed cells (Hagag *et al.*.1990). The actin microfilaments are represented by the polymeric form F-actin in the cytoplasm of non-muscle cells, and 40-70% of the cellular actin exists in the monomeric (G-actin) or the oligomeric storage form which polymerizes into filaments when needed for physiological function (Bershadsky and Vasiliev,1988; Preston *et al.*,1990).

Cytoskeletal elements such as microfilaments, intermediate filaments and microtubules were altered in many cell systems transformed by viruses, tumor promoters or oncogenes, and they have been suggested to be a potential marker for transformation events (Verderame *et al.*,1980; Holme,1990; Rao *et al.*,1990; Urbancikova and Grofova,1990; Carter *et al.*,1991; Rao and Cohen, 1991).

3) Contact - inhibited growth: Another feature of transformed cells is the loss of contact inhibition of growth (Stoker *et al.*,1966; Feramisco *et al.*,1984; Linstead *et al.*,1988; Hagag *et al.*,1990). This can be observed by growing cells past the exponential growth phase - the result of the loss of normal growth control mechanisms in the cell. As mentioned in the previous chapter, *ras* activation in CO25 cells inhibited the differentiation process independently of DNA synthesis or proliferation. Cells grew in a criss-crossed pattern by forming several layers on top of each other in contrast to forming a regular elongated cell-monolayer. Myotubes formed during cell differentiation.

2.3. MARKERS OF DIFFERENTIATION

1) In CO25 cells, the formation of multinucleated myotubes is the most important and easily observed morphological feature of differentiation. To observe the morphology of differentiated cells in this study, scanning electron microscopy and also phase-contrast microscopy were used.

2) During myogenesis, several structural muscle-specific proteins have been demonstrated. α-actin is expressed during CO25 cell differentiation process as mentioned in previous chapter. In this chapter, expression of total actin during differentiation and transformation will be investigated using immunostaining techniques.

2.4. MATERIALS AND METHODS

2.4.1 CELL LINES AND CULTURE CONDITIONS

The CO25 and C2 cell lines (donated by Dr. E. N. Olson, University of Texas) was maintained in DMEM obteined from Gibco Ltd., Paisley Scotland. This was supplemented with 20% FCS (Gibco), 1%L-glutamine and penicillin/streptomycin at 100 units/ml. Cells were gassed with 10%CO_2 / 90% air and incubated at 37°C in a Flow Laboratories CO_2 Incubator 1500.

2.4.2. INDUCTION OF TRANSFORMATION AND DIFFERENTIATION

To initiate differentiation, cultures at ~80% confluence were transferred to the same medium containing 10% HS (Gibco) as a fusion promoting medium, instead of FCS. The cells were incubated

for four days, at which time fusion and formation of myotubes were observed.

To induce the expression of the N-*ras* oncogene, the cells maintained in the 10% HS were exposed to 1μM dexamethasone for various periods of time. Dexamethasone was prepared as a 200μM stock in sterile deionized water and stored at -20°C.

2.4.3. GROWTH CURVES

The cells were seeded at a concentration of 1-2 x 10^6 cells in a 25 cm^2 flask. This was carried out in triplicate.To harvest, the medium was removed from each flask by aspiration, 2.0 ml trypsin-EDTA (ethylene diamine tetracetic acid) (0.2% : 0.04%) solution was added and incubated at 37°C for approximately 5 minutes or until the cells could be observed to be lifting from the flask. Addition of 2 ml medium inhibited any further breakdown of the cells and 0.5 ml of the resulting cell suspension was then counted. 0.5 ml of cells was added to 19.5 ml 0.9% saline solution containing 0.5% formaldehyde and counted with the aid of a Coulter counter (model ZB, Coulter Electronics Ltd., Luton, Beds) with the amplification set at 0.32.

2.4.4. LIGHT MICROSCOPY

Phase-contrast observation was carried out using a Nikon TMS inverted microscope fitted with a Nikon F-301 camera for photography. Kodak Tri-X-Pan 400 film was used.

2.4.5. SCANNING ELECTRON MICROSCOPY

Cells in growth, differentiation and transformation phase were used for scanning electron microscopy. The specimens were

prepared using a modification of the method described by Linstead *et al.* (1988). Cells grown on glass coverslips were washed in PBS and fixed in 2.5% glutaraldehyde in phosphate buffer saline (PBS), pH 7.6, for 15 minutes at room temperature.

They were rinsed in PBS for 15 minutes, post-fixed in 1% osmium tetroxide solution in PBS for further 15 minutes, and washed in distilled water for a few minutes. The coverslips were dehydrated in a series of graded alcohol solutions (30%, 50%, 70%, 90%, 100%). Critical point drying was performed using liquid carbon dioxide for 30 minutes in a Polaron Critical Point Dryer. The glass coverslips with attached cells were then stuck onto copper sample holders using double sided sellotape and gold coated in an Edwards sputter coater (model no.S105B) at 40 mA for 1 minute in an argon atmosphere. The specimens were examined in a Jeol CX100 electron microscope in scanning mode with an accelerating voltage of 20 kV and normal illuminating conditions. Photographs were taken using Ilford FP4 film.

2.5. NORTHERN BLOTTING

Non-disposable glass and plasticware were treated before use to ensure that they were RNase-free. Plasticware was thoroughly rinsed before use with 0.1 N NaOH and 1 mM (EDTA) followed by RNase-free water. Corex tubes and solutions were treated with 0.05% Diethylpyrocarbonate (DEPC) overnight at room temperature, and then autoclaved for 30 minutes to remove any trace of DEPC.

2.5.1. EXTRACTION OF TOTAL RNA

Extraction of total RNA was performed by the method described in Chomczynski and Sacchi (1987).The culture media was

aspirated off from each 100 mm diameter dish and the cell monolayers were washed twice with ice-cold PBS. Cells were lysed in the dish by addition of 2 ml denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and β-mercaptoethanol), and then transferred to a 50 ml polypropylene tube. 0.2 ml of 2 M sodium acetate pH 4.0, 2 ml of water saturated phenol, 0.4 ml of a chloroform: isoamyl alchohol (49:1) were added, and mixed after each addition. The final mixture was shaked vigorously for 10 seconds and centrifuged at 10.000 x g for 20 minutes at 4°C. The aqueous phase was removed to a fresh tube, and an equal volume of isopropanol was added, mixed, placed at -20°C for 4-5 hours. The RNA was pelleted by centrifugation at 10.000 x g for 20 minutes at 4°C. The pellet was resuspended in 0.3 ml of the denaturing solution and transferred to a 1.5 ml eppendorf tube. An equal volume of isopropanol was added to the sample, mixed and then placed at -20°C overnight. The RNA was pelleted by centrifugation at 12.000 x g for 10 minutes at 4°C. The pellet was washed with 1 ml of ice-cold 75% ethanol, dried under vacuum and resuspended in 0.5% sodium dodecyl sulphate (SDS).

Determination of RNAs quality and quantity was performed by spectrophotometer. The A_{260}/A_{280} ratios were 2.0 or more. A_{260}/A_{230} ratios were greater than 2.0 indicating no contamination with guanidine thiocyanate.

2.5.2. ELECTROPHORESIS OF RNA

RNA samples were denaturated and electrophoresed as described in Maniatis *et al.* (1982). A vertical gel in 3 mm depth was prepared containing a gel running buffer (40 mM morpholino-propanesulfonic acid, pH 7.0; 10 mM sodium acetate; 1 mM EDTA,

pH 8.0); 6% formaldehyde and 1% agarose. The gel was placed in a electrophoresis apparatus using the gel running buffer.

RNA samples in 4.5 µl volume were denatured in a mixture containing 2 µl of 5 x gel running buffer, 3.5 µl of formaldehyde and 10 µl of deionized formamide. The samples were heated at 55o C for 15 minutes, and then chilled on ice. After that 1 µl of 1 mg/ml Ethidium bromide and 2 µl of sterile loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol) were added, and then the samples were loaded on to the gel. The electrophoresis was performed at 100 V for 3-4 hours. After electrophoresis total RNAs which were stained with ethidium bromide and photographed under U.V. illumination.

2.5.3. TRANSFER OF RNA TO NITROCELLULOSE MEMBRANE

Before transfer, the gel was soaked for 5 minutes in several changes of DEPC treated water, for 30 minutes in 50 mM NaOH, 10 mM NaCl and neutralized by soaking in 0.1 M Tris.Cl, pH 7.5 for another 30 minutes at room temperature. Finally, the gel was soaked for an hour in 20 x sodium saline citrate (SSC), and then RNA was transferred on to a 0.45 um nitrocellulose filter (Bio- Trace N.T. Gelman Sciencies) using 20 x SSC as a transfer buffer overnight. After the transfer was completed, the filter was washed in 3 x SSC, air-dried and baked at 80° C for 2 hours. The gel and the filter were checked under U.V. after transfer in order to assess the efficiency of the transfer.

2.5.4. HYBRIDIZATION OF RNA

Prehybridization and hybridization were performed as described in Williams and Mason (1985). For prehybridization, the filter was placed in a sealed plastic bag with a buffer containing 50% deionized formamide; 5 x SSC; 50 mM sodium phosphate, pH 6.5; 250 µg/ml denatured, sonicated salmon sperm DNA and 10 x Dendhart solution (0.5% Ficoll, 0.5% Polyvinylpyriolidone, 0.5% BSA, bovine serum albumin) at 42°C for 4-6 hours.

Hybridization was carried on in the same buffer with the addition of 10% Dextran sulfate and heat denatured [α-^{32}P]CTP-labelled probe (specific activity > 10 c.p.m./µg) at 42°C overnight. The filter was washed four times for 15 minutes in 2 x SSC and 0,1% SDS at 50°C, and wrapped in a plastic film while still damp, then exposed to a pre-flashed Kodak X-OMAT X-ray film with an intensifying screen for different periods of time at -80°C.

The filters were re-used by removing the probe in DEPC treated distilled water at 100 °C for 5-10 minutes or until it cooled to room temperature, and hybridization was performed by the same method above.

2.5.5. PREPARATION OF THE PROBE

The probe was a 1.5 Kb fragment of human N-*ras* cDNA (Amersham), linearized with Hind III (Brown,1984) and isolated from a low melting temperature agarose (LMT) gel.The probes were labelled using a Random Primer Kit (Stratagene) by the method of Feinberg and Vogelstein (1983). Approximately 50 ng of DNA template in LMT agarose and 10 µl of random oligonucleotide primers (27 OD u/ml) were heated to 95-100 °C for 5 minutes, and then placed at 37°C. 10 µl of 5 x Primer buffer (dATP, dGTP and

dTTP, 0.1 mM each), 5 µl of [α-^{32}P]-dCTP at 3000 Ci/mmole and 1 µl of 2 u/µl T7 DNA Polymerase were added. The contents of the tube were mixed and incubated at 37°C for 2 hours. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA, pH 8.0, and then the labelled DNA was separated from unincorporated dNTPs by a column of Sephadex G-50 (Pharmacia LKB). The mixture was heated to 100°C for 5 minutes and cooled on ice before addition to the hybridization buffer.

2.6. WESTERN BLOTTING

2.6.1. CELL EXTRACTION

Cells were prepared for assays by seeding in to a 25 cm^2 flask in growth medium. When the cells reached 80% confluence, the medium was changed to fusion promoting medium with or without 1µM dexamethasone for periods of time.

Cell monolayer was rinsed twice with ice-cold PBS and then lysed in 0.5ml of extraction buffer containing; 1% NP-40, 50mM Tris Base, pH 8.0, 50mM NaCl, 50µg/ml Leupeptin and 1mM phenylmethyl sulphonyl fluoride (PMSF). The cells were transferred to a 1.5ml eppendrof tube after incubation on ice for 20 minutes with occasional rocking, vigorously vortexed for10 seconds and then centrifuged at 13000 g for 10 minutes at +4°C. Supernatant was kept for analysis. Total protein was estimated using the method by Bradford (Bradford,1976).

2.6.2. ELECTROPHORESIS AND BLOTTING

For each sample, 30 µg of protein in 20 µl volume was mixed with 5 µl of 4 x SDS sample buffer (0.4 M Tris, 0.4 M dithiothreitol,

8% SDS, 40% glycerol and 0.04% bromophenol blue, pH 6.8), denatured at 100°C for 2-3 minutes and then resolved on a 12% SDS- polyacrylamide mini gel. The Rainbow molecular weight marker (Amersham) was prepared in the similar way to the samples and loaded a parallel well on the gel. Electrophoresis was performed at 80V and 150V for the stacking gel and the resolving gel respectively, using a Tris-Glycine electrophoresis buffer (0.25 M Tris, 1.92 M Glycine, pH 8.5).

Proteins were transferred to 0.45 μm nitrocellulose (NEN Research) in a LKB Multiphor II apparatus using the Tris- Glycine buffer containing 20% methanol at a current of 15 V for 30 minutes and then the blot was air-dried .

2.6.3. IMMUNOLABELLING

After rinsing in a rinse buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.02% Tween-20), the blot was blocked for 1 hour in the rinse buffer containing 5% nonfat powdered milk and probed for 2 hours in the same buffer containing 1:100 monoclonal antibody 142EO5 to p21^{N-ras} (gift from P.Hawley, UEA). The blot was washed four times for 5 minutes in the rinse buffer, reblocked for 10 minutes in the blocking buffer and incubated with a Rabbit anti-mouse Ig (HRP),1:500, (Dako Ltd.) in the rinse buffer containing 0.2% nonfat powdered milk for 60 minutes, and then washed six times for 10 minutes each in the rinse buffer, finally twice for 10 minutes in the rinse buffer without Tween-20.

2.6.4. DETECTION OF PROTEINS

Detection of proteins was performed using the manufacturing method. After well washing, in a dark room, the blot was drained,

then incubated in a 1:1 mixture of enhanced chemiluminescence (ECL) Western blotting detection reagents A and B (Amersham) for 1-2 minutes. The blot was drained again, placed on a glass plate in a High-Speed cassette and covered with a Saran-Wrap. All excess reagents and air bubbles were squeezed out and the blot was exposed to a Fuji or Kodak X-ray film for 3-60 seconds.

2.7. IMMUNOSTAINING FOR F-ACTIN

For F-actin (polymeric actin) staining (Faulstich *et al.*,1983), cells were grown on a glass coverslip and first fixed with 4% paraformaldehyde for 15 minutes at 37°C. Paraformaldehyde fixer was prepared by dissolving in PBS at 60°C and pH was adjusted to 7.4 by 1M NaOH. After fixation, cells were permeabilised with acetone for 30 seconds at -20°C, and then washed three times in PBS containing 0.2% BSA. The cell were then stained with F-actin specific 1µg/ml rhodamine labelled phalloidin (gift from Prof. Th. Wieland) for 1 hour at 37°C in a humid chamber. The stained cells were examined under a Standard Zeiss epifluorescence microscope.

2.8. RESULTS

2.8.1. INHIBITION OF FUSION AND INDUCTION OF TRANSFORMATION OF CO25 MYOBLASTS BY THE EXPRESSION OF ACTIVATED *N-RAS*

C2 myoblasts proliferated and did not exhibit any of the biochemical or morphological manifestations of the myogenic phenotype when maintained at subconfluent densities in 20% FCS. After transfer of cultures at 80% confluence to medium with low mitogenic activity (10% HS; fusion promoting medium) in a few

days, the cells fused and formed multinucleate myotubes, and accumulated muscle-specific gene products such as muscle creatine kinase and Ach receptors (Yaffe and Saxel,1977; Olson *et al.*,1986; Olson *et al.*,1987). C2 cells were transfected with an activated human N-*ras* gene with a mutation at codon 61 (Gossett *et al.*, 1988). Transcription of N-*ras* gene was controlled by the MMTV-LTR, rendering the gene inducible by a steroid hormone such as dexamethasone. Concentrations of 1-2 µM dexamethasone are sufficient to cause levels of p21 expression which results in complete morphological transformation within 3-4 days. The effect is reversed upon removal of the dexamethasone (Gossett *et al.*, 1988).

Fig.2. shows the growth rate of CO25 cells in growth medium and fusion medium with or without dexamethasone. In growth medium, the cells proliferated up to 3 days at which time they reached confluence and ceased dividing. In the presence or absence of dexamethasone in 10% HS, there was no significant difference in the proliferation rate indicating that *ras* induced the transformation of CO25 myoblasts independent of cell proliferation, at least up to 5 days. These results confirm the observations by Gosset *et al.* (1988) and by Olson *et al.* (1987).

Fig.3. shows the morphology of CO25 and C2 cells under a series of culture conditions. In growth medium (20% FCS), CO25 cells display a normal morphology with a flattened, irregular shape with nuclei confined to a centrally located area of the sarcoplasm, comparable to C2 cells (Fig.3. a and b). Also both cell lines, in 10% HS, display similar morphology by fusing and forming myotubes after 4 days (Fig.3. c and d).
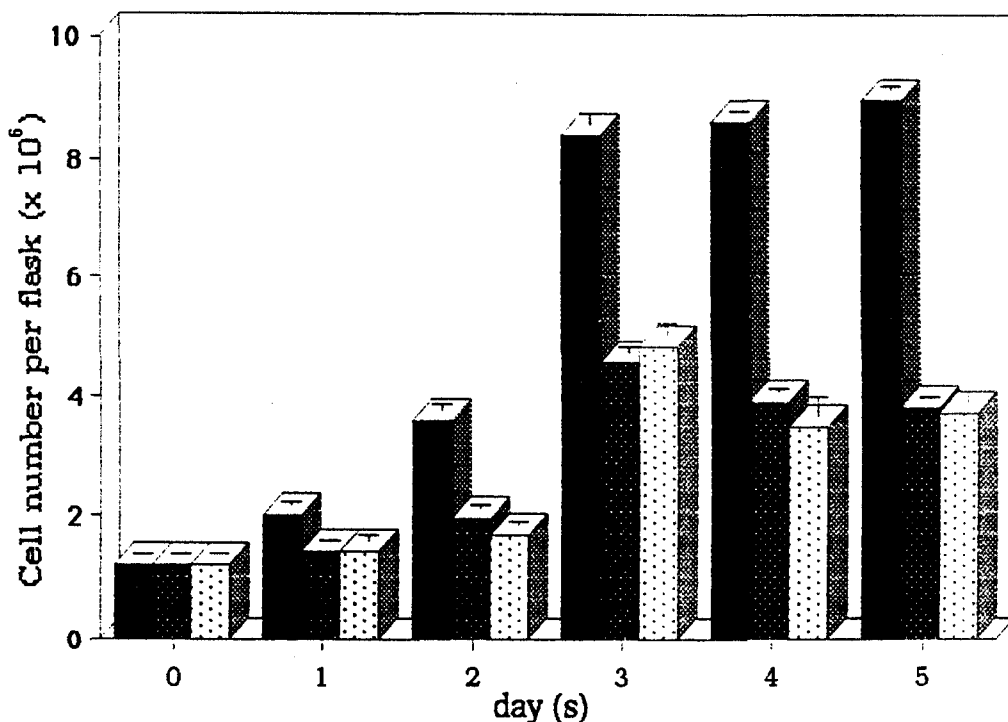
Fig.2. Growth rate of CO25 cells in growth medium (20%FCS) (■), in fusion promoting medium (10%HS) (■), and in the presence of 1mM dexamethasone in 10%HS (⊙). Plotted values represent the average of duplicate flasks from two separate experiments.

Induction of $p21^{N\text{-}ras}$ by DEX in CO25 cells correlated with marked morphological changes, unlike untreated CO25 cells in 10% HS after four days (Fig.4. a and b). Addition of 1 µM DEX to 10% HS, completely blocked the ability of CO25 cells to form myotubes. The cells became fusiform and grew in a criss-crossed fashion. After the addition of DEX for 7-10 days, the cells formed foci by growing over each other (arrow shows on Fig.3. e), and they completely failed to form myotubes. This indicates that the *ras* gene fully transforms the cells by inhibiting myogenic differentiation. After removal of the
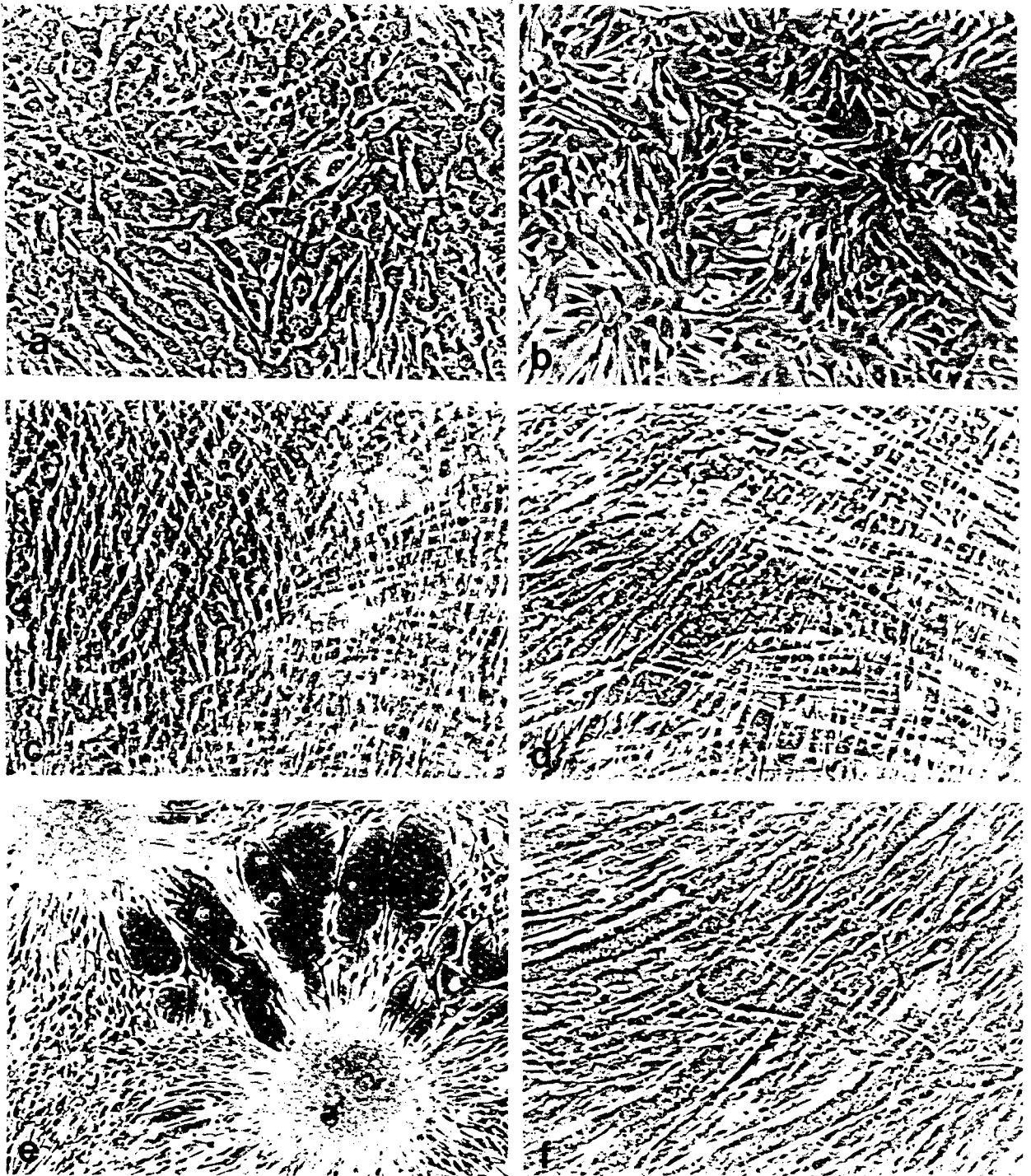
Fig.3. General view of CO25 and C2 cells in the growth medium and in the fusion promoting medium with or without dexamethasone :

(a) CO25 and (b) C2 cells in 20% FCS, magnification 240X;

(c) CO25 and (d) C2 cells in 10% HS for10 days, magnification 240X;

(e) CO25 and (f) C2 cells in 10% HS with 1mM DEX for 10 days, magnification 120X.

DEX, the cells started to fuse and formed myotubes (Fig.4. c) over a period of 7 days, indicating that the effects of DEX is reversible. Morever, parental C2 cells grew in flat monolayers, then stopped growing when they reached confluence and were morphologically unaffected by DEX. They formed myotubes (Fig.3. f) despite having endogenous *ras* genes.
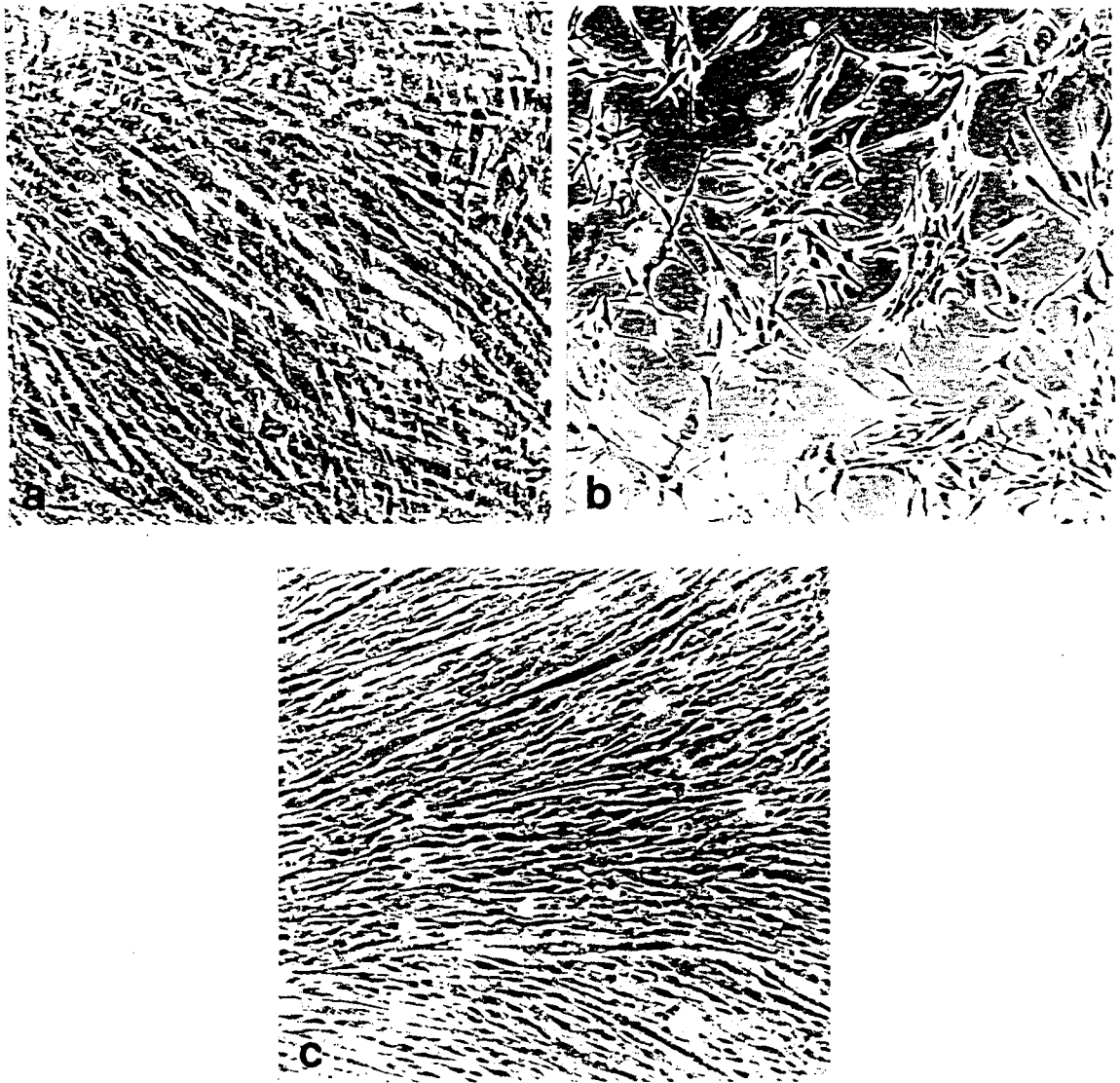
Fig.4. Reversibility of the inhibition of myogenic differentiation by activated N-*ras* oncogene in CO25 cells after removing the dexamethasone. (a) Cells were grown in 10% HS for four days, (b) Cells were grown in the presence of 1μM DEX for four days, (c) DEX was removed and cells were incubated in 10% HS for six days. Magnifications 120X.

2.8.2. THE FINE STRUCTURE OF CO25 MYOBLASTS BY SCANNING ELECTRON MICROSCOPY

The morphology of CO25 cells in different phases also was examined by scanning electron microscopy. Following activation of N-*ras* gene, there were significant ultrastructural alterations in the cells. Fig.5.a shows the flattened, irregular shape of the CO25 cells in growth medium at low magnification. A few cells in mitosis have a spherical shape, and appear as bright objects (arrowed in Fig.5.a). As seen in Fig.5.b, these cells had numerous long microvilli on their surface. Four days after *ras* activation by DEX, these cells assume a more rounded morphology as shown in Fig.6.a, and have long processes, thin ruffling membranes and numerous short and wide microvilli on their surface (Fig.6.b). In contrast, the cells incubated in the absence of dexamethasone in 10% HS, are more flattened and elongated in shape (Fig.7.a) with less microvilli on their smooth plasma membrane (Fig.7.b), and most of them started to fuse. At low magnification, densely growing cells were found with the usual artefacts in the form of a few cracks in the cytoplasm and fissures between adjacent cells.

After 7-10 days, *ras* activated cells rounded up and formed foci with piled up on top of each other (Fig.8.a) and exhibited extensive formation of microvilli and ruffling on their plasma membrane at high magnification (Fig.8.b). In contrast, cells grown in 10% HS without DEX, formed long, branched myotubes (Fig.8.c) and these myotubes have a smooth plasma membrane with a very few and short microvilli (Fig.8.d).
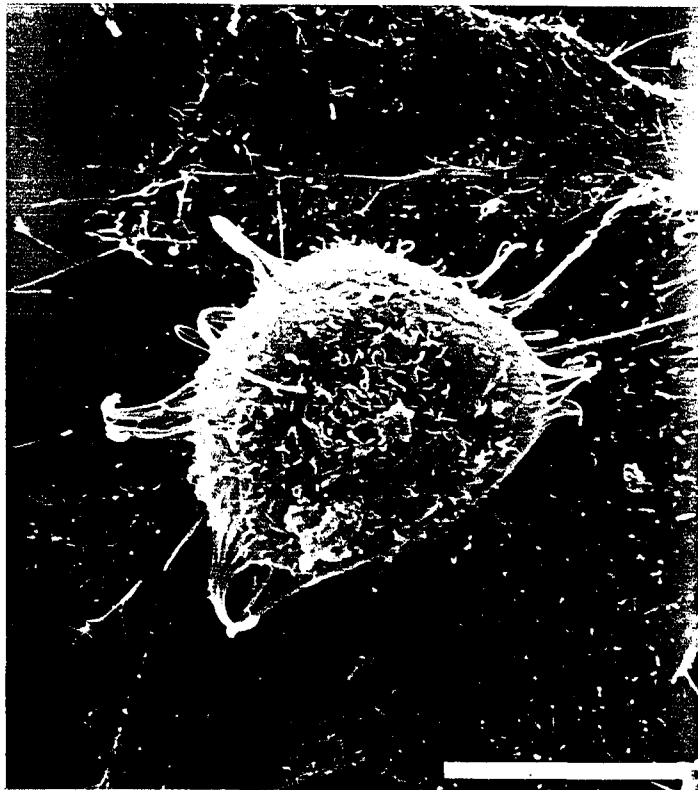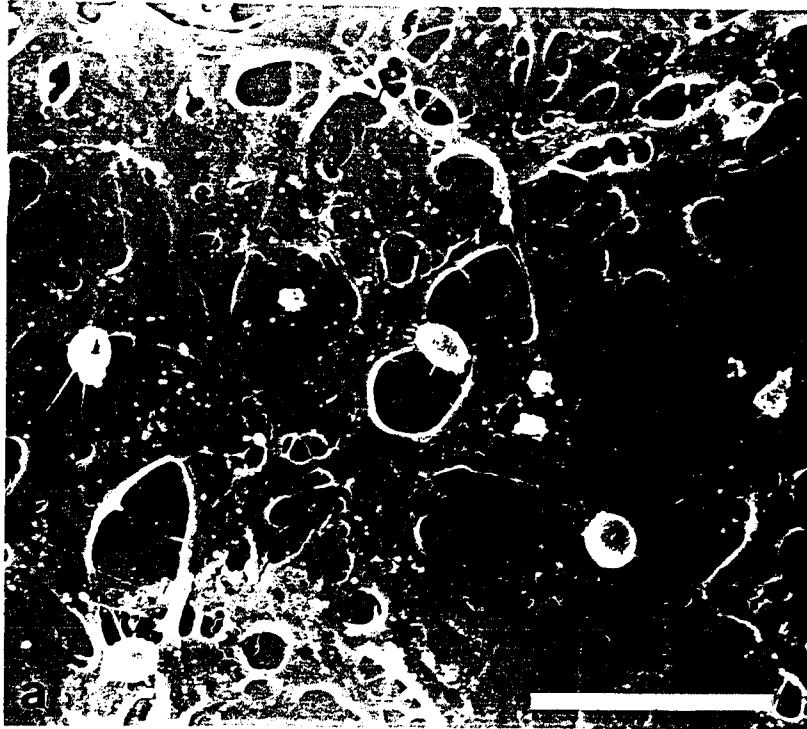
Fig.5. Morphology of CO25 myoblasts grown in 20% FCS was illustrated by scanning electron microscopy. (a) Cells at low magnification. Bar= 51 μm. (b) A cell undergoing division at high magnification. Bar= 10 μm.
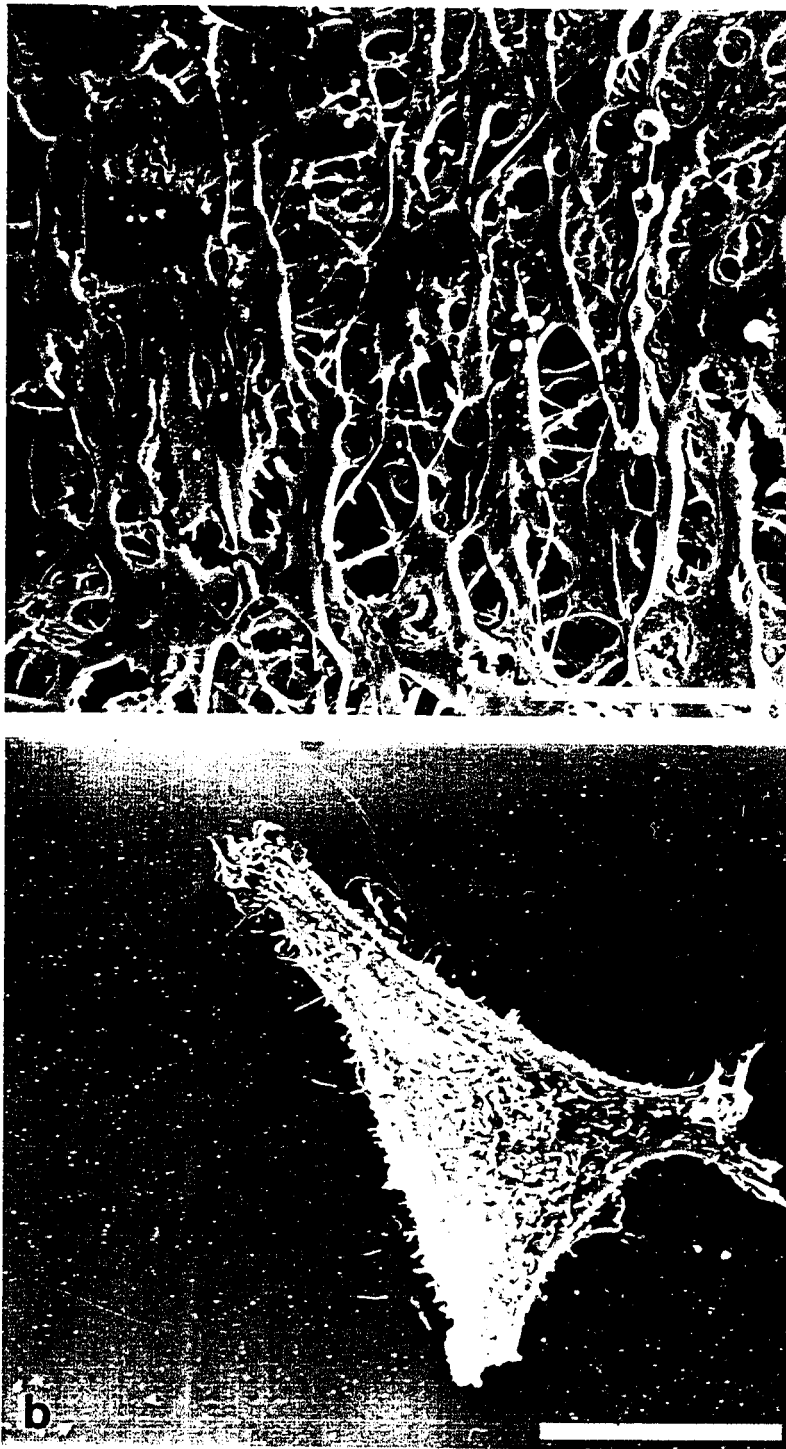
Fig.6. Morphology of CO25 myoblasts grown in 10% HS in the presence of DEX to induce expression of activated N-*ras* oncogene illustrated by scanning electron microscopy. (a) CO25 cells at low magnification. Bar= 50µm. (b) A cell at high magnification. Bar= 10 µm.
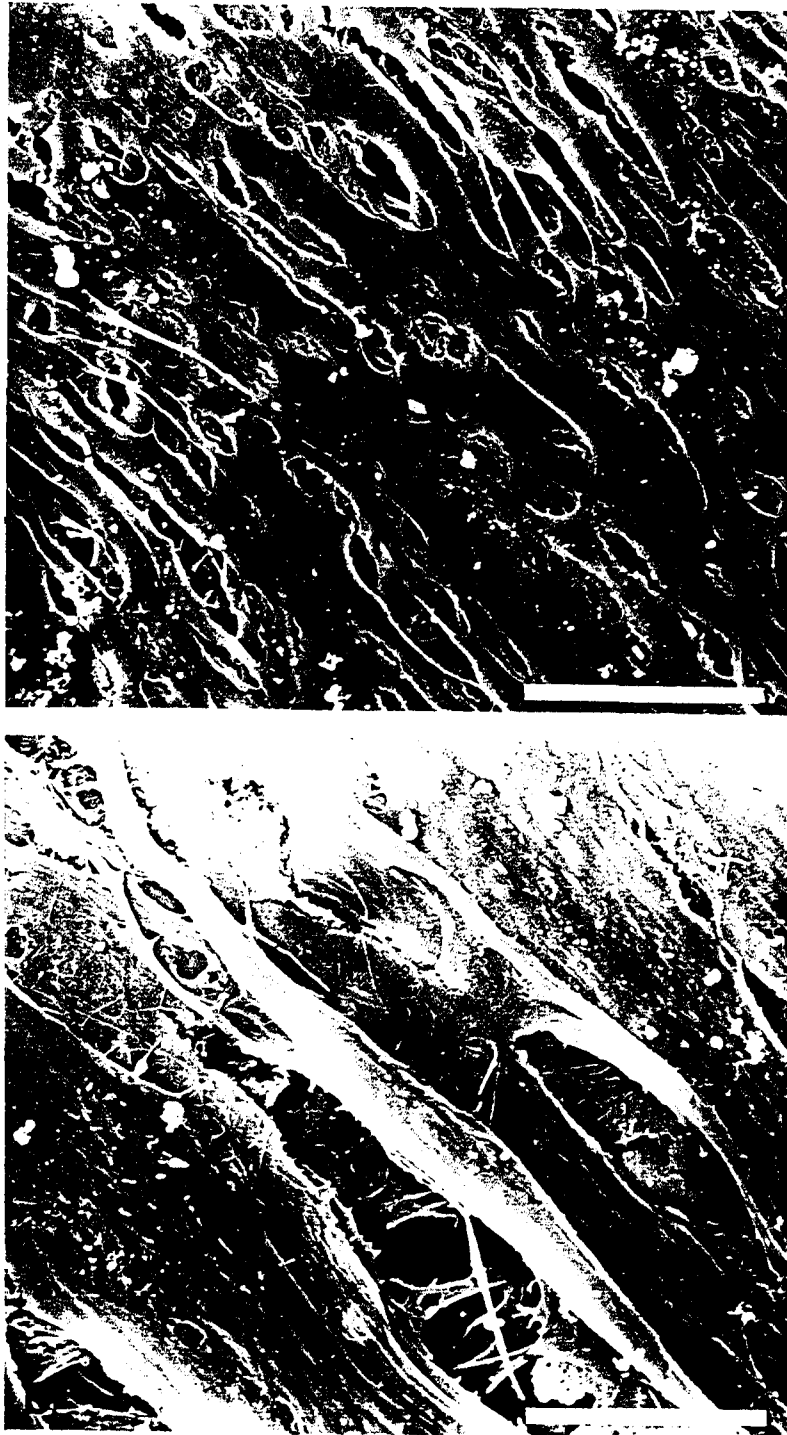
Fig.7. Morphology of CO25 myoblasts grown in 10% HS for four days to initiate the differentiation was illustrated by scanning electron microscopy. (a) CO25 cells at low magnification, arrows show elongated cells. Bar= 50μm. (b) Elongated CO25 cells at high magnification. Bar= 10 μm.

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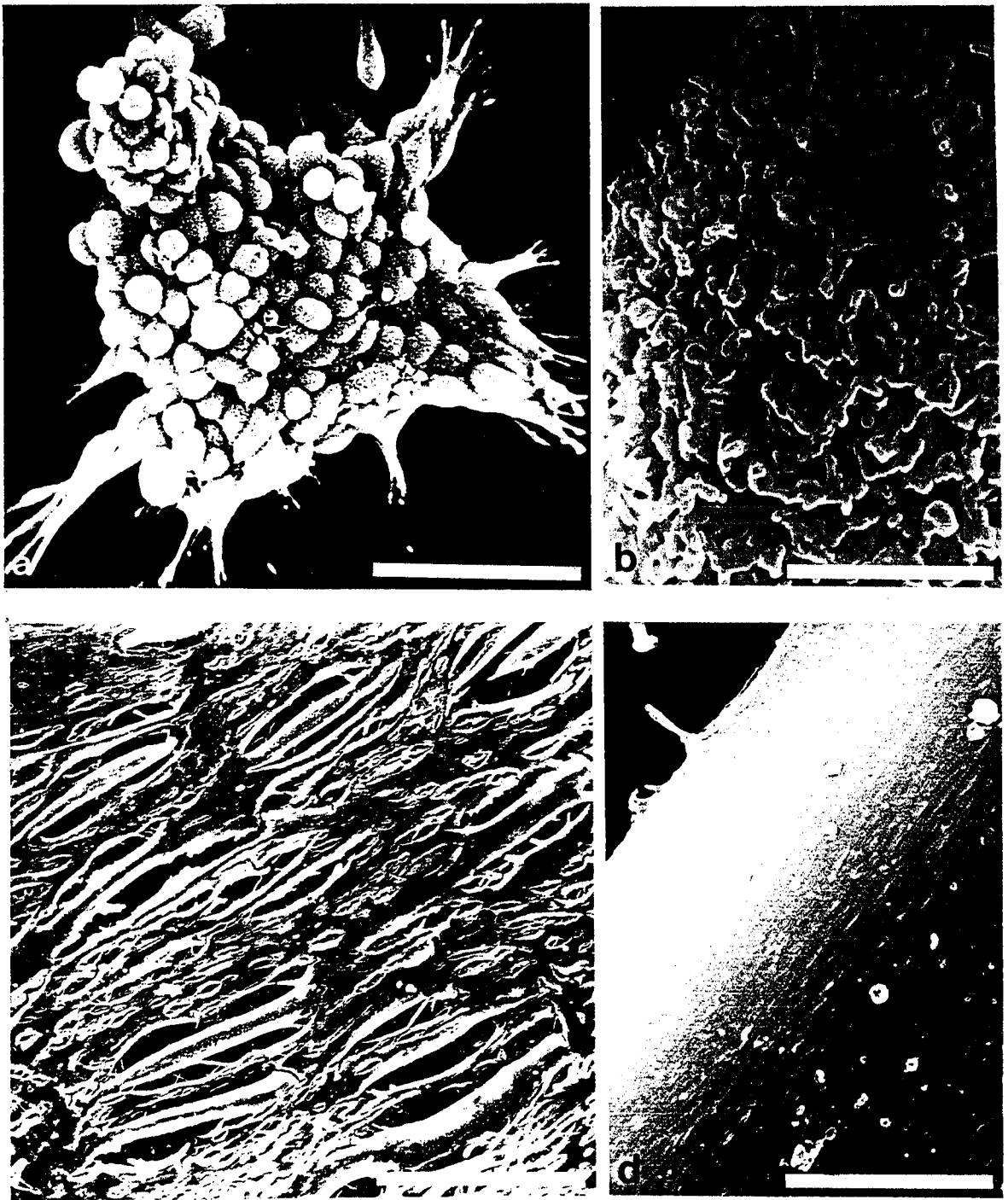
Fig.8. Induced and uninduced CO25 cells by DEX were illustrated by scanning electron microscopy after 10 days.

(a) A foci of cells in the presence of DEX. (b) The surface of a cell in the foci.

(c) Cells in the fusion promoting medium. (d) The surface of a myotube.

Bars ; (a) 600 μm, (c) 200 μm, (b) 3.8 μm, and (d) 2 μm.

2.8.3. INDUCTION OF N-*RAS* PROTEIN p21 AND mRNA BY
DEXAMETHASONE IN CO25 CELLS

CO25cells were exposed to 1μM DEX in 10% HS or grown in 10% HS without DEX to allow differentiation, and then cell extracts or RNA samples were prepared at various times for analysis of *ras* p21 or mRNA.

The level of p21^{N-ras} was determined by Western blot analysis using a monoclonal antibody to N-*ras* protein, 142EO5. The 21kD polypeptide increased in the presence of DEX over the 1-4 days period (Fig.9. lanes 5-8). Since the antibody recognized the endogenous *ras* proteins from mouse, the bands in the absence of DEX represent normal *ras* proteins (Fig.9. lanes 1-4).
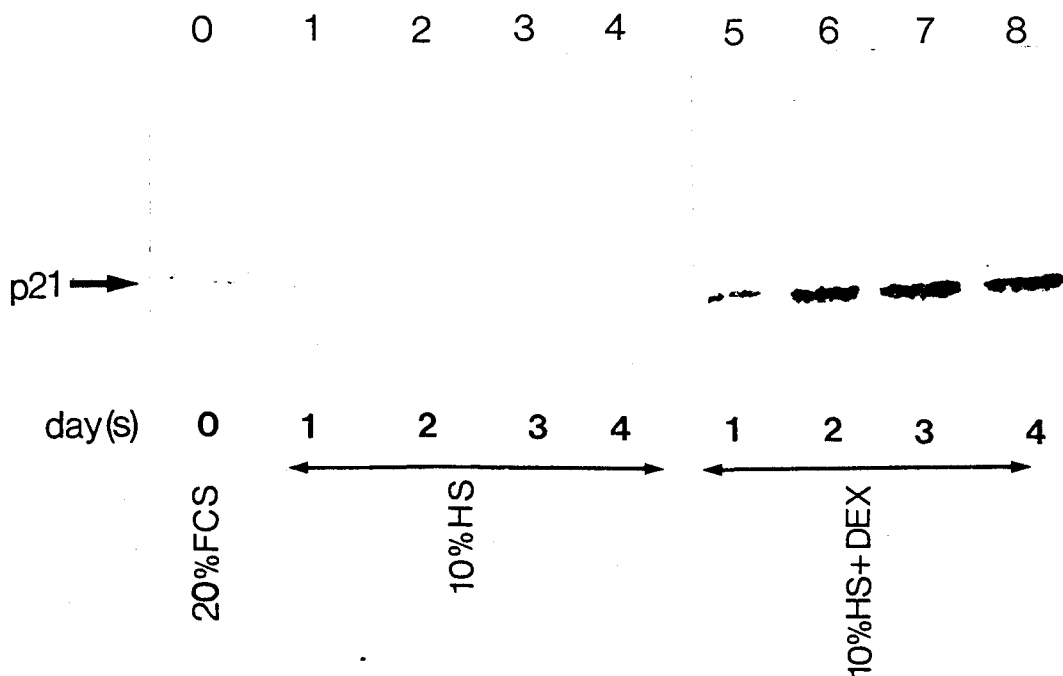
Fig.9. Expression of p21^{N-ras} protein in CO25 myoblasts bearing steroid-inducible N-*ras* oncogenes was determined by Western blot analysis as described in Materials and Methods, exposure time is 3 seconds.

The level of N-*ras* mRNA was determined by Northern blot analysis in similar growth conditions, using a ^{32}P-labelled human N-*ras* cDNA probe. The induction of N-*ras* mRNA followed similar kinetics to that of the p21$^{N\text{-}ras}$ protein over the time period 1 to 4 days (Fig.10. lanes 1-4). In the absence of dexamethasone, there were very few detectable mRNA transcripts (Fig.10. lanes 5-8).
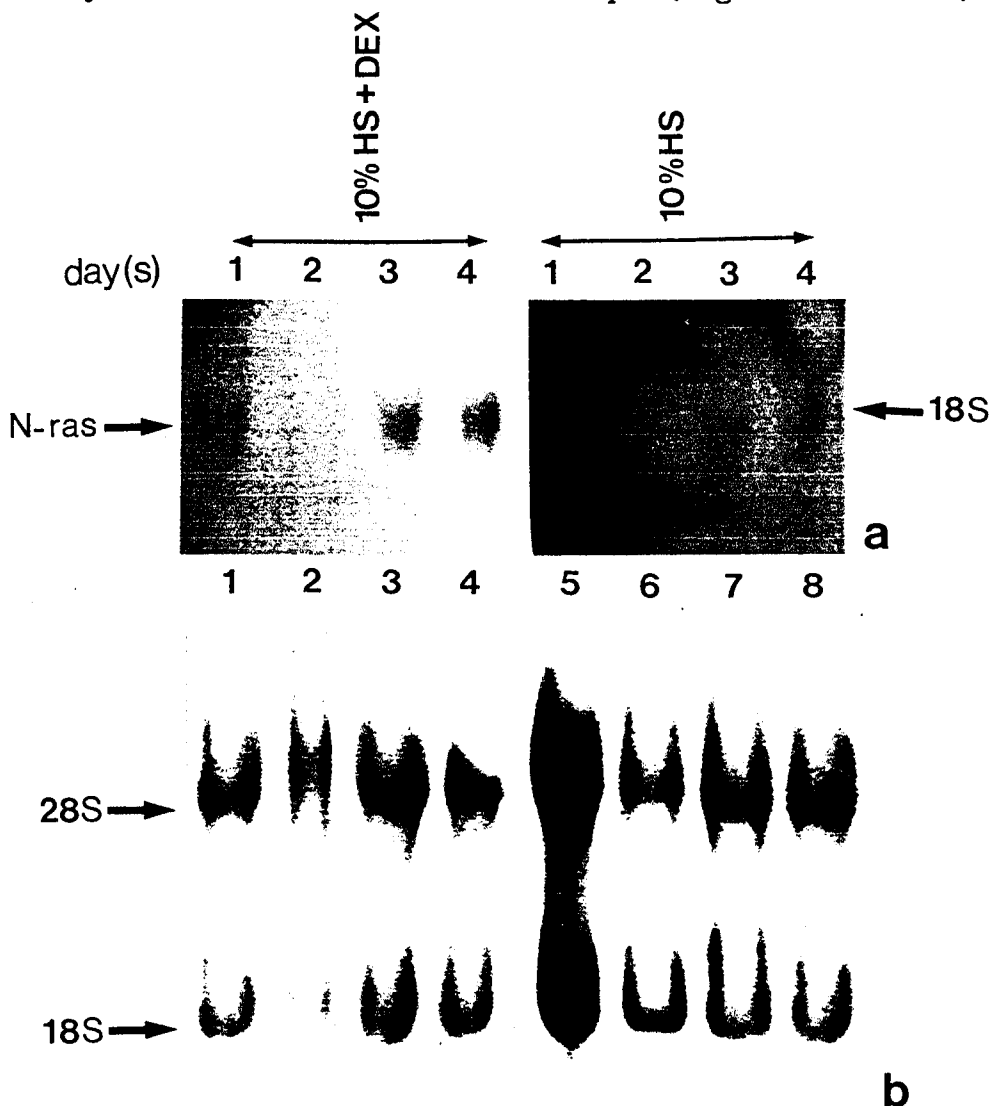
Fig.10. Northern blot analysis of total RNA. CO25 cells were incubated in 10% HS in the presence and absence of 1μM DEX, and the cells were harvested at 1,2,3 and 4 days. **(a)** Levels of the N-*ras* mRNA were determined by Northern blot analysis of 20 mg RNA at the indicated times. The position of 18S ribosomal RNA is indicated, exposure time is 96 hours. **(b)** Levels of total ribosomal RNAs were shown by re-probing blot A, and illustrated the quantity of rRNAs in each lane. Exposure time is 2 h .

2.8.4. ACTIN ORGANIZATION IN CO25 CELLS

To test whether expression of activated N-*ras* oncogene induces any changes in the cytoskeleton, rhodamine-conjugated phalloidin (rh-phalloidin) was used for these experiments which reacted *in vitro* with only F-actin. CO25 myoblast cells in the three different media, 20%FCS, 10%HS with or without DEX, showed very different patterns of stress fibre organization as shown in Fig.11. a, b and c. Phalloidin staining showed the presence of large numbers of stress fibres within the myoblasts growing in 20% FCS. The majority of flattened mononucleated myoblasts displayed parallel running microfilaments as criss-crossing bundles distributed throughout the cells (Fig.11.a).

This tendency for most of the microfilament bundles to run in parallel was much more apparent in newly formed myotubes after four day in 10% HS (Fig.11.b). The fused cells on the other hand were stained densely as arrowed on Fig.11.b.

In contrast, the distribution of actin cables in CO25 myoblasts was dramatically changed after four days induction of the N-*ras* oncogene by DEX. Polymerized actins were quite disorganized. Phalloidin brightly stained the edges of the smaller transformed cells (arrowed on Fig.11.c), and stress fiber became largely replaced by a diffuse bright fluorescence.

Further experiments were performed to observe the changing distribution of actin filaments in fully formed myotubes and in the foci of transformed cells. After ten days, when cells formed foci in the presence of DEX, the round cells in the foci had no actin fibres visible in the central area. But the cells showed only a bright diffuse fluorescence (thick arrows on Fig.12.a). In some cells at the bottom level of the foci which attached the foci to the substratum, cortical

F-actin was present at the edges and the long processes of cells (thin arrows on Fig.12.a).
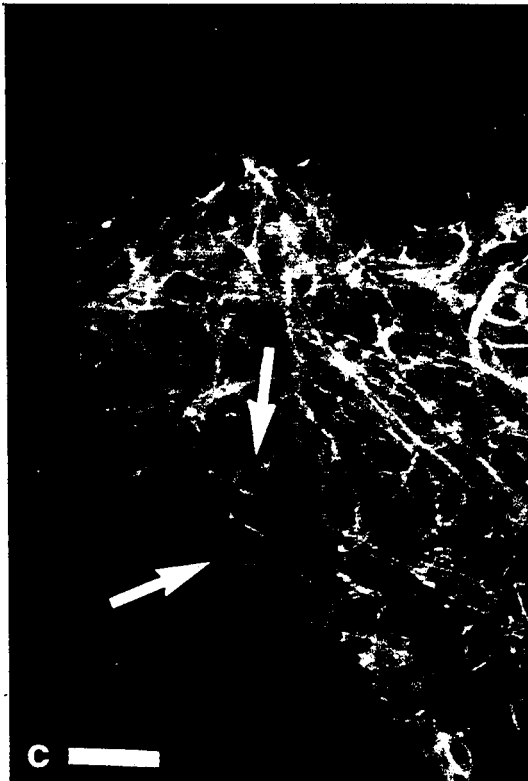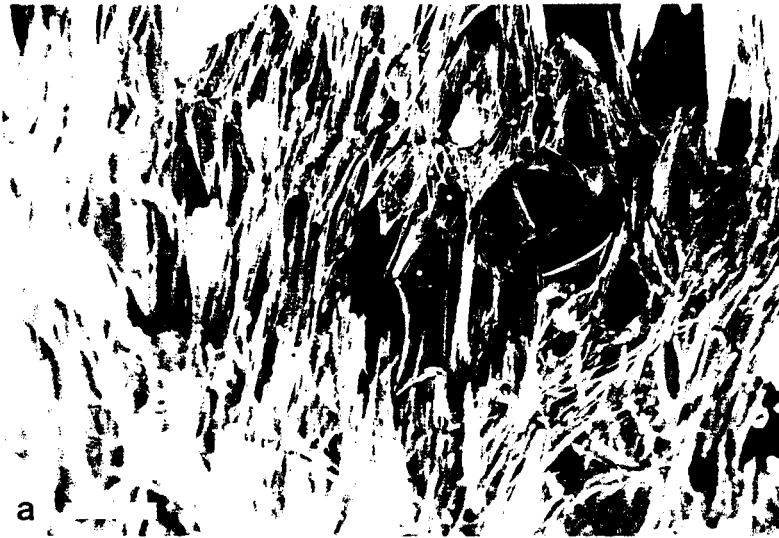
Fig.11. Distribution of F-actin in CO25 myoblast cells in the different media was examined using phalloidin-rhodamine fluorescence microscopy. (a) cells in the growth medium, (b) cells in the fusion promoting medium for 4 days. Arrow shows localization of F-actin in fused cells, (c) cells in the fusion promoting medium with DEX for 4 days. Arrows show localization of actin around the edges of transformed cells. Bars= 20 μm.

After ten days in 10% HS in the absence of DEX, most of the CO25 myoblasts formed myotubes. Following staining by rh-phalliodin, some of the myotubes showed a periodic fluorescence to the long axis of the myotubes. The stress fibres became redistributed in periodic sarcomeres in the myotubes (arrow shows on Fig.12.b).
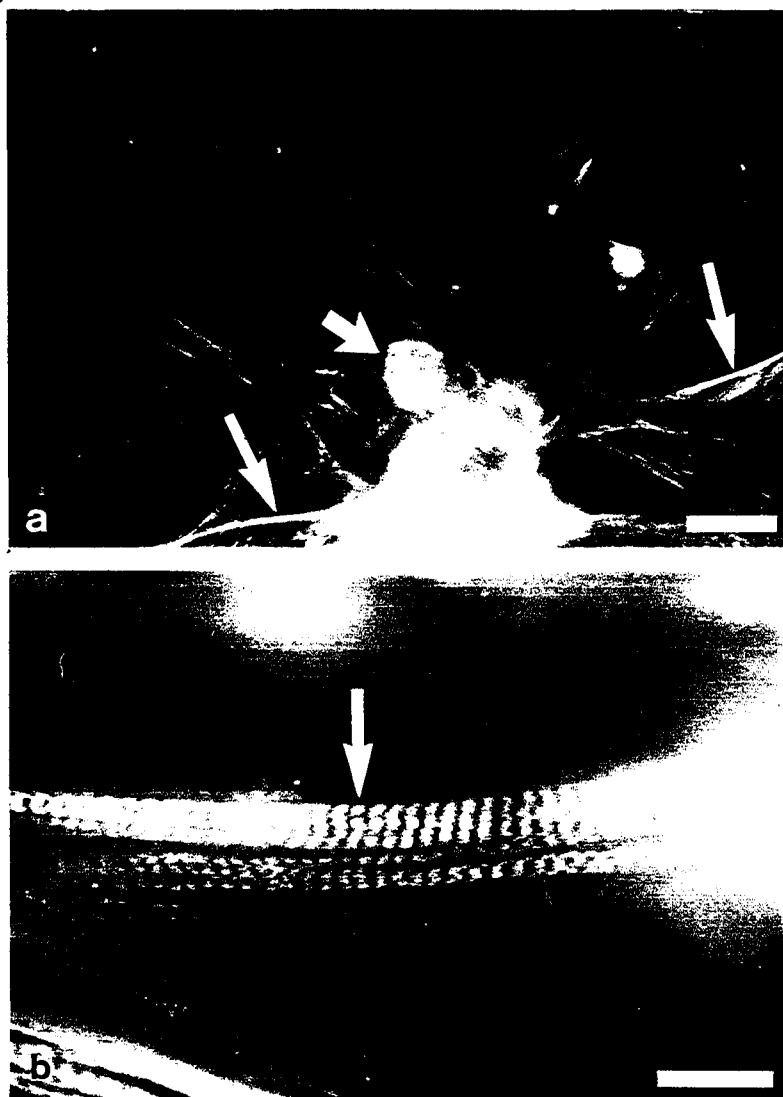
Fig. 12. Actin organization of CO25 myoblasts in the later stages of differentiation or transformation was examined using phalloidin-rhodamine fluorescence microscopy. (a) A foci of the transformed cells after dexamethasone treatment in 10% HS for ten days. Thick arrow shows bright diffuse stained cells, thin arrows show the localization of F-actin at the edges and processes of cells, (b) Fully formed myotubes after ten days in 10% HS. Arrow shows the periodic organization of F-actin. Bars= 20μm.

2.8.4. DISCUSSION

In this section, the ability of CO25 myoblasts transfected with a plasmid containing a steroid-inducible mutated N-*ras* oncogene has been studied in situations where cell differentiation was promoted or inhibited by the activation of the N-*ras* oncogene. CO25 cells differentiated into myotubes in the presence of 10% HS. The results of this study demonstrated clearly that the expression of an activated mutant N-*ras* oncogene suppressed the ability of CO25 myoblast to form myotubes in the fusion promoting medium. The inhibition of myogenic differentiation by the N-*ras* oncogene was reversible by removal of dexamethasone. Expression of the N-*ras* oncogene did not cause cell proliferation similar to the cells growing in the absence of DEX, whereas the cells growing in 20% FCS showed a high proliferation rate. These results are in agreement with the findings of Gossett *et al.* (1988) in which the inhibition of differentiation in CO25 cells by the N-*ras* oncogene was reversible and independent of cell proliferation. Results from several works suggested that *ras* oncogenes (H-*ras* and N-*ras*) inhibited the myogenic differentiation in various myoblast cell types by preventing the accumulation of regulatory factors, such as *mck, MyoD1* and *Ach*, required for the transcriptional induction of muscle-specific genes muscle cell line (Payne *et al.*,1987; Gossett *et al.*,1988; Sternberg *et al.*,1989; Lassar *et al.*,1989; Konieczny *et al.*,1989).

TGF-β and FGF are also known to inhibit the differentiation of myoblasts through a mechanism independent of cell proliferation, and also this inhibition was found to be reversible by the removal of these growth factors (Olson *et al.*,1986; Spizz *et al.*,1986; Clegg *et al.*,1987). It was well documented that the ras protein product p21 is localized on the plasma membrane where it functions as a signal

transducer between specific cell surface growth factor receptors and intracellular effectors. It was suggested that therefore *ras* oncogenes may prevent differentiation effecting the same signalling pathway which involve plasma membrane receptors for TGF-β and FGF.

Contradictory effects of an activated *ras* gene has been shown to be involved in the differentiation of pheochromocytoma cell line PC12. The *ras* oncogene induced the differentiation of these cells through a mechanism similar to that of the nerve growth factor (NGF) in the same cells (Bar-Sagi and Feramisco,1985; Noda *et al.*,1985; Muroya *et al.*,1992). Taken all together, *ras* protein may exert different biological effects in different cell types, by utilizing a common molecular mechanism which involves the coupling of growth factors to intracellular signal pathways, independent of cell proliferation.

Morphology of CO25 cells displayed significant changes during differentiation or transformation processes. The cells expressing the N-*ras* oncogene showed a transformed morphology in a rounded shape and extensive microvilli on the cell surface. Recently, it has been shown that over expression of H-*ras* p21 caused transformation of NIH-3T3 fibroblasts. These cells showed significant changes in the cell morphology, in the formation of microvilli on the cell surface, and in the disorganization of actin filaments (Hagag *et al.*,1990).

Also in the later stages of transformation in CO25 cells (for 10 days), foci were formed. Foci formation is a typical indicator of transformation and indicates loss of contact inhibition and anchorage dependence for growth of the cells (Linstead *et al.*,1988). Foci formation by the CO25 cells expressing the activated N-*ras*

oncogene has not been reported previously. In contrast, the cells grown in the fusion promoting medium showed a flattened morphology and fused to form myotubes with a few short microvilli on their surface. After three weeks growth in this medium, most of the cells formed long branched myotubes.

To test the effects of DEX on myogenic differentiation, morphological changes were also examined in parental C2 cells in the presence or in the absence of DEX. The addition of DEX to the C2 cells in the fusion promoting medium had no effect on the differentiation process in these cells. In both conditions, with or without DEX in 10%HS, C2 cells formed long branched myotubes. This observation suggests that the exogenous activated N-*ras* oncogene caused the transformation after induction by DEX in CO25 cells.

In this study, a cytoskeletal element, actin, was examined for its distribution during differentiation and transformation of CO25 cells to investigate any correlation with the morphological changes of these cells. F-actin bundles in CO25 cells were disrupted after expression of the N-*ras* oncogene, and they were seemed to be absent from the cell center, but were organized toward the cell margin (Fig.11.c and 12a). Previously, Lee *et al.* (1988) reported that actin and p21 both located in the cell membrane, and they still located together when actin was altered and relocated following treatment with cytochalasin A.

CO25 myoblasts showed a rounded morphology and formed foci following the expression of activated N-*ras* oncogene after ten days. Rh-phalloidin staining showed that F-actin reduced distribution in cells which were attached on the substratum, and completely dispersed in the rounded up cells which were without

attachment with the substratum (Fig.12.a). A comparison of normal cells and their transformed variants has been reported by numerous workers. Cells transformed with a *ras* oncogene (Hagag *et al.*, 1990) and or with SV40 T antigen (Verderame *et al.*, 1980; Carter *et al.*, 1991) displayed a decrease in the number and lenght of actin microfilaments and disorganization of actin. Disordered metabolism of microfilament proteins has also been demonstrated in mouse mammary cells expressing H-*ras* oncogene (Bhattacharya *et al.*, 1988). The cultured tumor cells derived from renal carcinoma (Rahilly and Fleming, 1992) or from mammary carcinocarcoma (Urbancikova and Grofova, 1990) also showed few or no actin cables. In these cells, weak diffuse staining of cytoplasm without distinct filaments was observed when compered to those normal cells. Others have reported however that *ras* oncogene alone was not sufficient to disrupt the actin filament bundle organization (Carter *et al.*, 1991). These observations suggest that the distribution of actin cables can be related to other factors such as shape of the cell and the adhesiveness to the substrate rather than directly to transformation. The rounded cell shape is usually found in motile cells and may be due to changed interactions with the substrate (Folkman and Moscona, 1978; Trinkaus, 1985). The actin cytoskeleton is known to change during the cell cycle. In the G_2-phase of a normal cell cycle, immediately before mitosis, depolymerization of microfilament bundles has been observed (Wessells, 1971; Wang and Goldberg, 1976; Celis *et al.*, 1986). Thus it is concluded that in the foci of CO25 cells, the cells not attached to the substratum lose all F-actin stress fibers as a consequence, while the cells remaining attached contain F-actin.

An actin binding protein, profilin, forms a stable 1:1 complex with G-actin which ensures actin remains in the monomeric form and therefore prevents polymerization of actin. The product of phosphatidylinositol metabolism, PIP_2 has been shown to interact and activate the protein profilin on the inner surface of the plasma membrane (Bershadsky and Vasiliev,1988; Lassing and Lindberg, 1988; Preston *et al.*,1990). It is also well documented that p21 is involved in the signal transduction from growth factor receptors to the intracellular effector molecules (Bishop,1988). Expression of *ras* oncogene increases the activity of PLC and consequently the hydrolyses of PIP_2 and the production of DAG and IP_3 (see Chapter 4). Also EGF receptor kinase hydrolysis profilin from PIP_2 via the phosphorylation of PLC-γ1 (Goldschmidt-Clermont *et al.*,1991). Therefore the release of profilin may sequester actin as G-actin and prevent stress fibre formation. The distribution of actin and its depolymerization in CO25 cells expressing the activated N-*ras* oncogene may be due to an alternative mechanism which involves the activation of profilin by activated *ras* protein.

CHAPTER.3.

EFFECTS OF THE *N-RAS* ONCOGENE ON THE TRANSCRIPTIONS OF CELLULAR ONCOGENES

3.1. INTRODUCTION

The molecular basis of transformation which occurs in stages involves the co-operative action of several oncogenes (Land *et al.*, 1983a; Franza *et al.*, 1986; Sinn *et al.*, 1987; Hunter, 1991). The mechanisms of transformation by oncogenes involved altered function of key regulatory proto-oncogenes, such as c-*myc*, *myb* and c-*fos*, by stimulating or repressing their expression (Leibovitch *et al.*, 1987; Owen *et al.*, 1987). For example, transformation of fibroblast cells by v-*abl* oncogene involved amplification of the c-*myc* locus (Nepveu *et al.*, 1985; Colledge *et al.*, 1989) or overexpression of the cellular protein *p53* (Rotter *et al.*, 1980). Recently, Jamal and Ziff (1990) showed that transformation of fibroblast cells by v-*raf* which is a constitutively activated oncogenic counterpart of *raf-1* gene (which encodes a cytoplasmic serine/ threonine kinase) can transactivate transcription of two early-response genes, c-*fos* and β–actin genes. In *ras* transformed fibroblasts, a v-*sis* related mRNA and a family of retrovirus-related VL30 mRNA molecules were increased whilst there was no change in the c-*myc* or c-*fos* levels of mRNA (Owen *et al.*, 1987). C-*myc* and c-K-*ras* proto-oncogenes have been shown to cooperate in the transformation of secondary rat embryo fibroblasts (Land *et al.*, 1983a, 1983b), and in chemically transformed fibroblasts their mRNA products were constitutively expressed (Campisi *et al.*, 1984). During the terminal differentiation of F9 teratocarcinoma stem cells, expression of c-*myc* decreased much more extensively than c-K-*ras* (Campisi *et al.*, 1984).

In contrast to the activation of these certain proto-oncogenes, in two neoplastic myogenic cell lines (M4 and RMS4) transformed by the v-*fos* oncogene, the loss of myogenic capacity correlated

with inactivation of certain proto-oncogenes; c-*fos*, c-*myc*, *erbB*, *erbA*, *src* and *sis* (Leibovitch *et al.*, 1987).

A primary response to serum and many growth factor induced transmembrane signals, such as PDGF, EGF and FGF, is the rapid activation of transcription of early-response genes including proto-oncogenes c-*fos* (Greenberg and Ziff, 1984; Muller *et al.*, 1984; Treisman, 1985; Jamal and Ziff, 1990), *fos-B* (Zerial *et al.*, 1989), c-*myc* (Kelly *et al.*, 1983; Campisi *et al.*, 1984), c-*jun* (Lamph *et al.*, 1988; Ryseck *et al.*, 1988) and *p53* (Reich and Levine, 1984).

Muller *et al.* (1984) reported that quiescent fibroblasts respond to stimulation with serum or individual growth factors (EGF, FGF and PDGF) by the rapid synthesis of c-*fos* mRNA followed by the appearance of c-*myc* mRNA. The levels of c-H-*ras* mRNA and c-K-*ras* mRNA also increased in the serum stimulated cells. In 3T3 cells, the abundance level of c-*myc* mRNA increased more than 40 fold following the addition of PDGF to the culture medium. Growth factors such as EGF and insulin had a little effect on c-*myc* expression (Kelly *et al.*, 1983). When the expression of the *myc* gene is placed under control of promotors, the growth requirement for PDGF is diminished (Armelin *et al.*, 1984).

The *p53* gene is required for the control of cell division and plays an important role in the suppression of abnormal cell proliferation and tumour development (Milner and Watson, 1990; Ullrich *et al.*, 1992). Increased levels of *p53* have been shown to induce apoptosis (programmed cell death) in a human colon tumor-derived cell line (Shaw *et al.*, 1992) and in myeloid leukaemic cells (Yonish-Rouach *et al.*, 1991). Wild-type *p53* has been found in association with the products of a number of other oncogenes including *myc*, *mdm-2*, H-*ras*, *HPV18 E6*, *hsc 70* (Hinds *et al.*, 1987;

Chen and Defendi, 1992; Momand *et al.*, 1992), and to repress the c-*fos*, retinoblastoma (*Rb*) and proliferating-cell nuclear antigen genes as well as a number of viral promotors (Kley *et al.*, 1992; Shiio *et al.*, 1992; Subler *et al.*, 1992). In contrast to the anti-oncogene functions of wild-type *p53*, certain point mutations within the *p53* coding sequence convert *p53* to a promotor of cell proliferation (Finlay *et al.*, 1989; Milner and Watson, 1990). The mutant forms of *p53* could fully transform rodent cells in co-operation with an activated *ras* oncogene (Eliyahu *et al.*, 1984; Rowinski and Benchimol, 1988; Hinds *et al.*, 1989). When a wild-type *p53* cDNA was co-transfected with two activated oncogenes *E1a* and *ras* (Finlay *et al.*, 1989) or *myc* and *ras* (Eliyahu *et al.*, 1989) into rat embryonic fibroblasts, the formation of foci was reduced.

Taken together, these observations suggest that alterations of additional proto-oncogenes such as c-*myc*, c-K-*ras* and *p53* may play some role in differentiation or in transformation events mediated by activated oncogenes. To investigate the transformation events mediated by an activated N-*ras* oncogene, I have analysed transcription levels of c-*myc*, *p53*, c-K-*ras* proto-oncogenes and β-actin genes during transformation and differentiation of CO25 myoblast cells bearing the inducible mutated human N-*ras* oncogene, using Northern blotting techniques.

3.2. MATERIALS AND METHODS

3.2.1. CELLS

CO25 myoblasts were cultered in 10% HS with or without 1μM DEX for periods of time as described in section 2.4.1.

3.2.2. NORTHERN BLOTTING

Extraction of total RNA, electrophoresis, blotting onto a nitrocellulose membrane and hybridization of the RNA samples were performed as described previously in section 2.5.

3.2.3. PROBES

Probes were used for measurement of individual mRNAs were as follows: c-*myc*, a 2.3 kb Eco RI fragment from pUC plasmid containing full-length human c-*myc* gene (Rabbits *et. al.*,1984); K-*ras*, a 1.1 kb Pst I fragment from human cDNA (Amersham) (Mc Coy *et. al.*,1984); *p53*, a 1.8 kb Xba I fragment of human cDNA (Baker *et al.*,1989); ß-actin, a 1.15 kb Pst I fragment from pBR322 plasmid containing the mouse ß-actin gene (Alonso *et. al.*,1986); and rRNA, a pXLrIOI plasmid (a gift from Dr. W.James, Oxford) containing the whole transcription unit from *Xenopus*.

3.3. RESULTS

To investigate whether *ras*-dependent inhibition of myogenic differentiation was accompanied by effects on normal cellular gene expression including the proto-oncogenes c-*myc*, *p53*, c-K-*ras* and the structural gene β–actin, cellular RNAs were extracted from CO25 cells cultured in 10% HS with or without 1µM DEX for 1,2,3 and 4 days. Samples were analysed by probing blots after electrophoresis in denaturing agarose gels as described in the Materials and Methods.

3.3.1. C-*myc* mRNA levels

The levels of c-*myc* mRNA have been found to decline during terminal differentiation of various myoblast cell lines (Sejersen *et*

al.,1985; Endo and Nadal-Ginard,1986; Leibovitch *et al.*,1987; Olson *et al.*,1987; Schneider *et al.*,1987). However, c-*myc* mRNA remained at a high level after withdrawal from the cell cycle in differentiation-defective myoblasts. I have examined the levels of c-*myc* mRNA in CO25 cells grown in the presence or absence of DEX. Appropriate samples were assayed using a 2.3 kb Eco RI fragment from pUC plasmid containing full-length human c-*myc* gene as a probe. C-*myc* mRNA bands were recognized above an18 S rRNA marker (Fig.13.a). As shown in Fig.13.a, during differentiation of CO25 cells growing in 10% HS, the c-*myc* mRNA levels fell in 2 days, disappeared by 3 days, then began to reaccumulate and returned to the basal level by 4 days. This result confirms previous studies in various cells (Lachman and Shoultchi,1984; Coppola and Cole,1986; Denis *et al.*,1987; Richon *et al.*,1989) who reported that the level of c-*myc* mRNA declined when the cells were stimulated to differentiate and then returned to the basal level.

During transformation of CO25 cells growing in the presence of DEX, the levels of c-*myc* mRNA were high on day 1, a slight decrease was observed in 2 days, a rise was observed in 3 days and finally it returned to the basal level in 4 days. In these cells, c-*myc* mRNA levels were generally higher than in the cells grown in the absence of DEX. The decrease in mRNA levels occurred in 2 days in both control and DEX treated cells. Similar results were obtained by Olson and cooworkers (1987) in C2 cells transfected with the oncogenic· forms of N-*ras* or H-*ras*. Thus, initiation of the differentiation programme was accompanied by a decline in the c-*myc* mRNA level, and a return to the basal level during terminal differentiation after 4 days growth. However, during

transformation, c-*myc* mRNA levels increased and remained at this level.
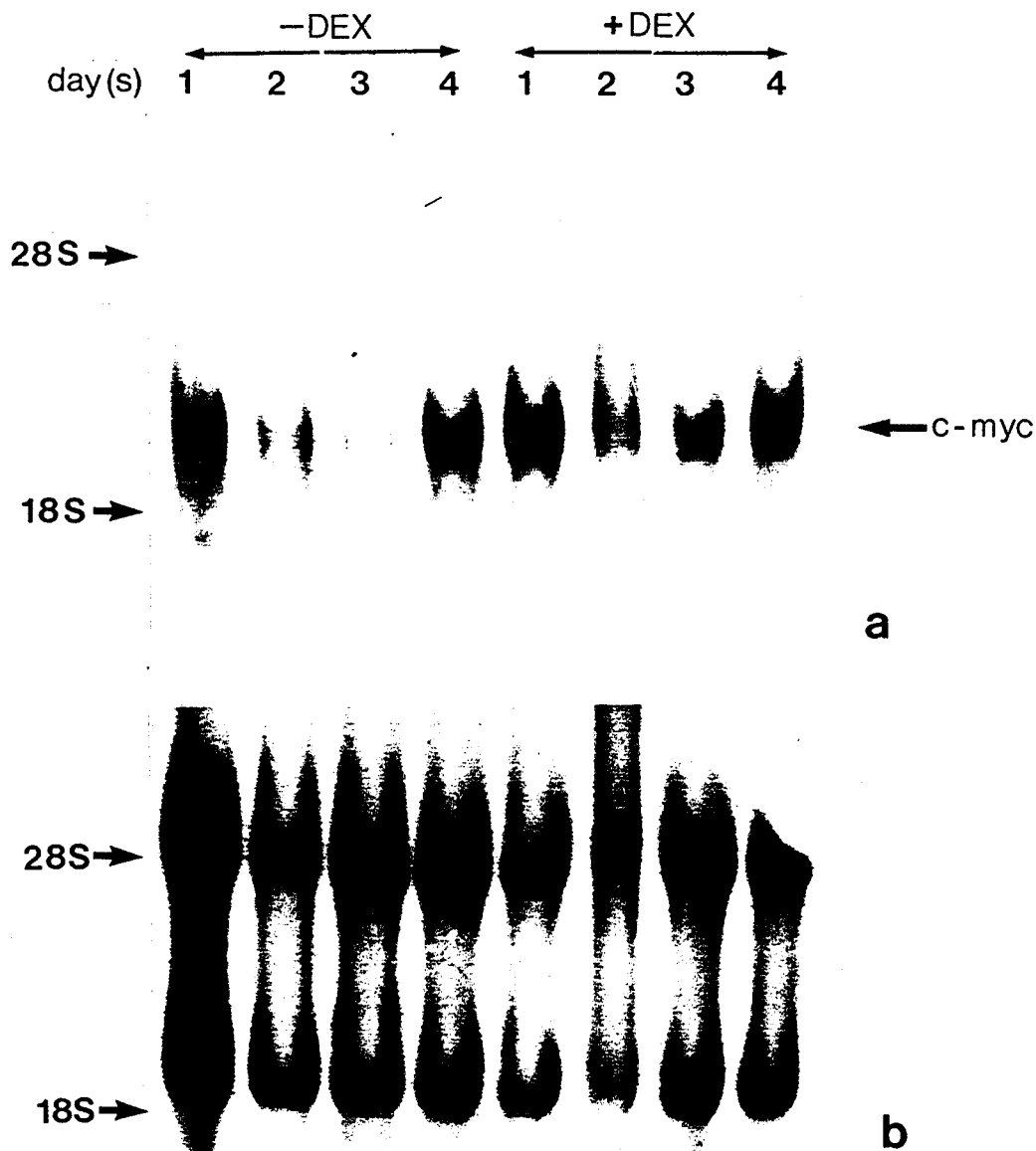
Fig.13. Northern blot hybridization analysis of c-*myc* mRNA levels in CO25 myoblasts treated or untreated with DEX over 1-4 days

a) Autoradiogram of c-*myc* mRNA. Exposure time is 4 days. 20 mg of cytoplasmic RNA was analysed as described in the Materials and Methods. The migration of rRNA size markers is indicated.

b) Autoradiogram of rRNA showing total RNA levels for each track. Exposure time is 3.5 hours. The blot was stripped from the c-*myc* probe and then reprobed with an rRNA probe.

3.3.2. c-K-*ras* mRNA levels

The c-K-*ras* oncogene has been shown to be expressed constitutively in transformed fibroblasts (Land *et al.*, 1983a, 1983b), and overexpressed in the neoplastic transformants of L6α1 myoblasts (Leibovitch *et al.*,1987). However, during differentiation of F9 teratocarcinoma cells (Campisi *et al.*,1984) and L6α1 cells (Leibovitch *et al.*,1987), c-K-*ras* mRNA decreased and gradually disappeared. I therefore analysed c-K-*ras* mRNA levels by Northern blots in CO25 cells grown in the presence or absence of DEX as described in the Materials and Methods.

A c-K-*ras* probe, 1.1 kb Pst I fragment from human cDNA, detected two mRNA bands on the blot containing the RNA samples (Fig.14.a). Similar transcription patterns of the c-K-*ras* gene have been reported by other workers (Campisi *et al.*, 1984). Both transcripts of c-K-*ras* appeared at a similar level in each sample. During both differentiation and transformation of CO25 cells, the expression of the c-K-*ras* gene revealed similar levels. c-K-*ras* mRNA levels were decreased in 2 days and disappeared in 3 days. They then rose in 4 days to 50% of the initial level during cell differentiation. In the transformed cells, the levels reached 80% of the initial levels.

Fig.14. (next page) Northern blot hybridization analysis of c-K-*ras* mRNA levels in CO25 myoblasts treated or untreated with DEX over 1-4 days.

a) Autoradiogram of c-K-*ras* mRNA levels. Exposure time is 2 days. 20 mg of cytoplasmic RNA was analysed as described in the Materials and Methods. The migration of rRNA size markers is indicated.

b) Autoradiogram of rRNA showing total RNA levels for each track. Exposure time is 3.5 hours. The blot was stripped from the c-K-*ras* probe and then reprobed with an rRNA probe.

day(s) — DEX +DEX

28S→ 18S→

←K-ras ←K-ras

a

28S→ 18S→

b
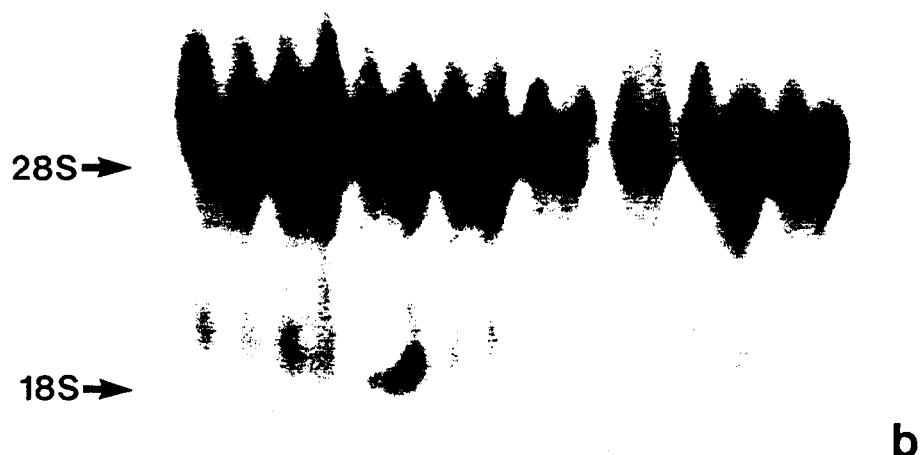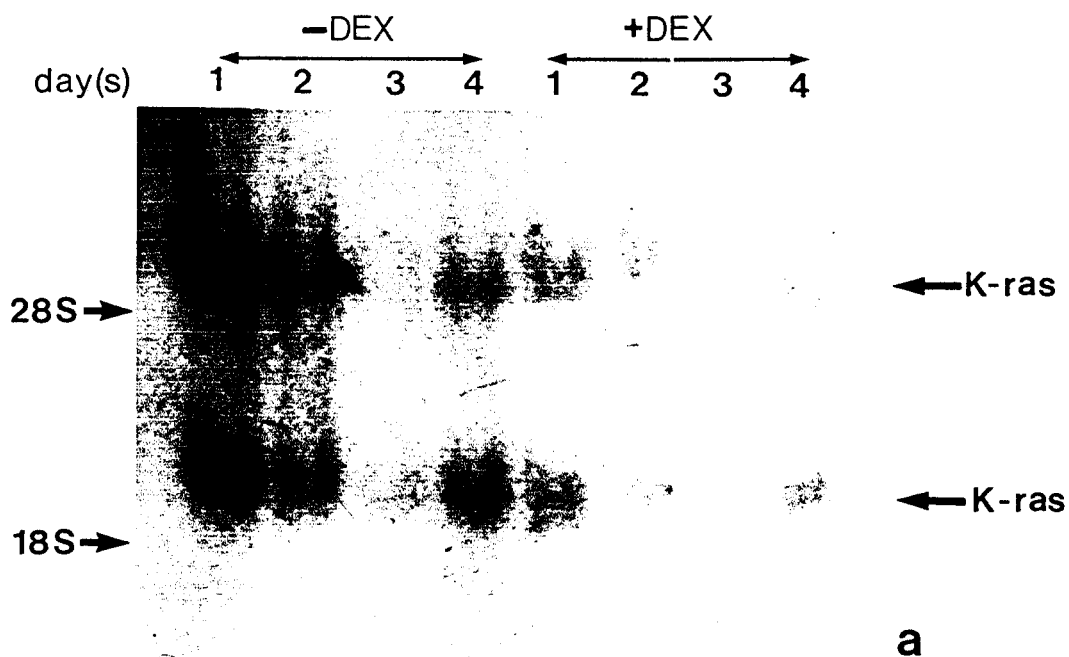
3.3.3. *p53* mRNA and *β- actin* mRNA levels

RNA samples from CO25 cells in either the differentiated or transformed states were analysed by Northern blotting as described in Materials and Methods. A *p53* probe in a 1.8 kb Xba I fragment of human cDNA was used. A single mRNA species was detected and it migrated just ahead of the 18S rRNA marker (Fig.15.a) as previously reported·by Reich and Levine (1984). I have found that CO25 cells express a small amount of *p53* mRNA as seen in Fig.3.a, needing a very long exposure time when compared with exposure times of other blots.

During the differentiation and transformation of CO25 cells, there was no significant change in the levels of *p53* mRNA, expressed at the similar levels during these processes. However, a very small decrease occurred in 3 days and an increase in 4 days similar to the c-*myc*, c-K-*ras* and β–actin mRNA levels also observed in this laboratory. Richon *et al.* (1989) reported similar results.

As shown in Fig.15.b, a probe for β–actin, a 1.15 kb Pst I fragment from a pBR322 plasmid containing the mouse β–actin gene detected a single band just above the 18S rRNA marker. After stripping off the *p53* probe, the blot was reprobed with β–actin as described in the Materials and Methods. The levels of β–actin mRNA decreased slightly in 2- 3 days, then returned to the initial level at 4 days during both the differentiation and transformation of CO25 cells. However, the β–actin mRNA levels were generally higher in differentiated cells than in the transformed. My findings disagree with those of Olson and Capetanaki (1989) who reported a decline in the β–actin mRNA levels during C2 myoblast differentiation, and the activity of an H-*ras* oncogene prevented the down regulation of β–actin gene. Similar results were reported also by Leibovitch *et al.* (1987).

Fig.15.(next page) Northern blot analysis of *p53* mRNA and β–actin mRNA levels in CO25 myoblasts treated or untreated with DEX over 1-4 days.

a) Autoradiogram of *p53* mRNA levels. Exposure time is 19 days. 30 mg of cytoplasmic RNA were analysed . The migration of rRNA size markers is indicated.

b) Autoradiogram of β–actin mRNA levels. Exposure time is 6 hours. The same blot was reprobed with β–actin after stripping of the *p53* probe.

c) Autoradiogram of rRNA showing total RNA levels for each track. Exposure time is 3.5 hours. The blot was stripped of from the β–actin probe and then reprobed with an rRNA probe.
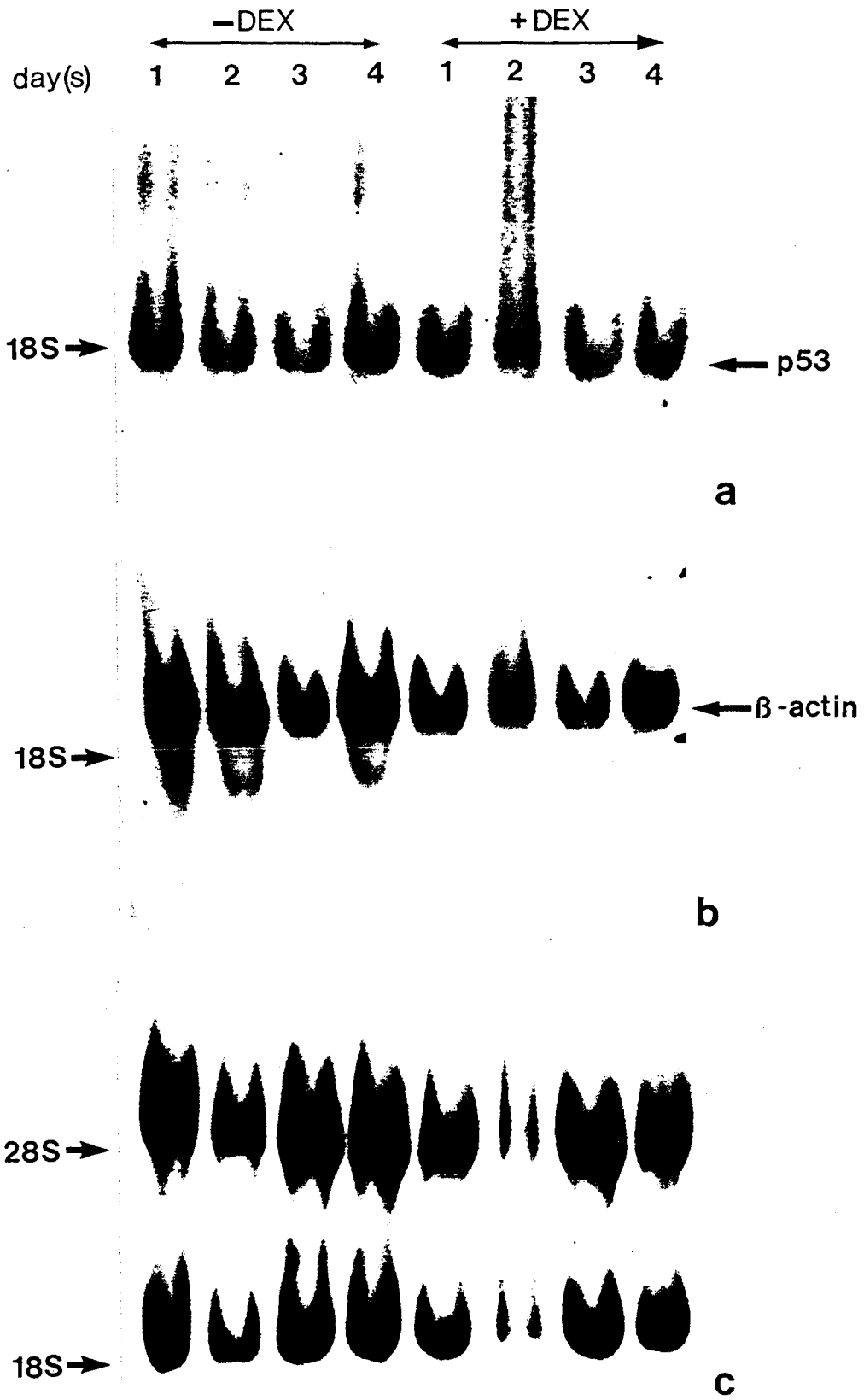
3.4. DISCUSSION

Induction of cell proliferation, transformation and differentiation appear to be mutually exclusive phenomena in various differentiating cell systems, including myogenic cells (Nguyen *et al.*,1983; Coppola and Cole,1986; Denis *et al.*, 1987; Gossett *et al.*,1988). In various cell, c-*myc* mRNA, c-K-*ras* mRNA, *p53* mRNA and β–actin mRNA are induced at an early stage when quiescent cells stimulated to proliferate in response to growth factors (Kelly *et al.*,1983; Campisi *et al.*,1984; Muller *et al.*,1984; Reich and Levine,1984; Endo and Nadal-Ginard,1986), whereas their cytoplasmic levels decrease markedly in terminally differentiated cells (Campisi *et al.*,1984; Sejersen *et al.*,1985; Endo and Nadal-Ginard,1986; Leibovitch *et al.*,1987; Olson *et al.*,1987; Olson and Capetanaki,1989; Richon *et al.*,1989). These observations suggest that a high level expression of these genes is necessary for the initiation of DNA synthesis and cell proliferation, and also their down-regulation is involved in the induction of specific genes for differentiation.

The relative transcription levels of protooncogenes, c-*myc*, c-K-*ras*, *p53* and β-actin, which have been observed in this section of the thesis were summerized in graphs as shown in Fig.16 and 17.

c-*myc* mRNA levels

The c-*myc* oncogene is expressed in the majority of proliferating normal cells, and altered expression of this gene has been implicated in the genesis of a wide variety of tumors (Taub *et al.*,1982; Little *et al.*,1983; Corcoran *et al.*, 1984). The results presented here demonstrate that the c-*myc* mRNA decreased within 2 days and disappeared following 3 days after culturing CO25
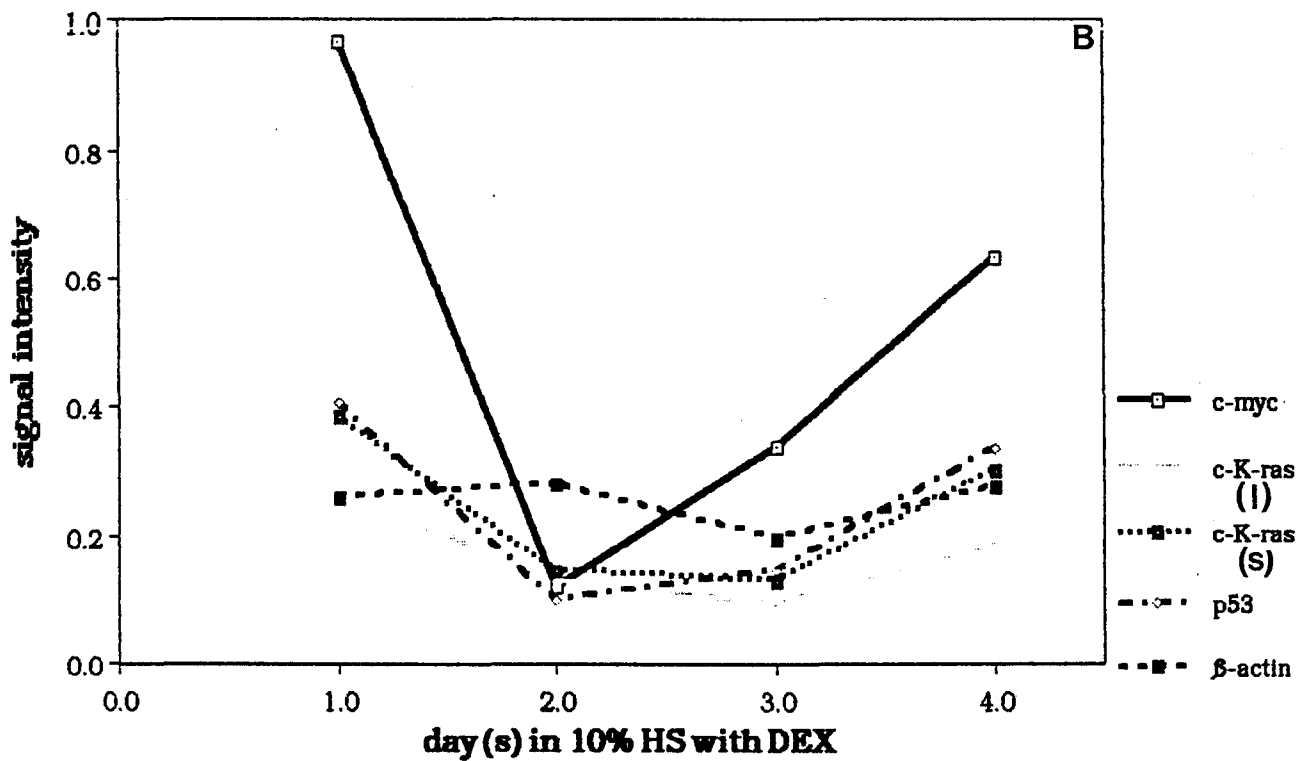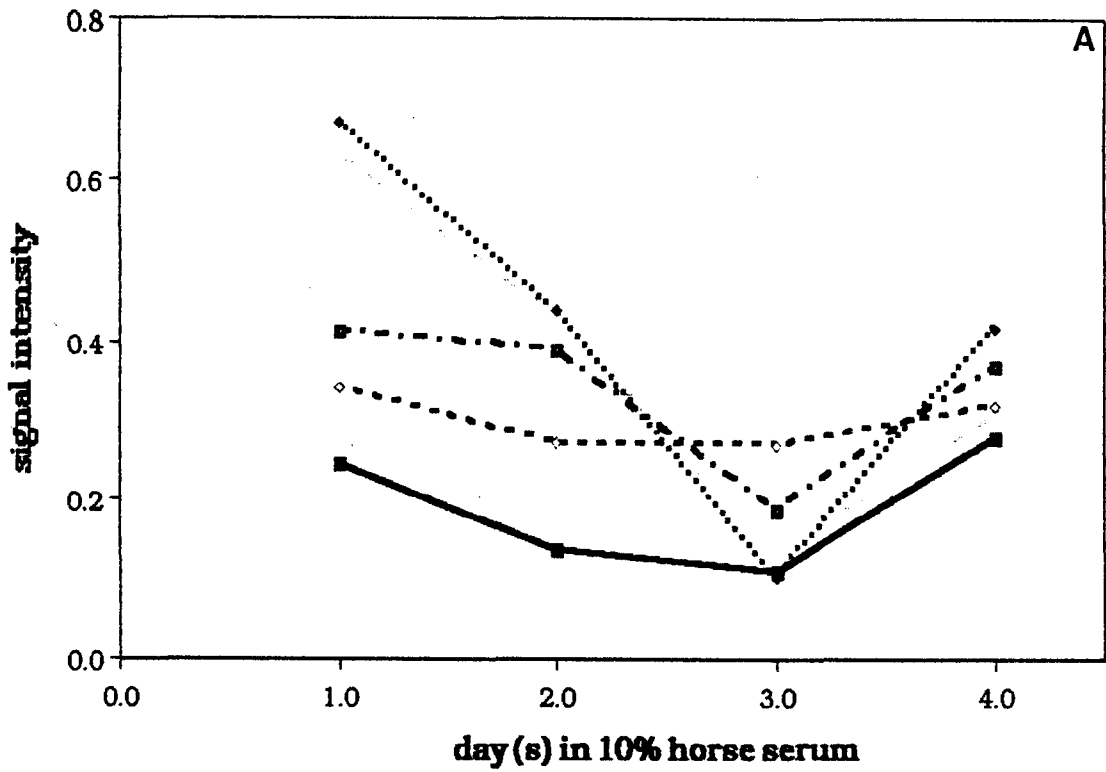
Fig.16. Comparative levels of RNA species during differentiation (A) or transformation (B) of CO25 cells. Autoradiograms were scanned with a Transmittance/Reflectance scanning densitometer (BIO TECN.Lt.). The peaks were integrated and graphically expressed in arbitary units which are ratios of the intensity of mRNA/rRNA. (l), large size; (s), small size K-*ras*.

myoblast cells in the fusion promoting medium (Fig.13.a). This decrease in c-*myc* expression seems to be required for the initiation of differentiation. After this critical period myotubes form within four days, and an increase in c-*myc* mRNA does not inhibit differentiation. This observation is in agreement with the results of Endo and Nadal-Ginard (1986) who showed that c-*myc* transcription was regulated independently of muscle-specific gene (Myosin heavy chain) transcription. My observation is also consistent with the results of Sejersen *et al.* (1985), Leibovitch *et al.* (1987) and Denis *et al.* (1987) who showed that in myogenic subclone lines, c-*myc* is expressed at high levels.

Previous studies involving the expression of exogenous c-*myc* constructs suggest that the rapid suppression and subsequent reexpression of c-*myc* may be involved in determining the kinetics of commitment to terminal differentiation (Coppola and Cole,1986; Lachman *et al.*,1986; Denis *et al.*,1987). For example, constitutive expression of the exogenous c-*myc* gene inhibits muscular differentiation (Denis *et al.*,1987; Schneider *et al.*,1987), but has no effect on endogenous *myc* expression which shows a decline within the first 24 hours after transfer of the cells to a differentiating medium (Denis *et al.*,1987). The expression of *mck*, Ach receptors (Schneider *et al.*,1987) and functional sodium and calcium channels which are characteristic of skeletal muscle cells (Caffrey *et al.*,1987) can coexist with the c-*myc* expression. Similar results were obtained by Coppola and Cole (1986) which indicate that the c-*myc* transfected MEL cells shows an early decrease in endogenous c-*myc* mRNA followed by a return to normal levels

similar to the control lines during the differentiation process. The level of exogenous *myc* transcription remains at the same level throughout of the cell stage.

Endo and Nadal-Ginard, (1986) reported that insulin and IGF-1 induce c-*myc* mRNA in the quiescent myogenic cells L6E9-B. It has been shown that the levels of these growth factors and their receptors dramatically increased during differentiation of C2 myoblasts (Tollefsen *et al.*,1989a,1989b). For that reason, c-*myc* reaccumulation by 4 days of differentiation may be regulated by an autocrine mechanism and involves the expression of growth factors and their receptors at this stage in the differentiation process.

It has been shown in this thesis that the induction of the mutated N-*ras* oncogene in CO25 myoblast cells following treatment with DEX, caused an increase in the expression levels of c-*myc* gene (Fig.13.a). However, a small decrease by 2 days occurred similar to the decline observed during the differentiation of CO25 cells, in the absence of DEX. In contrast with the disappearence of c-*myc* mRNA in 3 days during differentiation, c-*myc* mRNA was reaccumulated in 3 days and was expressed at the same level in 4 days in the N-*ras* induced cells. The reason for this decline in these cells in 2 days may be dependent on serum deprivation as found during the initiation of differentiation. At the later stages, the presence of increased *ras* p21 levels may overcome the effects of low serum conditions and interfere with the overexpression of c-*myc* mRNA. Thus the elevated expression of the c-*myc* gene in 3 days may have a negative effect on the initiation of differentiation, leading instead to the transformed state.

During the culture of cells, media changes were made every two days. Therefore old media after two days culture may cause a

reduction in c-*myc* mRNA, and this is not observed in the cells during differentiation at 3 days.

The increase in the level of c-*myc* mRNA after induction of the N-*ras* oncogene is in agreement with the results of Olson *et al.* (1987). They showed that c-*myc* mRNA declined in abundance during differentiation of C2 cells and that activated H-*ras* and N-*ras* oncogenes prevented the normal decline in this mRNA species. But, they obtained their results from the cells which had been grown for 5 days in the presence and in the absence of DEX, and did not analyse the levels of c-*myc* expression from day 1.

Taken together, these results suggest that a decrease in c-*myc* expression in CO25 myoblasts must occur at an early stage to allow differentiation, and activation of the N-*ras* oncogene inhibits the decline of c-*myc* expression, but does not inhibit its initial decline at the early stages of transformation process, and confirms the important role of c-*myc* in the transformation- differentiation switch.

c-K-*ras*, *p53* and β-actin mRNA levels

An early decline was observed in the levels of c-K-*ras*, *p53* and β-actin mRNAs in 2-3 days during both differentiation and transformation stages of CO25 cells.

In the *ras*-induced cells, reaccumulation of c-K-*ras* mRNA reached 80% of the initial 1 day level. In differentiating cells it reached 50% of the same level. In contrast, Leibovitch *et al.* (1987) showed that c-K-*ras* mRNA declined and became negligible throughout the process of L6α1 myoblast cell differentiation whilst the level of c-N-*ras* mRNA rose. The changes in c-K-*ras* expression during CO25 differentiation and transformation were in general agreement with the findings of Campisi *et al.* (1984). They

reported that the level of c-K-*ras* mRNA decreased after differentiation of F9 teratocarcinoma stem cells to endoderm. Both transcripts of this gene were elevated in transformed fibroblasts. They did not report any reaccumulation of c-K-*ras* mRNA during the differentiation processes of these cells.

The levels of *p53* and β-actin mRNA showed similar changes during differentiation and transformation of CO25 cells. Their mRNA transcripts decreased generally by day 3 and reaccumulated by day 4 when the c-*myc* and c-K-*ras* transcripts also declined during these stages. The early changes in c-*myc* and *p53* protein levels have been reported by Richon *et al.* (1989) and they declined during the differentiation of MEL cells. Similar results were obtained by Lachman and Skoultchi (1984) showing a decline in both β-actin and histone H3 mRNA while c-*myc* mRNA declined during MEL cell differentiation. Olson and Capetanaki (1989) reported that β-actin was downregulated in C2 myoblast differentiation, whereas oncogenic H-*ras* transfection of these cells prevented this downregulation. The results here contrast with the observation since I showed a slight decrease in the levels of β-actin mRNA after induction of the N-*ras* oncogene.

It should be noted, however, that the type of RNA analysis used here measures steady-state levels. We cannot therefore distinguish whether the dramatic dissappearance of these mRNAs and their reappearance are the result only of a modulated rate of transcription or whether it reflects also modulation of the mRNAs stability.

These results reported here suggest that there may be a specific time in the differentiation process during which down-regulation of c-*myc*, c-K-*ras*, *p53* and β-actin genes is necessary for

commitment to terminal differentiation, and that once this stage is passed, reexpression cannot reverse the commitment process. An activated N-*ras* gene by a mutation of a regulatory domain can prevent the down-regulation of some genes, eg. c-*myc* in the absence of mitogens. This may be regulated by an indirect mechanism which may involve phosphorylation of a factor(s) capable of regulating transcription of these genes (Korn,1987; Bishop,1988; Wakelam,1989; Sjölander *et.al.*,1991). Thus the oncogenic form of N-*ras* p21 may transform cells by constitutively activating early response proto-oncogenes such as c-*myc*.

CHAPTER.4.

EFFECTS OF ACTIVATED *N-RAS* ONCOGENE ON
PHOSPHORYLATION OF CELLULAR PROTEINS
AT TYROSINE RESIDUES FOLLOWING STIMULATION OF
CO25 MYOBLASTS BY PDGF-BB

4.1. INTRODUCTION

Tyrosine phosphorylation of proteins by tyrosine kinases has been implicated both in mitogenic signal pathways and in oncogenic transformation (Hunter and Cooper,1985; Hunter,1989). Tyrosine kinases are oncogene-encoded products (e.g. *src*, *abl*, *fbs*) or growth factor receptors (e.g. PDGF and insulin receptors) as given in more detail in section 1.2.2. They are believed to be key components of signal transduction systems connected to cell proliferation. Regulation of cell proliferation by some growth factors clearly involves tyrosine phosphorylation (Hunter and Cooper,1985; Hanks *et al*,1988; Hunter,1989). For example, autophosphorylation of the PDGF receptor or EGF receptor leads to the phosphorylation of tyrosine residues present in the catalytic domain of tyrosine kinases, and appears to modulate the interaction of the activated receptors with substrates and other cellular proteins (reviewed in Ullrich and Schlessinger,1990).

4.1.1. GROWTH FACTORS AND SIGNAL TRANSDUCTION BY THEIR RECEPTORS

Growth factors are extracellular polypeptides of various sizes and can act as positive or negative modulators of cell proliferation and differentiation of particular types of cells (Michell,1989; Aaronson,1991). The mitogenic effects of growth factors occur on cells in the resting or G_0 phase causing them to enter and proceed through the cell cycle. Growth factors such as PDGF, EGF, TGFs and IGFs, mediate their actions by binding to and activating specific membrane receptors which possess an intrinsic protein tyrosine kinase activity. The resulting phosphorylation of specific substrates triggers a cascade of intracellular biochemical signals, resulting in

the activation and repression of various subsets of genes (Michell, 1989; Ullrich and Schlessinger,1990; Aaronson,1991).

Each growth factor receptor with tyrosine kinase activity contains; (a) a large single glycosylated extracellular region that includes one or more ligand binding domains, (b) a single linear hydrophobic transmembrane region, and (c) a single linear peptide sequence that resides in the cytoplasmic domain of the cell and contains a tyrosine kinase catalytic domain (Ullrich and Schlessinger,1990).

When a growth factor binds to the extracellular domain of its specific receptor, it causes conformational changes in the receptor protein. Following the activation of intrinsic receptor tyrosine kinases in the cytoplasmic domains of the receptor, an array of cellular responses are propagated. These cellular responses include stimulation of Na^+/H^+ exchange, glucose and amino acid transport, and activation of enzymes called phospholipase C-gamma (PLC-γ) leading to the generation of phosphatidylinositol metabolites such as IP_3. This causes the release of Ca^{2+} from intracellular stores and also the generation of DAG the natural activator of PKC (Nishizuka,1988; Ullrich and Schlessinger,1990). Evidence from several reports showed that PI-3 kinase (Coughlin *et al.*, 1989; Varticovski *et al.*,1989), GAP (Molloy *et al.*,1989) and the *c-raf* protooncogene product (Morrison *et al.*,1989) were direct substrates for receptor tyrosine kinases. After ligand binding, receptor aggregates present in coated pits are rapidly internalized and subsequently entered a cellular sorting pathways, leading to either degradation or recycling to the cell surface (Ullrich and Schlessinger, 1990).

Genetic alterations in growth factor signalling pathways are inextricably linked to a variety of chronic diseases including cancer. As mentioned in Chapter 1, oncogenes have been shown to encode growth factors, receptor tyrosine kinases, or other enzymes that participate in mitogenic signalling. A large variety of alterations found in the products of these genes or overexpression of these genes lead to constitutive activation and consequently, subversion of molecular control mechanisms and alteration of receptor signals (Ullrich and Schlessinger,1990; Aaronson, 1991).

4.1.2. ROLE OF PLC IN SIGNAL TRANSDUCTION

The hydrolysis of PIP_2 to IP_3 and DAG can be catalysed by any one of what is emerging as a large family of enzymes termed phospholipase C (PLC). Four types of PLC have been found in mammalian tissues, following the new nomenclature proposed by Rhee. These were PLC-α (62-68 kDa), PLC-β (150-154 kDa), PLC-δ (85-88 kDa) and PLC-γ (145-148) (Rhee,1991). All of them are single polypeptides and have similar catalytic properties in hydrolysing PI, PIP and PIP_2 (Rhee,1991; Wahl and Carpenter, 1991). In addition to the growth factor receptors, a variety of signals from hormone receptors have been shown to regulate PLC activity through G proteins. In contrast with hormone receptors, little evidence exists that a G-protein is involved or interacts with growth factor receptors. Experimentally, an interaction has been shown between certain growth factor receptors (EGF, PDGF, FGF) and the PLC-γ1 isoenzyme (Meisenhelder *et al.*,1989; Rhee,1991; Wahl and Carpenter,1991).

PLC-γ was phosphorylated on multiple tyrosine and serine residues and this phosphorylation increased the activity of PLC-γ

following EGF and PDGF treatment of cells (Kumjian *et al.*,1989; Meisenhelder *et al.*,1989; Sultzman *et al.*,1991). Stable physical association of PDGF receptors (PDGF-R) with PLC-γ has been shown in PDGF stimulated fibroblasts (Kumjian *et al.*,1989). In human mammary carcinomas, an increased activation of PLC-γ was correlated with the expression of the EGF receptor or *erbB-2* oncogene (Arteaga *et al.*,1991). In contrast with the activation of PLC-γ by the growth factor receptor kinase, elevation of cAMP caused the inhibition of PLC-γ activity through a cAMP-dependent protein kinase (PKA) mechanism (Kim *et al.*,1989). Also recently, PLC-γ has been found to be phosphorylated on multiple tyrosine residues and activated by a nonreceptor tyrosine kinase coupled to the T-cell antigen receptor-CD3 complex (Park *et al.*,1991).

Ras p21 was considered to be an upstream effector of PLC activity in PI-specific signal transduction by leading to the coupling of certain growth factor receptors to the stimulation of PLC (Chiarugi *et al.*,1986; Wakelam, *et al.*,1986; Lloyd *et al.*,1989). Activation of PLC-γ was necessary for *ras*-mediated induction of DNA synthesis in fibroblasts (Smith *et al.*,1990). However, the inositol phospho-lipid response to PDGF was reduced in the absence of a change in receptor number in cells transformed by the c-H-*ras* gene (Paries *et al.*,1987) or by the *EJ-ras* gene (Benjamin *et al.*,1987).

4.1.3. GAP AND SIGNAL TRANSDUCTION

GAP is a 125 kDa cytosolic protein, expressed in all cells and tissues so far examined (Trahey and McCormick,1987; recently reviewed in McCormick,1990). The C-terminal part of GAP is sufficient for the GTPase activity function (Marshall *et al.*,1989; for

more detail see section 2.1.2), whereas the N-terminal part contains two SH2 (*Src* homology 2) domains, which are also found in PLC-γ, *src*-like cytoplasmic kinases and the v-*crk* oncoprotein. It is thought to be involved in the interactions of tyrosine kinases with their targets (Anderson *et al.*,1990; Ullrich and Schlessinger,1990; Liu and Pawson,1991). This finding suggests that GAP might serve as a link between tyrosine kinases and *ras* p21.

Recently, a significant amount of GAP was found in membrane fractions, and phosphorylated on tyrosine residues in cells that were stimulated by EGF, PDGF or CSF-1 (Molloy *et al.*,1989; Kaplan *et al.*,1990; Kazlauskas *et al.*,1990; Heidaran *et al.*,1992), and in the cells transformed by v-*src*, v-*fps* or v-*abl* oncogenes (Ellis *et al.*,1990). Furthermore, GAP was found to be associated with phosphotyrosine containing proteins, in particular the PDGF-R after stimulation of cells with the ligand (Kaplan *et al.*,1990; Kazlauskas *et al.*,1990). GAP has been found to form cytoplasmic multiple heteromeric protein-protein complexes with 62 kDa (p62) and 190 kDa (p190) phosphoproteins in v-*src* or v-*fps* transformed cells and also in EGF or CSF-1 induced cells (Ellis *et al.*,1990; Moran *et al.*, 1991; Heidaran *et al.*,1992; Pronk *et al.*,1992).

Mutated forms of PDGF-R were found to be defective in signalling and unable to bind GAP, suggesting the association with GAP may be a critical event in signal transduction by PDGF (Kaplan *et al.*,1990; Kazlauskas *et al.*,1990). Similarly, in *ras*-transformed cells, PDGF-R was inactive and GAP failed to bind following addition of PDGF, although the receptors bound GAP *in vitro* (Kaplan *et al.*,1990). This association of GAP with ligand-activated PDGF-R may directly link PDGF and *ras* signalling pathways. It has been shown that overexpression of GAP can suppress transformation of

cells by c-H-*ras*, c-*src* or mutated *src* genes through a mechanism of inhibition involving endogenous *ras* gene. This does not alter the enzymatic properties or cellular targeting of the *src* protein, and does not inhibit transformation by activated H-*ras* or v-*ras* genes even when cells overexpress GAP (Zhang *et al.*,1990; De Clue *et al.*, 1991b).

4.1.4. OTHER RECEPTOR TYPES FOR SIGNAL TRANSDUCTION

There are at least two classes of receptors distinct from membrane spanning tyrosine kinases which stimulate cell proliferation.

1. The receptors which are membrane glycoproteins with a single hydrophobic transmembrane domain, include the receptors for interleukins (IL-2-7), granulocyte-macrophage colony stimulating factor (GM-CSF), G-CSF and erythropoietin. Their activation can lead to the appearance of tyrosine phosphorylated proteins (e.g. *src*) and increased amounts of GTP bound *ras* (reviewed in Aaronson,1991).

2. The receptors which posses a seven transmembrane domain motif, an extracellular NH_2-terminal domain and a cytoplasmic COOH-terminal tail. These are neurotransmitters and hormones such as the $\alpha-$ or $\beta-$ adrenergic receptors, the bombesin receptors and the muscarinic acetylcholine receptors which couple with heterotrimeric G-protein (a guanine nucleotide binding protein consisting of three subunits α , β and γ) that interact with PLC, adenylate cyclase or K^+ channels (Taylor and Putney,Jr.,1987; Aaronson,1991; Cerione,1991).

4.1.5. PDGF AND ITS RECEPTOR

PDGF is a basic protein and exhibits multiple molecular weight forms ranging in size from 28.000 to 35.000 daltons. PDGF is a disulfide linked dimer of two homologous polypeptides termed A and B, and can be assembled in at least three isoforms as PDGF-AA, PDGF-BB and PDGF-AB. Distribution of these isoforms varies with cell type and each of them binds to a different spectrum of receptor phenotypes as shown in Fig.18. (Bowen-Pope et al.,1991). The BB homodimer amino acid sequence is closely related to the homodimeric form of PDGF encoded by the v-sis oncogene (Williams,1989).

In vitro, PDGF stimulates the proliferation of various cells including fibroblasts, smooth muscle cells and glial cells, and delays the early stages in differentiation of these cells. PDGF is synthesized and secre t ed by platelets and other normal cells. The growth-promoting properties of PDGF play an important role in tissue repair mechanisms *in vivo*. PDGF-like molecules are secreted by a variety of transformed cells into their growth media, and stimulate proliferation of tumor cells by binding to the PDGF receptors (Bowen-Pope and Ross,1985; Singh,1987; Bowen-Pope et al., 1991).

The PDGF receptor is a 170- to 190 kD membrane glyco-protein, and can be found on muscle cells, fibroblasts and glial cells. The PDGF receptor has intrinsic tyrosine kinase activity which allowed identification of the receptor using antibodies that recognize phosphotyrosine. As represented in Fig.17., the structural domains of PDGF receptor have been found to contain the five immunoglobulin like domains and the kinase insert region (Williams,1989).
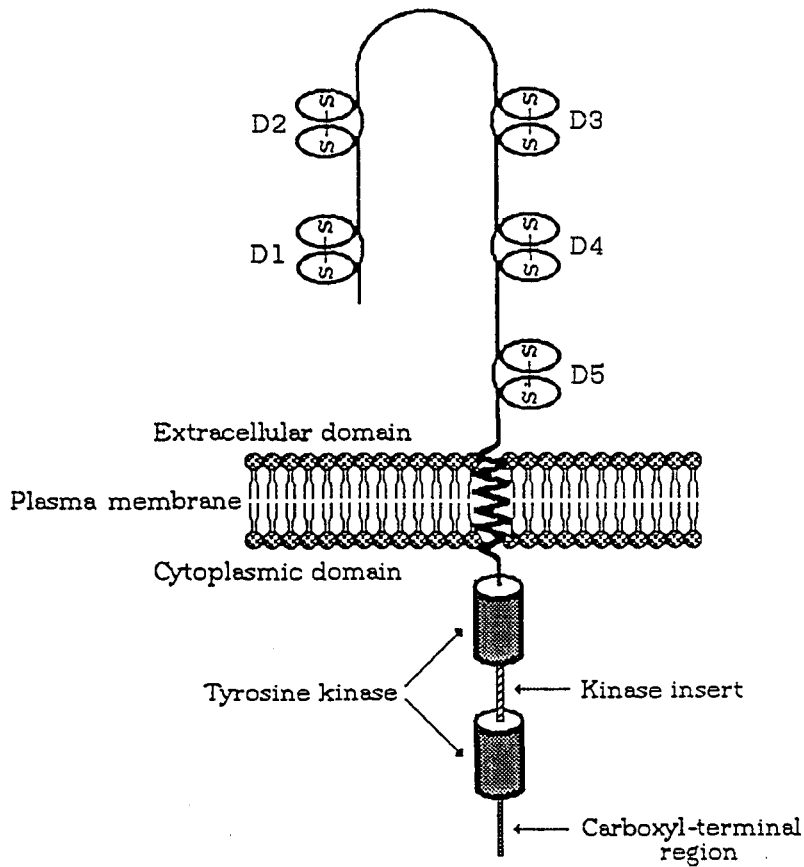
FIG. 17. The schematic representation of the structural domains of a PDGF receptor. D1 to D5 are immunoglobin-like domains of the extracellular part of the receptor. The transmembrane region of the receptor pass through the plasma membrane. In the cytoplasmic region, the tyrosine kinase sequences are interrupted by the kinase insert domain (modified from Williams,1989).

It is believed that there are two receptor subunits termed α and β which dimerize non-covalently to form a high-affinity binding site for the dimeric PDGF ligand. The α subunit of the receptor can bind both PDGF-A chain and PDGF-B chain, and the β subunit of the receptor can bind only PDGF-B chain as demonstrated in Fig.19. (Bowen-Pope *et al.*, 1991).
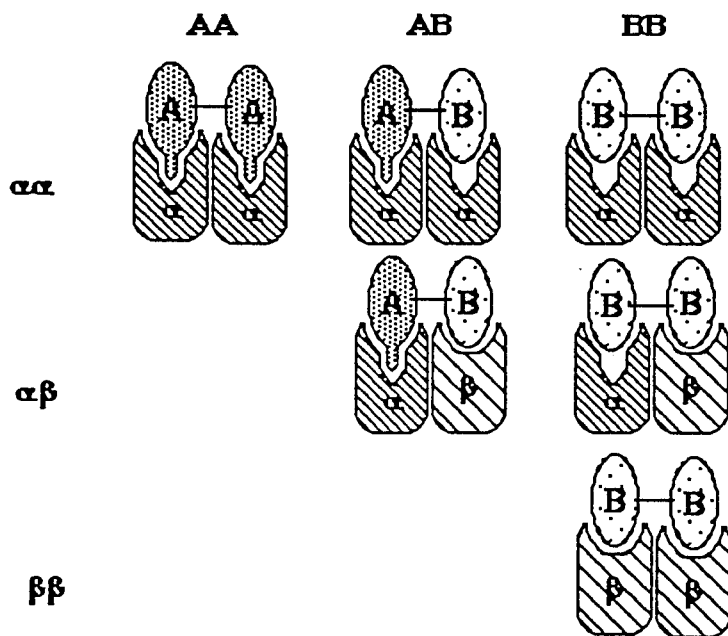
FIG. 18. Current model of dimeric PDGF receptors bound to the dimers of PDGF. Horizontal rows show the high affinity interactions between the three dimeric forms of PDGF, and vertical rows show the three noncovalent dimers of receptor subunits (modified from Bowen-Pope *et al.*,1991).

When PDGF binds to its receptor, there may be a conformational change in either the cytoplasmic part of the receptor or in the association of this region with other molecules to propagate the signal transduction into the cell (Williams,1989). Binding of PDGF ligand to its receptor rapidly triggers a group of early responses that occur in minutes as diagrammed in Fig.19. These include: **(i)** phosphorylation of the receptor itself and a number of other substrate proteins on tyrosine (Hunter and Cooper, 1985; Williams,1989); **(ii)** activation of PLC-γ, and following **(iii)** hydrolysis of PI; **(iv)** an increase in cytosolic calcium levels (Nishizuka,1988; Polverino *et al.*,1990). PDGF tyrosine kinase associates with some other substrates including PI$_3$ kinase which

Ligand

extracellular

Receptor

plasma membrane

$p21^{ras}$ ← GAP ← ATP → PLC-γ → PIP_2 → DAG → PKC

PI-3 Kinase

PIP_3

src

raf

Substrates

Substrates

IP_3

IP_3 R

Ca^{2+}

PKC
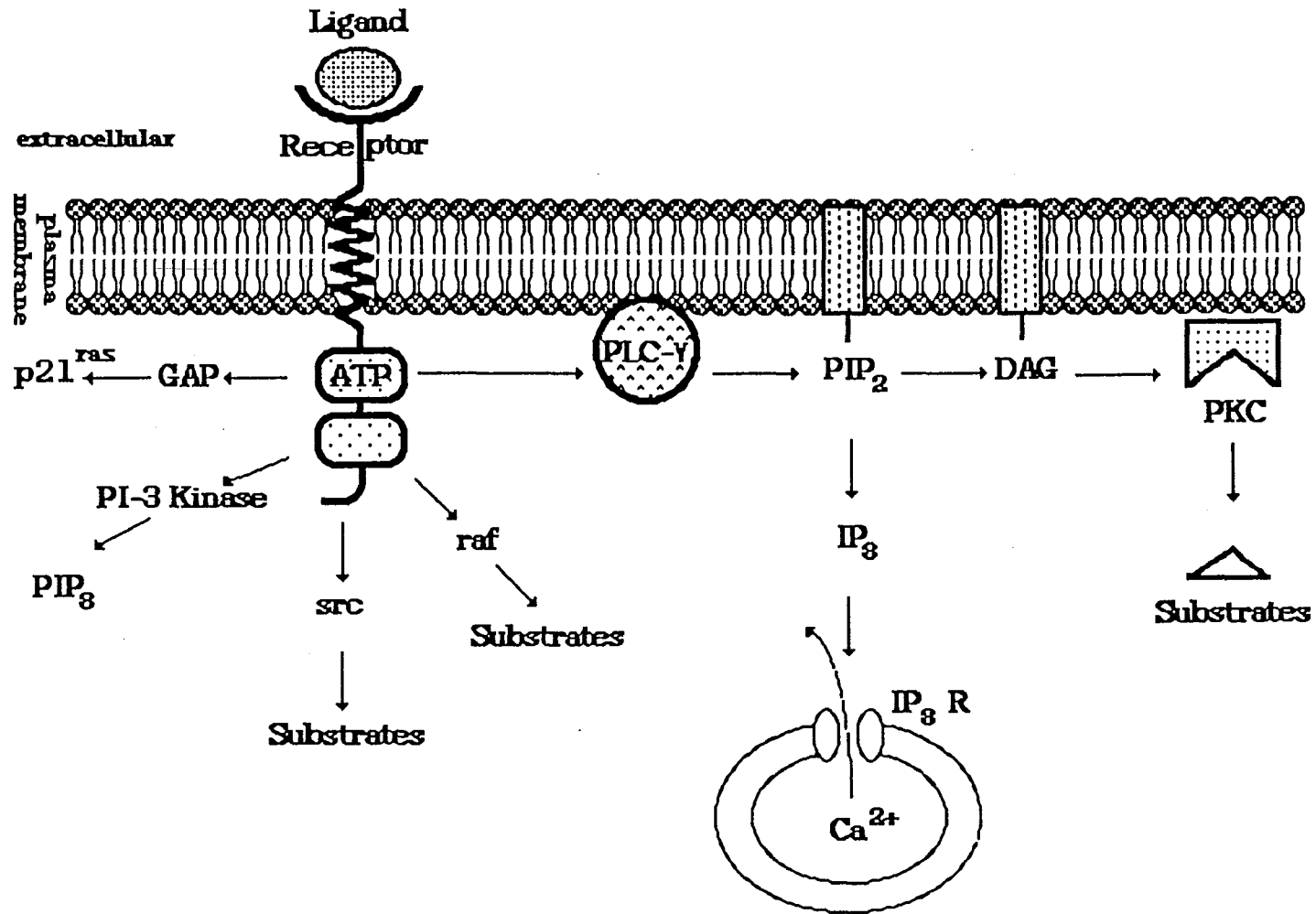
Substrates

Fig.19. Substrats of receptor tyrosine kinases, and activation of the phosphatidylinositol signalling pathway after binding of growth factors to their receptors (modified from Ullrich and Schlessinger, 1990 and

complex with other protein kinases and can regulate the transduction of mitogenic signals through p21.

In the myogenic cell system chosen in this study, inhibition of myogenic differentiation by a mutated N-*ras* oncogene and the transformed phenotype of these cells could be due to the effects of the p21 protein on the regulation of activity of phosphoproteins involved in this signal transduction from growth factor receptors.

The aim of this study in the present chapter was to investigate the phosphorylation of proteins on tyrosine residues following stimulation of *ras-* induced and - uninduced CO25 myoblasts, and in particular to describe the levels of expression and tyrosine phosphorylation of GAP and PLC-γ proteins by Western blotting techniques.

4.2. MATERIALS AND METHODS

4.2.1. WESTERN BLOTTING

4.2.1.1. Cell extraction

Cells were prepared for assays following growth in different media as detailed in section 2.4.1. and 2.4.2. They were incubated for a further 10- 12 hours in the same medium without serum to induce starvation. Cells were treated with PDGF-BB for 5 minutes and then rinsed twice with ice-cold PBS containing 0.1 mM Na_3VO_4. The cell monolayer was lysed in 0.5 ml of extraction buffer containing 1% NP-40, 50 mM Tris base pH 8.0, 50 mM NaCl, 1mM Na_3VO_4, 50 µg/ml Leupeptin and 1 mM PMSF, and the assay was carried out as described in section 2.6.1.

4.2.1.2. Electrophoresis and blotting

40 µg of protein from each lysate was resolved on a 8% SDS-polyacrylamide gel and electroblotted to nitrocellulose membranes as described in section 2.6.2.

4.2.1.3. Immunolabelling and detection of proteins

Immunolabelling was carried out by the method described in section 2.6.3, but 5% BSA was used in the blocking buffer instead of nonfat milk powder which may possess have phosphatase activity. Detection of antibody-antigen complexes on the blot was performed as described in section 2.6.4.

4.2.1.4. Immunoprecipitation

Cell lysates prepared as described above were subjected to immunoprecipitation by the method of Kaplan *et al.* (1990). Cell lysates were normalized for protein contents before the assay. Immunoprecipitations were performed by incubating lysates containing approximetly 250 µg protein with the GAP antibody (at a concentration 1:500) for four hours at 4ºC. Protein A- Sepharose beads (Sigma) were washed three times with the lysis buffer, and 10 mg of beads were added to the lysates to collect the antigen-antibody complexes. After an hour incubation at 4ºC, beads were pelleted, then washed once for 20 minutes and twice for a few minutes with cold lysis buffer. The last traces of the lysis buffer were aspirated carefully using a 0.5 mm x 16 mm needle. The pellet of Protein A- Sepharose beads with the antigen-antibody complexes was resuspended in 50 µl of the sample buffer (as given in section 2.6.2), boiled for 2-3 minutes and then supernatant was removed for analysis as described above.

4.2.1.5. Antibodies

The following antibodies were used in the present work; a monoclonal mouse anti-phosphotyrosine (Sigma) at a concentration of 1:4000 with a rabbit anti-mouse Ig (HRP) (Dako Ltd.) at 1:500; a rabbit polyclonal anti-PDGF-R (Oncogene Science, Inc.) at 2.5 μg/ml with a goat anti-rabbit Ig (HRP) (Dako Ltd.) at 1:500; a mixed monoclonal anti-PLC-γ 1 (Update Inc.) at 1 μg/ml with the goat anti-rabbit Ig; and a polyclonal affinity purified rabbit anti-rGAP (gift from Dr. F. McCormick, Cetus Corporation, Emeryville, CA) at 1:500 with the goat anti-rabbit Ig.

4.3. RESULTS

4.3.1. TYROSINE PHOSPHORYLATION OF CELLULAR PROTEINS IN PDGF-BB STIMULATED CO25 MYOBLASTS

Phosphorylation of proteins at tyrosine residues following growth factor stimulation, in particular PDGF, has been investigated in various cell types (Coughlin *et al.*,1989; Molloy *et al.*,1989; Kaplan *et al.*,1990; Brambilla *et al.*,1991; Downing and Reynolds, 1991; Lee and Donoghue,1991; Rake *et al.*,1991; Ueno *et al.*,1991; Walker *et al.*,1992). In this part of the present study, tyrosine phosphorylation (TP) of proteins following stimulation of CO25 myoblasts with PDGF-BB has been examined using a monoclonal anti-phosphotyrosine antibody. Na_3VO_4 was used as a potent inhibitor of tyrosine-specific protein phosphatases which could affect phosphoproteins (Swarup *et al.*,1982) by adding into PBS to wash cells and also into the lysis buffer. Addition of Na_3VO_4 was able to provide the signal of PDGF-R and other proteins phosphorylation.

To find the right incubation time of CO25 cells with PDGF-BB, after serum starvation, cells were tested following incubation times for 5 , 10, 20 and 30 minutes in the presence of PDGF-BB followed by Western blotting. In Fig.20, the results of this time-course experiment showed that after 20 minutes incubation with PDGF-BB, the 185 kDa and 160 kDa phospho-protein bands had disappeared. 5 or 10 minutes was adequate for incubation with PDGF-BB to phosphorylate the proteins. Cells were subsequently treated with PDGF-BB for five minutes during subsequent investigations.
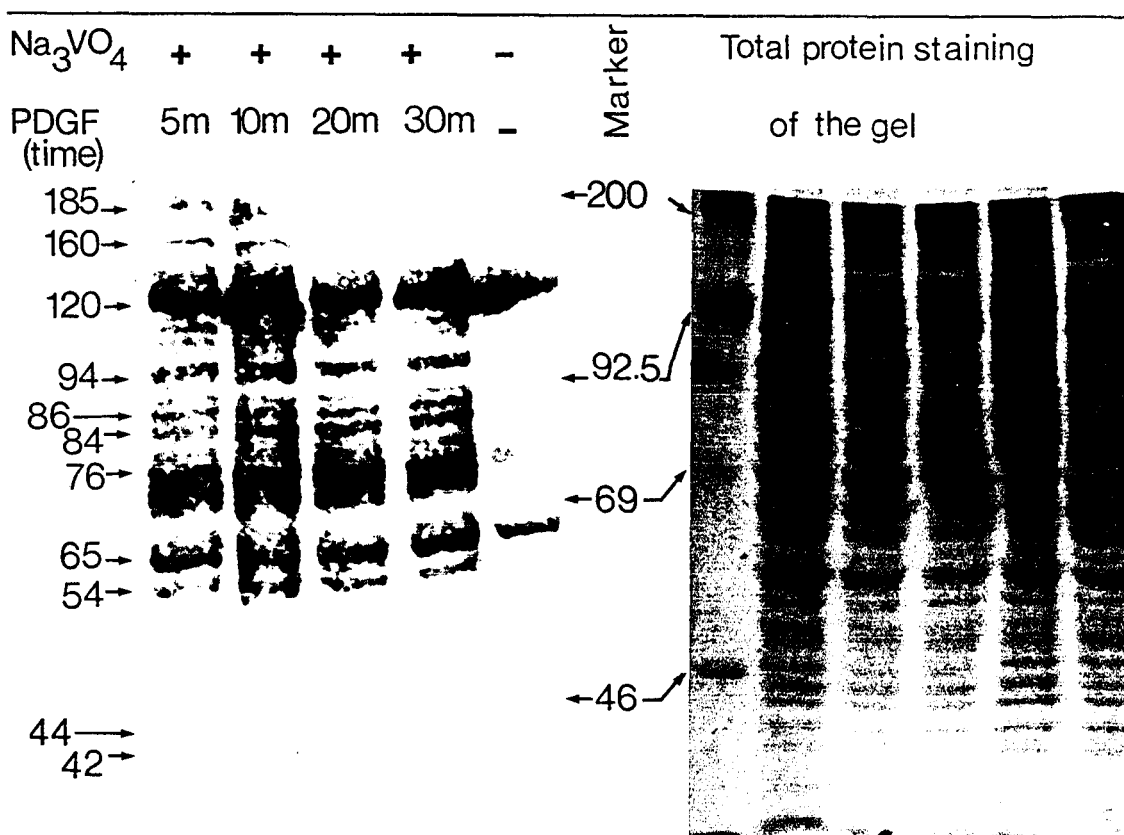
Fig.20. Time course analysis of tyrosine-phosphorylated proteins following PDGF-BB stimulation of CO25 cells. CO25 cells grown in 20% FCS were treated with PDGF-BB for the indicated times and analysed as described in Materials and Methods by immunoblotting with anti-P.Tyr antibody in the presence of orthovanadate. (-) indicates the absence of orthovanadate and PDGF-treatment. Numbers on the right indicate the molecular weight of the standards, and on the left the molecular weight of the tyrosine phosphorylated proteins.

Stimulation of CO25 cells with PDGF-BB for five minutes resulted in TP of 185 kDa, 160 kDa, 94 kDa, 54kDa, 44 kDa and 42 kDa proteins, and in significant increases in the TP of the 120 kDa, 84kDa, 76 kDa, 70 kDa, 65 kDa and 58 kDa proteins as seen in Fig.21. Some cells which were not treated with PDGF-BB prior to lysis were analysed in a parallel well as the control. As shown in Fig.21-A(marked as -), only weak TP of 120kDa, 76 kDa, 70 kDa, 65 kDa and 58 kDa proteins was observed.

To examine the specifity of the anti-phosphotyrosine antibody, a duplicate blot was incubated with the antibody in the presence of para-nitrophenyl phosphate which is a substrate for tyrosine. When the blot was incubated with the antibody plus para-nitrophenyl phosphate, there were a few very faint bands (Fig.22.b), indicating the specific binding of the antibody.

4.3.2. PHOSPHORYLATION OF PROTEINS ON TYROSINE RESIDUES FOLLOWING STIMULATION OF *RAS-* INDUCED CO25 MYOBLASTS WITH PDGF-BB

p21 has been shown to inhibit the response of fibroblast cells to stimulation with PDGF (Kaplan *et al.*,1990; Rake *et al.*,1991). For example, TP of some proteins or association of GAP with PDGF-R were inhibited. The effects of *ras* p21 on TP of proteins were investigated following PDGF-BB stimulation of CO25 myoblasts.

Cells were grown in 10% HS with or without DEX for four days, and analysed by Western blot using the anti-phosphotyrosine antibody as described in Material and Methods. In the cells grown in 10% HS, the pattern of TP of proteins were similar to the cells grown in 20% FCS, in both PDGF-BB treated and untreated cells (Fig.21.a). But there were differences in the density of some bands.

Phosphorylation of the 185 kDa band was weaker, whereas the 120 kDa, 84 kDa, 65 kDa, 58 kDa and 42 kDa bands were stronger. The 44 kDa protein was not phosphorylated in the PDGF-BB stimulated cells grown in 10% HS. In the cells untreated with PDGF-BB, the pattern of TP of proteins was similar to the untreated cells grown in 20% FCS, except that the 120 kDa band gave a stronger band.

In contrast with the cells grown in FCS or HS, cells expressing the N-*ras* oncogene showed a pattern of TP of proteins significantly different following stimulation with PDGF-BB. TP of the 185 kDa, 160 kDa, 58 kDa, 54 kDa, 44 kDa and 42 kDa proteins (Fig.21.a) did not occur in these cells. The TP of 120 kDa, 84 kDa, 76 kDa, 70 kDa and 65 kDa proteins however were stronger than in cells grown in HS. In contrast, phosphorylation of 86 kDa and 94 kDa proteins were weaker in these cells. There were no differences in the TP of proteins between the protein samples from the untreated- or treated- cells with PDGF-BB. Treatment of cells expressing the N-*ras* oncogene with PDGF-BB did not show any differences when compared to the other cells.

Fig.21. (next page) Tyrosine phosphorylation of cellular proteins in CO25 myoblasts. (A) CO25 cells grown in 20% FCS, 10% HS and HS+DEX starved overnight were stimulated (+) or unstimulated (-) with PDGF-BB. Cell lysates containing orthovanadate were analysed as described in Material and Methods by immunoblotting with anti-P.Tyr antibody. An autoradiograph of ECL labelled proteins of representative experiments is shown. Numbers on the right indicate the molecular weight of the standards, and on the left indicate the molecular weight of the tyrosine phosphorylated proteins. (B) Specificity of immunoblotting with anti-P.Tyr antibody. Immuno-blotting was carried out as described in (A) with 5 mM para- nitrophenyl phosphate.

PDGF − + − + − + + +

A

B

185→
160→
120→

94→
86→
84→
76→
70→
58→
54→

44→
42→

FCS   HS   HS+DEX   FCS   HS

200→

92.5→

69→

46→

Marker   Total protein staining of the gel
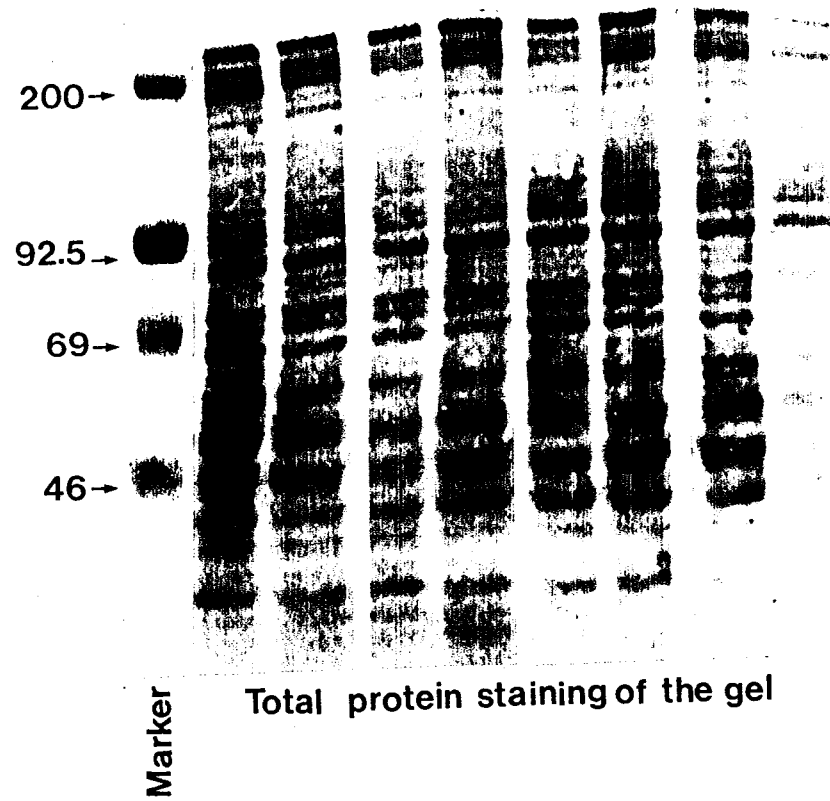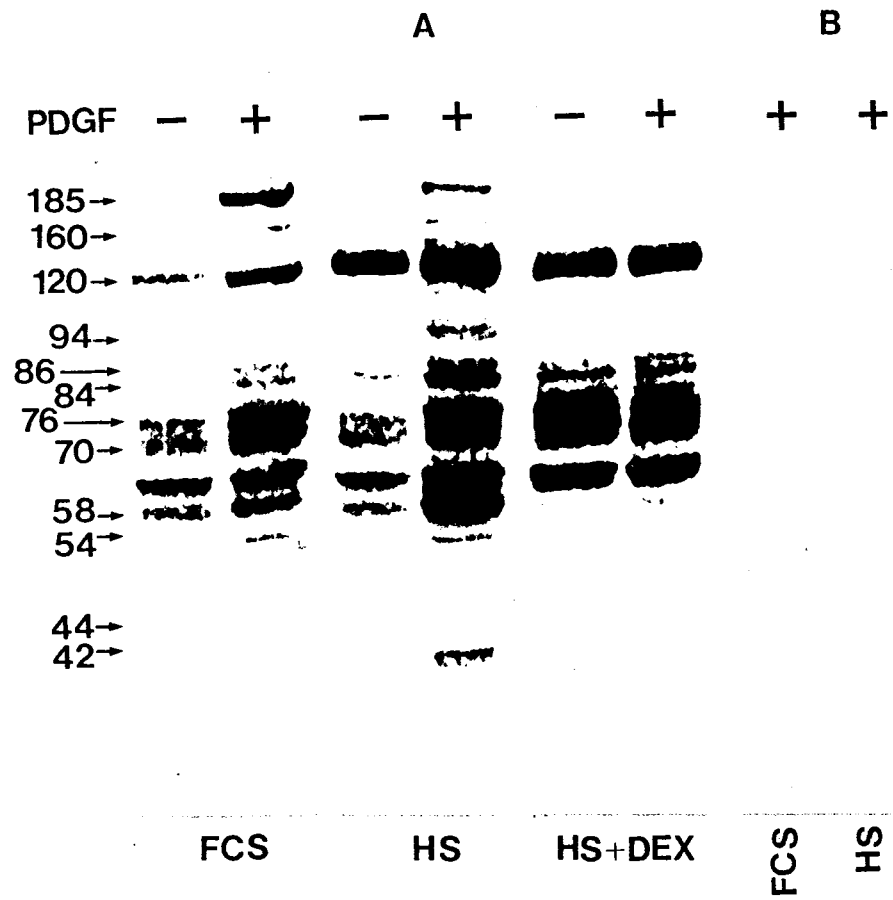
4.3.3. TYROSINE PHOSPHORYLATION OF PROTEINS AFTER PDGF STIMULATION OF C2 MYOBLASTS GROWN IN THE FUSION PROMOTING MEDIUM WITH OR WITHOUT DEX

To test whether DEX may have an effect on tyrosine phosphorylation of proteins stimulated with PDGF-BB, parental C2 cells were grown in 10% HS with or without DEX and analysed by Western blotting using P.Tyr antibody. Fig.22 shows that the pattern of phosphorylated proteins at tyrosine was similar to that found in CO25 cells grown in 10% HS. This was done both for PDGF-BB treated- and untreated-cells. To test the specificity of the P.Tyr antibody, para-nitrophenyl phosphate was added during the incubation of duplicate samples with the antibody. As shown in Fig.22 in lane 5,6, there were a few weak bands on the blot in contrast with the blot incubated with only the P.Tyr antibody.

4.3.4. THE LEVEL OF THE PDGF-RECEPTOR PROTEIN IN CO25 MYOBLASTS

In the previous experiment, expression of the N-*ras* oncogene inhibited the TP of the 185 kDa protein. The lack in the responses to the PDGF-BB stimulation in CO25 cells after induction of the N-*ras* oncogene may be correlated with the loss of PDGF-R. Therfore, in this study, the levels of PDGF-R protein were analysed using a polyclonal PDGF-R antibody which recognizes the extracellular portion of PDGF-R (Coughlin *et al.*,1989) in CO25 cells. Western blot analysis showed that in the cells grown in 20% FCS or 10% HS (for four days) stimulated or unstimulated with PDGF-BB, the levels of PDGF-R protein were similar those shown in Fig.23. lanes 1,2,3,4.

Fig.22. Tyrosine phosphorylation of cellular proteins in C2 myoblast cells. C2 cells grown ; **(1,3)** in 10% HS, **(2,4)** in HS+DEX, **(1,2)** stimulated or **(3,4)** unstimulated with PDGF-BB were analysed as described in Materials and Methods by immunoblotting with anti-P.Tyr antibody in the presence of orthovanadate. **(5,6)** show immunoblotting in the presence of 5 mM para-nitrophenyl phosphate.

In contrast, there was a significant difference in the level of PDGF-R protein in the cells expressing the N-*ras* oncogene (Fig.23, lanes 5,6). However, two bands were visible on the blot probed with PDGF-R antibody at molecular weights of 185 kDa and 130 kDa, indicating the presence of the mature and immature form of receptors, as shown elsewhere (Heldin *et al.*, 1985; Coughlin *et al.*,1989; Kumjian *et al.*,1989; Kaplan *et al.*, 1990; Walker *et al.*,1992; Larochelle *et al.*,1990; Ueno *et al.*,1992).
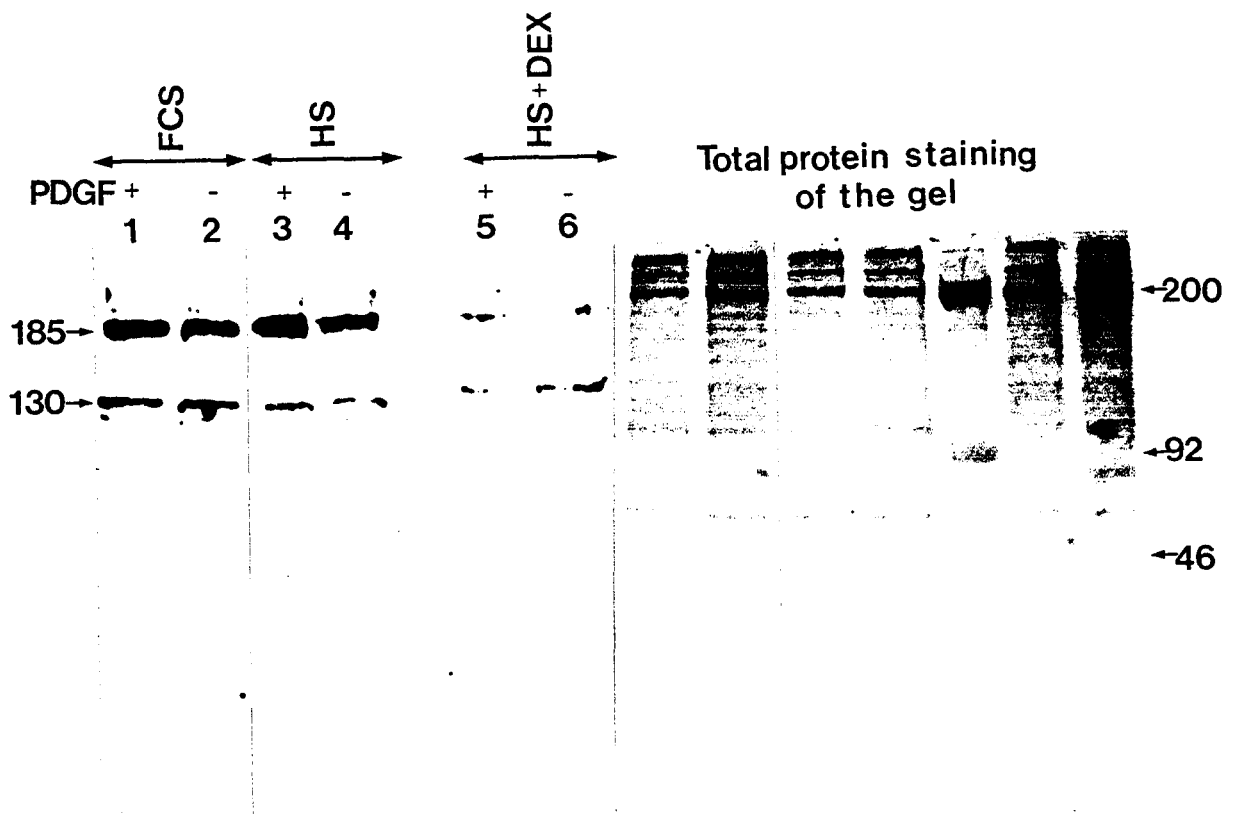
Fig.23. Expression of PDGF-R in CO25 cells analysed by Western blotting. CO25 cells grown in 20% FCS (**1,2**), 10% HS (**3,4**) and HS+DEX (**5,6**) were stimulated (+) or unstimulated (-) with PDGF-BB. Cell lysates were analysed as described in Material and Methods by immunoblotting with anti-PDGF-R antibody. An autoradiograph of ECL labelled proteins of representative experiments is shown. Numbers on the right indicate the molecular weight of standards, and on the left the molecular weight of PDGF-R proteins are indicated.

4.3.5. THE LEVELS OF GAP AND PLC-γ 1 PROTEINS IN CO25 CELLS

In section 4.3.1, TP of the 160 kDa protein was found following stimulation of the CO25 cells with PDGF-BB, but not in the cells expressing the N-*ras* oncogene (see 4.3.2). Thus levels of PLC-γ 1 (145 kDa) and GAP (110-125 kDa) were investigated, because one of them could be the 160 kDa tyrosine phosphorylated protein. CO25 cells grown in the three different conditions were analysed by Western blotting using a monoclonal anti-PLC-γ 1 and a polyclonal anti-GAP. Fig.24.b shows that the PLC-γ 1 antibody recognized a major protein at 160 kDa molecular weight similar to the band found by probing with the phosphotyrosine antibody. Thus this 160 kDa phosphorylated protein may be PLC-γ 1 in these cells. Also this antibody recognized another very weak band at 46 kDa. The levels of PLC-γ 1 protein were similar in all the cells, and incubation of the cells with PDGF-BB had no effect on the levels of PLC-γ 1 protein.

Fig.24.a shows that the GAP antibody recognized two main proteins on a duplicate blot. The major band was a 120 kDa protein which migrated the same distance as one of the major tyrosine phosphorylated proteins in the range of 120-135 kDa. The level of expression of GAP was similar in all the cells and also in cells with or without the addition of the PDGF-BB. The GAP antibody recognized a second band at molecular weight 44 kDa. This protein might be a proteolytic fragment of GAP.

The 120 kDa band obtained with the phosphotyrosine antibody was a thick band in PDGF-stimulated cells. This band migrated with the proteins between 120 and 135 kDa molecular

92

weight, and this is thought to represent more than one protein. GAP migrated a similar distance to this band.
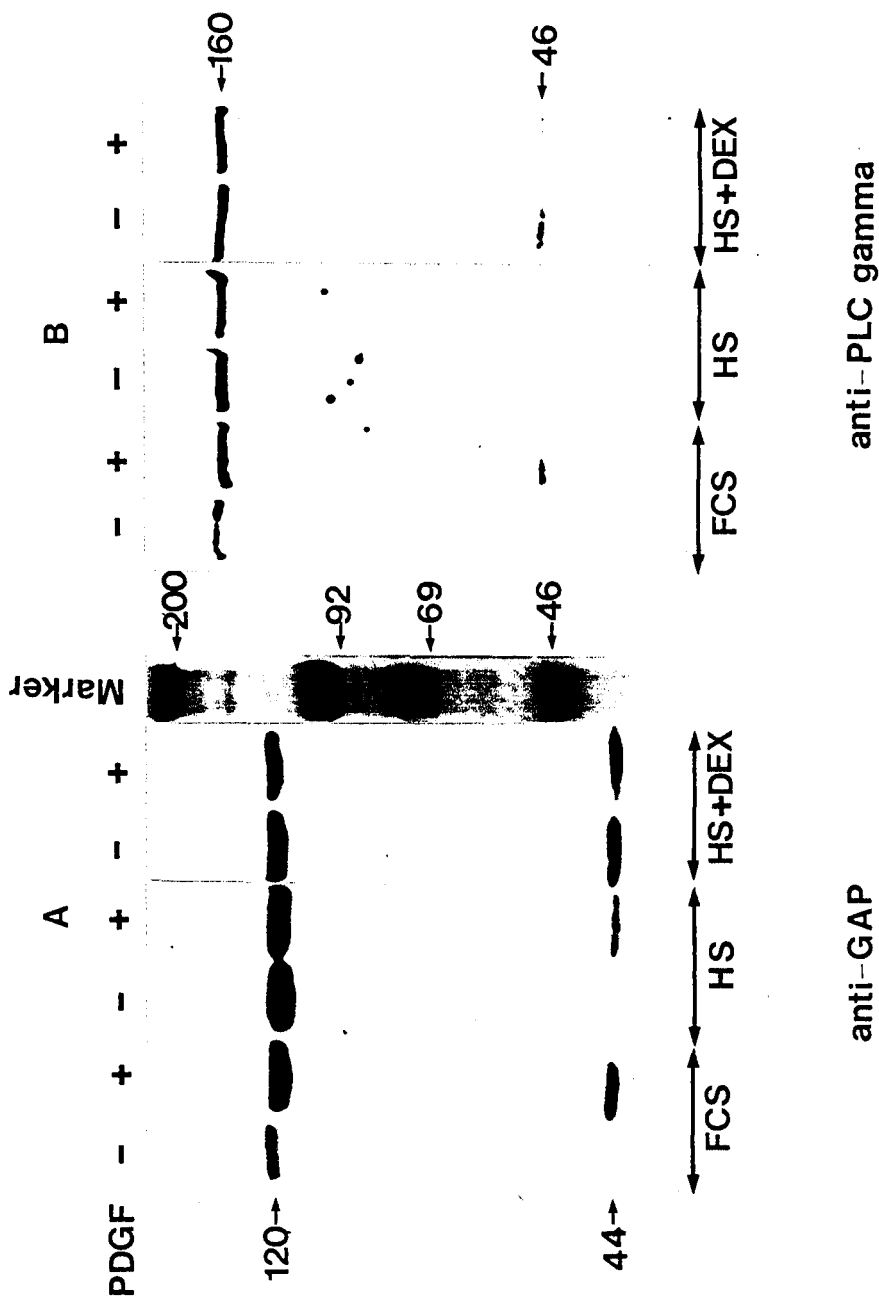
Fig.24.   Expression of GAP and PLC-γ were analysed by Western

blotting as described in Materials and Methods in CO25 cells grown in 20% FCS, 10%

HS and HS+DEX, stimulated (+) or unstimulated (-) with PDGF-BB. (A)

Immunoblotting with anti-GAP antibody, (B) Immuno-blotting of the same lysates with

anti-PLC-γ 1 antibody. Numbers in the middle indicate the molecular weight of

standards, those on the right indicate the molecular weight of PLC-γ 1, and on the left

the molecular weight of GAP.

It is reported that GAP was phosphorylated at tyrosines following PDGF stimulation of a 120kDa protein, and transformation by the *ras* oncogene inhibited its association with PDGF-R and its phosphorylation (Kaplan *et al.*, 1990). Thus, to investigate the TP of GAP in cells grown in the 10% HS with or without DEX, following stimulation of the cells with PDGF-BB, proteins were precipitated with the GAP antibody in the presence of Na_3VO_4 then probed with the phosphotyrosine antibody, and reprobed with the GAP antibody.

To test the nonspecific binding of proteins to the Protein A-Sepharose beads, duplicate protein samples were incubated under the same immunoprecipitation conditions in the absence of the GAP antibody. Fig.25.a, lane 1-2 show the control immunoprecipitated protein samples in the absence of the GAP antibody, indicating the unspecific binding of several proteins to Protein A- Sepharose beads. As shown in Fig.25.a, lane 4 (arrowed), a 120 kDa protein was detected with the phosphotyrosine antibody in cells expressing the N-*ras* oncogene. In contrast, there was no phosphoprotein in lane 3 cells grown in the absence of DEX.

The same blot was stripped and reprobed with GAP antibody to analyse the level of expression. In contrast to the TP of GAP, anti-GAP recognized the same 120 kDa proteins in both lanes. Also stronger bands were found at 58 kDa in these two lanes when compared to the control lanes (Fig.25.b,lanes, 1-2).
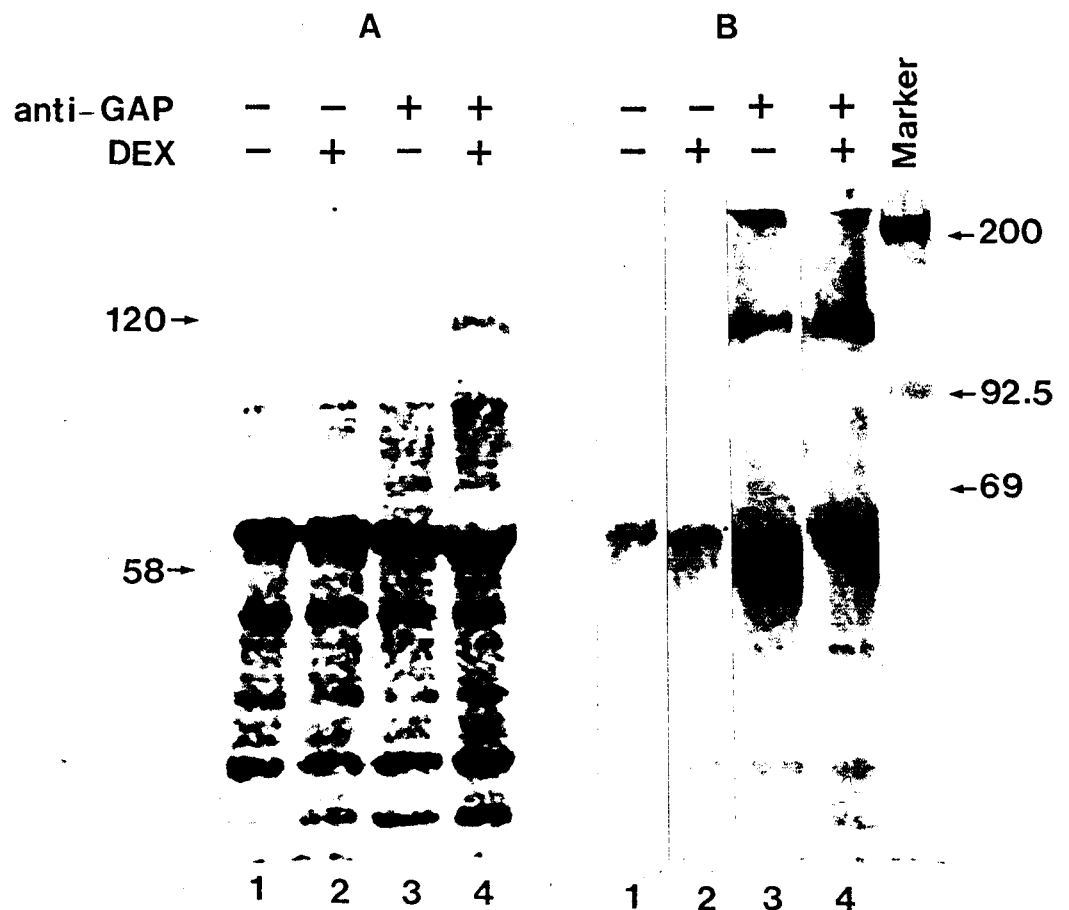
Fig.25. Immunoprecipitation of GAP from CO25 cells grown in 10% HS with (2,4) or without (1,3) DEX for four days. PDGF-treated cells were lysed and immunoprecipitated with anti-GAP antibody in the presence of orthovanadate and immunoblotted as described in Materials and Methods. The immunoprecipitates from the 250 µg total protein samples were probed with (**A**) anti-P.Tyr antibody, and reprobed with (**B**) anti-GAP antibody. (**1,2**) Immunoprecipitations in the absence of the antibody.

4.3.6. TYROSINE PHOSPHORYLATION OF CELLULAR PROTEINS IN CO25 CELLS IN THE ABSENCE OF NA$_3$VO$_4$

TP of proteins were investigated in CO25 cells without PDGF-BB treatment and in the absence of Na$_3$VO$_4$. Na$_3$VO$_4$ may have an

effect on cellular proteins, including *ras* p21. Also starvation of the cells for 10-12 hours could have effect on the cells. CO25 cells were therefore grown in 20% FCS, 10% HS with or without DEX, lysed in a buffer without Na_3VO_4, and analysed by Western blotting using the anti-phosphotyrosine antibody.

As shown in Fig.26.a, lane 1,2, four major bands were detected, a strong band at 65 kDa molecular weight and three weak bands at 185 kDa, 120 kDa and 42 kDa. In contrast to the samples from the cells grown in FCS and HS, the 120 kDa protein band disappeared in the sample from the cells expressing the N-*ras* oncogene (Fig.26.a, in lane 3). A few weak bands were observed at in a longer exposure time in the absence of tyrosine phosphatase inhibitor when compared with the blot in the presence of Na_3VO_4. Repeat experiments from different protein samples gave similar results. As seen in Fig.21, in the presence of Na_3VO_4, there was no significant difference in the TP of the 120 kDa protein between the normal and the *ras*-induced cells.

A polyclonal GAP antibody was used to determine the protein levels of GAP in these protein samples, since GAP is known to be a 110-125 kDa tyrosine phosphorylated protein and a substrate for PDGF-R (Molloy *et al.*,1989; Ellis *et al.*,1990; Kaplan *et al.*,1990; McCormick,1990). Western blot analysis showed that the GAP antibody recognized only two main bands (Fig.26.b). One of them was a 120 kDa protein and the mobility was identical to that of 120 kDa protein previously identified with anti-phosphotyrosine antibody in the same protein samples. Thus this protein could be GAP. Also, the expression levels of GAP were found to be equivalent in all cells grown in FCS, HS or in the presence of DEX.
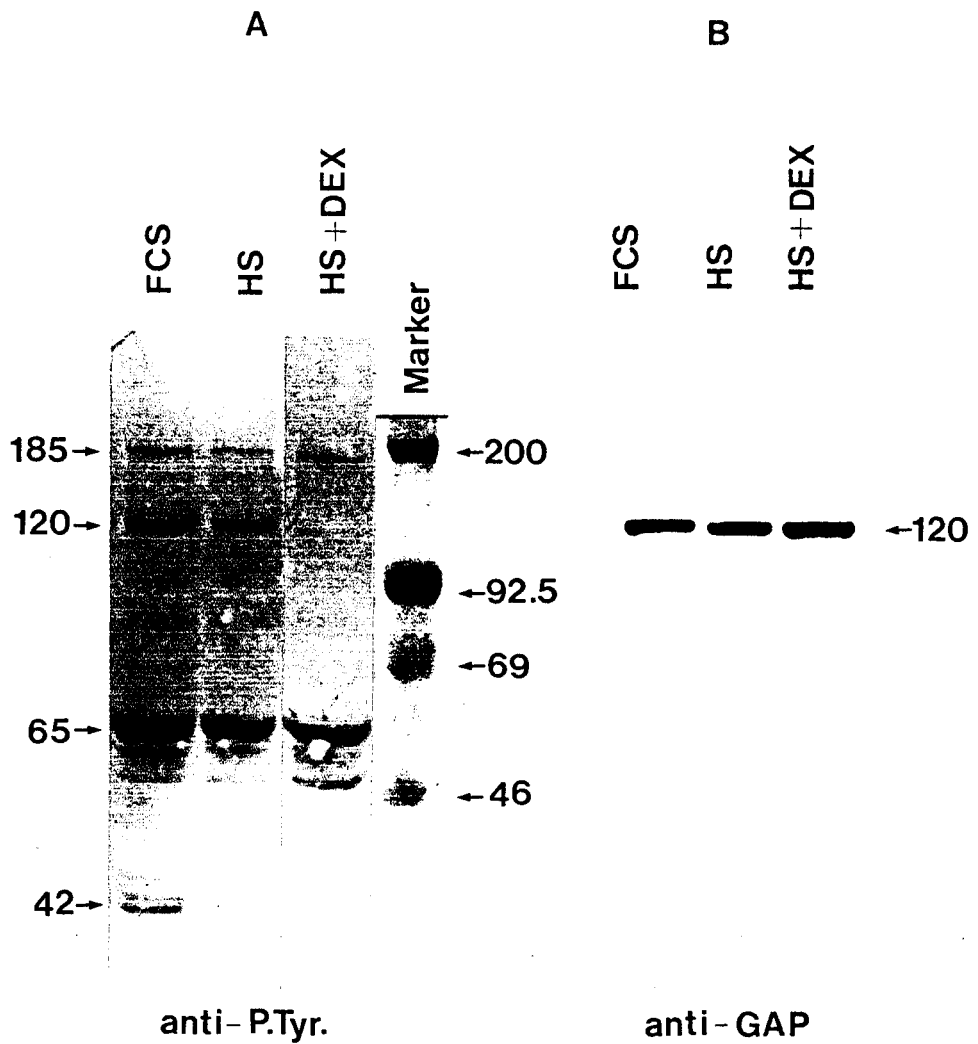
Fig.26. (A) Tyrosine phosphorylation of cellular proteins in CO25 cells in the absence of orthovanadate. Cells were grown in 20% FCS, 10% HS or in the presence of DEX, lysed in the absence of orthovanadate and immunoblotted with anti-P.Tyr antibody as described in Materials and Methods. (B) The same samples in a duplicate blot were immunoblotted with anti-GAP antibody. Numbers in the middle indicate the molecular weight of standards, at the right indicate the molecular weight of GAP, and at the left indicate the molecular weight of tyrosine-phosphorylated proteins.

4.4. DISCUSSION

Tyrosine phosphorylation is one of the key events in signal transduction following PDGF-stimulation of cells. Many proteins involved in this process are substrates of the ligand-activated receptor kinase which is itself tyrosine-phosphorylated (Hunter, 1989; Heldin and Westermark, 1990; Ullrich and Schlessinger, 1990). Several methods have been used to identify substrates for the PDGF-R or other growth factors. For example, Cooper *et al.* (1982) analysed lysates from metabolically labelled fibroblasts following PDGF-stimulation and found certain components at 45 kDa to 43 kDa. The use of anti-P.Tyr antibodies for immunoblotting or immunoprecipitation has been the most successful method for the identification of protein-tyrosine kinase substrates which are ~0.05% of cellular proteins in most cells (Hunter, 1989). The abundance of these proteins can be increased by pretreating the cells with the PTPase inhibitor vanadate, which may be helpful in identifying transiently phosphorylated proteins.

Using anti-P.Tyr antibodies, PDGF-R has been identified as the tyrosine-phosphorylated component at 170-185 kDa in various PDGF-stimulated cells (Ek and Heldin, 1984; Brambilla *et al.*, 1991; Downing and Reynolds, 1991). Following PDGF-stimulation of cells, several other components ranging from 22-150 kDa were also found to be tyrosine-phosphorylated (Ek and Heldin, 1984; Hunter, 1989; Molloy *et al.*, 1989; Kaplan *et al.*, 1990; Brambilla *et al.*, 1991; Downing and Reynolds, 1991).

In the present study, TP was monitored by immunoblotting with an anti-P.Tyr antibody. Proteins were extracted from the cells stimulated or unstimulated with PDGF-BB. Results showed that 185, 160, 94, 54, 44 and 42 kDa proteins were phosphorylated at

tyrosine residues in PDGF-BB-stimulated CO25 myoblasts grown in 20% FCS or in 10% HS (Fig.21.a). The tyrosine phosphorylated proteins were barely detectable in CO25 cells stimulated with PDGF-BB in the absence of orthovanadate which appears to act with PDGF in inducing and maintaining the phosphorylation of these proteins. Although vanadate has been reported to induce several cellular effects (Nechay,1984; Paris and Pouyssegur,1987), it was used as a specific inhibitor of phosphotyrosine phosphatases (PTPase).

In response to PDGF-BB, 185 kDa and 160 kDa proteins were phosphorylated at tyrosine within 5 minutes of ligand binding. Maximum phosphorylation occured at 10 minutes, and then they dephosphorylated. In spite of the presence of vanadate, the phosphorylation of these two proteins remained a transient event, but phosphorylation of the others appeared to be long-lasting. Brambilla *et al.* (1991) reported the transient phosphorylation of PDGF-R for 30 minutes following PDGF-stimulation of fibroblasts.

I have found that the 185 kDa protein was PDGF-R by immunoblotting with anti-PDGF-R antibody. Thus, the 185 kDa phosphorylated protein detected by PTyr. antibody may be the PDGF receptor. PDGF-R antibody recognized two proteins at 185 kDa and 130 kDa (Fig.23). The mature form and the immature or precursor form of PDGF-R were reported as 185 kDa and 160 kDa respectively by Coughlin *et al.* (1989), Kumjian *et al.* (1989), Kaplan *et al.* (1990), Larochelle *et al.* (1990), Ueno *et al.* (1991), and Walker *et al.* (1992). Heldin *et al.* (1985) reported the phosphorylation of 185 kDa and 130 kDa proteins which represent the PDGF-R proper which undergoes autophosphorylation and a fragment of the receptor formed after cleavage by an endogenous

Ca^{2+}-dependent SH-protease. Thus the fragment at 130 kDa which was recognized by anti-PDGF-R antibody may be an the immature form of the receptor.

PLC-γ was found as a 160 kDa protein by immunoblotting with anti-PLC-γ1 antibody (Fig.24.b). Previously, PLC-γ has been found at 145-150 kDa molecular size in various cell types (Meisenhelder *et al.*,1989; Smith *et al.*,1990; Meldrum *et al.*,1991; Rhee,1991). In the estimation of molecular sizes, different molecular weight markers may not be acurate. Small differences are possible in the calculation of protein sizes. Tyrosinphosphorylated 160 kDa protein which was detected after PDGF-BB stimulation might be PLC-γ. It has been shown that PDGF treatment of cells leads to a rapid increase in tyrosine phosphorylation of PLC-γ (Kumjian *et al.*,1989; Meisenhelder *et al.*, 1989; Kaplan *et al.*,1990; Morrison *et al.*,1990).

Several protein tyrosine kinase substrates are proteins associated with the plasma membrane and involved in the regulation of signal transduction by growth factors. They are also cytoplasmic proteins which are involved in intracellular signalling pathways (Hunter,1989). Several of these phosphorylated proteins have been identified following PDGF-stimulation of cells. For example; the 85 kDa protein is phosphatidylinositol kinase (PI-K), the 75 kDa protein is the raf proto-oncogene product (Kaplan *et al.*,1990; Rapp,1991), the 42 kDa protein is MAP (microtubule associated proteins) kinases (ERK1 and ERK2) (Swenson *et al.*,1990), and the 39 kDa protein is calpactin I (annexin II) (Brambilla *et al.*,1991). Many other proteins have been found to be phosphorylated at tyrosine by growth factor stimulation or by oncogenic tyrosine kinases. For example; ezrin (cytovillin, 81 kDa),

talin (76 kDa), paxillin (68-76 kDa), connexin (Gap junction proteins, 43 kDa), acetylcholine receptor (42 kDa), and cdc^2 (protein-serine kinases, 34 kDa) (recently reviewed in Hunter, 1989; Glenney, 1991).

PDGF-BB-treatment of CO25 cells resulted in the TP of 94 kDa, 54 kDa, 44 kDa and 42 kDa proteins and in an increase in the TP of 84 kDa, 76 kDa, 70 kDa, 65 kDa and 58 kDa proteins which are at similar sizes with the tyrosine-phosphorylated proteins given above. In the CO25 cells, PDGF might be causing the TP of some of these proteins.

In other experiments, I have observed the stimulation of TP of a 185 kDa protein, and the TP of 94, 54, 44 and 42 kDa proteins were markedly reduced (Fig.21.a). They were not visible in the lysates from the *ras*-induced CO25 cells as compared with those from cells grown in 20% FCS or 10% HS. Even in the presence of orthovanadate, PDGF-BB-treatment did not produce a change in the phosphorylation of proteins when compared with the PDGF-BB-untreated cells. The TP of 120, 84, 76, 70 and 65 kDa proteins were strong in the *ras*-induced cells with or without PDGF. This was similar to their TP in the PDGF-stimulated cells grown in FCS or HS. The 58 kDa protein which was tyrosine-phosphorylated in the presence or absence of PDGF dissapered after the induction of the N-*ras* oncogene.

These results in the *ras*-induced cells can be summarized as 1- The TP of proteins were similar in both PDGF-stimulated or unstimulated cells, 2- the TP of the proteins in the unstimulated cells was as strong as those proteins in the PDGF-stimulated cells, and 3- the 58 kDa protein was not tyrosine-phosphorylated in

either the PDGF stimulated or unstimulated cells, whereas it was phosphorylated following PDGF-stimulation of the control cells.

All these observations suggest that *ras* p21 is involved in a signalling pathway by phosphorylation of some cellular proteins on tyrosine residues independent of the PDGF-induced signalling pathway, perhaps by inhibiting the expression or posttranslation of PDGF-R and consequently its signalling pathway. If the PDGF activation of PLC and the other proteins are dependent on PDGF-R, then a defect in receptor autophosphorylation could account for the lack of PDGF stimulation, or if the PDGF-R level is decreased. Consequently the autophosphorylation of the receptor and the other substrates could be reduced.

My findings in the present study show that the levels of PDGF-R are significantly reduced after induction of the N-*ras* oncogene in CO25 cells, in conflict with the findings by Kaplan *et al.* (1990) and Rake *et al.* (1991) who found that the ras-transformed cells which showed a lack in responses to PDGF-stimulation had the same level of PDGF-R as the control cells, indicated either by Western blot or by radioimmunoassay for receptors. Rake *et al.* (1991) reported that inhibition of responses to PDGF-stimulation by *ras* p21 occurred in the early stages of signalling pathway at the levels of PDGF-R autophosphorylation. Previous studies by Benjamin and co-workers (1987) showed that in *ras*-transformed fibroblasts, PDGF was unable to stimulate PLC activity even though the PDGF-Rs in these cells were present in relatively normal numbers and bound PDGF normally.

I conclude that the lack in the responses to PDGF-BB in *ras*-induced CO25 myoblasts could be due to reduced levels of PDGF-R in these cells, and *ras* p21 may act in the transformation or in the

inbition of differentiation by effecting these levels. This effect of p21 might be at the expression level of the receptor gene or at the posttranslational level of the receptor gene expression. In the absence of PDGF-R or activated PDGF-R, *ras*-induced CO25 cells are able to proliferate and later form foci. There may be multiple signal transduction pathways where *ras* p21 acts in these events to mediate the different downstream effects of PDGF.

In the CO25 cells grown in the fusion promoting medium for four days where cell differentiation occurs, PDGF-Rs were still present and PDGF-BB treatment of the cells stimulated the phosphorylation of proteins at tyrosine similar to the cells grown in the 20% FCS. Previously, it has been shown that during differentiation of several kinds of skeletal muscle cells, EGF, FGF and TGF-β receptors were decreased in the cells (Olwin and Hauschka,1988; Ewton *et al.*,1988), whereas the level of insulin receptors, insulin-like growth factor receptors and also their ligands were increased (Tollefsen *et al.*,1989a; 1989b; Standaert *et al.*,1984). This suggests that IGFs may have autocrine effect on skeletal muscle.The presence of PDGF-Rs may not affect the differentiation process of CO25 cells in the serum deficient medium (10% HS) which has very low mitogenic activity, whilst they regulate the differentiation process by an autocrine mechanism by expressing insulin or IGFs (Ewton *et al.*,1988; Florini *et al.*,1991).

I have analysed the TP of cellular proteins by Western blotting in the absence of orthovanadate in the PDGF-untreated CO25 cells. A 120 kDa protein was not phosphorylated at tyrosine in *ras*-induced CO25 cells when compared to those grown in 20% FCS or 10% HS (Fig.26.a). In the absence of orthovanadate,

immunoblotting with anti-P.Tyr antibody gave very weak protein bands on the blot. The lysis buffer in these samples was without phosphatase inhibitor, thus endogenous phosphatases may be affect all phosphoproteins. In contrast, a major band at 120 kDa was present in the lysates in the presence of orthovanadate (Fig.21.a) in these cells. In the presence of phosphatase inhibitor vanadate, p21 did not affect the phosphorylation of this protein.

Immunoblotting of cell lysates with P-Tyr antibody resulted in a broad band at 120-135 kDa, probably including more than one phosphorylated protein. Thus, I investigated specificly the phosphorylation of GAP by the immunoprecipitation assay. The phosphotyrosine containing proteins precipitated by anti-GAP were probed first with anti-P.Tyr antibody, and then reprobed with anti-GAP antibodies. Anti-P.Tyr recognized a 120 kDa band in the *ras*-induced cells, but did not recognize it in the cells grown in 10% HS (Fig.25.a). The expression of GAP, in contrast to its phosphorylation was present in equivalent amount in both cells as detected by anti-GAP antibody (Fig.25.b).

These results suggest that *ras* p21 may act as a phosphatase on GAP and inactivate its phosphorylation as observed in the absence of orthovanadate. The presence of phosphatase inhibitor vanadate may overcome this effect of p21, and GAP could be phosphorylated at tyrosine residues. The reason for the absence of phosphorylation of GAP in the cells grown in the 10% HS is not clear. It must be investigated by immunoprecipitation of all the samples in the presence or in the absence of orthovanadate. According to Kaplan *et al.* (1990), GAP was present in *ras* immunoprecipitates from activated c-H-*ras*-transformed fibroblast cells but GAP failed to associate with PDGF-R. Furthermore, GAP

was not phosphorylated following PDGF-treatment. My findings also show that GAP was phosphorylated in the ras-induced cells which also showed lack of phosphorylation of PDGF-R and its substrates, suggesting there might be different mechanisms of *ras* action independent from PDGF-R activation.

CHAPTER.5.

ALTERATION OF THE INTRACELLULAR Ca^{2+} LEVEL AFTER PDGF-BB STIMULATION AND THE CHANGES IN THE NUMBER OF THE PDGF RECEPTORS DURING DIFFERENTIATION OR TRANSFORMATION OF CO25 MYOBLASTS.

5.1. INTRODUCTION

5.1.1 EFFECTS OF *RAS* ONCOGENE ON SIGNALLING PATHWAY INDUCED BY GROWTH FACTOR RECEPTORS

The role of the *ras* oncogene product in the transduction of receptor signal stimulated by growth factors and hormons is still unclear. Overexpression of normal N-*ras* p21 in 3T3 cells, increases IP production and DNA synthesis in response to a number of growth factors (Bishop,1988). However reports from several laboratories have yielded conflicting results in *ras* transfected cells. Wakelam *et al.* (1986) found no significant change in basal IP$_3$ levels in control cells when compared to cells expressing high levels of normal N-*ras* p21.

The cells expressing *ras* oncogene showed a decrease in the cellular responses to PDGF. These effects may be due to changes in the properties of the PDGF receptors or to changes in the coupling of the receptors to effector molecules. It has been demonstrated that binding of PDGF to its receptor is uncoupled from certain downstream events, including induction of transcription of the growth-related genes c-*myc* and c-*fos* (Lin *et al.*,1988; Zullo and Faller,1988), stimulation of PLC and mobilization of Ca^{2+} in fibroblasts expressing a transforming *ras* gene when compared with the action of the agonists on normal cells (Benjamin *et al.*,1987; Parries *et al.*,1987; Benjamin *et al.*,1988; Kaplan *et al.*,1990; Polverino *et al.*,1990). Further, scrape loading of v-*ras* into fibroblasts inhibits PDGF-dependent PI turnover (Price *et al.*,1989). Rake *et al.* (1991) recently reported that the blockade by v-*ras* of PDGF signal transduction occurs at a very early stage at the level of

PDGR receptor autophosphorylation. Also, activated c-H-*ras* transfected 3T3 and CHO cells have been found to be defective in certain responses to PDGF such as association of activated receptors with GAP, raf-1, and PI-3 when compared to that of control cells (Parries *et al.*,1987; Kaplan *et al.*,1990).

The growth factor, bradykinin (BK) is a peptide of the kinin family, and is released during tissue damage, causing localized pain, inflammation and vasodilation (Pisano,1975; Regoli and Barabé, 1980). BK has been demonstrated to have a mitogenic effect on Rat 1 cells (Roberts and Gullick,1989). It stimulated the elevation of IP metabolism (Tilly *et al.*,1987; Fu *et al.*,1988), and the activation of PLC (Portilla *et al.*,1988), and consequently Ca^{2+} relase from intracellular stores (Higashida *et al.*,1986; Peres *et al.*,1990).

BK stimulated signalling pathway in various cells has been affected by the expression of the *ras* oncogene, displaying elevated BK receptors (Downward *et al.*,1988; Roberts and Gullick,1989), and increased the production of IP_3 (Downward *et al.*,1988; Parries *et al.*,1987). Bombesin stimulated signal transduction has also been affected by the *ras* expression in fibroblasts. In T15 cells overexpressing the N-*ras* proto-oncogene, bombesin stimulation of IP_3 generation and Ca^{2+} release from intracellular stores was amplified when compared to uninduced cells (Lloyd *et al.*,1989). In fibroblasts transformed with the activated *ras* oncogene, however the ability of bombesin to stimulate Ca^{2+} inflow was inhibited (Polverino *et al.*, 1990).

Most of these findings indicate that *ras* p21 is involved in coupling of growth factor receptors to phosphoinositide hydrolysis via the phosphorylation of several enzymes and substrates, and the

altered *ras* protein may lead to altered regulation of this signalling pathway, probably at the early stages of the signal transduction.

The aim of the present study in this chapter was to determine whether the signal transduction by PDGF or BK or other some growth factors and hormones (such as EGF, insulin, bombesin) was affected by the differentiation of CO25 cells or by the expression of a mutated N-*ras* oncogene during the transformation of CO25 myoblasts. Investigation of the relationship between PDGF and BK stimulated Ca^{2+} mobilization and growth in CO25 during differentiation or transformation have been carried out, and also the number of receptors present on these cells were determined.

5.1.2. CALCIUM SIGNALLING AND THE MEASUREMENT OF CYTOSOLIC Ca^{2+}

Changes in Ca^{2+} consentrations in the cytosol by movements either across the plasma membrane or across intracellular membranes regulate metabolic processes by the association or dissociation of Ca^{2+} with a wide variety of ligands such as proteins and phosphate groups (reviewed in Dawson, 1990).

The cytosolic Ca^{2+} is elevated by the two categories of stimuli hormones and neurotransmitters which work by different signals. One of the first events appears to be electrical signalling which works via channels at the plasma membrane. For example, in mammalian skeletal muscle, the binding of acetylcholine to the nicotinic acetylcholine receptors opens Na^+/K^+ channels, depolarize the membrane leading to propagation of an action potential across the sarcolemma, and then releases Ca^{2+} from the sarcoplasmic reticulum. Secondly, there is the transmembrane chemical signalling which results in   PI   hydrolysis. PI

comprises about 10% of plasma membrane phospholipid in animal cells. Also two phosphorylated PIs, PIP and PIP_2 which are formed from PI by two kinases are present in lesser quantities in the membranes (Dawson,1990). PIP_2 is the substrate for receptors, and hydrolysed by PLC to the second messengers IP_3 and DAG which initiate DNA replication after stimulation by ligands such as PDGF or vasopressin (for more detail see section 4.1.5). DAG stays in the membrane and activates PKC which affects the activation of a Na^+/H^+ exchanger, resulting in the rapid alkalinization of the cytosol.

IP_3 is released to the cell interior and mobilizes intracellular calcium by releasing Ca^{2+} from intracellular pools. IP_3 binds a receptor on a non-mitocondrial intracellular membrane and opens a IP_3- sensitive Ca^{2+} channel to release Ca^{2+} into the cytosol. This release of calcium is counteracted by calcium pumps which would act to lower the level of free calcium by pumping it back into the calcium stores (Dawson,1990; Berridge,1984; Berridge *et al.*, 1985). IP_3 is rapidly degraded to $(1,4)IP_4$ and IP which do not affect Ca^{2+} release, these dephosphorylations rapidly and effectively inactivate the signal molecule (Taylor and Putney,1987).

The increase in cytosolic Ca^{2+} activates a Ca^{2+}- calmodulin-dependent protein kinase, and this complex may act by stimulating protein phosphorylation which may then induce cells to grow by stimulating the synthesis of specific proteins that accumulate early in G_1 (Berridge,1984).

The methods for the measurement of free intracellular Ca^{2+} levels have been improved over the years. Four distinct classes of calcium probes have been used in these methods according the investigations. These probes include; (1) the tetracarboxylate

fluorescent dyes, (2) the bisazometallochromic indicators, (3) the bioluminescent proteins, and (4) the Ca^{2+}- selective micro-electrodes.

The use of the fluorescent Ca^{2+} indicators are most preferable in cells which show cytosolic Ca^{2+} changes in a few seconds to minutes. They are tetracarboxylic acid derivatives of the calcium chelator EGTA. These chelators increase the intensity of their fluorescence upon binding Ca^{2+} and change the profile of their fluorescence spectrum (Tsien,1980). By using fura-2 AM (Fig.27.a) which is the one of these Ca^{2+} indicators, small changes in intracellular Ca^{2+} can be measured in systems in which such changes were previously undetected.

As diagramed in Fig.27.b, fura-2 AM penetrates cell membranes and is sequestered after hydrolysis of the ester groups by cytosolic esterase (Tsien et al., 1985; Dawson,1990).

Compared to other fluorescent Ca^{2+} indicators, fura-2 has higher fluorescent intensity and resistance to photobleaching. It can be used in lower consentration, reducing the toxicity or cell damage (Maxfield,F.R.,1989). Therefore, fura-2 AM was chosen as a Ca^{2+} indicator in the present part of this study, to investigate the changes in the level of intracellular Ca^{2+} following stimulation of CO25 cells.

5.2. MATERIALS AND METHODS

5.2.1. CELL CULTURE

CO25 cells were cultured as described in section 2.4.1. The cells were maintained in the growth medium or in the fusion promoting medium with or without dexamethasone. After five days
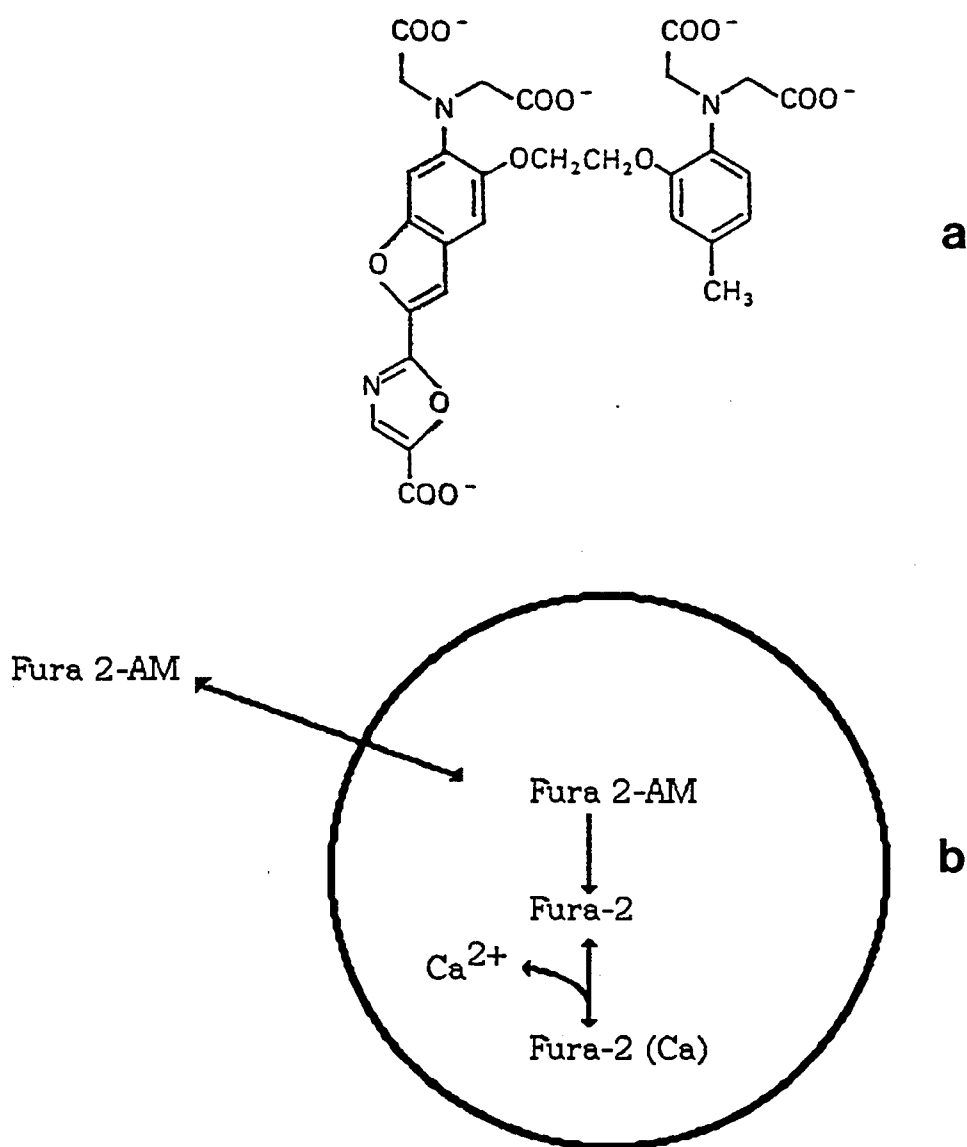
Fig.27. Structure and use of Fura-2. (a) Structural formula of Fura-2; (b) Tetraacetoxymethyl ester derivative of Fura-2 passes through the plasma membrane and intracellular esterases hydrolyse off the ester groups, and Fura-2 is too polar to pass through the plasma membrane. After washing Fura 2-AM, a stable Fura-2 is left inside to measure intracellular Ca^{2+}.

in the fusion promoting medium with or without dexamethasone, the cells were then starved for 10-12 hours in culture medium

(DMEM) in the absence of the serum to increase the sensitivity of the cells to ligands.

To investigate the mitogenic effects of PDGF-BB on CO25 cells, they were incubated in 10% HS in the presence and in the absence of 20nM PDGF-BB for six days, and then examined under the phase-contrast miroscope as described in section 2.4.4.

5.2.2. FLUORESCENCE MEASUREMENT OF CYTOPLASMIC FREE Ca^{2+}

5.2.2.1. Fura-2 AM loading

Fura-2 loading was performed by the modifications of the method as described in Lloyd *et al.* (1989).The cells grown in 145 cm^2 flasks were removed by treatment with trypsin, resuspended in DMEM and transferred to plastic tubes, and the suspension was spun down at 800 rpm for 5 minutes, washed once more with DMEM and resuspended at a concentration of $5X10^6$ cells/ml in DMEM. The cells were loaded by the addition of the calcium indicator acetoxymethl ester of fura-2 (fura-2 AM) (Sigma) at a final concentration $3\mu M$. Fura-2 AM was prepared as a 1mM stock solution in dimethyl sulfoxide. The cell suspension was incubated at 37^oC in the tissue culture incubator with occasionaly mixing. After 30 minutes, the cells were spun down and resuspended in 10 ml of DMEM, and then incubated for a further 45 minutes at 37^oC to allow deesterification of the indicator. Fluorimetric measurements demonstrated that this consentration of fura-2 AM and this time for incubation were adequate for complete intracellular hydrolysis of the fura-2 AM. The cells were then washed twice in HBS (140 mM NaCl, 5 mM KCl, 1.8 mM $MgCl_2$, 10 mM glucose, 1mM $CaCl_2$ and

10 mM Hepes, pH 7.4), and resuspended in HBS at a concentration of 2×10^6 cells/ml. Before each experiment, the viability of cells were examined by staining with trypan blue. Generally, 80% of the cells were found to be viable.

5.2.2.2. Fluorescence measurements of cells loaded with fura-2

Fluorescence measurements were performed in a Shimadzu RF-5000 recording spectrofluorophotometer equipped with a magnetic stirring device and a thermostatically controlled cell housing set. For each measurement, 1,5 ml of the cell suspension was placed in a quartz cuvette and incubated at 37°C for 2-3 minutes before the addition of agonists. Triplicate or further determinations were performed in each experiment. Fluorescence was continuously recorded at an excitation wavelength of 340 nm and 380 nm, and at an emission wavelength of 508 nm using a 5 nm band width.

Digitonin (BDH Lt.) was prepared in DMSO and used at a concentration of 50 µg/ml, to determine maximum fluorescence of the calcium-saturated dye. To determine the fluorescence of free dye, EGTA (Sigma), pH 9.0, was used at aconsentration of 4 mM.

5.2.2.3. Agonists

PDGF-BB and bradykinin were obtained from Sigma, and used at a concentration 13 ng/ml and 1µM respectively. FCS was obtained from Gibco and used as 1% (v/v) concentration. Ionomycin was a gift from Dr. G.Duncan (University of East Anglia).

5.2.3. PDGF-RECEPTOR BINDING

CO25 and C2 cells were prepared by culturing 1×10^4 cells in each well of a 24-well tissue culture plate in the growth or fusion promoting medium with or without dexamethasone as described in section 2.4. The cells were starved by incubation in DMEM without serum for 10-12 hours before binding experiments were carried out. Radioreceptor assay was performed by the modification of the method as described in Singh (1987).

Cell monolayers were washed once with cold PBS, and covered with cold DMEM containing 1 mg/ml BSA and varying amounts of ^{125}I-PDGF-BB (specific activity 1000-1800 Ci/mmol, NEN research) in triplicate wells. Incubation was carried out for 3 hours with gentle rocking in a CO_2 gassed container in a cold room at 4^oC. To determine unspesific binding, unlabelled PDGF-BB at a concentration 20 ng/ml was added in to the some of wells (Bowen-Pope and Ross, 1985). For competition experiment, increasing amounts of unlabelled PDGF-BB (ranging from 0-600 ng/ml) were added in to the wells. The cells were incubated for 1 hour at 4^oC and then 2 nm of ^{125}I-PDGF-BB was added in to the each well, incubated further 2 hours. At the end of the incubation period, unbound ^{125}I-PDGF-BB was removed by aspiration of the assay mixture and washing 3 times with cold DMEM/BSA (1 mg/ml). The cells were then incubated with 1 ml of 1% Triton X-100 containing 0.1% BSA at room temperature for 15 minutes. The contents of the wells were mixed by pipetting up and down a few times and transferred to polystyrene tubes (LP/3, Luckham) for gamma counting. The radioactivity in the cell lysate was quantitated in a 1275 MiniGamma (LKM, Wallac) gamma counter which was found to have 93% efficiency.

To test the unspecific binding of ^{125}I-PDGF-BB to the culture well, labelled PDGF-BB was added to some wells without cells, and same procedure was performed. Little PDGF-BB was bound to the well.

5.3. RESULTS

5.3.1. MITOGENIC EFFECTS OF PDGF-BB ON CO25 MYOBLASTS

To investigate the effects of PDGF-BB on differentiation of CO25 cells, the cells were incubated in 10% HS to induce differentiation, and in 10% HS in the presence of PDGF-BB for several days. They were examined under a phase-contrast microscope as described in section 2.4. After four days in the HS, myoblasts began to fuse and to form myotubes as shown in Fig.28.a. But in the presence of PDGF-BB, CO25 myoblasts did not form myotubes in 10% HS (Fig.28.b). They grew in a criss-cross pattern similar to those grown in the presence of DEX (Fig.28.c).

5.3.2. CHANGES IN AGONIST STIMULATED INTRACELLULAR FREE Ca^{2+} IN AN ACTIVATED N-*RAS* TRANSFECTED CO25 MYOBLASTS

A direct consequence of PDGF-stimulated PLC activation is Ca^{2+} mobilization which plays an important role as an intracellular messenger in the action of mitogens (Berridge,1984). Measurements on single cells and populations of cell using fura-2 have shown an increase in intracellular Ca^{2+} (Cobbold and Rink,1987; Martinez-Zaguilan *et al.*,1991; Uto *et al.*,1991). In principle then, reduced PDGF-stimulated PLC activity or reduced
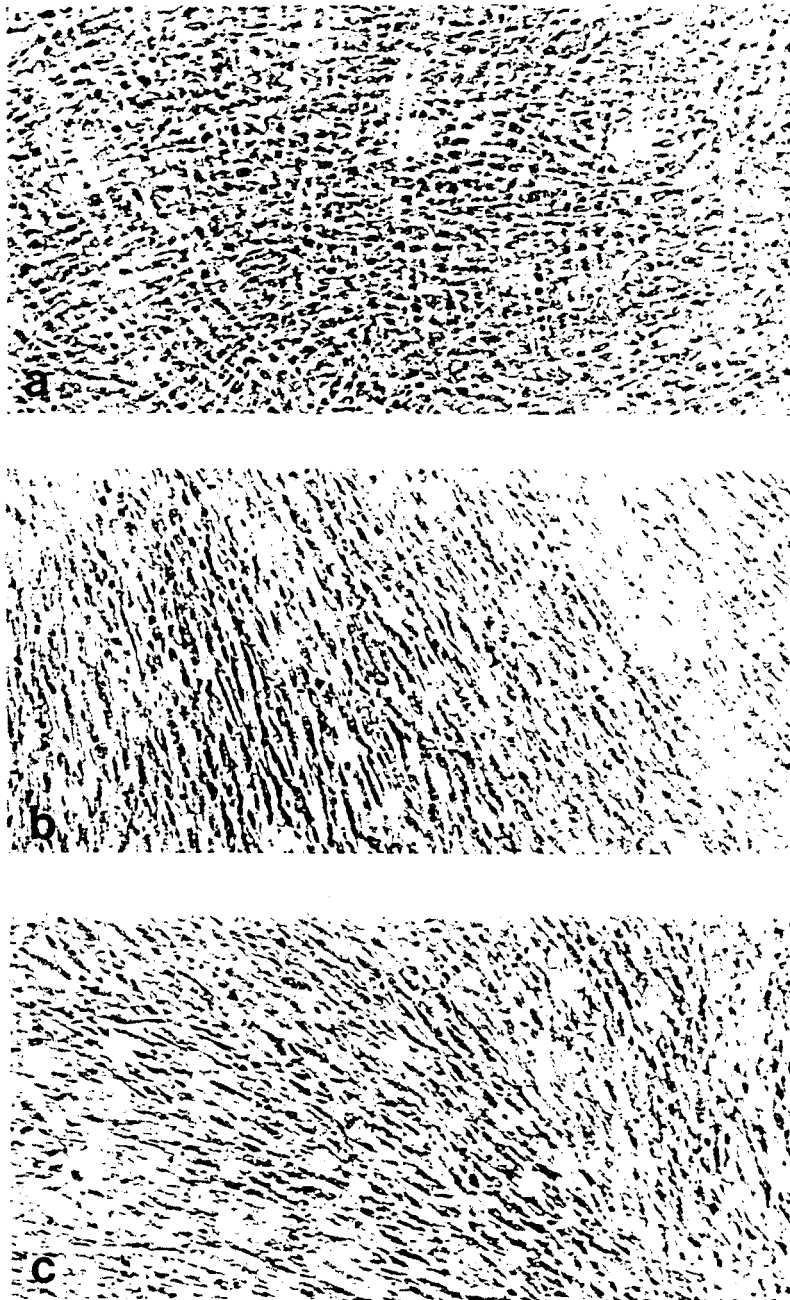
Fig.28. Mitogenic effects of PDGF-BB on the CO25 cells were illustrated by the light microscopy as described in Materials and Methods. Cells (a) in 10% HS, (b) in the presence of PDGF-BB, and (c) in the presence of DEX, for four days. Magnifications: 120X.

expression of PDGF receptors might result in diminished changes in intracellular Ca^{2+} since PLC or PDGF receptors are not activated. Consequently, I measured the levels of intracellular calcium $[Ca^{2+}]_i$

by changes in fura-2 fluorescence in control and in *ras* induced CO25 cells exposed to PDGF-BB.

Also BK has been shown to realase Ca^{2+} from intracellular stores via the production of IP_3 by binding to its receptors in various cell systems including their transformed types (Fu *et al.*,1988; Peres *et al.*,1990). Therefore, effects of BK and also FCS on $[Ca^{2+}]_i$ were investigated in CO25 cells.

Before the measurement of $[Ca^{2+}]_i$ level, the cells were grown in 20% FCS or in 10% HS with or without DEX for five days, and then incubated for 10-12 hours in the absence of the serum. This incubation in the absence of the serum was performed in order to reduce desensitization of plasma membrane receptors and to allow the concentrations of intracellular messengers to return to basal values. Incubation of cells for several hours in the absence of serum has been employed in of other studies in which the properties of cells transformed with a *ras* oncogene have been compared with those of wild-type cells (e.g. Chiarugi *et al.*,1986; Parries *et al.*, 1987; Benjamin *et al.*,1988; Polverino *et al.*,1990; Rake *et al.*, 1991). EGTA was used to chelate extracellular calcium and to test whether an extracellular source of the ion was necessary for the effects of the agonists on $[Ca^{2+}]_i$ level.

As shown in Fig.29, the assay of fluorescence measurements of $[Ca^{2+}]_i$ was generally performed in the presence (Fig.29.a) or in the absence (Fig.29.b) of extracellular Ca^{2+}. At the end of the experimental incubation, 1μM ionomycin as an ionophore was added to the cell suspension to determine the level of stored Ca^{2+} in intracellular stores (Smith *et al.*,1989). After the cells were exposed to agonists, the maximum fluorescence of calcium-saturated dye (F_{max}) was determined by the addition of digitonin

which makes the plasma membrane permeable by binding to membrane steroids (Dawson, 1990). The fluorescence of the free dye (virtual absence of Ca^{2+}) (F_{min}) was determined by the addition of EGTA.

a

b
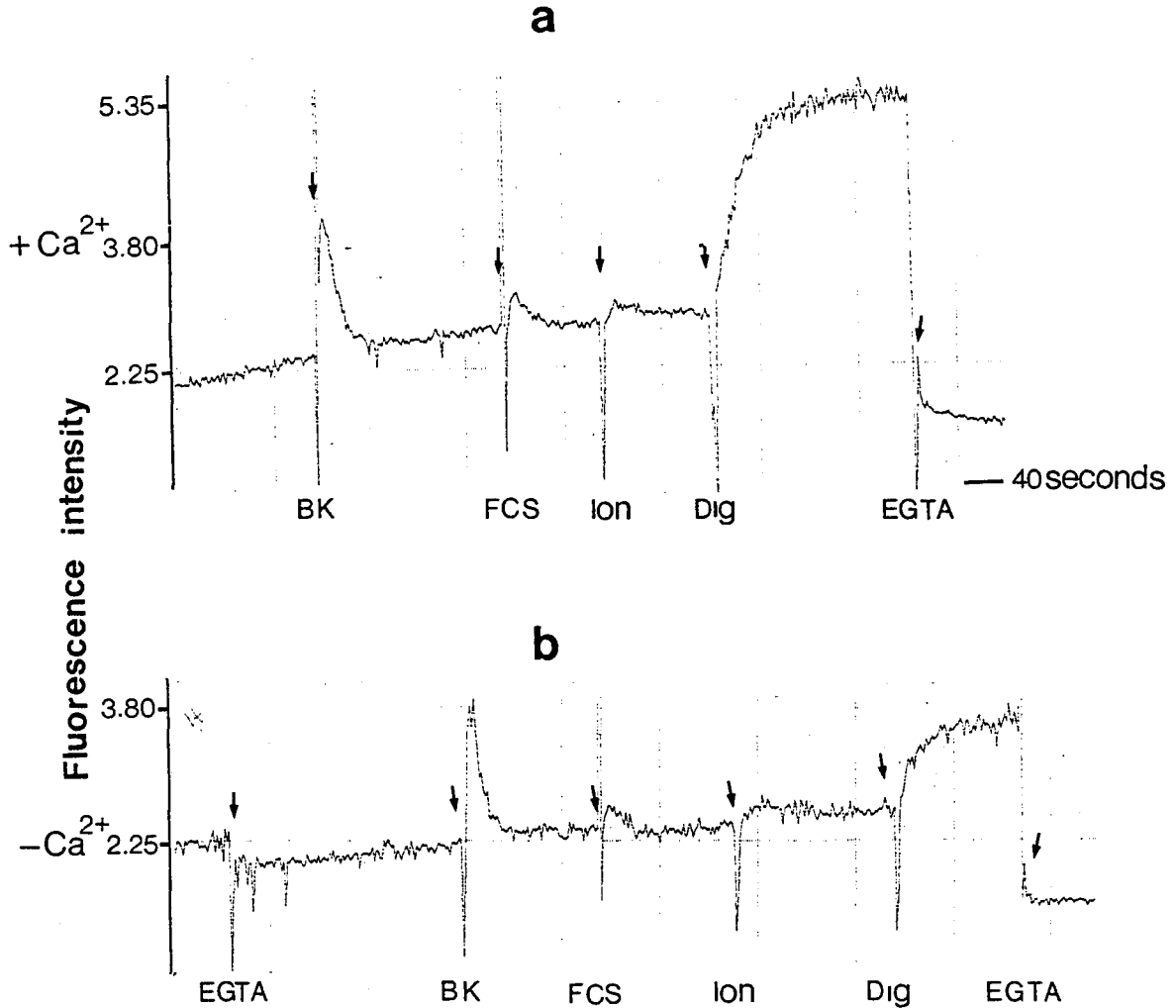
Fig.29. General demonstration of the measurements of $[Ca^{2+}]_i$ by fura-2 fluorescence in CO25 myoblasts. **a)** Cells were exposed to BK, FCS and ionomycin in the presence of 1mM extracellular Ca^{2+}. **b)** EGTA (2mM, pH 7.0) was added to the cell suspension to obtain Ca^{2+} free buffer and then cells were exposed to the agonists as described in Materials and Methods. F_{max} and F_{min} levels were determined by the addition of digitonin and EGTA, respectively. The times of addition of the agonists are indicated by the arrows. Each plot is a representative of the plots obtained for at least three repeats of 3-5 similar experiments (separate loadings with fura-2 AM).

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In CO25 myoblasts, the addition of 1μM BK and 1% FCS to the cells caused a rapid increase in $[Ca^{2+}]_i$ which, after reaching a maximum value, decreased to the nearly basal value both in the presence (Fig.29.a) or in the absence (Fig.29.b) of extracellular Ca^{2+}. However, the magnitude of the stimulated Ca^{2+} responses by FCS after stimulation by BK was quite low. Addition of ionomycin to the cells after BK and FCS, caused very small increases in $[Ca^{2+}]_i$, showing that Ca^{2+} was maintained in the internal stores during the experiment.

As reported previously for other types of cells such as fibroblasts or glioblastoma cells (Polverino et al.,1990; Szöllös et al.,1991), Fig.30.a and b show that the addition of 13 ng/ml PDGF-BB to CO25 cells incubated in the presence of 1mM free Ca^{2+} caused a rapid increase in $[Ca^{2+}]_i$ which, after reaching a maximum value, decreased to a value above the basal value. After addition of the PDGF-BB, the cells were exposed to 1μM BK, and the cells responded to BK in the similar pattern to the responses obtained in Fig.29. However, the magnitude of the stimulated Ca^{2+} responses by PDGF and BK were much greater in the CO25 cells grown in 20% FCS (Fig.30.a) than the cells grown in 10% HS (Fig.30.b).

In contrast to the results obtained for these cells grown in 20% FCS or 10% HS, PDGF-BB had no effect on $[Ca^{2+}]_i$ in the CO25 cells when ras was activated by dexamethasone (Fig.30.c). But the addition of BK to these cells after the addition of PDGF-BB increased the $[Ca^{2+}]_i$ to a higher level than in cells grown in 10% HS (Fig.30.b). The level was similar to that shown by cells grown in 20% FCS (Fig.30.a). At the end of the experimental incubation period, the addition of ionomycin caused a similar increase of

$[Ca^{2+}]_i$ levels in the cells grown in 20% FCS and 10% HS, but it had less an effect in the *ras*-induced cells.

Cells treated with 2mM EGTA before the addition of PDGF-BB responded with slightly longer delay times and lower inital increases in $[Ca^{2+}]_i$ than the control cells (Fig.31.a-b). Also the responses to BK (Fig.31.a-b-c), FCS (Fig.29.b) and ionomycine (Fig.31.a-b) were affected very little by the removal of extracellular calcium by addition of 2mM EGTA, demonstrating that the ion was most being released from intracellular stores.

The patterns of the Ca^{2+} response induced by PDGF-BB and BK and FCS were slightly different (Fig.30.a-b and 29.a). The PDGF-BB stimulation of the cells increased the $[Ca^{2+}]_i$ slowly after a short delay of 20 seconds and then this slowly decreased. BK stimulation of the cells caused a rapid increase in $[Ca^{2+}]_i$ in less than 10 seconds, and then a decrease in 20-30 seconds. The first addition of BK, FCS and ionmycine to the cells stimulated a similar rapid response in the control cells and in the *ras*-induced cells (Fig.32.c-f and b-e, respectively).

The shapes of the curves for the transient increase in $[Ca^{2+}]_i$ induced by BK and FCS in the cells are similar, but in contrast the magnitude of the response was higher in the *ras*-induced cells. The magnitude of the response stimulated by FCS was similar in the control cells and in the *ras*-induced cells (Fig.32.b-e). The magnitude of the transient increase and the shapes of the curves for the transient increase in $[Ca^{2+}]_i$ induced by BK and FCS in the cells were similar.
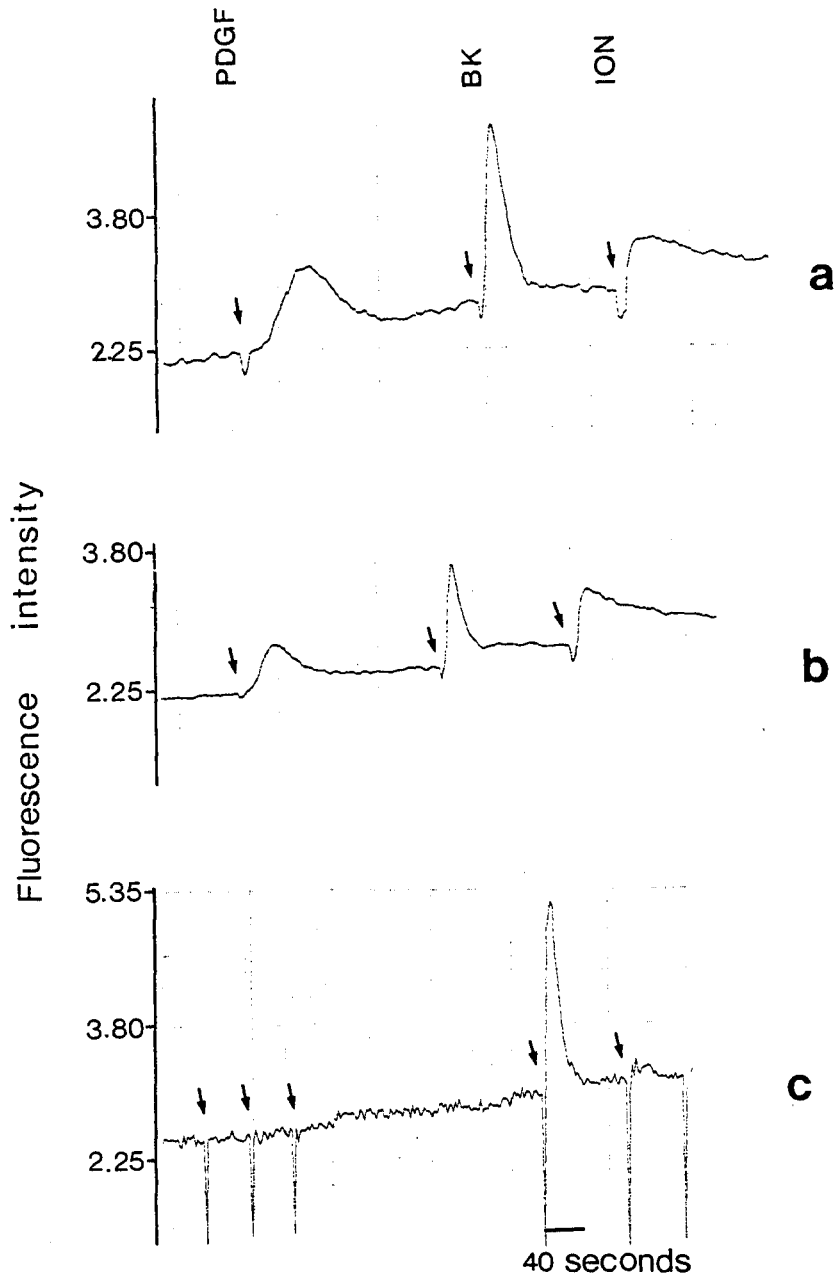
Fig.30. Effects of PDGF-BB and BK on $[Ca^{2+}]_i$ in CO25 cells cultured in different media using fura-2 fluorescence dye. $[Ca^{2+}]_i$ was measured in the presence of 1mM extracellular Ca^{2+} as described in Materials and Methods.

a) Cells in 20% FCS for 24 hours; b) Cells in 10% HS; or c) in 10% HS with DEX for four five days. The times of addition of the agonists are indicated by the arrows. Each plot is a representative of the plots obtained from at least three repeats of 3-5 similar experiments (separate loadings with fura-2 AM).
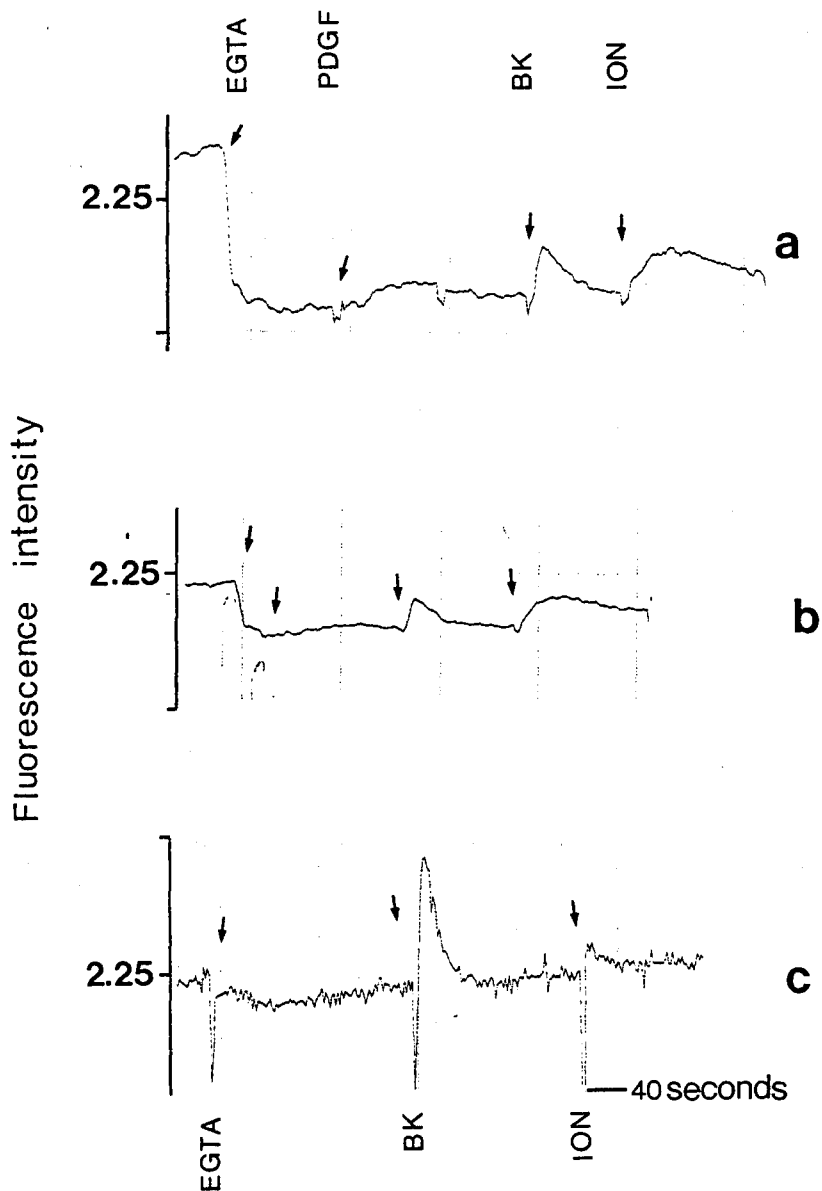
Fig.31. Effects of PDGF-BB and BK on $[Ca^{2+}]_i$ in the CO25 cells using fura-2 fluorescence in the absence of extracellular Ca^{2+} by addition of EGTA to the cell suspansion. a) Cells in 20% FCS for 24 hours. b) Cells in 10% HS for five days. c) Cells in 10% HS for five days with DEX. The growth factors were employed as described in Material and Methods. The times of addition of the agonists are indicated by the arrows. Each plot is a representative of the plots obtained for at least three repeats of 3-5 similar experiments (separate loadings with fura-2 AM).
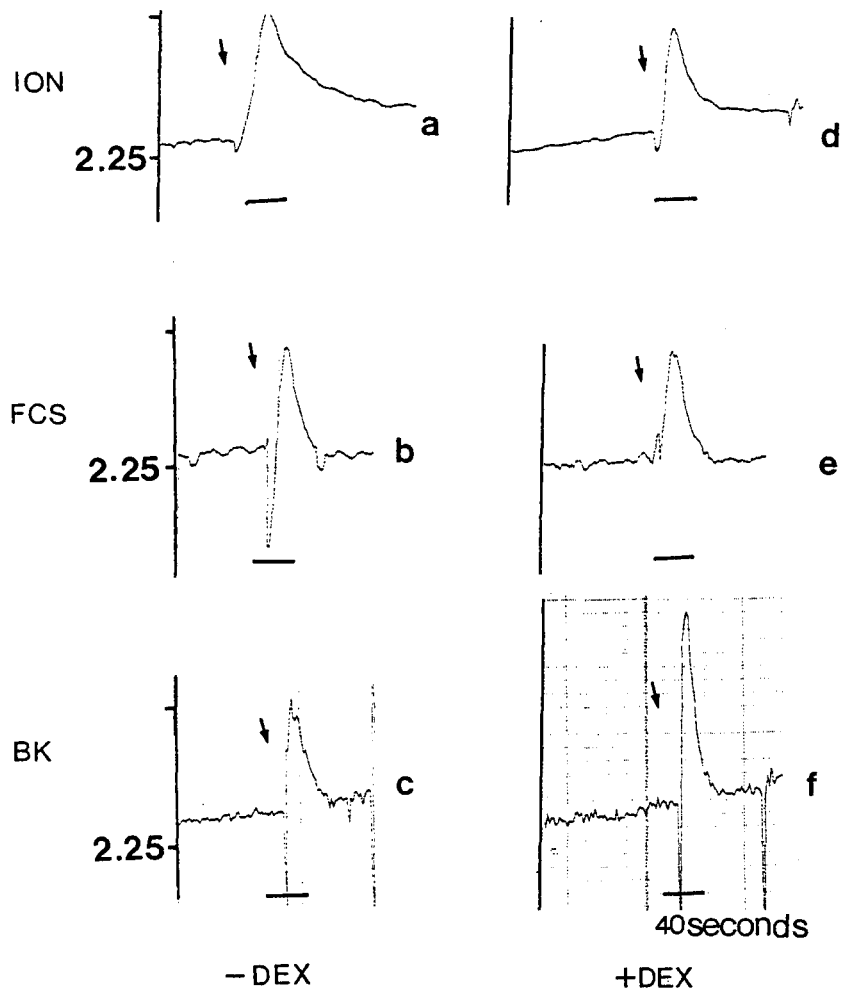
Fig.32. The magnitude and the shapes of the curves of $[Ca^{2+}]_i$ stimulated by FCS, BK and ionomycine in CO25 cells loaded with fura-2 AM and incubated in the presence of 1mM extracellular Ca^{2+}.

(a, b, c) Cells in 10% HS or; **(d, e, f)** in 10% HS with DEX for five days. **(a, d)** $[Ca^{2+}]_i$ stimulated by 1mM ionomycin ; **(b, e)** by 1% FCS (v/v) or **(d, f)** by 1mM BK. The times of addition of the agonists are indicated by the arrows. Each plot is a representative of the plots obtained for at least three repeats of 3-5 similar experiments (separate loadings with fura-2 AM).

At the beginning of the present work, the effects of EGF, bombesin and insulin on the mobilization of Ca^{2+} in CO25 cells were examined using the fura-2 AM assay to investigate any possible role of *ras* expression on the signal transduction via these ligands. Stimulation of fura-2 loaded CO25 cells by these ligands did not result in an increase in $[Ca^{2+}]_i$ level.

5.3.3. THE NUMBER OF PDGF RECEPTORS IN THE CO25 MYOBLASTS

Receptor binding experiments with $[^{125}\text{-}I]$-PDGF-BB were performed to investigate whether the lack of PDGF response in $[Ca^{2+}]_i$ levels and in the phosphorylation of some proteins (as described in Chapter 4) in the ras-induced CO25 cells might be due to an absence of cell-surface receptors. Therefore, the numbers of PDGF receptors were measured on CO25 myoblasts (1) during the differentiation, or (2) during the transformation by inducing the expression of the N-*ras* oncogene, as well as (3) in the cells incubated in the growth medium, also (4) in parental C2 cells. Cells were incubated with $[^{125}\text{-}I]$-PDGF-BB after the starvation for 10-12 hours in the serum free DMEM.

$[^{125}\text{-}I]$-PDGF-BB bound to the CO25 myoblasts in a specific and saturable manner (Fig.33.a). Specific binding of $[^{125}\text{-}I]$-PDGF-BB was quantitated by measuring the non-specific binding in the presence of a 10 fold excess of cold PDGF-BB, and substracting from total binding. The non-specific binding was low as compared to the total binding of the $[^{125}\text{-}I]$-PDGF-BB. In CO25 cells grown in 10% HS for five days, $[^{125}\text{-}I]$-PDGF-BB bound at the same level as cells incubated in the FCS (Fig.33.b). Four days after the induction of N-*ras* oncogene expression by DEX, the binding of $[^{125}\text{-}I]$-PDGF-BB to the

cells was diminished (Fig.33.c). In all cells at the different stages of growth, saturation binding of [^{125}-I]-PDGF-BB showed that the level of specific binding (eg. at 550 pM) in *ras*-induced CO25 cells was 30-40% of that in the uninduced cells. The levels of specific binding at 550 pM were 17.71 ± 0.902 (in 20% FCS), 18.56 ± 0.632 (in 10% HS) and 4.97 ± 0.54 fmoles/well of cells (n=2) (in the *ras*-induced cells).
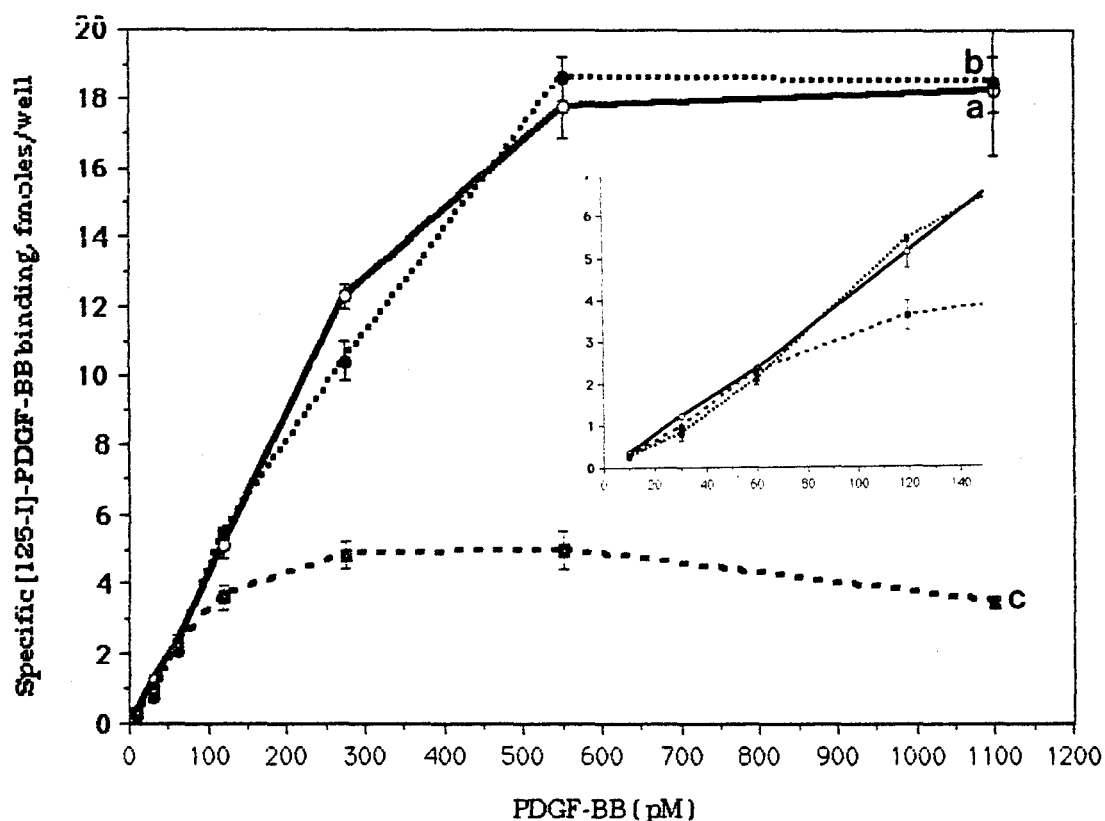
Fig.33. [^{125}I]-PDGF-BB binding to CO25 cells grown; a) in the growth medium (20% FCS), b) in the fusion promoting medium (10% HS) for five days, and c) in 10% HS plus 1 μM DEX for five days. The cells were incubated with indicated concentrations of [^{125}I]-PDGF-BB and the specific binding was calculated as described under Materials and Methods. The small graph shows the specific binding at the low concentration (from big graph) in more detail. Each point represents triplicate determinations with non-specific counts subtracted. Two independent experiments gave identical results.

In order to investigate whether the decreased levels of PDGF-BB binding in *ras*-induced cells might be an effect of the presence of dexamethasone, the parental C2 cells (which do not possess the mutated N-*ras* oncogene) were incubated under the same conditions as the CO25 cells, and then quantitatation of [^{125}-I]-PDGF-BB was carried out. The levels of specific binding were found to be similar to those obtained in all C2 cells (Fig.34.a-b-c) and also CO25 cells grown in the absence of dexamethasone (Fig.32.a-b).
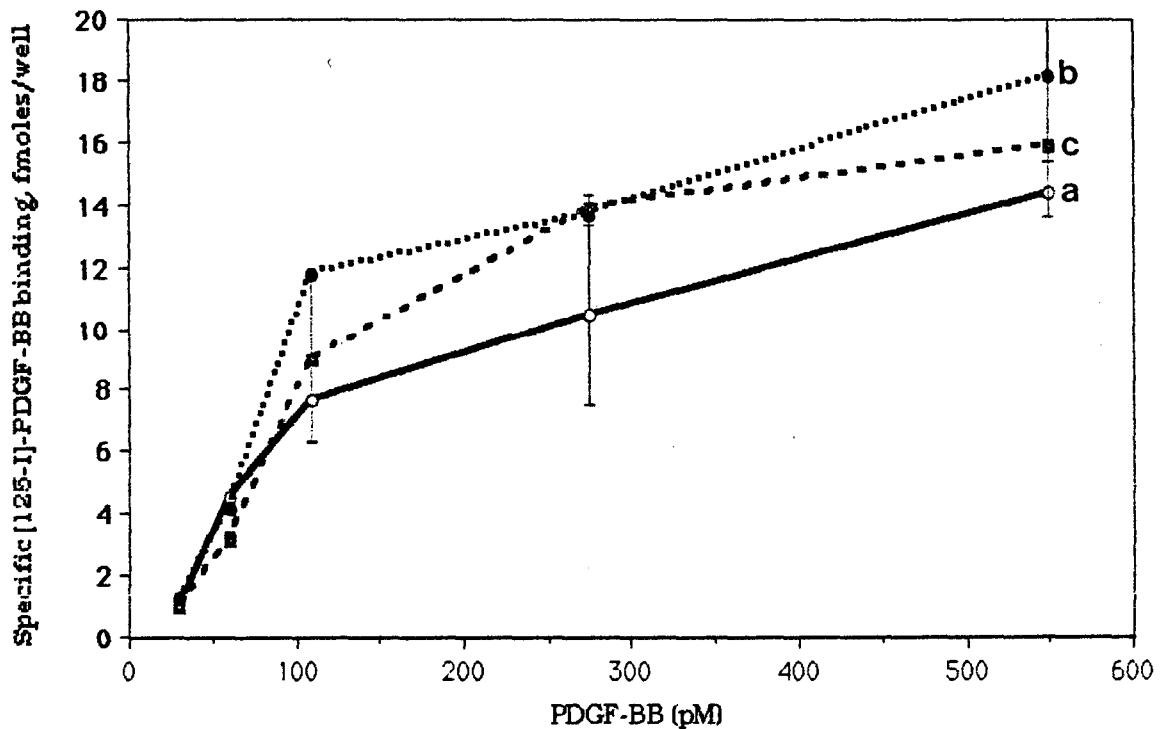
Fig.34. [^{125}I]-PDGF-BB binding to C2 cells grown; a) 20% FCS, b) in 10% HS for five days, and c) in 10% HS with DEX for five days.The cells were incubated with indicated concentrations of [^{125}I]-PDGF-BB. The specific binding was calculated as described under Materials and Methods. Each point represents triplicate determinations with non-specific counts subtracted. Two independent experiments gave identical results.

The levels of specific binding at 550pM were 14.4 ± 0.004 (in 20% FCS), 18.1 ± 2.78 (in 10% HS) and 15.8 ± 2.21 fmoles/well (in the presence of DEX) (n=2) in C2 cells.

In order to determine the specificity of the [^{125}-I]-PDGF-BB binding, the unlabelled PDGF-BB (ranging from 0-400 pM) was added in competition experiments with 2 nM of [^{125}-I]-PDGF-BB. Binding to the C2 (Fig.36) and CO25 cells (Fig.35) grown in 20%FCS (Fig.36.a and 35.a) or in 10%HS with (Fig.36.b and 35.b) or without (Fig.36.c and 35.c) dexamethasone was determined. Analysis of these competition curves indicated a specific binding affinity of the PDGF-BB to the cells by their competition with [^{125}-I]-PDGF-BB.
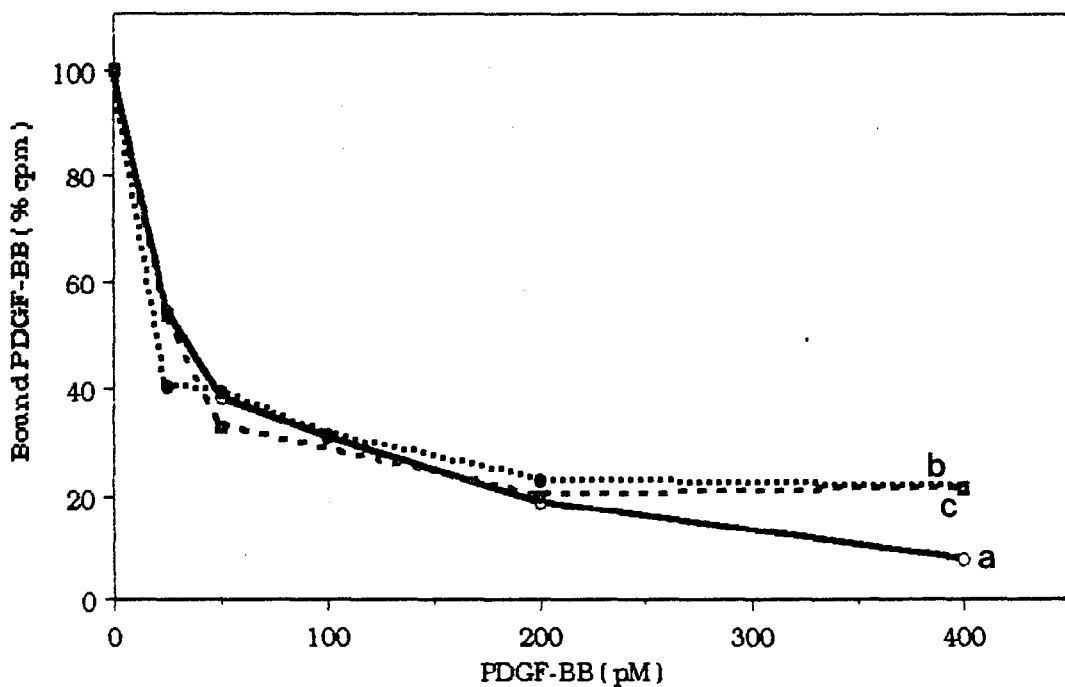
Fig.35. Competition of binding of [^{125}-I]-PDGF-BB to CO25 cells by increasing concentrations of unlabelled PDGF-BB. Cells in (a) 20% FCS, (b) 10% HS for five days, and (c) 10% HS with DEX for five days. Points are the means of three determinations. Two independent experiments gave identical results.

The binding affinity was similar in all the cells grown in the different media, in the absence or in the presence of dexamethasone, indicating the receptors have similar properties before and after cell transformation, and also during cell differentiation.
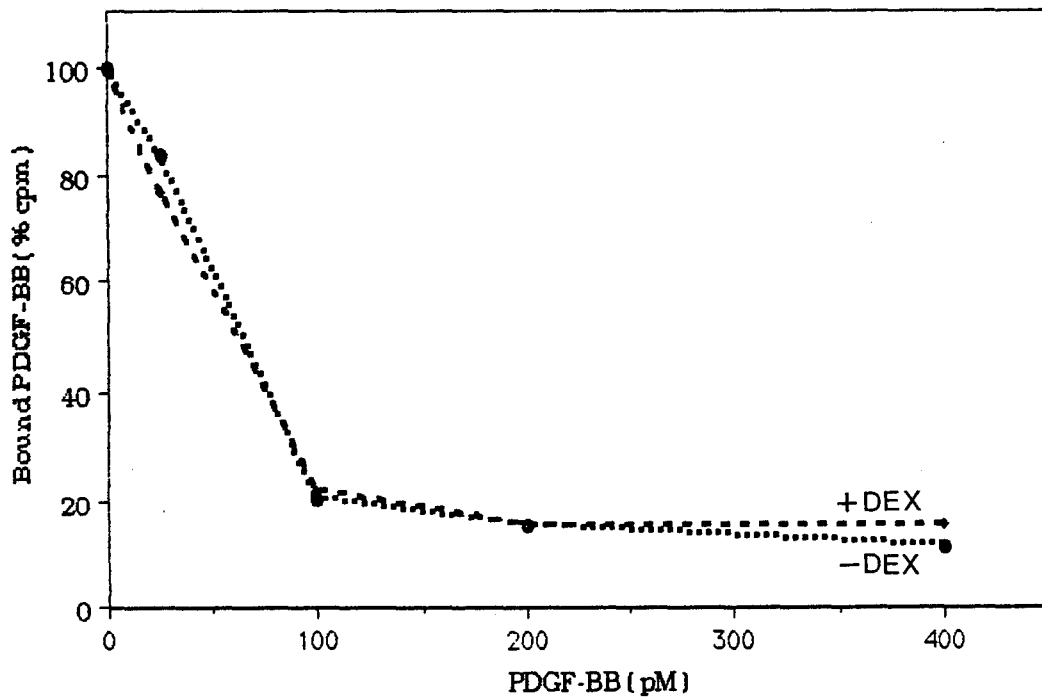
Fig.36. Competition of binding of [^{125}-I]-PDGF-BB to C2 cells by increasing concentrations of unlabelled PDGF-BB. Experiments were carried out as described in Materials Methods. Points are the means of three determinations. Two independent experiment gave identical results.

DISCUSSION

In this work, the effects of mitogens, PDGF-BB and BK, on [Ca^{2+}]$_i$ concentration in CO25 myoblasts bearing a steroid-inducible mutated N-*ras* oncogene have been examined.

PDGF-BB acts through both α and β receptor type. β type has been found majority of total PDGF receptors (about 80%) in the C2 parental cells (Yablonka-Reuveni et al.,1990). BK acts through the B_1 and B_2 receptors in fibroblasts (Etscheid and Villereal, 1989), but it is unknown in these cells. Two receptors trigger different mechanisms in the signalling pathway, although common signalling pathways are eventually generated. PDGF receptors phosphorylate PLC-γ and other substrates on tyrosine residues (Hunter and Cooper, 1985; Williams, 1989), but coupling of BK receptors of PLC-γ appears to be mediated by a G protein (Etscheid and Villereal, 1989; Higashida et al.,1986). Both PDGF-BB and BK activate PI turnover and induce rises in cytoplasmic free Ca^{2+} (Downward et al.,1988; Burch and Axelrod, 1987; Peres et al.,1990; Polverino et al.,199.

The results described have shown that in the CO25 cells grown in the growth medium, PDGF-BB, BK and FCS each caused a rapid increase in $[Ca^{2+}]_i$ (above the basal value) in the presence of Ca^{2+} (Fig.29.a and 30). An enhanced rate of increase in $[Ca^{2+}]_i$ was observed after the addition of BK and FCS to cells incubated in the presence of EGTA (Fig.29.b and 31). These results suggest that Ca^{2+} is released from intracellular stores by stimulation with BK and FCS. Most of the investigations in this area have been carried out in fibroblast cells. Previously, Lang et al. (1991) showed in fibroblast cells that BK induced a transient increase in the release of $[Ca^{2+}]_i$ in the presence of EGTA.

The rate of increase in $[Ca^{2+}]_i$ observed after addition of PDGF-BB to CO25 cells incubated in the presence of EGTA was much lower than that which found in the absence of EGTA. As shown by others in fibroblasts (Moolenaar et al.,1984; Polverino et al.,1990), the plateau in $[Ca^{2+}]_i$ induced by PDGF was abolished

when the experiment was performed in the presence of excess EGTA. They suggested that this intracellular Ca^{2+} movement following PDGF stimulation occured by plasma membrane Ca^{2+} inflow. Recently, Szöllös et al. (1991) reported that the increase of $[Ca^{2+}]_i$ resulted from inital release of intracellular stores, and subsequent calcium influx across the plasma membrane after addition of PDGF-BB to human glioblastoma cells in the presence of EGTA. Also, Peres et al. (1990) showed that Ca^{2+} was released from an intracellular source in fibroblasts following stimulation with PDGF and BK, and both agonists induced very similar early responses.

Intracellular free calcium has a very critical role in the regulation of cell growth. Ca^{2+} binds several proteins such as calmodulin, activates the kinase and phosphorylates other proteins, resulting in DNA replication. The role $[Ca^{2+}]_i$ in inducing mitogenesis in PDGF-stimulated cells has been investigated by several authors (Williams et al.,1988; Tucker et al.,1989). Tucker et al. (1989) showed that PDGF-induced increases in $[Ca^{2+}]_i$ correlate with subsequent DNA synthesis, showing PDGF stimulation of mitogenesis depends on $[Ca^{2+}]_i$.

During differentiation of CO25 cells, the increase in $[Ca^{2+}]_i$ stimulated by PDGF-BB or BK was diminished, indicating the reduced activation by these ligands in differentiation. Some other growth factor receptors such as FGF, EGF and TGF-β have been shown to be deactivated or to decrease in number (Olwin and Hauschka,1988; Lim and Hauschka,1984; Ewton et al.,1988). This decrease in the response to PDGF-BB and BK may be correlated with the loss of proliferation of CO25 myoblasts in the differentiation process.

In *ras* transformed fibroblasts, changes in $[Ca^{2+}]_i$ concentration stimulated by the mitogen bombesin have been investigated. Lloyd *et al.* (1989) showed that overexpression of N-*ras* gene caused the amplification of IP_3 generation and subsequently increased the intracellular Ca^{2+} release following bombesin stimulation of fibroblasts, while the number and affinity of the bombesin receptors on each cell remained unchanged. In contrast, Polverino *et al.* (1990) found in v-K-*ras* and H-*ras* transformed fibroblasts, Ca^{2+} release from internal stores by bombesin stimulation was completely inhibited and a substiantial decrease in the number of bombesin receptors were found.

The complete loss of the ability of PDGF-BB to stimulate increase of $[Ca^{2+}]_i$ in the mutant N-*ras* transformed CO25 cells is most probably due to the decreased binding of PDGF-BB to its receptor, since the amount of ^{125}I-PDGF-BB bound by these cells was considerably lower than that which bound to the control cells (parental C2 and CO25 cells grown in the growth medium or fusion promoting medium). The low binding of PDGF-BB to its receptor in the *ras* induced CO25 cells was not due to some defect or change in the receptors, since the competition experiment indicated that PDGF-BB bound ti its receptor with a similar affinity in control and *ras*-induced cells (Fig.35).

These results are in agreement with the results of Parries *et al.* (1987), Polverino *et al.* (1990) and Rake *et al.* (1991) who found a lack in the response to PDGF stimulated signalling pathway in *ras* transformed fibroblasts. But they are in conflict with the results of Parries *et al.* (1987) and Rake *et al.* (1991) who found no change in the PDGF receptor number and expression, although the cells failed to respond to PDGF. They suggested that the *ras* p21 exerts an

effect by blocking the signal transduction induced by PDGF in the early stages of signalling pathway. Moreover, the ability of PDGF to increase $[Ca^{2+}]_i$ level is lost in an activating N-*ras* transformed cell line, but the expression of normal N-*ras* p21 in fibroblasts leads to the coupling of PDGF receptors to stimulated IP production via the stimulation of PLC (Wakelam *et al.*,1986).

In the presence of the PDGF-BB in the fusion promoting medium, the CO25 cells failed to form myotubes, indicating the inhibition of differentiation by PDGF-BB (Fig.28). Yablonka-Reuveni *et al.* (1990) showed that PDGF-BB exerts a mitogenic effect on C2 myoblasts. It has been proposed that in myoblasts, PDGF stimulates proliferation and delays differentiation of precursors (Bowen-Pope *et al.*,1991). Also the mitogenic effects of PDGF on other cell lines such as many connective tissue cells and fibroblasts, have been reported by those others (Ross *et al.*,1986; Parries *et al.*,1987; Peres *et al.*,1990).

In contrast to the action of PDGF, the ability of BK to release Ca^{2+} from intracellular stores was only slightly elevated in *ras* induced CO25 cells, whereas the ability of PDGF to stimulate $[Ca^{2+}]_i$ increase was substantially inhibited in the transformed cells. It is concluded that the increased action of BK on Ca^{2+} release in the *ras*-induced cells may be due to an increase in BK receptors. It has previously been observed that both normal and mutant *ras* transfection of fibroblasts increases the number of BK receptors by Parries *et al.* (1987, in v-H-*ras*, K-MuSV and *EJ-ras* transfected cells), Downward *et al.* (1988, in normal and mutant N-, K- and H-*ras* transfected cells) and by Roberts and Gullick (1989, in mutant H-*ras* transfected cells). In other work, the *dbl* (human diffuse B-cell lymphoma) oncogene, v-K-*ras* and human H-*ras* oncogenes were

also reported to increase the expression of BK receptors in fibroblasts, whereas v-*src*, v-*abl*, v-*mos*, v-*raf*, v-*fos*, v-H-*ras*, human N-*ras* and K-*ras* oncogenes did not have this effect as reported by Ruggiero *et al.* (1989).

These results suggest that the effects of the mutant *ras* p21 on cellular responses stimulated by BK may due the changes in receptor number rather than to direct coupling by *ras* p21 with the receptor. Expression of normal and mutated N-*ras* p21 in fibroblasts, increased the coupling of BK receptors to stimulated IP production (Downward *et al.*,1988; Wakelam *et al.*, 1986).It has been shown that BK stimulates the generation of IP_3 in fibroblasts (Fu *et al.*,1988) and consequently the release of Ca^{2+} from intracellular stores into the cytoplasm (Higashida *et al.*,1986). Ruggiero *et al.* (1989) found that BK stimulated PI turnover, but did not stimulate mitogenesis in the same transformed cells. Roberts and Gullick (1989) showed that BK is a potent mitogen for Rat-13 cells transfected with a mutant H-*ras*, in contrast to the parental Rat-1 cells which responded weakly to BK as a mitogen.

BK is released during tissue damage causing localized pain and inflammation (Pisano,1975; Higashida *et al.*,1986; Joris *et al.*,1987) and this may imply a role for BK in cell growth control during tumorigenesis. This may be particularly pertinent to the mitogenic effects and increased number of BK receptors in cells containing activated *ras* oncogenes. The mitogenic effects of BK and the number of BK receptors remain to be investigated in C2 and CO25 cells system.

6. GENERAL DISCUSSION

Mammalian *ras* proto-oncogenes encode membrane associated proteins which bind guanine nucleotides and posses GTPase activity alternating between the active GTP bound form and the inactive GDP form. The function of the *ras* products, the p21 proteins, are not understood but it is generally agreed that they are involved in intracellular signal transduction. *Ras* has a role in mitogenic signal transduction and in the differentiation of certain cell types eg. neuronal cells induced by the nerve growth factor.

The mouse myoblast cell line CO25 normally cease cell proliferation and develops myotubes in the serum deficient medium, but in the presence of an activated N-*ras* mutant gene, cell differentiation is blocked and the cells are capable of divide.

In this regard it is interesting that certain oncogenes eg. *myc* increase their activity during the initial stages of cell proliferation. It would be interesting to compare these *ras* inhibited cultures with normally proliferating cells from the point of view of oncogene expression and in particular the expression of the *fos, jun* and *myc* genes. These are active during proliferation.

The failure of cells to proliferate following *ras* activation may involve the mechanisms by which these genes are activated. For example, the *myc* gene product remains elevated in N-*ras*-transformed CO25 cells despite the fact that these cells stop dividing upon achieving confluency. Effects of N-*ras* expression on DNA synthesis in these cells is clearly required.

One serious complication is the possibility that dexamethasone via the glucocorticoid receptor may inhibit proliferation and stimulate differentiation. These inhibitory effects may be confused with *ras* effects on differentiation. Cloning *ras*

with another promoter would help distinguish the effects. Using different cell lines eg. keratinocytes might also prove illustrative.

The inactivation of or failure to produce PDGF receptors following the stimulation of *ras* is intriguing. This is particularly interesting since bradykinin receptors are unaffected. Other receptors eg. these for the insulin, insulin growth factors (IGFs), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β) have not been examined. The PDGF receptor with tyrosine kinase activity is linked to *ras* p21 and other substrates, eg. phosphatidylinositol 3-kinase (PI-3K), and phospholipase C (PLC). The mobilization of Ca^{2+} results from the stimulation of PLC with the subsequent formation of the secondary messages diacylglycerol and inositol 1,4,5-triphosphate and activation of protein kinase C (PKC). It is a possibility that p21 interacts with PLC.

The increase or decrease of receptors is observed in many systems following mitogen stimulation and differentiation. Direct experiments on PDGF in this system remain to be carried out and in particular following the activation of N-*ras*. It would be of some interest to determine at which level in the gene- receptor protein pathway *ras* has its effects. Inhibition of the processing of the p21 protein eg. by preventing its accumulation in the membrane utilizing various inhibitors might also unravel the level of activity of *ras*.

This system offers many interesting pointers to the processes involved in the switch from cell differentiation to proliferation.

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