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TRANSCRIPTIONAL REGULATION OF THE HUMAN PDGFα RECEPTOR GENE,

&

THE EFFECT OF BRADYKININ ON PHENOTYPIC TRANSFORMATION OF NRK CELLS

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TRANSCRIPTIONAL REGULATION OF THE HUMAN PDGFα RECEPTOR GENE,

AND THE EFFECT OF BRADYKININ ON PHENOTYPIC TRANSFORMATION OF NRK CELLS

een wetenschappelijke proeve op het gebied van de Natuurwetenschappen

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Katholieke Universiteit Nijmegen, volgens besluit van het College van Decanen in het openbaar te verdedigen op maandag 8 januari 1996, des namiddags te 1.30 uur precies

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PROLOGUE

The work described in this thesis was initiated as part of a NWO funded project, within the BION/Biofysica theme 'intracellular signal transduction, entitled:'Signal transduction of TGFß and retinoic acid in phenotypic transformation of normal rat kidney cells. Biochemical and biophysical aspects of density-dependent inhibition of growth'. This thesis consists of two parts. A: 'The effect of bradykinin on phenotypic transformation of NRK cells', and B: 'Transcriptional regulation of the human PDGFα receptor gene'. Based on these titles, there is no apparent correlation between the two parts. However, all the work described in this thesis has been originally designed to investigate the signal transduction routes of TGFß.

In part A, the original focus was on the molecular mechanisms involved in the TGFß-induced phenotypic transformation of NRK cells. The inhibitory effect of bradykinin was used as a tool to gain more insight in this process. Although this approach has lead to a better understanding of the mechanisms involved in the inhibitory action of bradykinin, no specific information was obtained about TGFß signal transduction. Therefore, an additional approach has been undertaken as described in part B.

In part B, the PDGFα receptor-mediated inhibitory effect of TGFß on several cell lines was used as a model system to investigate TGFß signal transduction. This work has resulted in a much better insight in the regulation of PDGFα receptor gene expression, but so far no direct studies have been performed with respect to TGFß signal transduction.

Thus, the work described in this thesis does not meet its original goal as reflected in the project title. However, it has resulted in two pieces of research, which relation can only be appreciated from this prologue.

NWO

PART A

Effect of bradykinin on phenotypic transformation of NRK cells

CHAPTER 1

Introduction

Neoplastic transformation of cells is accompanied by large alterations in cellular behaviour. *In vitro*, transformed cells display a reduced requirement for externally added growth factors, they have acquired an indefinite life span, and have a reduced ability to differentiate. In addition, transformed cells have an altered morphology compared to their normal counterparts, are not limited by density-dependent growth inhibition, and have acquired the ability to grow under anchorage-independent conditions (Folkman and Moscona, 1978; Ozanne *et al.*, 1982). This last characteristic is considered to be the best *in vitro* correlate with the capacity of transformed cells to induce tumors in nude mice (Freedman and Shin, 1974; Colburn *et al.*, 1978; Barret *et al.*, 1979) and therefore, anchorage-independent growth may reflect a fundamental characteristic of tumor cells. Neoplastic transformation is generally a result of genetic mutations. However, certain non-transformed cells can acquire a transformed phenotype in response to externally added growth factors. These phenotypically transformed cells display the same morphology, loss of density-dependent growth inhibition and anchorage-independent growth as neoplastically transformed cells. Normal rat kidney (NRK) cells are a widely used model system to investigate the role of growth factors in phenotypic transformation.

Phenotypic transformation of NRK cells

NRK cells typically grow as immortalized, non-transformed cells which require the addition of growth factors to their culture medium for proliferation. However, these cells undergo phenotypic transformation in response to transforming growth factor ß (TGFß) in the additional presence of either epidermal growth factor (EGF) or transforming growth factor α (TGFα) (Roberts *et al.*, 1981; Anzano *et al.*, 1982; 1983) and thereby they acquire characteristics similar to neoplastically transformed cells, including morphological alterations, loss of density-arrest and anchorage-independent growth. In contrast to neoplastic transformation, the phenotypic transformation of NRK cells is reversible. Upon removal of TGFß the cells continue to grow with a normal, non-transformed phenotype.

The mechanisms involved in the TGFß-induced phenotypic transformation of NRK cells and the concomitant anchorage-independent growth and loss of density-arrest are not

completely understood. However, several observations have indicated that modulation of EGF receptor numbers at the cell surface seems to play an essential role in this process. The involvement of EGF/TGFα and the EGF receptor in the TGFß-induced anchorage-independent growth of NRK cells has already evolved from the earliest studies on the characterization of the transforming growth factors. The so-called sarcoma growth factor, isolated from conditioned medium of murine sarcoma virus-transformed cells, stimulated cell division in normal fibroblasts and interacted with the EGF receptor (Todaro *et al.*, 1976; 1977; De Larco and Todaro, 1978). In addition, it was able to induce soft-agar growth of NRK cells. Subsequent studies have shown that the sarcoma growth factor consist of two componenents: TGFα, which competes with EGF for receptor binding, and TGFß, which strongly synergizes with EGF to induce anchorage-independent growth of NRK cells (Roberts *et al.*, 1982; Anzano *et al.*, 1982; 1983). However, the involvement of the EGF receptor in this process became evident when it was demonstrated that TGFß upregulates EGF receptor numbers in confluent NRK cells (Assoian *et al.*, 1984; Assoian, 1985).

A similar mechanism has been described for retinoic acid (RA). Like TGFß, RA is a potent inducer of phenotypic transformation in NRK cells, in combination with EGF, as characterized by its capacity to induce anchorage-independent growth and loss of density-arrest. This effect of RA has also been correlated with an increase of EGF receptor numbers on the cell surface (Jetten, 1983; Jetten and Goldfarb, 1983; Roberts et al; 1984). Together with the observations of Rizzino *et al.* (1988; 1990), which show that EGF receptor numbers on the cell surface decrease with increasing NRK cell densities, these data have lead to a model that describes the phenotypic transformation of NRK cells as a function of EGF receptor numbers. In short: 'In subconfluent cultures, NRK cells have sufficient EGF receptors on their cell surface to proliferate in response to EGF. However, at a critical cell density this EGF receptor number has dropped to a level at which the cells become unresponsive to EGF for proliferation and are density-arrested in their G_1/G_0- phase. When additional TGFß or RA is presented to the cells, the number of EGF receptors increases again above a treshold level and as a result the cells reinitiate proliferation in response to EGF'.

A point of consideration in the EGF receptor model is that this model does not completely cover all the aspects of phenotypic transformation of NRK cells. As shown by van Zoelen *et al.* (1988; 1993) and in chapter 3 of this thesis, platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA) are not completely dependent on the presence of EGF for their transforming effects on NRK cells. In addition, PDGF and LPA are very well capable to induce a mitogenic response in serum-starved, quiescent NRK cells. This in contrast to RA and TGFß, which do not elicit a mitogenic response in

quiescent cells, but are only capable to induce phenotypic transformation (in combination with EGF). Another observation that supports the notion that PDGF transforms NRK cells through a fundamentally different mechanism than TGFß and RA, is provided by the kinetics of the proliferative response. Compared to PDGF, TGFß and RA display delayed kinetics in the induction of loss of density-arrest (Van Zoelen *et al.*, 1992), which reflects the time necessary to induce EGF receptors. Thus, PDGF provides a direct proliferative signal to the cells. Together these observations have lead to a slightly modified version of the EGF receptor model (Van Zoelen, 1991), which is summarized in figure 1.1.

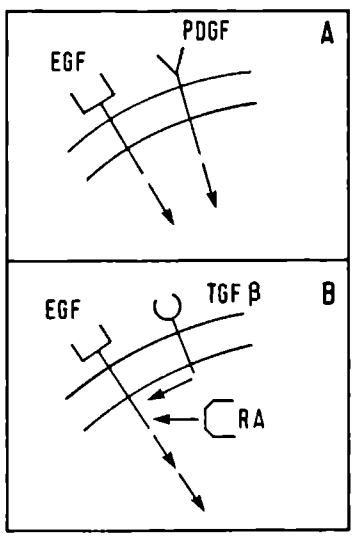

figure 1.1 Model for growth factor-induced loss of density-dependent inhibition of growth of NRK cells (Van Zoelen, 1991). NRK cells cultured in the presence of EGF become density inhibited at high cell densities because the EGF-induced growth-stimulating signals (indicated by the single arrow from the EGF receptor) are insufficient for induction of cell proliferation. Cells can be restimulated to proliferate by the activity of a growth factor such as PDGF acting in parallel (A), such that the growth-stimulating signals of the two factors combined are sufficient to induce proliferation. Alternatively (B), factors such as TGFß and RA can be added, which enhance the number of EGF receptors and thereby the level of growth-stimulating signals induced by EGF (double arrow).

Besides the proliferative response as a result of EGF receptor upregulation, this model also includes the possibility of another, direct growth stimulatory pathway parallel to that of EGF. Together these stimuli may also be sufficient to overcome density-dependent growth inhibition and thus initiate phenotypic transformation.

There are also some restrictions that apply to the EGF receptor model in general. Based on the observations of Van Zoelen *et al.* (1988), which demonstrate that the growth factor requirements for both loss of density-arrest and the induction of anchorage-independent growth are quite similar, it has been assumed that the molecular basis for both these phenomena is similar. However, nothing is known about regulation of EGF receptor expression under anchorage-independent conditions. In addition, other mechanisms than EGF receptor modulation have been proposed to be involved in the phenotypic transformation of NRK cells. Ignotz and Massagué (1986) have postulated that

stimulation of extracellular matrix components by TGFß is responsible for the anchorage-independent growth of NRK cells. Also the occurrence of a specific factor in the plasma membrane of NRK cells, capable of triggering density-arrest has been reported to play a role (Nagasaki *et al.*, 1994). Furthermore, anchorage-independent growth has been associated with an enhanced intracellular pH (Schwartz *et al.*, 1989; 1990), a phenomena that may also occur in the TGFß-induced phenotypic transformation of NRK cells (Van Zoelen and Tertoolen, 1991). Recently, it has been demonstrated that cyclins are also important determinants of density-arrest and anchorage-independent growth in several cell lines, incuding NRK cells (Guadagno *et al.*, 1993; Deffie *et al.*, 1995). However, not one of these mechanisms has been investigated extensively. It is therefore difficult to appreciate their contribution to the phenotypic transformation of NRK cells, and to determine whether they act in concert, or are a result of EGF receptor modulation.

Intracellular signaling pathways involved in phenotypic transformation

The role of the different intracellular signaling routes activated by the above mentioned factors in the phenotypic transformation of NRK cells has not been established yet. TGFß, RA, PDGF/EGF and LPA are representatives of growth factors which are linked to completely different receptor and signal transduction systems.

TGFß and RA

TGFß and RA share many characteristics in their ability to transform NRK cells, in particular their dependence on EGF to establish anchorage-independent growth and loss of density-arrest (van Zoelen *et al.*, 1986; 1988). However, their intracellular signal transduction mechanisms are completely different.

TGFß belongs to a large family of related growth factors, which also includes, among others, members of the bone morphogenetic proteins, activin, inhibin and Müllerian inhibitory substance (reviewed by Massagué, 1992; Attisano *et al.*, 1994). TGFß displays a wide variety of effects (reviewed by Barnard *et al.*, 1990; Roberts and Sporn, 1991), but its inhibitory and stimulatory effects on cell proliferation are the most extensively studied (Moses *et al.*, 1990). Although many membrane-bound proteins have been identified that bind TGFß, it appears that only the so-called TGFß receptors I and II are directly involved in TGFß signaling (Massagué, 1992; Lin and Lodish, 1993; Segarini, 1993). Both the type I and II receptors are transmembrane serine/threonine kinases, which form a heteromeric complex upon TGFß binding. A general model for TGFß receptor signaling depicts that TGFß first binds to the type II receptor, which in turn recruits the

type I receptor by means of bound TGFß The type I receptor becomes subsequently phosphorylated on serines and threonines by the the kinase activity of the type II receptor and this allows the type I receptor to propagate the signal to downstream substrates (Wrana *et al* , 1994) However, there are no downstream substrates for the TGFß receptor identified yet

RA also displays a pleiotropy of effects, but is best characterized in its differentiating effects on cells (reviewed by De Luca, 1991) In contrast to TGFß, RA does not bind to a transmembrane receptor, but to a nuclear RA receptor Two families of these nuclear RA receptors exist retinoic acid receptors (RARs) and retinoic X receptors (RXRs), each consisting of an α, ß and γ receptor type (reviewed by Leid *et al* , 1992) These RARs and RXRs are transcription factors and after RA binding they heterodimerize and bind to RA-responsive DNA elements within target genes and affect transcription of these genes (Leid *et al* , 1992, Stunnenberg, 1993)

Although their signal transduction routes differ completely, both TGFß and RA have been shown to increase EGF receptor gene expression through enhanced transcriptional activation (Thompson *et al* , 1988, Thompson and Rosner, 1989) The mechanism by which this enhanced transcription is accomplished is not understood It has recently been demonstrated that TGFß increases EGF receptor transcription through a loss of protein binding to a negatively regulatory element (Hou *et al* , 1994) However, there is currently no information whether RA affects the same regulatory element or binds directly through its receptors to the EGF receptor gene There is also no additional information about signal transduction mechanisms more upstream from the TGFß- and RA induced transcription of the EGF receptor gene These observations clearly illustrate the gap of information between activation of TGFß and RA receptors and the increase of EGF receptor gene transcription

The induction of another growth factor or growth factor receptor is being considered as a typical characteristic of the TGFß-induced transformation of cells In AKR-2B fibroblasts, TGFß induces the production of a growth factor (PDGF-B) to elicit a proliferative response in these cells (Leof *et al* , 1986)

PDGF and EGF

In contrast to RA and TGFß, EGF and PDGF are mitogenic to serum-starved, quiescent NRK cells (Rizzino *et al* , 1986, van Zoelen *et al* , 1988) In addition, they appear to elicit a parallel growth stimulatory response in density-arrested NRK cells, which together is sufficient to induce phenotypic transformation of these cells (van Zoelen, 1991) Thus it appears that EGF and PDGF also share some common mechanisms in the phenotypic transformation of NRK cells This similarity is also reflected in their mode of signal

transduction. Both EGF and PDGF bind to receptors which belong to the large family of transmembrane tyrosine kinase receptors (Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993). Upon ligand binding these receptors dimerize and become phosphorylated on specific tyrosine residues due to the receptors' own kinase activity. This autophosphorylation results in the recruitment of several signal transduction molecules, which bind by virtue of their SH2-domain to the phosphorylated tyrosine residues. These signal transduction molecules take care of the receptors downstream signaling, which finally results in activation of the genes necessary for cell proliferation.

LPA

It has not been until recently that it was recognized that LPA, the simplest natural occurring phospholipid, is a potent mitogen for quiescent fibroblasts (Van Corven *et al.*, 1989). In addition, in chapter 3 of this thesis it will be shown that LPA is also a potent inducer of phenotypic transformation in NRK cells, as characterized by its ability to induce loss of density-dependent growth inhibition.

Stimulation of fibroblasts with LPA results in activation of signal transduction pathways typically correlated to activation of G protein-coupled receptors. This includes: activation of phospholipase C and the subsequent hydrolysis of phosphoinositides, leading to Ca^{2+}-mobilization and activation of protein kinase C (Van Corven *et al.*, 1989; Jalink *et al.*, 1990); a GTP-dependent release of arachidonic acid, presumably as a result of phospholipase A_2 activation (Van Corven *et al.*, 1989); activation of phospholipase D (Van der Bend *et al.*, 1992a); inhibition of adenylate cyclase via an activation of a pertussis toxin-sensitive G_i protein (Van Corven *et al.*, 1989). In addition, a putative LPA receptor has been characterized which has at least the molecular mass of a G protein-coupled receptor (Van der Bend *et al.*, 1992). However, the exact contribution of these signal transduction pathways to the effects of LPA is not completely understood, but recent observations have stressed the importance of G_i proteins and Ras in the mitogenic signaling route of LPA (reviewed by Moolenaar, 1995).

The following chapters of part A will deal with the role of EGF receptor levels and signal transduction pathways (in particular those elicited by G protein-coupled receptors) in the process of phenotypic transformation of NRK cells. Firstly, in chapter 2 additional evidence will be provided that modulation of EGF receptor levels is indeed the major determinant for RA- and TGFß-induced phenotypic transformation of NRK cells. This evidence is based on the correlation between the growth inhibitory effects of bradykinin and its effect on cell-surface EGF receptor and EGF receptor mRNA levels in NRK cells. Secondly, in chapter 3 the molecular mechanisms involved in the inhibitory effect of

bradykinin are studied in more detail. The intracellular responses elicited by bradykinin are compared with those elicited by LPA and prostaglandin $F_{2\alpha}$. All three compounds mediate their effects through G protein-coupled receptors, but have completely different, and even opposite effects on the phenotypic transformation of NRK cells.

CHAPTER 2

Bradykinin-induced growth inhibition of normal rat kidney (NRK) cells is paralleled by a decrease in epidermal growth factor receptor expression

ABSTRACT

Normal rat kidney fibroblasts, grown to density arrest in the presence of epidermal growth factor (EGF), can be induced to undergo phenotypic transformation upon treatment with transforming growth factor β or retinoic acid Here we show that bradykinin blocks this growth stimulus-induced loss of density-dependent growth arrest by a specific, receptor-mediated mechanism The effects of bradykinin are specific, and are not mimicked by other phospho-inositide mobilizing agents such as prostaglandin $F_{2\alpha}$ Northern analysis and receptor binding studies demonstrate that bradykinin also inhibits the retinoic acid-induced increase in EGF receptor levels in these cells These studies provide additional evidence that EGF receptor levels modulate EGF-induced expression of the transformed phenotype in these cells

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INTRODUCTION

Normal rat kidney (NRK) fibroblasts have been widely used as a non-transformed indicator cell line for studying the role of polypeptide growth factors in phenotypic transformation (Van Zoelen, 1991). When cultured in the presence of epidermal growth factor (EGF) as the only growth stimulating polypeptide, these cells have a normal phenotype and undergo density-dependent growth inhibition. When cultured in the additional presence of transforming growth factor β (TGFβ), retinoic acid (RA) or platelet-derived growth factor (PDGF), however, cells acquire a transformed phenotype characterized by loss of density-dependent growth inhibition (Van Zoelen *et al.*, 1988). These cells therefore form an attractive model system for studying the control mechanisms involved in density-dependent growth regulation, since they can first be grown to density arrest, from which they can be released upon treatment with specific additional growth factors (Van Zoelen *et al.*, 1992).

Exponentially growing NRK cells in sparse cultures have low EGF receptor levels already, but EGF binding is reduced even further at high cell densities (Rizzino *et al.*, 1990). Neither TGFβ nor RA by itself has a growth stimulatory effect on NRK cells (Van Zoelen *et al.*, 1988), but both are able to increase the number of EGF receptors these cells express (Roberts *et al.*, 1984; Assoian *et al.*, 1984; Assoian, 1985) by transcriptional activation of the receptor gene (Thompson *et al.*, 1988; Thompson and Rosner, 1989). These observations have led to the model that EGF receptor levels control density-dependent growth arrest of NRK cells cultured in the presence of EGF (Van Zoelen, 1991). Upon increasing cell density, EGF receptor levels decrease to such a level that EGF-induced growth stimulating signals are insufficient for induction of cell proliferation. Factors such as TGFβ or RA enhance these growth stimulating signals induced by EGF by increasing EGF receptor levels.

In this study we have investigated the effects of the nonapeptide bradykinin on phenotypic transformation of NRK cells. Bradykinin is a regulatory peptide with a variety of physiological and pharmacological activities, ranging from vasodilation and control of neurotransmission to regulation of cell proliferation (Roberts, 1989). Here we show that bradykinin inhibits the loss of density-dependent growth arrest of NRK cells induced by TGFβ or RA during the first twenty hours of incubation, a property which is not shared by other phosphoinositide mobilizing agents. The observed growth inhibition induced by bradykinin is paralleled by a repression of the upregulation in EGF receptor expression, strongly suggesting that EGF-induced proliferation of NRK cells is a direct function of EGF receptor density. These data are discussed in the light of the current hypotheses on the molecular mechanism involved in density-dependent growth control.

RESULTS

Effect of bradykinin on loss of density-dependent growth inhibition

When confluent, serum-deprived cultures of NRK cells are treated with EGF and insulin, cells are stimulated to undergo one additional cell cycle, after which they become quiescent in the G_1/G_0-phase as a result of density-dependent growth inhibition (Van Zoelen *et al* , 1988) In the continuous presence of EGF and insulin, these density-arrested cells can be restimulated to proliferate in a synchronous manner by treatment with additional growth factors, such as TGFβ, RA, PDGF or basic fibroblast growth factor (Van Zoelen *et al* , 1992) In contrast to the latter two factors, TGFβ and RA as such have no growth stimulating activity on NRK cells, and can only exert their transforming activity in the additional presence of EGF (Van Zoelen *et al* , 1988)

Figure 2 1A shows the effect of bradykinin on the loss of density inhibition of NRK cells induced by TGFβ, RA and the tumor promotor tetradecanoyl phorbol acetate (TPA) Cells were density-arrested in the presence of EGF and insulin, and cumulative thymidine incorporation was subsequently measured during the first 20 hours following addition of the modulating factors indicated It is shown that although bradykinin by itself has no significant effect on the proliferation of these density-arrested cells, it almost completely abolishes the growth stimulating effect of TGFβ and RA As a comparison it is shown that

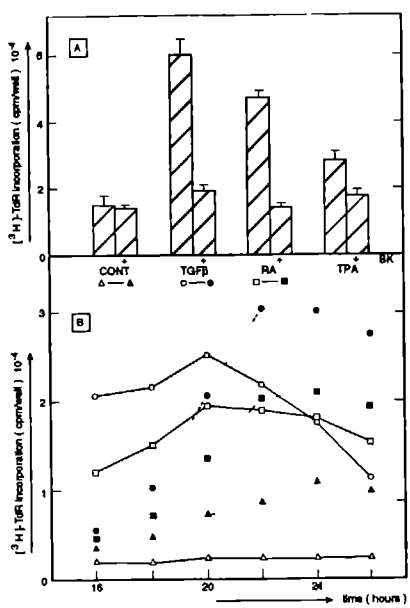

Figure 2 1: Effect of bradykinin on growth factor-induced loss of density inhibition of NRK cells

A Density arrested cells were incubated with 2 ng/ml TGFβ 50 ng/ml RA (retinoic acid), 50 ng/ml TPA (tetradecanoyl phorbol acetate) or without stimulus (CONT), in the additional presence (+) or absence ( ) of 1 μM bradykinin (BK) Incorporation of [^{3}H] thymidine (TdR) was determined between 4 and 20 hours after growth factor addition Indicated standard errors of the mean are based on at least triplicate experiments

B Time course of [^{3}H] thymidine (TdR) incorporation (2 hour pulses) into density arrested NRK cells after the following treatments (△ —— △) no addition (▲ - ▲) 1 μM bradykinin, (○ —— ○) 2 ng/ml TGFβ, (● ●) 2 ng/ml TGFβ + 1 μM bradykinin, (□ —— □) 50 ng/ml RA, (■ ■) 50 ng/ml RA + 1 μM bradykinin

TPA is only a poor inducer of phenotypic transformation of NRK cells under these conditions, but that the effect induced is also sensitive to the inhibitory action of bradykinin.

Figure 2.1B shows in a time course experiment that the growth inhibitory effect of bradykinin depends on the time of incubation. Previous studies have indicated that 10 hours after addition of TGFβ or RA to density-arrested NRK cells, the first cells from these synchronized populations enter the S-phase (Van Zoelen *et al.*, 1992). By studying 2-hour pulses of [^{3}H]-thydimidine incorporation, the present data show that in the case of TGFβ and RA a maximum rate of thymidine incorporation is reached within 20 hours of growth factor addition, in agreement with previous data (Van Zoelen *et al.*, 1992). In the additional presence of bradykinin, however, these curves are shifted in time to reach a maximum around 24 hours of incubation. Within the first 20 hours of growth factor treatment, bradykinin therefore appears to have a strong inhibitory effect on loss of density-dependent growth arrest induced by TGFβ or RA. At later time points, however, bradykinin is shown to have growth stimulatory effects by itself, and to enhance proliferation induced by TGFβ and RA.

Bradykinin effect on cell proliferation is receptor mediated

Bradykinin is known to exert its action by activation of a set of G-protein coupled receptors, designated the B_1 and B_2 receptor types (Roberts, 1989). Dose-response curves showed that half-maximum inhibition of RA-induced loss of density inhibition of NRK cells was obtained at a concentration of 30 nM bradykinin (data not shown), which is indicative of a process mediated by a high affinity receptor. Figure 2.2 shows that the inhibition of TGFβ and RA-induced loss of density inhibition by 0.1 μM bradykinin is counteracted by the B_2-receptor antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin in a

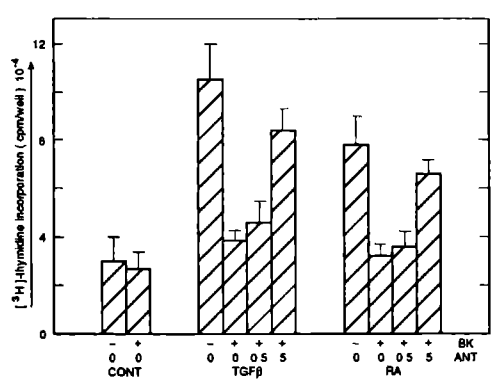

Figure 2.2: Reversal of bradykinin-induced growth inhibition of NRK cells by bradykinin receptor antagonist [^{3}H]-thymidine incorporation into density-arrested NRK cells was measured following incubation with 2 ng/ml TGFβ, 50 ng/ml RA or no addition (CONT), in the additional presence (+) or absence (-) of 0.1 μM bradykinin (BK), and the indicated concentrations in μM of the antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-bradykinin (ANT) Indicated standard errors of the mean are based on at least triplicate experiments.

dose dependent fashion. The bradykinin effect was also antagonized, although to a lesser extent, by [Des-Arg^9, Leu^8]-bradykinin (data not shown), which is known to have B_1-receptor antagonist effects (Patel and Schrey, 1992). Although the present data do not give direct information on the type of bradykinin receptor involved, they show that the observed biological effects of bradykinin are receptor mediated.

Bradykinin effect is not related to degradation of inositol-containing phospholipids

Upon receptor activation bradykinin is known to induce degradation of inositol-containing phospholipids through activation of a phospholipase C, and to induce release of fatty acids including arachidonic acid by activation of a phospholipase A_2 (Roberts, 1989). It has been shown before that bradykinin is able to mobilize Ca^{2+} from intracellular sources in NRK cells (Marks *et al.*, 1988). Figure 2.3A shows that under the conditions tested bradykinin is a relatively poor inducer of inositol polyphosphate formation, when compared to prostaglandin $F_{2\alpha}$ and fetal calf serum. As shown in figure 2.3B, however, prostaglandin $F_{2\alpha}$ is unable to mimic the growth inhibitory effect of bradykinin on NRK cells, and generally by itself induces slight mitogenic effects. Also with respect to release of [^{3}H]-arachidonic acid, prostaglandin $F_{2\alpha}$ was found to be far more potent than bradykinin in NRK cells (G.B. Afink, D.C.J.G. van Alewijk and E.J.J. van Zoelen, unpublished observation). Also other agents such as the protein kinase C activator TPA and the protein kinase C inhibitor sphingosine did not mimic the growth inhibitory effect of bradykinin on NRK cells (data not shown). Taken together these data show that the inhibitory effect of bradykinin on loss of density-dependent growth inhibition is specific, and is not a general property of phosphoinositide mobilizing agents.

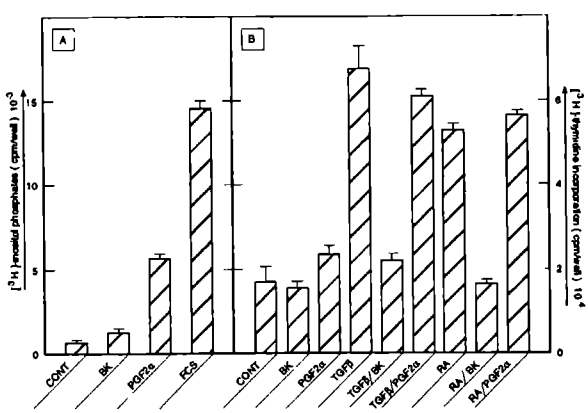

Figure 2.3: Comparison of bradykinin (BK) and prostaglandin $F_{2\alpha}$ (PGF2α) in inducing degradation of inositol-containing phospholipids (A) and growth inhibition (B) of NRK cells Indicated stimuli were added to density-arrested NRK cells at the following concentrations. CONT, no addition, BK, 10 μM (A) or 1 μM (B); PGF2α, 1 μM, FCS, 9% fetal calf serum; TGFβ, 2 ng/ml; RA, 50 ng/ml Incorporation of [^{3}H]-thymidine was determined between 4 and 20 hours after addition of the growth stimuli Indicated standard errors of the mean are based on at least triplicate experiments.

Effects of bradykinin on EGF receptor density

It has been established in a number of cell lines including NRK cells, that treatment with RA results in an immediate increase in the number of EGF receptors per cell (Jetten, 1982; Roberts et al, 1984). In the case of TGFβ treatment, EGF receptor density in NRK cells first decreases, and then increases after more than four hours of incubation (Assoian,1985). Figure 2.4 shows that bradykinin blocks the RA-induced increase in EGF binding sites during the first four hours of incubation. Subsequently, bradykinin synergizes with RA in increasing EGF receptor levels, resulting in enhanced EGF binding after incubation for more than six hours. During the time period investigated, bradykinin does by itself not affect EGF receptor densities in NRK cells, which contrasts with observations by Earp *et al.* (1988) in WB cells, that hormones stimulating phosphoinositide hydrolysis may enhance levels of EGF binding.

The insert of figure 2.4 shows Scatchard plots for binding of EGF to density-arrested NRK cells, before and after treatment with RA. Both plots have been fitted to a single receptor affinity model, although the curves show a slight tendency toward curvilinearity, in agreement with data of Assoian (1985). The present Scatchard plots show that before RA treatment density-arrested NRK cells contain approximately 1100 EGF receptors with a dissociation constant of 0.5 nM, while after 20 hours of incubation with RA cells contain approximately 9500 EGF receptors with a dissociation constant of 0.6 nM. These data underline the low number of EGF receptors in density-arrested NRK cells and the significant effect of RA on these EGF receptor levels.

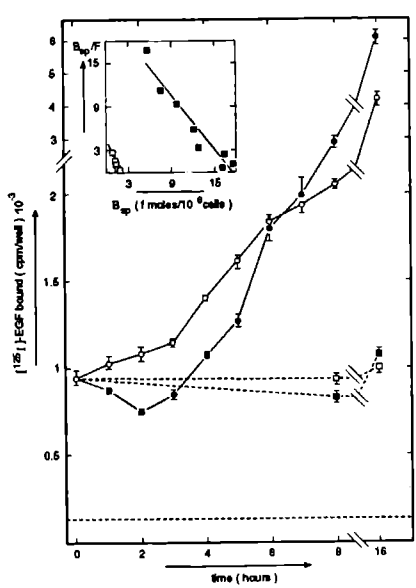

Figure 2.4: Effect of bradykinin on RA-induced EGF receptor levels in density-arrested NRK cells. Time course of ^{125}I-EGF (4 ng/ml) binding after addition of 50 ng/ml RA (○ —— ○), 50 ng/ml RA + 1 μM bradykinin (● —— ●), 1 μM bradykinin (■----■) or no addition (□----□). The dashed horizontal line represents the level of non-specific binding obtained by additional treatment with a hundred-fold excess of unlabelled EGF. The insert shows a Scatchard plot for specific binding (B_{sp}) of 1 ng/ml ^{125}I-EGF and variable concentrations of unlabelled EGF, without (□ —— □) and with (■ —— ■) pretreatment for 20 hours with 50 ng/ml RA. Free ligand concentrations (F) have been expressed in nM. Indicated standard errors of the mean are based on at least triplicate experiments.

Figure 2.5 shows the effect of bradykinin on EGF receptor mRNA levels in a Northern analysis. Rat EGF receptor mRNA chromatographs at a size of 9.6 kb, with an occasional additional transcript at 5 kb (Oberg and Carpenter, 1991). The data presented here show that the steady-state mRNA levels for the EGF receptor rapidly increase with time upon RA treatment, but that within the period of four hours tested this effect is completely abrogated by bradykinin. In TGFβ-treated cells, an increase in EGF receptor mRNA levels was only visible after four hours of incubation, while PDGF, which in combination with EGF also induces phenotypic transformation of NRK cells, had no effect on EGF receptor mRNA levels (data not shown). The data of figure 2.5 also show that in

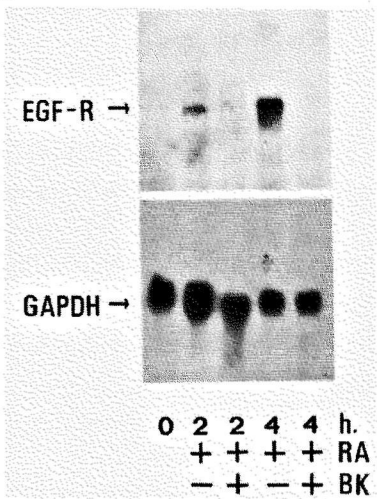

Figure 2.5: Northern blot of EGF receptor (EGF-R) mRNA levels in density-arrested NRK cells following time incubations (0,2,4 hours) with 50 ng/ml RA in the absence (-) and additional presence (+) of 1 μM bradykinin (BK). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control; mRNA sizes EGF-R 9.6 kb, GAPDH 1.4 kb.

NRK cells density-arrested in the presence of EGF, little expression of the EGF receptor gene is detectable under the present experimental conditions. This observation agrees with the EGF binding data of figure 2.4, and is in line with the concept that EGF receptor levels decrease with increasing cell density (Rizzino *et al.*, 1990). Interestingly, however, it has been shown in a variety of studies that EGF is able to enhance expression of its own receptor gene by a combination of both transcriptional and post-transcriptional controls (Clark *et al.*, 1985; Earp *et al.*, 1988; Thompson and Rosner, 1989; Fernandez-Pol *et al.*, 1989; Hudson *et al.*, 1989). The present data show that in NRK cells which have undergone density-dependent growth inhibition EGF is unable to induce EGF receptor expression to a detectable level, which may be an important aspect of the molecular mechanisms involved in density-dependent growth control. In conclusion, the present data show that, in parallel with a four hours shift in RA- and TGFβ-induced release from density arrest (see figure 2.1B), bradykinin also blocks the RA-induced increase in EGF receptor levels during the same period of time.

DISCUSSION

In the present study we have shown that bradykinin selectively inhibits the expression of a transformed phenotype in NRK cells. We have used this observation to demonstrate that a close correlation exists between EGF receptor levels in these cells, and the ability of growth modulating agents such as TGFβ and RA to release these cells from density-dependent growth arrest. Earlier studies have shown that EGF receptor levels decrease with increasing cell density (Holley *et al.*, 1977; Rizzino *et al.*, 1988; 1990), and that agents such as TGFβ and RA enhance the number of EGF receptors in NRK cells (Roberts *et al.*, 1984; Assoian *et al.*, 1984; Assoian, 1985; Thompson *et al.*, 1988; Thompson and Rosner, 1989). Taken together with the present results on the effect of bradykinin on EGF receptor expression and density-dependent growth control, these data strongly suggest that EGF receptor density is one of the major controlling parameters in density-dependent growth inhibition and phenotypic transformation of NRK cells. It is well established that EGF receptor expression can be controlled at both the transcriptional and post-transcriptional level (Hadcock and Malbon, 1991). Further experiments are required to investigate if under the present experimental conditions bradykinin inhibits RA-induced transcriptional activation of the EGF receptor gene, or decreases EGF receptor mRNA stability.

Bradykinin has been associated with both growth stimulation (Owen and Villereal, 1983; Roberts and Gullick, 1989; Roberts, 1989 and references therein) and growth inhibition (Newman *et al.*, 1989; Patel and Schrey, 1992) on a variety of cells. On still other cell lines, no direct growth effect of bradykinin has been observed in spite of the presence of specific receptors and bradykinin mediated second messengers (Van Corven *et al.*, 1989; Ruggiero *et al.*, 1989). Here we have shown that bradykinin induces both short term growth inhibition and long term growth stimulation of NRK cells. The short term growth inhibitory effects are paralleled by repression of TGFβ- and RA-induced enhancement of EGF receptor levels. These inhibitory effects are specific for density-arrested NRK cells, since no such effects were observed on growth-stimulated NRK cells which were first made quiescent by serum deprivation. In the case of prostaglandin $F_{2\alpha}$, only long term growth stimulatory effects were observed similar to bradykinin, but no growth inhibitory effects. Bradykinin exerts its action through a set of high and low affinity receptors, which results in degradation of choline and inositol-containing phospholipids and release of arachidonic acid (Van Blitterswijk *et al.*, 1991, 1991a; Kast *et al.*, 1991), as well as in enhancement of tyrosine phosphorylation (Leeb-Lundberg and Song, 1991). Degradation of inositol-containing phopholipids and release of arachidonic

acid are also induced in NRK cells by prostaglandin $F_{2\alpha}$, endothelin (Kusuhara *et al.*, 1992) and lysophosphatidic acid (data not shown; see also Van Corven *et al.*, 1989), even to larger extent than observed for bradykinin. However, only bradykinin is a growth inhibitory factor for NRK cells, also when added in combination with one of the other above factors. These observations suggest that bradykinin must induce a so far unknown second messenger relevant for growth inhibition, which is not induced by other phosphoinositide mobilizing agents. Bradykinin-induced effects on cell proliferation generally result from the induction of prostaglandin derivatives (Patel and Schrey, 1992). Preliminary results indeed show that the observed growth inhibitory effects of bradykinin can be largely blocked by inhibitors of cyclo-oxygenase activity such as indomethacin (G.B. Afink, D.C.G.J. van Alewijk and E.J.J. van Zoelen; unpublished). It is presently unclear, however, which growth inhibitory prostaglandin is specifically induced by bradykinin in NRK cells. The observed inhibitory effects could not be mimicked by addition of well-characterized prostaglandins such as $F_{2\alpha}$, E_2, A_2 or D_2 to NRK cells (data not shown).

NRK cells form an attractive model for studying the molecular mechanisms involved in density-dependent growth control since depending on the growth factors added, cells display either a normal or a transformed phenotype (Van Zoelen, 1991). When cells are cultured in the presence of insulin and EGF they become arrested at a saturating density, from which they can be released upon treatment with additional growth factors including TGFβ and RA (Van Zoelen, 1991). Still relatively little is known about the molecular mechanisms involved in density-dependent growth inhibition, and the loss of this density control upon tumorigenic transformation. The following processes, however, have been proposed as playing a possible role. It has been shown that normal cells can become quiescent at high densities because of production of autocrine growth inhibitory factors (Holley *et al.*, 1978; Harel *et al.*, 1985). In the case of NRK cells, however, no evidence for production of such factors by density-arrested cells was found (Van Zoelen, 1991). In the case of the epithelial NRK cell line 52E an abrupt increase in the membrane potential has been observed as the cells reach a saturating density (Binggeli and Weinstein, 1985). In line with this observation we found that NRK-49F fibroblasts released from density arrest by TGFβ rapidly depolarize, underlining the importance of the membrane potential in controlling density-dependent growth inhibition (A.P.R. Theuvenet, P.H.J. Peters and E.J.J. van Zoelen; unpublished observation). In addition, gap junction-mediated intercellular communication has been considered to control growth arrest by direct intercellular contacts (Loewenstein, 1979), but in the case of NRK cells no significant intercellular communication was detected in density-arrested cells (Van Zoelen and Tertoolen, 1991). Wieser *et al.* (1990) have published evidence that a membrane

glycoprotein known as contactinhibin, is involved in contact-dependent growth inhibition, but as yet no effects of this factor have been described in NRK cells.

The present and previous studies (Holley *et al.*, 1977; Rizzino *et al.*, 1988, 1990) have shown that modulation of growth factor receptor densities may provide an additional mechanism for controlling cell proliferation. This may be paralleled by a density-dependent modulation of protein-tyrosine phosphatase activity; together with a modulation of growth factor receptor levels this may control growth factor-induced phospho-tyrosine levels in the cell (Pallen and Tong, 1991). In support of this idea we have recently observed that the phosphatase inhibitor vanadate mimics TGFβ in preventing EGF-stimulated NRK cells from becoming density-arrested (Rijksen *et al.*, 1993; 1993a). It is likely that all of the above mechanisms in fact play a distinct role in the regulation of density-dependent growth control. Based on the observation of Rizzino *et al.* (1990) that in NRK cells EGF receptor levels decrease with increasing cell density, and the present observations on the effect of bradykinin on EGF receptor expression, it seems that for NRK cells modulation of receptor densities is the most appropriate mechanism for describing density-dependent growth regulation. This is also underlined by the observation that mutant NRK-49F cells with only a three-fold reduction in EGF receptor levels, are fully unresponsive to the growth stimulating activity of EGF (Hamanaka *et al.*, 1990). It is well realized, however, that the other above described mechanisms may play a more dominant role in other cell types. For example, in the case of human glial cells, it has been shown that density-dependent growth inhibition is associated with even an increase in EGF receptor levels (Westermark, 1977).

MATERIALS AND METHODS

Growth stimulation assays

Normal rat kidney (NRK) fibroblasts, clone 49F, were plated at a density of 15,000 cells/cm^2, and grown to confluence in serum-containing medium for four days, and subsequently cultured in serum-free medium for three days, as described (Van Zoelen *et al* , 1988). These cells are referred to as serum-deprived, quiescent cells. These cells were then density-arrested by incubation in 5 ng/ml EGF and 5 μg/ml insulin for an additional 48 hours (Van Zoelen *et al.*, 1988) Density-arrested cells were restimulated to proliferate by treatment with additional growth factors, and incorporation of [^{3}H]-thymidine (0 5 μCi/ml added; Amersham) was measured either cumulatively between 4 and 20 hours of growth factor addition, or as a pulse during consecutive 2-hour periods (Van Zoelen *et al.*, 1992).

EGF binding studies

Density-arrested NRK cells (10 cm^2 wells) were incubated for 24 hours in fresh serum-free medium without insulin and EGF Subsequently factors to be tested were added during the indicated time period, after which

the medium was changed for 0 5 ml binding buffer (Dulbecco's modified Eagle's medium, containing 0 1% bovine serum albumin and 50 mM BES, pH 6 8) Subsequently 1-4 ng/ml ^{125}I-EGF (10^5 cpm/ng) and various concentrations of unlabelled EGF were added After incubation for 1 hour at room temperature cells were washed with phosphate buffered saline containing 0 1% bovine serum albumin, and extracted with a 1% Triton X-100 solution Receptor-ligand binding data were analysed using the linear subtraction method (Van Zoelen, 1989)

Lipid degradation studies

Confluent NRK cultures (10 cm^2 wells) were incubated for 24 hours with 4 μCi [^{3}H]-inositol (Amersham) in serum-containing medium After subsequent incubation for 24 hours in serum-free medium, cells were grown to density arrest by incubation for 48 hours in the presence of 5 μg/ml insulin and 5 ng/ml EGF Subsequently agents to be tested were added together with 10 mM LiCl, and after 30 min the medium was changed for 1 ml 10% trichloro-acetic acid. Inositol phosphates were determined by elution from an AG 1X8 anion exchange column (BioRad) with 0 8 M ammoniumformate/0 1 M formic acid (pH 4 5), as described in detail by Tilly *et al* (1987)

Northern analysis

Poly-A$^+$ RNA was isolated from density-arrested and restimulated NRK cells, as described previously (Van Zoelen *et al* , 1992) Samples (20 μg) were denatured, electrophoresed on agarose gels and transferred to nitrocellulose blots as described (Van den Eijnden-Van Raaij *et al* , 1989), and hybridized with a mixture of a 0 77 kb and a 1 84 kb EcoRI cDNA fragment of the human EGF receptor

Materials

^{125}I-EGF was a generous gift from Dr T Benraad (Department of Endocrinology, University of Nijmegen) TGFβ_1 was isolated from human platelets as described (Van den Eijnden Van Raaij *et al* , 1988) RA, TPA and prostaglandin $F_{2\alpha}$ were from Sigma, bradykinin from Boehringer and bradykinin-related compounds from Peninsula Laboratories

CHAPTER 3

Lysophosphatidic acid and bradykinin have opposite effects on phenotypic transformation of normal rat kidney cells

ABSTRACT

The bioactive lipid lysophosphatidic acid is besides a strong mitogen for quiescent fibroblasts, a potent inducer of phenotypic transformation of normal rat kidney cells The lysophosphatidic acid-induced loss of density-arrest is strongly inhibited by bradykinin Although their effects on normal rat kidney cell proliferation are opposite, bradykinin mimics many of the intracellular effects induced upon lysophosphatidic acid receptor activation, including phosphoinositide turnover, Ca^{2+}-mobilization and arachidonic acid release Bradykinin does not counteract the lysophosphatidic acid-induced reduction of cAMP levels in normal rat kidney cells However, bradykinin inhibits the lysophosphatidic acid and other growth factor-induced phenotypic transformation through the induction of a so far uncharacterized prostaglandin G/H synthase product The growth inhibitory effect of bradykinin is limited to density-arrested cells, while upon prolonged treatment bradykinin itself is capable to induce the loss of density-dependent growth control It is concluded that bradykinin is a bifunctional regulator of normal rat kidney cell proliferation and that its inhibitory effects are mediated via the induction of a prostaglandin derivative

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INTRODUCTION

Density-dependent growth inhibition or contact-inhibition is an important mechanism by which normal non-transformed cells become limited in their growth However, upon tumorigenic transformation this density-dependent growth control is lost and cells become able to proliferate without restrictions Density-dependent growth control is one of the most prominent differences between the growth characteristics of transformed and non-transformed cells The precise molecular mechanisms that underly this growth inhibition at increasing cell densities are not completely understood (reviewed in Van Zoelen, 1991) Several polypeptide growth factors are capable to induce the loss of density-dependent growth inibition and thus phenotypically transform cells The cell line NRK-49F, derived from normal rat kidney fibroblasts, provides an excellent model system to study the role of growth factors in this transformation process NRK cells become density-arrested when cultured in the sole presence of epidermal growth factor (EGF) However, the cells are reversibly transformed and lose their density-dependent growth inhibition if additional transforming growth factor ß (TGFß), retinoic acid (RA) or platelet-derived growth factor (PDGF) is present (Van Zoelen *et al* , 1988) At the molecular level this TGFß- and RA-induced loss of density-dependent growth inhibition in NRK cells can be explained by the regulation of EGF receptor numbers EGF receptor densities on the surface of NRK cells decrease with increasing cell densities (Rizzino *et al* , 1988, 1990) At the stage of density-dependent growth arrest these EGF receptor numbers have been decreased to a level at which EGF is no longer able to provide a mitogenic signal to the cells However, addition of TGFß or RA, which by themselves are not mitogenic for these cells, results in an increase of EGF receptor numbers in NRK cells (Roberts *et al* , 1984, Assoian *et al* , 1984, Assoian, 1985, Van Zoelen *et al* , 1994) As a result the cells become respondent to EGF again and resume growing with a transformed phenotype

The lipid lysophosphatidic acid (LPA) is a potent mitogen for a number of different fibroblasts (Van Corven *et al* , 1989) A putative LPA receptor has been described, which is most likely G-protein coupled (Van der Bend *et al* , 1992, Van Corven *et al* , 1993, Howe and Marshall, 1993) The intracellular second messenger systems affected upon LPA activation include phosphatidyl inositol (PI)-hydrolysis, Ca^{2+}-mobilization, PLA_2-activation and a decrease in cAMP levels (Van Corven *et al* , 1989, Jalink *et al* , 1990) Recently Van Corven *et al* (1993) have also reported that the proto-oncogene $p21^{RAS}$ is rapidly activated upon LPA stimulation In these studies the nonapeptide bradykinin has been shown to mimic a large variety of the intracellular messengers induced by LPA However, in contrast to LPA, bradykinin has no growth stimulatory effect on the investigated fibroblasts Moreover we have recently shown that bradykinin is a strong

inhibitor of the RA- and TGFß-induced increase in EGF receptor number and the concomitant loss of density-dependent growth inhibition of NRK cells (Van Zoelen *et al.*, 1994).

To gain more insight in the working mechanisms of LPA and bradykinin we studied the effects of these growth regulatory agents on the phenotypic transformation of NRK cells. LPA showed to be both a strong mitogen and a very potent inducer of phenotypic transformation in these cells. Bradykinin on the contrary inhibits not only the TGFß- and RA-induced phenotypic transformation but also the induction of this process by LPA. This inhibitory effect of bradykinin is specific for density-arrested cells. Quiescent, serum-starved NRK cells are not affected by bradykinin. The specific LPA-induced reduction in cAMP levels is not the target for the inhibitory effect of bradykinin, because bradykinin is not able to prevent this decrease in cAMP. However, the inhibition of growth factor-induced phenotypic tranformation by bradykinin is mediated through an arachidonic acid (AA) metabolite and more specifically via the induction of a prostaglandin G/H (PGH) synthase product.

RESULTS

Effect of LPA and bradykinin on the loss of density-dependent growth arrest

We have previously shown that bradykinin acts as a potent inhibitor of RA- and TGFß-induced phenotypic transformation of NRK cells (Van Zoelen *et al.*, 1994). The inhibitory action of bradykinin has been correlated with a specific inhibition of EGF receptor mRNA formation. LPA has been described as a very potent mitogen for serum-starved quiescent

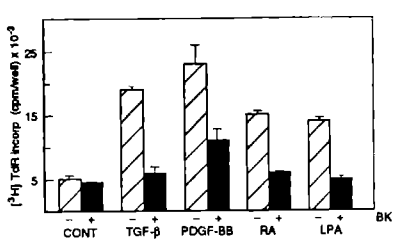

Figure 3.1. Effect of bradykinin on growth factor-induced loss of density-arrest.
Density-arrested NRK cells were incubated with 2 ng/ml TGFß, 5 ng/ml PDGF-BB, 50 ng/ml RA, 100 μM LPA or without stimulus (CONT) in the absence (-) or presence (+) of an additional 1 μM bradykinin [^{3}H]-TdR incorporation was determined between 4 and 19 hours after the additions. Values represent the mean $\pm$ s.e.m. of triplicate determinations.

fibroblasts (Van Corven *et al.*, 1989, 1992). Figure 3.1 shows the effect of LPA on the [^{3}H]-TdR incorporation in density-arrested NRK cells, compared with the transforming effects of TGFß, RA and PDGF-BB. LPA induces the loss of density-dependent growth arrest to the same extent as RA. The transforming capacities of LPA were confirmed by

induction of anchorage-independent growth (data not shown) Also shown in this figure is the inhibitory effect of bradykinin on this growth factor-induced loss of density-arrest Bradykinin does not only inhibit the RA- and TGFß-induced loss of density-arrest, but it abolishes also the transforming capacities of LPA and to a lesser extent those of PDGF-BB These results might suggest that bradykinin is a general inhibitor of NRK cell growth, but this is not the case The inhibitory action of bradykinin is specific for density-arrested cells as shown in figure 3 2 This figure compares the effects of LPA and bradykinin on the [^{3}H]-TdR incorporation in both serum-starved, quiescent cells (figure 3 2A) and density-arrested cells (figure 3 2B) LPA displays besides its transforming effect on density-arrested cells also a strong mitogenic response on quiescent NRK cells More importantly however is the observation that bradykinin does not inhibit this mitogenic action of LPA on quiescent cells, while it completely inhibits the transforming effect of LPA on density-arrested cells

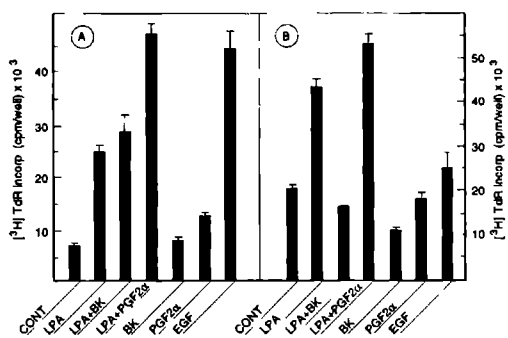

Figure 3 2. The inhibitory effect of bradykinin on NRK cells is specific for density arrested cells
Serum starved quiescent (A) or density arrested (B) NRK cells were incubated with the indicated substances Final concentrations 100 μM I PA 1 μM bradykinin 1 μM PGF2$_{\gamma_\alpha}$ and 5 ng/ml EGF or no addition (CONT) [^{3}H] TdR incorporation was determined between 3 and 22 hours (A) or between 2 and 16 hours (B) after addition of the growth factors Values represent the mean ± s e m of triplicate determinations

It is concluded that bradykinin is a potent inhibitor of growth factor-induced phenotypic transformation, but this inhibition is specifically limited to the conditions of density-arrest Furthermore LPA shows to be both a potent mitogen and a transforming growth factor for NRK cells

The opposite effects of bradykinin and LPA are not correlated to the induction of inositol phosphates, arachidonic acid, Ca^{2+}-release or changes in cAMP levels

In Rat-1 and HF cells bradykinin mimics several intracellular responses evoked by LPA, including PI-hydrolysis, Ca^{2+}-mobilization and AA-release (Van Corven *et al* , 1989, Jalink *et al* , 1990) However the biological effects of LPA and bradykinin on density arrested NRK cells are completely opposite This observation can be used to characterize the intracellular mechanisms involved in the process of phenotypic transformation To

investigate the role of these second messenger systems in the cell proliferation of density-arrested NRK cells, the effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on NRK cell growth was studied, which is also shown in figure 3 2 $PGF_{2\alpha}$ is like LPA and bradykinin a G-protein coupled agonist $PGF_{2\alpha}$ however does not display a remarkable effect on either quiescent or density-arrested cell proliferation It also does not inhibit the LPA-induced cell growth, but it rather synergises with LPA to stimulate NRK cell growth

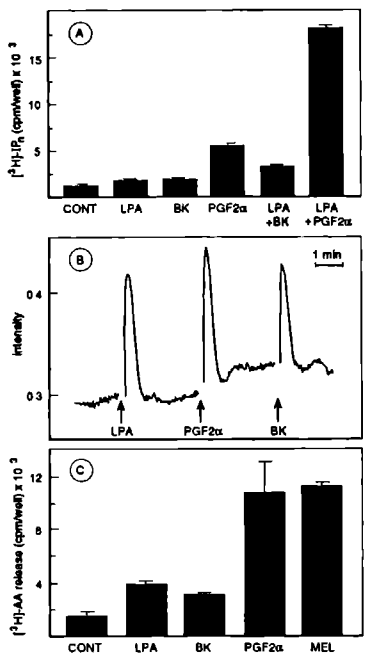

Figure 3.3. Induction of inositol phosphates, arachidonic acid or Ca^{2+}-mobilization by LPA, bradykinin and $PGF_{2\alpha}$ in NRK cells

A Density-arrested, [^{3}H] inositol labeled NRK cells were incubated for 30 minutes with the indicated agonists and the cumulative total inositol phosphate mobilization was determined Final concentrations LPA 100 μM, bradykinin (BK) 10 μM, $PGF_{2\alpha}$ 10 μM or no addition (CONT) Values represent the mean $\pm$ s e m of duplicate determinations from a representative experiment

B Typical Ca^{2+}-responses to sequential addition of LPA (10 μM), $PGF_{2\alpha}$ (1 μM) and bradykinin (BK, 1 μM) to density arrested NRK cells Ca^{2+} levels are represented as Fura2 fluorescence intensities

C [^{3}H] arachidonic acid labeled, density arrested NRK cells were incubated with the indicated agonists The [^{3}H] arachidonic acid release into the medium was measured after 1 hour incubation at 37 °C Final concentrations are the same as under A, with the addition of 1 μg/ml mellitin (MEL) Values represent the mean $\pm$ s e m of duplicate determinations from a representative experiment

The stimulation of PI-hydrolysis, Ca^{2+}-mobilization and AA-release by LPA, bradykinin and $PGF_{2\alpha}$ does not correlate with their effects on cell growth as shown in figure 3 3 Figure 3 3A displays the data of the cumulative PI-hydrolysis in density-arrested NRK cells after treatment with these agonists LPA and bradykinin generate a similar, rather weak PI-response when compared to $PGF_{2\alpha}$ Although $PGF_{2\alpha}$ elicits a much stronger PI response than LPA and bradykinin, the resulting Ca^{2+} transients are the same as shown in figure 3 3B Typical is the very strong PI-response when LPA and $PGF_{2\alpha}$ are added together This synergistic action was also observed in the [^{3}H]-TdR incorporation assays (figure 3 2) Figure 3 3C gives a comparable picture for the LPA-, bradykinin- and $PGF_{2\alpha}$-stimulated AA release $PGF_{2\alpha}$ is also the most potent inducer of AA-release compared to LPA and bradykinin $PGF_{2\alpha}$ generates the same amount of AA-release as the

phospholipase A_2 activator mellitin. Again, bradykinin and LPA give a equal response in AA-release. The same experiments on serum-starved, quiescent NRK cells show similar results (data not shown). Although $PGF_{2\alpha}$ generates the strongest PI and AA response, it does not show a pronounced effect on NRK cell proliferation compared to LPA and bradykinin. LPA and bradykinin however display a similar response in both AA-release and PI-hydrolysis, but they have completely opposite effects on NRK cell growth. Taken together these data strongly suggest that there is no correlation between the potency of bradykinin and LPA to generate PI-hydrolysis, Ca^{2+}-mobilization or AA-release and their effect on density-arrested NRK cell growth.

Another second messenger pathway that might be involved in the growth regulatory actions of LPA and bradykinin is cAMP. Increases in intracellular cAMP levels can inhibit fibroblast proliferation (Hollenberg and Cuatrecasas, 1973; Heldin *et al.*, 1989, Burgering *et al.*, 1989). As shown in figure 3.4A, proliferation of NRK cells is also inhibited when intracellular cAMP levels are elevated. The adenylate cyclase agonist forskolin inhibits the growth factor-induced loss of density-arrest of NRK cells in a concentration dependent manner. Decreases in cAMP levels have been reported as an

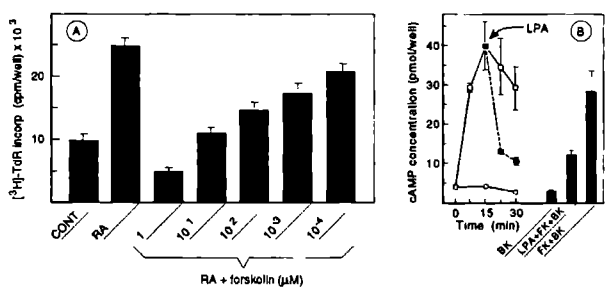

Figure 3.4. A Effect of forskolin on loss of density-arrest
Density-arrested NRK cells were treated with 50 ng/ml RA in the absence or additional presence of indicated concentrations of forskolin CONT represents untreated cells [^{3}H]-TdR incorporation was determined between 4 and 19 hours after the additions Values represent the mean ± s e.m of triplicate determinations from a representative experiment

B Effect of LPA and bradykinin on cAMP levels in NRK cells
Time course of cAMP levels in density-arrested NRK cells after treatment with 0 1 μM forskolin (□ ——□), 15 min. 0 1 μM forskolin followed by the addition of 100 μM LPA (arrow, ■-- ■) or no addition (○ ——○) The bargraphs on the right show the cAMP levels after a 30 min treatment with 1 μM bradykinin (BK) or a 15 min treatment with 0 1 μM forskolin followed by a subsequent 15 min incubation in the additional presence of 1 μM bradykinin (FK+BK) or 100 μM LPA and 1 μM bradykinin (LPA+FK+BK) All data are from the same experiment. Values represent the mean ± s e m. of duplicate determinations from a representative experiment

important early signaling event for LPA (Van Corven *et al.*, 1989; Kumagai *et al.*, 1993). The data in figure 3.4B clearly demonstrate that also in NRK cells LPA dramatically decreases forskolin-stimulated cAMP levels. However bradykinin does not show any effect on either basal, elevated or decreased cAMP levels. Thus it seems unlikely that cAMP plays a role in the inhibitory effect of bradykinin on NRK cells. The rapid decrease in elevated cAMP levels is specific for LPA, but not required for phenotypic transformation. The other investigated transforming growth factors RA, TGFß and PDGF-BB do not affect cAMP levels in NRK cells (data not shown).

The combined data on intracellular messengers suggest that PI-hydrolysis, Ca^{2+}-mobilization, AA-release and changes in cAMP levels per se, do not mediate the inhibitory effect of bradykinin. PI-hydrolysis, Ca^{2+}-mobilization and AA-release are also not sufficient for the transforming effect of LPA. The only specific LPA effect is the decrease in forskolin-stimulated cAMP levels, but this effect is not the target for the inhibitory action of bradykinin.

The inhibitory effect of bradykinin is mediated through a PGH synthase product

In previous reports on bradykinin mediated inhibition of cell growth, this effect could always be abolished by indomethacin, an inhibitor of PGH synthase (Straus and Pang, 1984; Patel and Schrey, 1992; McAllister *et al.*, 1993). It is known that several products of the metabolic conversion of AA by PGH synthase can be potent inhibitors of cell growth (reviewed in Fukushima, 1992). In our system indomethacin also completely abolished the inhibitory effect of bradykinin on growth factor-induced loss of density-arrest (figure 3.5), as did other PGH synthase inhibitors such as flurbiprofen and ibuprofen (data not shown). It is also shown in figure 3.5 that inhibitors of lipoxygenase (nordihydroguaiaretic acid) or epoxygenase (2-methyl-1,2-di-3-pyridyl-1-propanone), two

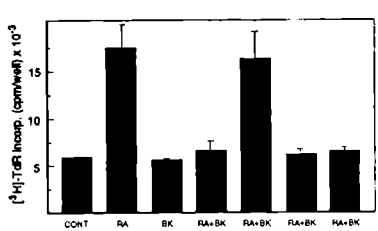

Figure 3.5. Inhibitory effect of bradykinin is abolished by indomethacin.
Density-arrested NRK cells were incubated with the indicated growth factors at the following concentrations: 50 ng/ml RA, 1 μM bradykinin (BK) or no addition (CONT). In the indicated lanes the cells were incubated with 1 μM indomethacin (INDO), 1 μM nordihydroguaiaretic acid (NDGA) or 1 μM 2-methyl-1,2-di-pyridyl-1-propanone (MDPP) before the addition of RA and bradykinin. [^{3}H]-TdR incorporation was determined between 4 and 19 hours after the addition of the growth factors. Values represent the mean $\pm$ s.e.m. of triplicate determinations.

other AA converting enzymes, had no effect on the bradykinin inhibition of phenotypic transformation. These results give a strong indication that one or more PGH synthase products mediate the inhibitory effect of bradykinin.

To test whether the observed effect was mediated by one of the well characterized PGHsynthase products several AA metabolites were added to RA-induced density-inhibited NRK cells, including the prostaglandins A_2, D_2, E_1, E_2, $F_{2\alpha}$, I_2, and the thromboxane A_2 agonist U46619. However, no inhibition of phenotypic transformation by any of these compounds was observed (data not shown). Preliminary data with conditioned medium from bradykinin-stimulated, density-arrested NRK cells however indicate that a growth inhibitory compound is released from the cells (DHTP Lahaye and EJJ Van Zoelen, unpublished observation). These observations suggest that bradykinin induces the formation of a so far uncharacterized PGH synthase product which is responsible for the inhibition of phenotypic transformation.

Bradykinin is a potential transforming growth factor for NRK cells

An important observation in the phenotypic transformation assays was that the inhibitory effect of bradykinin was fading after prolonged incubation times. To investigate this phenomenon in more detail, time courses of [^{3}H]-TdR incorporation into NRK cells were made under different growth conditions. In figure 3.6A the time course of [^{3}H]-TdR incorporation after the addition of LPA to density-arrested cells with or without additional bradykinin is shown. LPA displays a typical stimulation of [^{3}H]-TdR incorporation with a maximum incorporation rate between 19-21 hours after the addition. These kinetics are similar to those reported for TGFß, but are delayed when compared to the PDGF-stimulated [^{3}H]-TdR incorporation in density-arrested NRK cells (Van Zoelen *et al.*, 1992). These delayed kinetics may reflect that the LPA-induced loss of density-arrest is a indirect mechanism, like that of TGFß. However, LPA does not require EGF for its transforming activity (data not shown). Van Corven *et al.* (1992) have also shown that the kinetics for LPA-induced mitogenesis in quiescent Rat-1 fibroblasts are slower than for EGF-induced mitogenesis. Thus it might be possible that the relative slow kinetics of LPA rather reflect a slow working mechanism than a indirect mechanism.

In the additional presence of bradykinin the LPA-induced loss of density-arrest is inhibited, but not permanently. Bradykinin causes a delay of the LPA-stimulated growth curve of approximately 8 hours. Importantly, bradykinin alone displays a growth stimulatory effect with the same kinetics as LPA plus bradykinin, although the maximum incorporation rate is somewhat lower. This slow growth stimulatory effect of bradykinin explains the fading of the inhibitory effect in the [^{3}H]-TdR incorporation assays. When RA-, TGFß-, LPA- or PDGF-BB-stimulated density-arrested cells in these cumulative

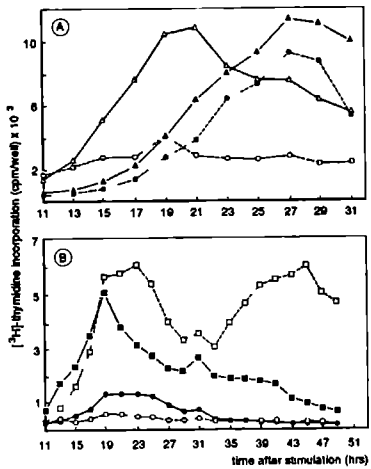

Figure 3.6. Kinetics of the growth stimulatory and inhibitory effect of bradykinin

A Time course of the [^{3}H] TdR incorporation (2-hour pulses) into density-arrested NRK cells after the addition of 100 μM LPA (▵ ——▵), 100 μM LPA and 1 μM bradykinin (▲ ——▲), 1 μM bradykinin (●- ●) or without stimulus (○---○)

B Time course of the [^{3}H]-TdR incorporation (2 hour pulses) into serum-starved, quiescent NRK cells after the addition of 5 ng/ml EGF (■ ——■) 5 ng/ml EGF and 1 μM bradykinin (□ --□), 1 μM bradykinin (● ——●), or no stimulus (○ --○) Values represent the mean ± s e m of triplicate determinations

assays were incubated for more than 19 hours in the presence of bradykinin, the growth stimulatory effect of bradykinin became visible and compensates its inhibitory effect

The fact that bradykinin is able to induce the loss of density-dependent growth inhibition makes it a potential transforming growth factor The question remains whether this bradykinin-induced loss of density-arrest is EGF dependent, similar to the transforming activities of RA and TGFß (Van Zoelen *et al* , 1988) To address this problem the kinetics of bradykinin-stimulated [^{3}H]-TdR incorporation in serum-starved, quiescent NRK cells were investigated in the presence and absence of EGF (figure 3 6B) Addition of EGF to quiescent NRK cells induces a single round of DNA synthesis, after which the cells become density-arrested However, in the additional presence of bradykinin the cells continue for another round of DNA synthesis, which is indicative for the loss of density-dependent growth inhibition (Van Zoelen, 1991) It is clear from the data in figure 3 6B that bradykinin needs the presence of EGF for its transforming effect When added alone, bradykinin is not able to induce [^{3}H]-TdR incorporation These data are very similar to those obtained with TFGß and RA as described in detail previously (Van Zoelen *et al* , 1988)

In conclusion, bradykinin displays a biphasic effect on density-arrested NRK cell growth Initially bradykinin transiently inhibits the growth factor-induced loss of density-arrest However, in the additional presence of EGF, extended incubation periods with bradykinin cause the phenotypic transformation of these cells

DISCUSSION

The bioactive lipid LPA has obtained much attention in recent years for its role in fibroblast proliferation (Van Corven *et al* , 1989, 1992) and regulation of cytoskeletal elements (Ridley and Hall, 1992, Edwards *et al* , 1993, Jalink *et al* , 1993) However, the intracellular signals that convey the LPA actions still remain obscure In the present study the intracellular effects of LPA were compared with those of bradykinin Bradykinin is a small peptide with a wide range of reported biological effects including neurotransmission, pain perception, smooth muscle contraction and cell proliferation (Roberts, 1989) Bradykinin mimics many of the LPA-induced intracellular responses, including PI-hydrolysis, Ca^{2+}-mobilization and AA-release as described in this paper and previously by others (Van Corven *et al* , 1989, Jalink *et al* , 1990) However their effect on density-arrested NRK cell proliferation is completely opposite LPA induces the loss of density-arrest in NRK cells, while this process is strongly inhibited by bradykinin The differential growth effects of LPA and bradykinin could not be correlated to differences in PI-hydrolysis, Ca^{2+}-mobilization or AA-release The specific effect of LPA on forskolin-stimulated cAMP levels was not counteracted by bradykinin, indicating that the growth inhibitory effect of bradykinin is not mediated by cAMP Although the inhibitory action of bradykinin is not correlated with the level of AA per se, it appears to be mediated through the formation of a specific AA metabolite by PGH synthase Other studies on the inhibitory effect of bradykinin on fibroblast proliferation have also indicated the involvement of a PGH synthase product in this process (Straus and Pang, 1984, Patel and Schrey, 1992, McAllister *et al* , 1993) The exact nature of the PGH synthase product has not been characterized yet, but externally added prostaglandins tested so far did not mimic the bradykinin inhibition of NRK cells This is in contrast to the observations of Patel and Schrey (1992), and McAllister *et al* (1993) In both studies bradykinin could be replaced by externally added prostaglandin E_2 to elicit an inhibitory effect on fibroblast cell growth This indicates that in NRK cells another prostaglandin is involved in the inhibitory action of bradykinin Current studies are underway to identify this prostaglandin

An intriguing observation is the specificity of the bradykinin inhibition for density arrested cells McAllister *et al* (1993a) have also reported a cell density-dependent inhibitory effect of bradykinin on human gingival fibroblasts but not as strict as in NRK cells These authors correlate the bradykinin responsiveness to the increase in bradykinin receptor numbers at increasing cell densities It remains to be investigated whether the bradykinin receptor number increases upon density arrest in NRK cells, but bradykinin-induced PI-hydrolysis and AA-release are similar in both quiescent and density-arrested cells Another option is an induction of a different bradykinin receptor subtype when NRK

cells become density-arrested. In general, bradykinin receptors are defined in two subtypes. The type 2 subtype is widely expressed in many cell types and seems to be responsible for the bradykinin-induced PI-response and stimulation of cell growth (Roberts, 1989; Roberts and Gullick, 1989). The type 1 subtype is in general not expressed, but its involvement in bradykinin mediated inhibition of human breast fibroblasts has been reported (Patel and Schrey, 1992). The exact contribution of either subtype in the inhibition of NRK cells has not been established yet (Van Zoelen *et al.*, 1994). Regulation of the phosphorylation state of the bradykinin receptor (Jong *et al.*, 1993) or regulation of processes downstream of the receptor upon density-arrest can also be reponsible for the specificity of the bradykinin inhibition. However at present not much is known about these processes and possible modes of regulation.

Upon prolonged incubation bradykinin induces the loss of density-dependent growth arrest of NRK cells. The mechanism by which bradykinin phenotypically transforms NRK cells is similar to that of RA and TGFß, although the kinetics are much slower (Van Zoelen *et al.*, 1988). The EGF dependence of this transforming effect of bradykinin suggests that bradykinin, like RA and TGFß, upregulates EGF receptor numbers to release the cells from density-arrest. ^{125}I-EGF binding is indeed increased upon bradykinin treatment of density-arrested NRK cells (unpublished observation). Stimulation of PI-turnover might be responsible for this effect on EGF receptor levels and the concomitant loss of density-arrest. The correlation between agents linked to the phosphoinositide signaling system and upregulation of EGF receptors has been reported previously by Earp *et al.* (1988). The observation that $PGF_{2\alpha}$ is also able to induce loss of density-arrest, in a EGF dependent manner, with the same kinetics as bradykinin (data not shown) further support this hypothesis. LPA also increases ^{125}I-EGF binding in density-arrested cells (unpublished observation). However, the phenotypic transformation of NRK cells is not dependent on EGF. This suggests that the transforming effect of LPA is mediated through another, as-yet-unidentified pathway.

METHODS

Phenotypic transformation assay

NRK-49F cells were grown to confluency in 24-well tissue culture dishes in Dulbecco's modified Eagle medium (Gibco), supplemented with 10% newborn calf serum (Hyclone). Confluent cells were made quiescent by a subsequent three days incubation in serum-free medium, as described previously (Van Zoelen *et al* , 1988) These cells were grown to density-arrest by an additional 48 hours incubation in the presence of 5 ng/ml EGF (Collaborative Research) and 5 µg/ml insulin (Sigma) After treatment with additional growth factors, the capacity of these growth factors to induce phenotypic transformation was determined by the measurement of [^{3}H]-thymidine (TdR) incorporation (0 5 µCi/ml, Amersham), either in a cumulative

manner between 4 19 hours after agonist addition, or in consecutive 2-hours pulse intervals

Inositol phosphate determination

Confluent NRK cultures in 6 well tissue culture dishes were labeled for 24 hours with 4 μCi/ml [^{3}H]-inositol (Amersham) in serum-containing medium After a subsequent 24 hours incubation in serum-free medium, cells were grown to density-arrest by a 48 hours treatment with 5 ng/ml EGF and 5 μg/ml insulin The cells were stimulated with agonists for 30 minutes in the additional presence of 10 mM LiCl The reactions were terminated by changing the medium for 10% trichloro-acetic acid (TCA) Inositol phosphates were determined by elution from a AG 1X8 anion exchange column (BioRad) with 0 8 M ammonium formate/0 1 M formic acid (pH 4 5), as described in detail by Tilly *et al* (1987)

cAMP measurements

Density-arrested NRK cells in 6-well tissue culture dishes were preincubated for 10 minutes with 0 5 mM 3 isobutyl-1-methylxanthine (Sigma), followed by the addition of agonists After the appropriate incubation times the cells were rinsed with ice-cold phosphate-buffered saline and the reactions were stopped by treatment with 1 ml 10% icecold TCA The TCA was removed by a fivefold extraction with diethylether and the samples were lyophilysed cAMP content was determined using the Amersham [^{3}H]-cAMP kit, according to the instructions of the manufacturer

[^{3}H] arachidonic acid release

Density-arrested NRK cells in 6-well tissue culture dishes were labeled with 1 μCi/ml [^{3}H]-arachidonic acid (180-240 Ci/mmol, NEN) for 15 hours After the labeling the medium was replaced by 1 5 ml HEPES buffered Dulbecco's modified Eagle medium with additonal 0 1% fatty acid free bovine serum albumin (Sigma) This medium was changed several times to remove unincorporated label Agonists were added and after 1 hour 0 5 ml of the culture medium was withdrawn and total radioactivity released into the medium was determined by liquid scintillation analysis

Measurements of intracellular Ca^{2+}

Cells were grown on gelatin-coated glass coverslips instead of tissue culture plastic as described above Density-arrested NRK cells were loaded for 90 minutes with 5 μg/ml Fura-2AM (Molecular Probes) and 0 01% pluronic acid in Hepes/bicarbonate-buffered serum free medium at room temperature Subsequently the cells were washed and incubated in Hepes/bicarbonate buffered saline (in mM 25 NaCl, 5 KCl, 2 $CaCl_2$, 10 glucose, 15 Hepes, 17 6 $NaHCO_3$) at 25 °C Intracellular calcium measurements were performed using a spectrofluorometer (SPF 500 Aminco), at an excitation wavelength of 340 nm (4 nm bandwidth) and an emission wavelength of 480 nm (8 nm bandwidth) Data were sampled and stored at a rate of 0 1 Hz using a data processor Growth factors were added directly to the incubation medium At the end of the experiment, ionomycin (5 μM) and Mn^{2+} (1 mM) were added to obtain an estimate of maximum and background calcium values

Materials

TGFß was purified from human platelets according to the protocol of Van den Eijnden-Van Raaij *et al* (1988) Recombinant PDGF-BB was a generous gift from Dr C-H Heldin (Uppsala, Sweden) LPA was purchased from both Sigma and Serdary RA, indomethacin nordihydroguaiaretic acid, 2 methyl-1,2 di pyridyl-1-propanone and $PGF_{2\alpha}$ were from Sigma, bradykinin was from Boehringer

CHAPTER 4

Discussion & references

Phenotypic transformation of NRK cells is a widely used model system to investigate the molecular mechanisms involved in the process of cellular transformation. In the previous two chapters, some aspects of these molecular mechanisms have been described. In chapter 2, the growth inhibitory effect of bradykinin was used to demonstrate the strong correlation between EGF receptor levels and the phenotypic transformation of NRK cells. These data provided additional support for the model that describes the phenotypic transformation of NRK cells as a function of EGF receptor numbers (Van Zoelen, 1991). The molecular mechanism by which bradykinin elicits its inhibitory effect is described in more detail in chapter 3. From the experimental data it appeared that a PGH synthase product is responsible for the bradykinin-induced growth inhibition. Although these studies clearly demonstrate the central role for the EGF receptor in the transformation process, some important questions remain unanswered.

Firstly, are the described observations applicable to all aspects of phenotypic transformation ? The present data are derived from studies on the loss of density-dependent growth arrest, which is only one aspect of phenotypic transformation. Whether bradykinin also inhibits for instance anchorage-independent growth of NRK cells is not possible to determine. The inhibitory effect lasts too short to study its effect on NRK growth in semi-solid medium, and only the long-term transforming effects are visible in these kind of experiments. Therefore, it is more appropriate to apply the model of EGF receptor modulation currently only to the control of density-dependent growth arrest. In this respect it may also be important to question whether growth-arrest in the presence of EGF can be considered as density-dependent growth inhibition. NRK cells have a very low level of EGF receptors and a further decrease of EGF receptor numbers at increasing cell densities will indeed stop the cells from proliferation in response to EGF. However, will NRK cells also become density-arrested at the same cell density in the presence of other growth factors ? From studies with PDGF it appears that NRK cells in the presence of either PDGF-AB or PDGF-BB reach higher cell densities before density-arrest than in the presence of EGF or PDGF-AA (Van Zoelen *et al.*, 1993).

Secondly, how does bradykinin inhibit EGF receptor levels ? In chapter 2, it has been shown that the inhibitory effect of bradykinin is paralleled by an inhibition of EGF receptor mRNA formation. From the data in chapter 3, it appears that bradykinin inhibits

the loss of density-arrest, and thus the formation of EGF receptor mRNA formation, through the formation of a PGH synthase product. In addition, subsequent studies have indicated that prostaglandin J_2 may be the intermediate responsible for this inhibitory effect (Lahaye et al., 1994). Prostaglandins, including prostaglandin J_2, are potent inhibitors of cell proliferation (reviewed by Fukushima, 1992). It has been postulated that this anti-proliferative effect is mediated through an induction of heat-shock proteins (Ohno et al., 1988; Santoro et al., 1989), thereby arresting the cells in their G_1-phase. Whether the decrease of EGF receptor mRNA formation and the resulting inhibition of phenotypic transformation is indeed a consequence of prostaglandin-induced cell cycle arrest still needs to be investigated. In addition, prostaglandins may regulate Ras-GAP (Han et al., 1991), and thus affect Ras activity. Ras has been shown to be involved in the transformation of many cell types (Satoh and Kaziro, 1992; Downward, 1992). Therefore, when activation of the EGF receptor gene would be located downstream of Ras in the process of phenotypic transformation, prostaglandins can block both these processes through modulation of Ras-GAP. However, both these models are rather speculative and additional experimental data is necessary to propose a good working mechanism for the inhibitory action of bradykinin.

A third question that remains is what differences in signal transduction are responsible for the observed differences between LPA, bradykinin and prostaglandin $F_{2\alpha}$ in the phenotypic transformation of NRK cells ? As already indicated in chapter 3, these differences in effects are apperently not due to differences in PI-turnover, Ca^{2+}-mobilization, cAMP levels or AA-release. A likely explanation for the growth stimulatory effects of LPA on NRK cells is that the LPA receptor couples to a G_i protein, in contrast to the bradykinin and prostaglandin $F_{2\alpha}$ receptors. However, not the resulting decrease in cAMP levels (Van Corven et al., 1989; chapter 3), but the G_i protein-mediated activation of Ras (Van Corven et al., 1993), triggered by the $G_{\beta\gamma}$ subunits (Koch et al., 1994), is believed to be responsible for the LPA-induced growth stimulation of fibroblasts and may also be the mechanisms by which LPA induces phenotypic transformation of NRK cells. The signal transduction route that leads to the typical inhibitory effect of bradykinin is, however, more difficult to imagine, based on the data presented in chapter 3. There is currently no information about unique intracellular events that are generated upon bradykinin receptor activation, but not by other G protein-coupled receptors. Therefore it would be a blind guess to speculate about possible signaling cascades involved in this process. Additional research is currently being performed to gain more information about the complete mechanism by which bradykinin elicits its inhibitory effect on NRK cells. The resulting information may also provide more insight in the molecular mechanisms involved in the process of phenotypic transformation in general.

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PART B

Transcriptional regulation of the human PDGFαR gene

CHAPTER 5

Introduction

Platelet-Derived Growth Factor

As its name already indicates, platelet-derived growth factor (PDGF) was originally characterized as the platelet-derived source for fibroblast and smooth muscle cell proliferation (Kohler and Lipton, 1974; Ross *et al.*, 1974). Subsequent studies have shown that the production of PDGF is not only restricted to platelets, but that a wide variety of both normal and tumor cells is able to produce PDGF (reviewed in: Raines *et al.*, 1991; Westermark, 1993). In addition to its mitogenic activity, PDGF is able to elicit many different biological effects, ranging from vasoconstriction to chemotaxis (Ross *et al.*, 1990; Raines *et al.*, 1991). As a result of this widely distributed expression and pleiotropy of effects, PDGF has been postulated to play a role in many biological processes. This includes embryogenesis, growth and development, atherosclerosis, wound-healing and neoplasia (Ross *et al.*, 1986, 1990; Bowen-Pope *et al.*, 1991; Raines *et al.*, 1991; Raines and Ross, 1993).

PDGF is a dimer, composed of two different, but highly homologous PDGF chains, denoted A and B. The A and B chains can either form homo- or hetero-dimers by disulfide bonds, which results in three PDGF isoforms: AA, AB and BB. These isoforms can bind to two different PDGF receptors, denoted α (PDGFαR) and ß (PDGFßR). The PDGF receptors belong to the family of protein tyrosine kinase receptors, which, among others, also contains the receptors for epidermal growth factor (EGF), insulin-like growth factor (IGF) and fibroblast growth factor (FGF). Like their ligands, the PDGF receptors are different, but highly homologous molecules, and they form homo- and hetero-dimers upon ligand binding (Claesson-Welsh, 1994, 1994a). The PDGFαR displays high affinity for all three PDGF isoforms. However, the PDGFßR preferentially binds the BB-isoform. As a result, $\alpha\alpha$, αß and ßß receptor dimers will be formed, depending on the PDGF isoform (Seifert *et al.*, 1989).

The events following receptor dimerization have been extensively studied, particularly in relation to the PDGFßR. Nevertheless, the exact order and importancy of events have still not been absolutely proven. According to Claesson-Welsh (1994a), the following scenario is most likely: 'receptor dimerization and accompanying conformational changes allow a basal kinase activity to phosphorylate a critical tyrosine residue, thereby "unlocking" the kinase, leading to full enzymatic activity directed toward other tyrosine

residues in the receptor molecules as well as other substrates for the kinase' Phosphorylation of these tyrosine residues within the receptor molecule attracts many signal transduction molecules by virtue of their SH2-domain, including Src family kinases (Src, Fyn and Yes), phosphatidylinositol 3'-kinase (PI3-kinase), Nck, GTPase-activating protein (GAP) of Ras, Shb, protein tyrosine phosphatase 1D (Syp), phospholipase C-γ (PLC-γ), Grb2 and Shc (reviewed by Claesson-Welsh, 1994, 1994a) These signal transduction molecules either carry an enzymatic activity themselves or they function as adaptor molecules for other proteins of the signal transduction cascade

As mentioned above, most of this information comes from studies on the PDGFßR Due to their structural homology, many of these events are thought to be similar for the PDGFαR Recently, binding of PI3-kinase, Syp and PLC-γ, but not GAP to the activated PDGFαR has been demonstrated (Bazenet and Kazlauskas, 1993, Inui *et al* , 1994) Whether there are genuine differences in the signal transduction between the α and ß receptor has still to be demonstrated In most cell lines tested, the reported differences in mitogenic sensitivity of cells to the different PDGF isoforms mainly reflects the difference in ratio of PDGFαR and PDGFßR numbers in these cells (Seifert *et al* , 1989) However, some observations indicate that there is a functional difference between PDGFαR and PDGFßR signaling in processes like actin organization of porcine aortic endothelial cells (Eriksson *et al* , 1992), chemotaxis of human foreskin fibroblasts (Vassbotn *et al* , 1992), proliferation of vascular smooth muscle cells (Inui *et al* , 1994), and transformation of NIH 3T3 fibroblasts (Heidaran *et al* , 1993, Kim *et al* , 1994), but the molecular background of these differences is currently unknown

Structure of the PDGFαR protein and gene

As predicted from the cDNA sequence, the human PDGFαR contains 1066 amino acids after cleavage of the 24 amino acids signal peptide (Claesson-Welsh *et al* , 1989, Matsui *et al* , 1989) Several structural and functional domains can be distinguished within the α-receptor protein The extracellular ligand binding part contains five immunoglobulin-like domains and eight potential N-linked glycosylation sites Several of these glycosylation sites are being used, increasing the molecular mass of the receptor from 140 kDa of the precursor form to approximately 170 kDa of the mature PDGFαR protein (Claesson-Welsh, 1994a) The intracellular part, which is separated from the extracellular by a single membrane spanning amino acid stretch, contains the catalytic tyrosine-kinase domain This tyrosine-kinase region consists of two parts, separated by a non-catalytic kinase insert

As already mentioned above, the PDGFαR is highly homologous to the PDGFßR. At the amino acid level this homology is almost 50% overall identity, and reaches almost 90% identity for the first tyrosine kinase domain (Claesson-Welsh *et al.*, 1989; Matsui *et al.*, 1989; Do *et al.*, 1992). Based on the sequence similarities and structural characteristics, the PDGFαR and PDGFßR belong to the class III subfamily of tyrosine kinase receptors, which also includes the colony stimulating factor-1 (CSF-1) receptor (or c-fms), c-kit and FLT3 (or FLK2) (Ullrich and Schlessinger, 1990; Rosnet and Birnbaum, 1993). These subfamily members share about 60% amino acid identity in their kinase domains, but are less related in their transmembrane and extracellular domains. They all share the structural features of the characteristic five immunoglobulin-like domains and the split tyrosine kinase domain. The PDGFαR sequence and structure is very well conserved among the different species. The human PDGFαR overall amino acid sequence shows more than 76% identity with that of the mouse (Do *et al.*, 1992), rat (Lee *et al.*, 1990) and Xenopus (Jones *et al.*, 1993).

The gene encoding the PDGFαR has been localized to human chromosomal region 4q11-q12 (Matsui *et al.*, 1989; Stenman *et al.*, 1989; Gronwald *et al.*, 1990), and to a homologous region on mouse chromosome 5 (Smith *et al.*, 1991; Stephenson *et al.*, 1991) in close proximity to c-*kit*. Recently Spritz *et al.* (1994) have shown that the human *PDGFαR* gene and c-*kit* are juxtaposed in a tandem head-to-tail array in chromosome segment 4q12. A similar orientation has been described for the *PDGFßR* gene and c-*fms* on human chromosome 5 (Roberts *et al.*, 1988) and mouse chromosome 18 (Sundaresan and Francke, 1989), and these data strongly suggest that the class III receptor tyrosine kinases are derived from one ancestral gene.

Although currently no effort has been made to characterize the complete exon-intron structure of the *PDGFαR* gene, it most likely resembles the structure of its class III family members. Recent genomic sequence data on parts of the *PDGFαR* gene (Mosselman *et al.*, 1994; Wang and Stiles, 1994; chapter 6 of this thesis) indicate that the *PDGFαR* gene is indeed structurally similar to c-*fms* and the *PDGFßR* gene (Sherr, 1990). This implies a non-coding exon 1, followed by a relatively large (>10 kb) intron 1. The translational start codon ATG is located within exon 2, and as predicted from the homology with the well characterized c-*fms* (Hampe *et al.*, 1989), the complete *PDGFαR* gene may encompass 22 exons.

Functional expression of the PDGFαR

A prerequisite for a cell to respond to PDGF is the expression of the appropriate PDGF receptor at the cell surface Expression of the PDGFαR renders the cell responsive to all PDGF isoforms, while the ß-receptor only contributes to PDGF-B responsiveness Due to the widely distributed expression of both PDGF and its receptors, it has been difficult to discriminate between the exact contribution of either receptor subtype in the different biological responses to PDGF However, several observations indicate a specific role for the PDGFαR in embryonic development and tumorigenesis

PDGFαR in embryonic development

Both PDGFαR and PDGF-A are present in all cells of the mouse pre-implantation embryo from the two-cell stage onwards, creating an autocrine PDGF loop (Rappolee *et al* , 1988, Palmieri *et al* , 1992) A similar observation has been made in bovine pre-implantation embryos (Watson *et al* , 1992) In addition, the presence of both PDGF-A and the PDGFαR has been demonstrated in early Xenopus embryos and at this stage no PDGF-B or ß-receptor could be detected (Mercola *et al* , 1988, Jones *et al* , 1993, Palmieri *et al* , 1993) In the mouse PDGF-A and α-receptor expression persist after implantation, but the expression patterns of ligand and receptor become separated PDGF-A expression becomes limited to the ectoderm, whereas the PDGFαR becomes restricted to the mesoderm (Orr-Urtreger and Lonai, 1992, Palmieri *et al* , 1992) This lineage specific expression of ligand and receptor is also apparent in Xenopus embryonal development (Jones *et al* , 1993) Thus, upon formation of the different germ layers a paracrine mode of interaction between the ectodermal produced PDGF-AA and the adjacent mesoderm can be envisaged Expression of the ß-receptor becomes also detectable in mouse early post-implantation development, but to a lesser extent and at a later time-point than the PDGFαR (Mercola *et al* , 1990) During subsequent developmental stages, PDGFαR expression remains characteristic for many mesoderm-derived structures (Orr-Urtreger *et al* , 1992, Orr-Urtreger and Lonai, 1992, Schatteman *et al* , 1992) In addition, the PDGFαR is expressed in a non-neural subset of neural crest-derived cells (Morrison-Graham *et al* , 1992) This lineage-specific expression of the PDGFαR indicates a functional role for the α-receptor subunit in the development of these tissues This functionality is clearly demonstrated in studies with *Patch* mice, which carry a deletion of the *PDGFαR* gene (Smith *et al* , 1991, Stephenson *et al* , 1991) Mice homozygous for this mutation display severe developmental defects in mesoderm- and neural crest-derived structures, eventually resulting in death of the embryo (Morrison-Graham *et al* , 1992, Orr Urtreger *et al* , 1992, Schatteman *et al* , 1992)

PDGFαR in tumorigenesis

The v-*sis* oncogene of simian sarcoma virus (SSV) is the viral homologue of the gene encoding the PDGF-B chain (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983; Devare *et al.*, 1983). Transformation of cells by SSV involves the v-*sis* product and subsequent binding of this protein to the cells' own PDGF receptors, thus creating an autocrine PDGF loop (Owen *et al.*, 1984; Johnson *et al.*, 1985; Leal *et al.*, 1985; Keating and Williams, 1988; Fleming *et al.*, 1989). This concept of autocrine (but also paracrine) PDGF stimulation is still the basis for the suggested role of PDGF in tumor development. Many human tumor cell lines express PDGF and several express at the same time the appropriate PDGF receptor (reviewed by Westermark, 1993). Gliomas are the best studied example which support the idea for a functional role for the PDGFαR in tumor development. Most human gliomas express high levels of PDGFαR together with its ligand, thus creating autocrine loops within these tumors (Nistér *et al.*, 1991; Hermanson *et al.*, 1992). In certain gliomas amplifications of the *PDGFαR* gene occur (Kumabe *et al.*, 1992; Collins 1993; Hermanson *et al.*, 1995), while in addition a glioma-specific *PDGFαR* gene mutation has been described (Kumabe *et al.*, 1992). Even in the absence of *PDGFαR* gene amplification, a large subgroup of gliomas overexpress the PDGFαR at the mRNA level (Hermanson *et al.*, 1995). These findings together with the recent observation that a truncated, dominant-negative PDGF receptor inhibits glioma cell proliferation (Strawn *et al.*, 1994) strongly suggest a prominent role for the PDGFαR in glioma development.

Besides glioma development, several other tumorigenic processes have been described in which the PDGFαR is thought to play a role. An example is the metastasizing capacity of 3LL Lewis lung carcinoma and T10 sarcoma cells. In these cells the PDGFαR is not involved in the growth of the primary tumor, but its expression is strongly correlated with the capacity of the cells to metastasize to the lungs. Autocrine PDGF stimulation is not involved in this process, but PDGF is supplied in a paracrine fashion by the lung tissue (Do *et al.*, 1992; Fitzer-Attas *et al.*, 1993). As in gliomas, the concomitant expression of PDGF and the α-receptor is related to the progression of ovarian tumors (Henriksen et al,. 1993) and has been described in neuroendocrine tumors of the digestive system (Chaudry *et al.*, 1992). Recently Mosselman *et al.* (1994, 1995) have demonstrated that *PDGFαR* gene expression serves as an excellent indicator of human testicular tumor development. The highly-malignant undifferentiated embryonal carcinoma cells do not express the full lenght 6.4 kb PDGFαR transcript, but instead they display a 1.5 kb and 5.0 kb α-receptor messenger, as a result of both alternative splicing and alternative transcription initiation. In differentiated, non-malignant embryonal carcinoma cells the 6.4 kb PDGFαR mRNA is expressed together with a 3.0 kb transcript, which may encode a truncated PDGFαR. Although no functional role for the 1.5 kb and 5.0 kb α-receptor transcripts has been

established yet, these transcripts appear to be specific markers for early stages of testicular tumors, including carcinoma *in situ*, and both seminoma and non-seminomatous tumors.

Regulation of PDGFαR expression

The important question remains what mechanisms are involved in this specific regulation of PDGFαR expression. As already mentioned above, gene amplification may account for abberantly high PDGFαR expression in certain tumors, but this cannot be considered as a general mechanism. Both embryonic development and tumorigenesis are highly complex systems in which many regulatory mechanisms are involved. Thus it seems more likely that timing and abundancy of PDGFαR expression is regulated by a complex interaction between signals from both outside and inside the cells. In this respect, the observation that PDGFαR expression *in vitro* is affected by several growth factors is of particular interest. This mechanism by which membrane receptors are modulated by heterologous ligands is referred to as transmodulation (Zachary and Rozengurt, 1985).

Transforming growth factor ß

Transforming growth factor ß (TGFß) was the first growth factor described that transmodulates PDGFαR expression. TGFß-treated mouse Swiss 3T3 fibroblasts display a reduced mitogenic response towards PDGF and show reduced PDGF-AA binding when compared to untreated cells (Gronwald *et al.*, 1989). Similar observations have been made in human foreskin fibroblasts (Yamakage *et al.*, 1992; Paulsson *et al.*, 1993), human smooth muscle cells (Battegay *et al.*, 1990), and human osteoblastic cells (Yeh *et al.*, 1993a). In addition to reduced PDGF-AA binding upon TGFß treatment, these studies also demonstrated a reduction in PDGFαR mRNA levels. However, downregulation of the PDGFαR and a parallel reduction in mitogenic response is not a general mechanism of TGFß. Adult human skin fibroblasts or scleroderma fibroblasts show an increase in PDGFαR mRNA expression in response to TGFß treatment (Ishikawa *et al.*, 1990; Yamakage *et al.*, 1992). Whether this positive or negative transmodulation of the PDGFαR by TGFß is regulated transcriptionally or post-transcriptionally is currently unknown.

Interleukin-1

Interleukin-1 (IL-1) is a potent modulator of PDGFαR expression in osteoblastic cells. Treatment of fetal rat osteoblastic cells or the mouse osteoblast-like MC3T3-E1 cells with IL-1 induces PDGFαR expression and stimulates in this way the chemotactic and

mitogenic response of these cells to PDGF (Tsukamoto *et al.*, 1991; Centrella *et al.*, 1992). However, similar experiments with normal human osteoblasts and human MG63 osteosarcoma cells showed a decrease in PDGFαR expression and a reduced migrational and mitogenic response to PDGF upon IL-1 treatment (Gilardetti *et al.*, 1991; Yeh *et al.*, 1993a). There is not a good explanation for these contrasting results other than that it reflects the difference in species. Recently, Xie *et al.* (1994) have demonstrated by nuclear run-on analysis that at least the inhibitory effect of IL-1 on *PDGFαR* gene expression is regulated at the transcriptional level.

Retinoic acid

Mouse F9 embryonal carcinoma cells provide a widely used model system for studying early embryonic events *in vitro*. Exposure of undifferentiated, pluripotent F9 cells to retinoic acid (RA) causes these pluripotent stem cells to differentiate into cells resembling extraembryonic endoderm (Strickland and Mahdavi, 1978; Strickland *et al.*, 1980; Hogan *et al.*, 1980). This differentiation is accompanied by a strong induction of PDGFαR gene expression, which is largely due to a RA-induced enhancement of transcription of the *PDGFαR* gene (Mercola *et al.*, 1990; Wang *et al.*, 1990). The human embryonal carcinoma cell line Tera-2 clone 13 displays a similar induction of *PDGFαR* gene expression by RA (Mosselman *et al.*, 1994). In addition, it has been reported that RA induces *PDGFαR* gene expression in mouse osteoblastic MC3T3-E1 cells, but it is currently unknown whether RA induces this effect by increased transcription as in F9 cells (Tsukamoto *et al.*, 1994).

FGF

Four completely different cell systems have been described in which FGF specifically upregulates PDGFαR expression. Firstly, basic FGF (bFGF) maintains a high level of PDGFαR expression in rat O-2A oligodendrocyte precursor cells, which correlates with the capacity of bFGF to block oligodendrocyte differentiation of these cells (McKinnon *et al.*, 1990). Another cell type in which bFGF selectively increases PDGFαR expression are bovine and human smooth muscle cells, thereby rendering these cells responsive to PDGF (Schöllmann *et al.*, 1992). Also in mouse osteoblast-like MC3T3-E1 cells the PDGFαR expression is increased by FGF (Tsukamoto *et al.*, 1991), and the same observation has been reported for human dermal fibroblasts (Ichiki *et al.*, 1995). There are currently no indications at what level this regulation takes place.

PDGF-BB, EGF and insulin

Less well documented are the actions of PDGF-BB, EGF and insulin on the PDGFαR expression. Eriksson *et al.* (1991) have reported that PDGF-BB transiently increases PDGFαR mRNA levels in human foreskin fibroblasts through a post-transcriptional mechanism. Like IL-1, FGF and RA (see above), EGF upregulates α-receptor expression in mouse MC3T3-E1 cells (Tsukamoto *et al.*, 1991). In addition, one observation describes the inhibition of PDGF-AA binding to mouse Swiss 3T3 fibroblasts by insulin, apparently through a downregulation of PDGFαR numbers (Gronwald *et al.*, 1989).

As indicated above, the RA-induced increase and IL-1-induced decrease in PDGFαR expression is controlled at the level of gene transcription. Transcriptional regulation of the *PDGFαR* gene as the target for transmodulation of α-receptor expression by the other factors mentioned can not be excluded, although many post-transcriptional alternatives are possible.

The following three chapters will deal with some aspects of the transcriptional regulation of the *PDGFαR* gene. Chapter 6 describes the first step towards a better understanding of this process, namely the molecular cloning and functional characterization of the human *PDGFαR* gene promoter. The functionality of the promoter is assessed by its capacity to drive reporter gene expression in a orientation dependent way and its ability to respond to RA. Chapter 7 deals in more detail with the molecular mechanisms that are involved in the RA-induced transcriptional activation of the *PDGFαR* gene. Finally, in chapter 8 some *in vivo* aspects of the *PDGFαR* gene promoter in relation to embryonic development will be addressed using a promoter/*lacZ* reporter gene in a transgenic mouse model.

CHAPTER 6

Molecular cloning and functional characterization of the human platelet-derived growth factor α receptor gene promoter

ABSTRACT

Expression of the platelet-derived growth factor α receptor (PDGFαR) is strictly regulated during mammalian development and tumorigenesis The molecular mechanisms involved in the specific regulation of PDGFαR expression are unknown, but transcriptional regulation of the *PDGFαR* gene is most likely to be involved This study describes the molecular cloning of the non-coding exon 1 and approximately 2 kb of 5' flanking region of the human *PDGFαR* gene This 5' flanking region is a functional promoter of the *PDGFαR* gene as concluded from its capacity to drive luciferase reporter gene expression in an orientation dependent way Analysis of 5' promoter deletion mutants revealed that the region from -441 to +118 is sufficient to establish high level promoter activity In addition, the morphogen retinoic acid, in combination with dibutyryl cAMP, gives a 22-fold induction of *PDGFαR* gene promoter activity in human embryonal carcinoma cells This indicates that the cloned *PDGFαR* gene promoter contains the necessary elements to regulate PDGFαR expression in response to cell growth regulatory factors

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INTRODUCTION

Platelet-derived growth factor (PDGF) is a potent mitogen for mesenchymal and glial cells and is thought to play an important role in wound-healing, atherosclerosis, neoplasia and embryogenesis (for reviews see Ross et al., 1990; Bowen-Pope et al., 1991; Raines et al., 1991; Westermark & Heldin, 1993). PDGF can occur as three isoforms, AA, AB and BB, which bind with different affinities to the PDGFα and ß receptor. PDGF BB is the only high affinity ligand for the PDGFß receptor (PDGFßR), but the PDGFα receptor (PDGFαR) can bind all three isoforms. Both the α and ß receptor belong to the family of tyrosine kinase receptors. Upon ligand binding PDGF receptors dimerize, which results in kinase activation and receptor transphosphorylation on specific tyrosine residues. The phosphorylated receptors subsequently attract SH2-domain containing molecules for downstream signaling (for review see Claesson-Welsh, 1994).

Although the exact contribution of either receptor subtype to the different biological responses of PDGF is still unclear, accumulating evidence suggests a specific role for the α receptor subunit in early embryonic development and tumorigenesis. This notion is supported by the observation that both the PDGF A-chain and the α receptor are already expressed in the preimplantation embryo (Mercola et al., 1988; Rappolee et al., 1988; Watson et al., 1992). In addition, both PDGF A-chain and PDGFαR mRNA are expressed prior to and more abundantly than PDGF B-chain and PDGFßR mRNA in early mouse postimplantation development (Mercola et al., 1990). Additional information concerning the importance of PDGFαR expression during early embryonic development comes from studies on *Patch* mice, which lack the *PDGFαR* gene (Smith et al., 1991; Stephenson et al., 1991). Mice carrying this mutation display severe developmental defects (Orr-Urtreger et al., 1992; Schatteman et al., 1992). Although the impact of PDGFαR expression in later development seems to decline, expression of the *PDGFαR* gene still correlates with the development of specific tissues. Glia cell specific PDGFαR expression in the developing central nervous system is the most extensively studied example (Yeh et al., 1993; Pringle et al., 1992). Tumors arising from glial cells also display a specific pattern of PDGFαR expression (Nistér et al., 1991; Hermanson et al., 1992) or contain *PDGFαR* gene mutations (Kumabe et al., 1992). Another observation that stresses the relevance of the PDGFαR subunit in tumorigenesis is its specific expression in highly metastatic murine tumor cells (Do et al., 1992; Fitzer-Attas et al., 1993), malignant ovarian tumors (Henriksen et al., 1993) and neuroendocrine tumors (Chaudhry et al., 1992).

The regulatory mechanisms underlying the specific expression of the *PDGFαR* gene in development and tumorigenesis are unknown, but they will probably reflect a combination of both transcriptional and posttranscriptional control. In mouse F9

embryonal carcinoma cells, which resemble cells from early embryonic stages, *PDGFαR* gene expression can be induced by the morphogen retinoic acid (RA) (Mercola et al., 1990). This induction of *PDGFαR* gene expression is transcriptionally regulated (Wang et al., 1990) and occurs also in human embryonal carcinoma cells, as shown previously by our group (Mosselman et al., 1994). To gain more insight in the transcriptional regulation of the *PDGFαR* gene in development and tumorigenesis, we have cloned and characterized exon 1 and approximately 2 kb of 5' flanking sequences of the human *PDGFαR* gene. This 5' flanking region is able to drive luciferase reporter gene expression in both human and mouse cells. In addition, this *PDGFαR* gene promoter displays responsiveness to RA and dibutyryl cAMP (Bt_2cAMP), which are important regulators of embryonal carcinoma cell differentiation.

RESULTS

Cloning and nucleotide sequence analysis of exon 1 and 5' flanking region of the human PDGFαR gene

A 255 nucleotides EcoRI fragment, representing the 5' end of the human PDGFαR cDNA (kindly provided by Dr. L. Claesson-Welsh, Ludwig Institute, Uppsala, Sweden), was used to screen a human genomic DNA library. Southern blot analysis of restriction enzyme digested DNA from three out of thirty positively hybridizing phages indicated that all three phage clones contained the same region of the *PDGFαR* gene (data not shown). Subsequent subcloning of this phage DNA (from two independent phage clones) into pBluescript and reselecting for EcoRI probe positive clones resulted in the 1.4 kb pPART101 and 4.5 kb pPARP9 genomic subclones as outlined in figure 6.1. Additional Southern blot analysis (data not shown) and partial sequencing of these subclones revealed that both pPART101 and pPARP9 contained sequences corresponding to the 5' end of the human PDGFαR cDNA (Claesson-Welsh et al., 1989; Matsui et al., 1989). This sequence corresponding to the cDNA sequence does not contain the ATG initiation codon and, analogous to the c-*fms* proto-oncogene (Hampe et al., 1989), a close gene family member of the *PDGFαR* gene, reflects the non-coding exon 1 of the human *PDGFαR* gene. The sequence of exon 1 and approximately 2.1 kb of 5' flanking DNA is shown in figure 6.2. The exon-intron boundary sequence of exon 1 is conform to the AG-GT rule and matches almost perfectly to the overall boundary consensus sequence *exon*..AG/GTPuAG..*intron* (Mount, 1982). From these sequence data it follows that the EcoRI fragment, used to screen the genomic library, also encompasses sequences extending at least into exon 2. However, Southern blot analysis of exon 1 containing phage DNA never indicated the

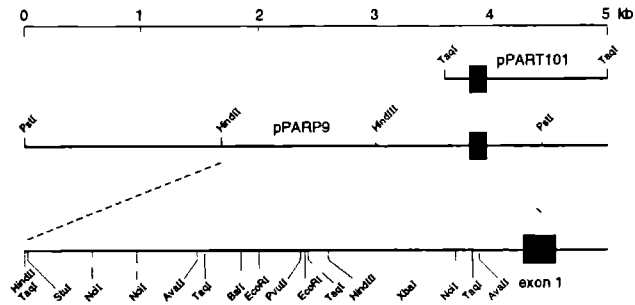

Figure 6.1 Schematic representation of the genomic subclones pPART101 and pPARP9 which together encompass approximately 5 kb of genomic sequences. Both subclones contain sequences homologous to the 5' end of the human PDGFαR cDNA (Claesson-Welsh et al. 1989; Matsui et al. 1989) indicated by the black box. In addition, the restriction map of the region used for promoter studies is shown at the bottom part of the figure. Indicated are those restriction enzyme sites that were used for subcloning into pSI A4 (promoter luciferase constructs) or pBluescript (sequence analysis).

presence of additional exons on the same genomic clone (data not shown).

Analysis of the *PDGFαR* gene promoter sequence revealed several elements that might bind proteins of the transcription machinery. An obvious element is the TATA box-like sequence located approximately 30 nucleotides upstream of the sequence corresponding to the 5' end of the PDGFαR cDNA (boxed sequence, figure 6.2). The TATA-box typically directs the transcription initiation 30 nucleotides downstream of the TATA motif (Zawel & Reinberg, 1992). To test whether the transcription initiation of the *PDGFαR* gene actually starts approximately 30 nucleotides downstream of the TATA motif, as suggested by the 5' end of the cDNA, primer extension analysis was performed to identify the transcription start location. As shown in figure 6.3, a major primer extension product was detected, which size indicates a transcription initiation site at the position marked by the hooked arrow in figure 6.2. This initiation site however, is located 34 nucleotides upstream of the 5' end of the cDNA sequence. This position of transcription initiation makes it very unlikely that the TATA-motif directs the transcription start of the *PDGFαR* gene. Analysis of the promoter sequence for other putative transcription factor binding sites (Faisst and Meyer, 1992) revealed several consensus motifs, including those for TFIID, GATA, AP2 and PEA3 (figure 6.2). It remains to be established however, whether these elements play a functional role in the *PDGFαR* gene transcription regulation.

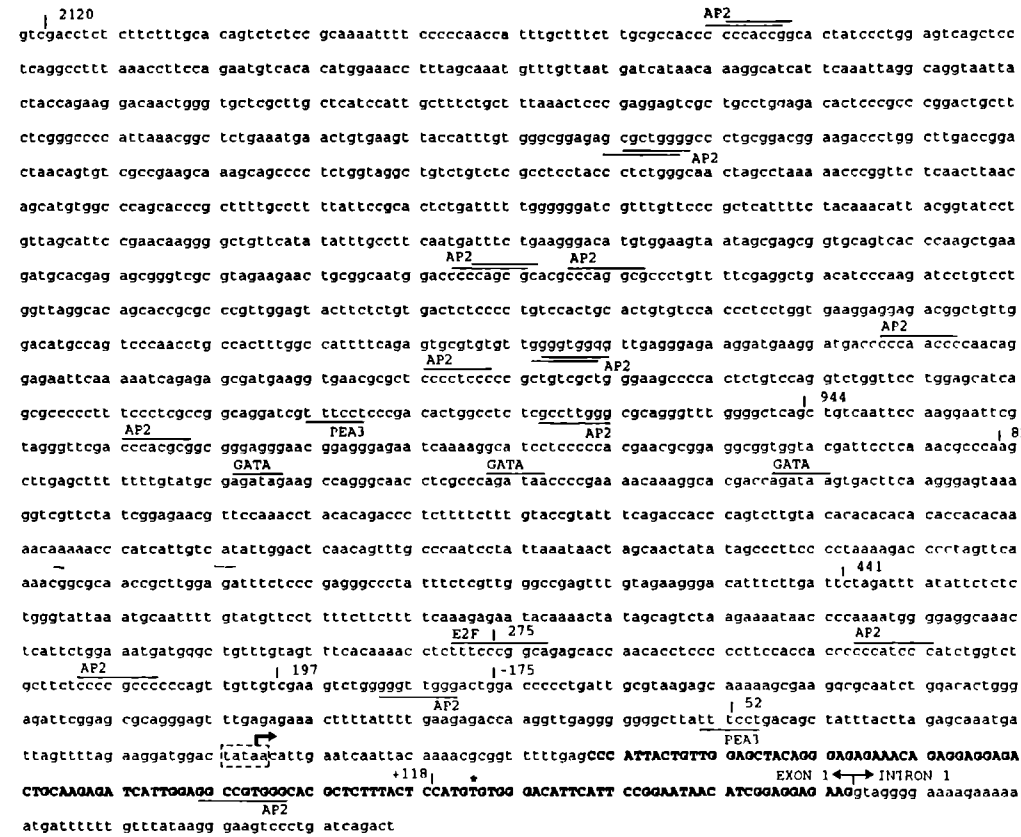

Figure 6.2. Nucleotide sequence of exon 1 and 5' flanking region of the human PDGFαR gene
Nucleotide sequence analysis was performed on multiple overlapping subclones from both pPARP9 and pPART101 The sequence homologous to the PDGFαR cDNA (Claesson-Welsh et al , 1989, Matsui et al , 1989) is shown bold face, and the position of the transcription start (+1) is indicated by the hooked arrow The 5' and 3' ends of the promoter deletion mutants are represented by vertical lines with nucleotide position number Putative transcription factor binding sites for AP2, PEA3, GATA (Faisst and Meyer, 1992) and for E2F (Ouelette et al , 1992) are marked by a horizontal line above (indicating 5'-3' orientation) or below (indicating 3'-5' orientation) the nucleotide sequence The TATA-box-like element (see text) is boxed The asterix marked T nucleotide at +123 has been mutated to a G in PCR experiments to generate a unique NcoI restriction enzyme site within exon 1 for subcloning into pSla4 (see materials and methods)

Functional characterization of the PDGFαR gene promoter

To perform a functional analysis of the *PDGFαR* gene promoter, approximately 2 kb of 5'flanking region and part of exon 1 was cloned in front of a luciferase reporter gene (see

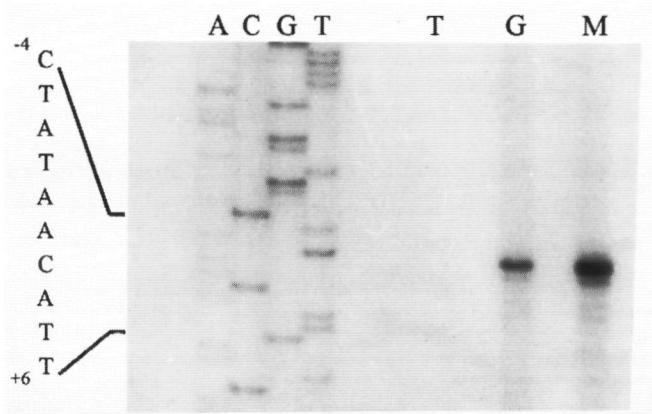

Figure 6.3. Primer extension analysis on PDGFαR RNA.
RNA (50 μg) was reverse transcribed using an end-labeled oligonucleotide, complementary to nucleotides 45-70 of exon 1 (figure 6.2). The products were analysed on a 6% polyacrylamide-ureum gel and visualized by autoradiography. T=tRNA, G=Germa II RNA, M=MA-90 RNA. As size marker, a parallel nucleotide sequence reaction on plasmid DNA, containing exon 1 and 5' flanking sequences, was performed with the same oligonucleotide. Indicated is the nucleotide sequence from -4 to +6.

materials and methods). In addition, a series of 5' deletion mutants were constructed and the promoter activity of these constructs was determined by transient transfection into several mammalian cell lines and monitoring luciferase activity. Figure 6.4 shows the results of the transfections into two PDGFαR expressing cell lines, RA-differentiated human Tera-2 cells (Mosselman et al., 1994) and mouse Swiss 3T3 cells (Gronwald et al., 1989). It is clear from these data that the 5' flanking region of the human *PDGFαR* gene is able to drive luciferase gene expression from a reporter construct in both human and mouse cells. Thus, the 5' flanking region can act as a functional promoter. The pattern of promoter activity of the different 5' deletion mutants is similar in both human and mouse cells. The highest promoter activity was observed in the -441/+118 LUC construct. Shortening this construct to -275/+118 dramatically decreased luciferase expression, but further 5' deletions up to -175 restored much of the promoter activity. Additional deletions from -175 to -52 reduced the luciferase expression to a level $\leq 5\%$ of the maximal expression, but well above background (pSLA4). Extending the -441/+118 LUC construct at the 5' end also results in a decrease of promoter activity. This complex activity pattern of the 5' deletion mutants suggests that both positive and negative promoter elements are involved in basal *PDGFαR* gene transcription. However, it can be concluded that the human *PDGFαR* gene 5' flanking region up to nucleotide -441 contains all the elements necessary for high level promoter activity in both human and mouse cells.

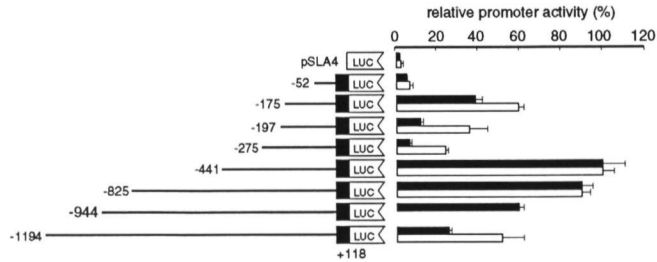

Figure 6.4. Promoter activity of 5' deletion mutants.
A series of 5' promoter deletion mutants, fused to a luciferase reporter gene, were transiently transfected into differentiated Tera-2 cells (black bars) or mouse Swiss 3T3 cells (open bars). Luciferase activity in the cell lysate was determined 24 hrs. (Tera-2), or 48 hrs. (Swiss 3T3) post transfection. The luciferase activity was normalized to the ß-galactosidase activity of the co-transfected pCH110 plasmid. Values represent mean promoter activity relative to the activity of -441/+118 LUC (error bars indicate sample standard deviation of triplicate determinations). pSLA4 represents the luciferase vector without promoter insertion and the activity of the -944/+118 LUC was not determined in Swiss 3T3 cells.

In addition to Tera-2 and Swiss 3T3 cells, several other cell lines have been tested for *PDGFαR* gene promoter activity. When transfected with promoter-luciferase constructs, the human malignant glioma cell lines U-1242 MG, U-343 MGa 35L (Nistér et al., 1991), mouse normal osteoblast-like cells MC3T3-E1 (Tsukamoto et al., 1991) and NIH 3T3 cells showed to be positive for luciferase activity (data not shown). This indicates that the promoter constructs are active in a wide variety of PDGFαR expressing cells.

An important observation that confirms the promoter activity of the cloned 5' flanking region of the *PDGFαR* gene is the orientation dependence of the promoter

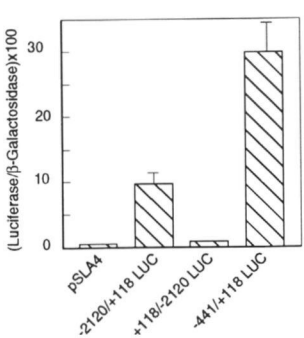

Figure 6.5. The promoter activity is orientation dependent.
The activity of the PDGFαR gene promoter was determined in both the 5'-3' (-2120/+118 LUC) and the 3'-5' (+118/-2120 LUC) orientation. Values are mean ± sample standard deviation of triplicate determinations.

constructs. When the -2120/+118 promoter fragment is cloned in the reverse orientation in front of the luciferase gene, the promoter activity is abolished, as shown in figure 6.5.

RA and cAMP strongly induce PDGFαR gene promoter activity

Mouse F9 embryonal carcinoma stem cells can be differentiated into endoderm-like cells by treatment with RA or RA in combination with Bt_2cAMP (Hogan et al., 1981). This differentiation is accompanied by an induction of PDGFαR expression, which is completely absent in undifferentiated F9 cells (Mercola et al., 1990; Wang et al., 1990). Enhanced transcription plays a prominent role in the RA/Bt_2cAMP-induced PDGFαR expression (Wang et al., 1990). We have recently shown that RA-induced differentiation of human embryonal carcinoma Tera-2 cells is also paralleled by an induction of *PDGFαR* gene expression (Mosselman et al., 1994). The data in figure 6.6 demonstrate that the above described effects can be explained by RA/Bt_2cAMP-induced promoter activity. Treatment of -825/+118 LUC transfected Tera-2 cells with RA increased the promoter activity 10-fold, and the combination RA/Bt_2cAMP enhanced promoter activity 22-fold over the unstimulated control levels. In contrast to the PDGFαR expression in mouse F9 cells, Bt_2cAMP alone has also a pronounced effect on the *PDGFαR* gene promoter activity. The RA or RA/Bt_2cAMP-induced *PDGFαR* gene promoter activity seems to be specific for embryonal carcinoma cells. When applied to transfected Swiss 3T3 cells, no effect of RA or Bt_2cAMP could be detected (data not shown).

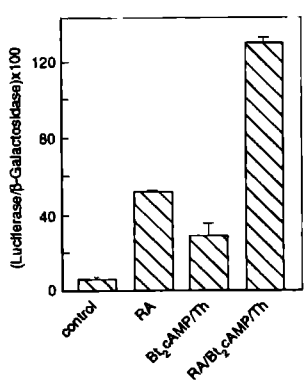

Figure 6.6. RA and Bt_2cAMP induce PDGFαR gene promoter activity

The -825/+118 LUC construct was transfected into undifferentiated Tera-2 cells (3 10^3 cells/cm2) The cells were treated for 48 hrs with the indicated compounds (control = no treatment), after which the cells were lysed and luciferase/ß-galactosidase activities of the lysate were determined The phosphodiesterase inhibitor theophylline was added in combination with Bt_2cAMP, as described as standard conditions for PDGFαR gene expression in mouse F9 cells (Wang et al , 1990) Values represent mean ± sample standard deviation of duplicate determinations Final concentrations 350 μM theophylline (Th), 5 μM RA, 100 μM Bt_2cAMP

DISCUSSION

The *PDGFαR* gene belongs to a family of tyrosine kinase receptor genes which also includes the *PDGFβR* gene, c-*fms* and c-*kit*. These four genes are pairwise localised on human chromosomes 4 and 5, and it has become evident that they must have originated from the same ancestral gene (Sherr, 1990). The current data on exon 1, and our previous data on exon 13 to 16 (Mosselman et al., 1994), suggest that the *PDGFαR* gene is structurally similar to the *PDGFβR* gene and c-*fms* (Sherr, 1990). All three genes are characterized by a non-coding exon 1, which is not present in c-*kit* (Giebel et al., 1992; Vandenbark et al., 1992). Although the size of the first intron of the *PDGFαR* gene has not been established, the lack of additional exons in exon 1 containing phage DNA indicates that the *PDGFαR* gene probably contains the typical large intron 1, like the other family member genes.

The 5' flanking region of the *PDGFαR* gene functions as a promoter, based on three criteria. **1.** When fused to the firefly luciferase reporter gene, the 5' flanking sequences are able to drive the expression of this luciferase gene. **2.** Luciferase reporter gene expression is not accomplished when the promoter is in the reverse orientation. **3.** RA and Bt_2cAMP greatly enhance the luciferase expression of the promoter-luciferase constructs. Although this promoter is able to drive luciferase expression in a variety of cell lines, it does not necessarily have to be the only promoter of the *PDGFαR* gene. The c-*fms* proto-gene has two seperate promoters that confer tissue specific transcription of the gene. One promoter is located upstream of the non-coding exon 1, and another is present just upstream of exon 2 (Roberts et al., 1992). Due to the non-coding nature of exon 1, transcription initiation from both promoters results in identical protein products. Since the *PDGFαR* gene and c-*fms* are closely related, this regulatory mechanism may also occur in the *PDGFαR* gene. Currently there are no indications for an additional *PDGFαR* gene promoter just upstream of exon 2. In all the cell lines tested so far, the 6.4 kb PDGFαR mRNA hybridizes with an exon 1 specific probe (unpublished observation). However, in addition to the 6.4 kb PDGFαR transcript, alternative transcripts are generated in undifferentiated human embryonal carcinoma cells (Mosselman et al., 1994). These transcripts initiate from a promoter within intron 12 of the *PDGFαR* gene. This promoter is also capable to drive luciferase reporter gene expression in undifferentiated cells (Kraft, H.J., Mosselman, S., Hohenstein, P., Overdijk, E.J. & Van Zoelen, E.J.J., manuscript in preparation).

The regulation in embryonal carcinoma cells by RA and Bt_2cAMP is a very important characteristic of the PDGFαR promoter. Although *PDGFαR* gene expression is affected by a number of growth regulating factors, including interleukin-1 (Tsukamoto et

al , 1991, Yeh et al , 1993a), PDGF BB (Eriksson et al , 1991), TGFß (Gronwald et al , 1989, Battegay et al , 1990, Ishikawa et al , 1990, Yamakage et al , 1992, Paulsson et al , 1993), bFGF (McKinnon et al , 1990, Schollmann et al , 1992), and RA (Mercola et al , 1990, Wang et al , 1990, Mosselman et al , 1994), only for RA it has been shown that it affects the transcription of the *PDGFαR* gene (Wang et al , 1990) The current study confirms this transcriptional effect of RA on the *PDGFαR* gene expression and the synergizing effect of Bt_2cAMP However, in contrast to the data of Wang et al (1990), Bt_2cAMP itself also generates a strong induction of promoter activity The exact reason for this discrepancy it not known, but it may reflect the difference in the cell system used

The molecular mechanisms by which RA and Bt_2cAMP increase the *PDGFαR* gene promoter activity are unknown The promoter sequence does not contain any putative RA receptor or cAMP responsive elements However, the activity of many transcription factors can be modulated by RA and Bt_2cAMP (Faisst and Meyer, 1992) In particular AP2, for which many putative binding sites are available in the human *PDGFαR* gene promoter, is of interest It has been shown that AP2 activity is induced upon RA differentiation of human embryonal carcinoma cells (Williams et al , 1988, Luscher et al , 1989) Another transcription factor that could mediate the RA inducibility of the *PDGFαR* gene promoter is GATA This factor is involved in the transactivation by RA of the J6 Serpin gene in mouse embryonal carcinoma cells, as recently described by Wang (1994) Current studies are underway to characterize the precise sequence elements involved in the RA- and Bt_2cAMP-induced promoter activity

Two other putative transcription factor binding sites attract attention The sequenced promoter region contains two PEA3 consensus sites High PEA3 expression, like high PDGFαR expression, has been correlated with the metastatic potential of tumour cells (Do et al , 1992, Fitzer-Attas, 1993, Trimble et al , 1993) More interesting however, is the E2F-like sequence element (Ouelette et al , 1992) located at nucleotide position -280/ 271 Deletion of the region -441 to -275, which partially includes this putative E2F site, results in a dramatic decrease of promoter activity In addition it has been hypothesised that the inhibitory effect of TGFß on the *c-myc* promoter is mediated through E2F (Moses, 1992), which makes this transcription factor a potential target for the TGFß regulated inhibition of *PDGFαR* gene expression (Gronwald et al , 1989, Battegay et al , 1990 Paulsson et al , 1993) Future experiments will have to establish the role of E2F in both basal and regulated transcription of the *PDGFαR* gene

In conclusion, the cloned 5' flanking region represents a functional promoter of the *PDGFαR* gene The promoter activity can be regulated by factors that play an important role in cell growth and differentiation Thus, the cloned PDGFαR promoter region may be a useful tool for studying the molecular mechanisms that are involved in *PDGFαR* gene

expression in development and in tumorigenesis

MATERIALS AND METHODS

Cell Culture

The human embryonal carcinoma cell line Tera 2 clone 13 was maintained in α-modification of minimal essential medium lacking nucleosides and deoxynucleosides, supplemented with 10% fetal calf serum (Gibco BRL) Mouse Swiss 3T3 cells were grown in Dulbecco's Modified Eagle Medium, supplemented with 10% newborn calf serum (Gibco BRL)

Genomic DNA cloning and sequencing

An amplified human genomic DNA library in phage λEMBL3 (kindly provided by Dr G Grosveld, Erasmus University, Rotterdam, The Netherlands) was screened with an EcoRI fragment containing nucleotide 1 255 of the human cDNA clone pSV7d15 1+5 (Claesson-Welsh et al , 1989) The EcoRI fragment was labeled using a random priming labeling kit (Amersham) and hybridization and washing was carried out as described previously (Mosselman et al , 1989) Using standard molecular cloning techniques (Sambrook et al , 1989), three out of thirty positively hybridizing plaques were analyzed in more detail Southern blot analysis of phage DNA with the EcoRI probe revealed that all three phages contained an identical region of the *PDGFαR* gene Phage DNA was subcloned into pBluescript SK (Stratagene) and DNA sequence analysis was performed using the Pharmacia T7 sequencing kit

Primer extension analysis

A 26-mer oligonucleotide complementary to the nucleotides 1 to 26 of the human PDGFαR cDNA (Claesson Welsh, 1989) was end-labeled with ^{32}P-ATP using T4 polynucleotide kinase (Boehringer) Total RNA (50 μg) from the differentiated human embryonal carcinoma cell lines Germa II (Walt and Hedinger, 1984), MA 90 (Hamers et al , 1991) or tRNA was ethanol-precipitated together with labeled oligonucleotide (5 10^5 cpm), and the precipitate was dissolved in 50 μl hybridization buffer (10 mM Tris pH 8 0, 1 mM EDTA and 500 mM NaCl) The samples were heated at 95 °C for 15 minutes, incubated at room temperature overnight and ethanol-precipitated Reverse transcription was performed in a 30 μl reaction (50 mM Tris pH 8 3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol) using 200 U Superscript (Gibco BRL) at 37 °C for 45 minutes and subsequently at 42 °C for an additional 45 minutes After treatment with 25 μg RNAse at 37 °C for 30 minutes, the samples were extracted with phenol-chloroform, precipitated with ethanol and analyzed on a 6% polyacrylamide ureum sequencing gel

Construction of promoter-luciferase plasmids

The 2120/+118, -825/+118, -52/+118 PDGFαR promoter fragments were amplified from the genomic subclone pPARP9 (figure 6 1), using the polymerase chain reaction (PCR) technique with primers introducing a unique NcoI restriction enzyme site within exon 1 (figure 6 2) PCR fragments were cloned into the T tailed pT7 Blue vector (Novagen) and subsequently cloned in front of the luciferase gene of the plasmid pSLA4 pSLA4 is a modification of the luciferase gene containing plasmid pSLA3 (Van Dijk et al , 1991) in which the BamHI-SalI part of the polylinker has been replaced by the BamHI SalI part of the pBluescript SK polylinker All other promoter fragments were isolated from either 2120/+118 or -825/+118 in pT7 Blue, using the appropriate restriction enzymes and were cloned in front of the pSLA4 luciferase gene Correctness of the promoter-luciferase constructs was confirmed by restriction enzyme analysis and partial sequencing

Transfections

Tera 2 cells were seeded in 0 1% gelatin coated 6-well tissue culture dishes, at a density of 5 10^3 cells/cm^2 The cells were treated with 5 μM retinoic acid (Sigma) for 6 days to induce differentiation before transfection Swiss 3T3 cells were seeded at a density of 3 10^4 cells/cm^2 in a 6 well tissue culture dish, one day prior to transfection Both Tera-2 and Swiss 3T3 cells were transfected using the calcium phosphate technique as described (Sambrook et al , 1989) Transfections were carried out using 5 μg (Tera-2) or 10 μg (Swiss 3T3) luciferase construct DNA, and a 4 hrs (Tera-2) or 24 hrs (Swiss 3T3) calcium phosphate-DNA incubation The cells were harvested 24 hrs (Tera-2) or 48 hrs (Swiss 3T3) post-transfection and luciferase activity was assayed in a LKB 1250 luminometer, using a luciferase assay kit (Promega) To assess for transfection efficiency, 1 μg (Tera-2) or 5 μg (Swiss 3T3) of the *lacZ* gene containing plasmid pCH110 (Pharmacia) was cotranfected with the luciferase construct DNA ß-Galactosidase activity was determined using a chemiluminescent assay as described (Jain and Magrath, 1991) All transfections were performed in several independent experiments, with at least two different batches of DNA

CHAPTER 7

An AP1-like element is involved in the retinoic acid-induced transcription of the human PDGFα receptor gene in embryonal carcinoma cells

ABSTRACT

Platelet-derived growth factor α receptor (PDGFαR) gene expression is strongly induced upon retinoic acid and cAMP treatment of embryonal carcinoma cells as a result of an increased transcription rate The molecular mechanisms behind this process are currently not known, but it apparently does not involve a direct binding of RA receptors to the *PDGFαR* promoter region The data from the present study demonstrate that an AP1-like regulatory element is involved in the RA- and cAMP-induced *PDGFαR* gene transcription in embryonal carcinoma cells In addition, this induction can also be accomplished by ectopic expression of c-*jun*, which suggests a role for this transcription factor in this process

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INTRODUCTION

Platelet-derived growth factor (PDGF) is a widely expressed growth factor and is thought to be involved in many biological processes, including embryonic development, atherosclerosis, wound-healing and tumorigenesis (Ross *et al.*, 1990; Bowen-Pope *et al.*, 1991; Raines *et al.*, 1991; Raines and Ross, 1993; Westermark and Heldin, 1993). Three isoforms of PDGF exist (AA, AB and BB), which bind with different affinities to two PDGF receptors, denoted α and ß. The α-receptor (PDGFαR) binds all three isoforms, whereas the ß-receptor (PDGFßR) displays only high affinity for PDGF-BB. The PDGF receptors dimerize upon ligand binding, which results in activation of their intracellular tyrosine kinase domain. The subsequent phosphorylation of tyrosine residues within the dimerized receptors themselves initiates a cascade of signaling events, finally resulting in a biological response (Claesson-Welsh, 1994a).

Expression of the PDGFαR, and thus responsiveness to all PDGF isoforms, is strictly regulated during embryonic development. Although present in all cells of the pre-implantation embryo from the two-cell stage onwards (Palmieri *et al.*, 1992), PDGFαR expression becomes limited to mesoderm- and certain neural crest-derived structures after implantation (Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992; Orr-Urtreger and Lonai, 1992; Schatteman *et al.*, 1992). However, loss of this tight regulation may have taken place in the process of tumorigenesis. Many tumors and tumor cell lines (in particular gliomas) express high levels of the PDGFαR together with its ligands, resulting in autocrine PDGF loops (Nistér *et al.*, 1991; Hermanson *et al.*, 1992; reviewed by Westermark, 1993).

An important mechanism in the regulation of PDGFαR expression is the transcriptional control of the *PDGFαR* gene. The best studied example in this is the RA-induced PDGFαR expression in embryonal carcinoma cells. Both human and mouse embryonal carcinoma stem cells do not display the 6.4 kb PDGFαR mRNA, but its expression is strongly induced upon RA treatment, as a result of increased transcription (Mercola *et al.*, 1990; Wang *et al.*, 1990; Mosselman *et al.*, 1994). We have recently cloned and characterized the promoter region of the human *PDGFαR* gene. Although RA strongly induced the promoter activity, sequence analysis revealed no putative binding site for RA receptors (Afink *et al.*, 1995). A similar observation has been made for the mouse *PDGFαR* gene promoter (Wang and Stiles, 1994). Thus, the RA-induced transcription of the *PDGFαR* gene does not seem to be mediated via direct binding of a RA receptor to the promoter region.

This chapter describes the possible mechanism responsible for the RA-induced transcription of the human *PDGFαR* gene in human embryonal carcinoma cells. Promoter

deletion studies and gel-retardation assays indicate the involvement of an AP1-like element in this process. However, the experimental data indicate that in embryonal carcinoma cells not the fos/jun dimer binds to this element. Another, as-yet-unidentified protein diplays high affinity binding for this AP1-like element and is a candidate transcription factor to mediate the RA-induced activation of *PDGFαR* gene transcription in embryonal carcinoma cells.

RESULTS

Mapping of RA and cAMP responsive elements

As we have recently shown, the human *PDGFαR* promoter activity is strongly induced by RA in embryonal carcinoma cells A similar effect can be obtained with the cAMP analogue dibutyryl cAMP (Bt_2cAMP), and together RA and Bt_2cAMP display a synergism in activation of the promoter (Afink *et al.*, 1995) Since the promoter region does not contain an obvious sequence with similarity to the known RA or cAMP responsive elements, a panel of progressive 5' deletion mutants of the promoter, cloned into a luciferase reporter gene vector (see materials and methods), was used to map the cis-element(s) involved in the RA- and cAMP-induced promoter activity. The promoter-

	Tera-2 EC		Tera-2 RA	
construct	RA	RACT	RA	RACT
-52/+118LUC	9.3 ± 0.1	16.5 ± 0.0	2.5 ± 0.2	10.7 ± 0.2
-175/+118LUC	7.9 ± 0.1	22.4 ± 1.0	3.1 ± 0.3	11.8 ± 0.3
-197/+118LUC	6.1 ± 0.7	25.8 ± 0.7	2.6 ± 0.0	13.6 ±1.7
-275/+118LUC	9.3 ± 0.4	16.3 ± 0.9	2.2 ± 0.4	18.6 ± 0.6
-441/+118LUC	9.2 ± 0.7	21.9 ± 1.8	3.8 ± 0.3	8.8 ± 0.2
-825/+118LUC	6.2 ± 0.0	18.8 ± 2.1	3.2 ± 0.1	11.9 ± 1.0

table 7.1: RA- and cAMP-induced activity of *PDGFαR* promoter deletion mutants.
A panel of 5' promoter-LUC deletion mutants was transfected into Tera-2 EC or Tera-2 RA cells The cells were subsequently treated with 10 μM RA or a combination of 10 μM RA, 1 mM Bt_2cAMP and 350 μM theophylline (RACT) for 48 hrs After this incubation period, luciferase activity in the cells was determined and corrected for transfection efficiency (see materials and methods) The values represent the fold induction of luciferase activity after treatment, relative to the luciferase activity of unstimulated cells and the sample standard deviation (duplicate determinations)

luciferase constructs were transfected into undifferentiated Tera-2 (Tera-2 EC) cells, which are derived from a spontaneous human testis tumor. The cells were subsequently treated with either RA or a combination of RA, Bt_2cAMP and theophylline (RACT). The resulting fold induction of promoter activity (determined as luciferase activity) of the different promoter mutants is shown in table 7.1.

All the promoter deletion mutants transfected into Tera-2 EC cells display an approximately 9-fold and 20-fold induction in response to treatment with RA and RACT respectively. Although not tested in detail, Bt_2cAMP alone also induces the complete panel of promoter deletion mutants to a similar extent as RA (data not shown). This implies that both RA and Bt_2cAMP mediate their effect through elements within the -52/+118 region of the human *PDGFαR* gene. In addition, the same results were obtained when the transfections were performed in RA-differentiated Tera-2 (Tera-2 RA) cells. Since these cells already display a high basal *PDGFαR* promoter activity (Afink *et al.*, 1995), RA and RACT enhance, rather than induce, the promoter activity.

To characterize the transcription factors that bind to this promoter region, gel-retardation assays were performed using the -52/+118 promoter fragment as a probe. As shown in figure 7.1, treatment of Tera-2 EC cells with RA or RACT results in a complex, but typical change in protein binding to the -52/+118 promoter fragment. The RACT-treated cells display a similar, although more pronounced change in protein binding than the cells treated with RA alone. This difference in potency of effect between RA and RACT is also apparent on promoter activity (Afink *et al.*, 1995 and table 1). Thus, the gel-retardation data correlate well with the promoter activity and suggest that RA and cAMP use the same transcription factors to stimulate *PDGFαR* gene expression.

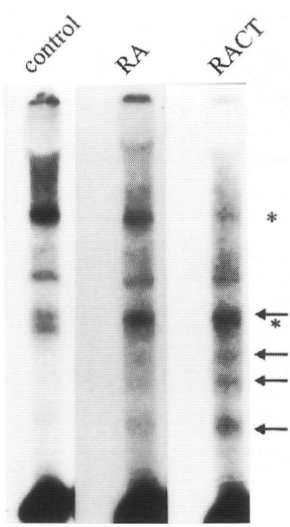

figure 7.1: Gel retardation analysis of nuclear extracts from untreated, RA- and RACT-treated Tera-2 cells.
A ^{32}-P-labelled *PDGFαR* promoter fragment, encompassing nucleotide -52 to +118 was incubated with nuclear extracts from Tera-2 EC cells treated 24 hrs with the indicated compounds (see table 1 for concentrations). The resulting protein-DNA complexes were analyzed by polyacrylamide electrophoresis and visualized on X-ray film. Six specific protein-DNA complexes were observed. Four of these complexes are induced upon RA or RACT treatment (arrows), whereas two protein-DNA complexes decrease in intensity after treament of Tera-2 EC cells with RA or RACT (asterisks).

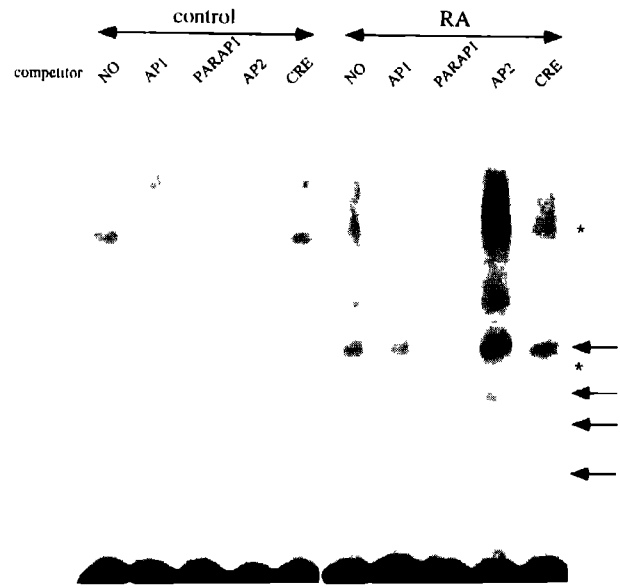

figure 7.2: A PARAP1 oligonucleotide competes with the -52/+118 promoter fragment for specific protein binding.
A similar gel retardation experiment as described in figure 7.1 was performed with the -52/+118 promoter fragment as a probe and nuclear extracts from either untreated (CONTROL) or 24 hrs RA-treated Tera-2 EC cells. In addition, an approximately 500-fold excess of unlabelled competitor DNA (as indicated) was added to the reaction mixtures. The same specific protein-DNA complexes as in figure 7.1 are marked by arrows and asterisks.

RA induces changes in protein binding to an AP1-like element

As described previously, the *PDGFαR* gene promoter contains many putative binding sites for the RA-inducible transcription factor AP2 (Afink *et al.*, 1995) and one of these AP2 sites ($^{+97}$*GCCGTGGG*$^{+104}$) is located within the -52/+118 region. In addition, the sequence $^{+6}$*TGAATCA*$^{+12}$ (denoted as PARAP1) bears a strong homology with the consensus binding site for the transcription factor complex AP1 (*TGASTCA*; Angel *et al.*, 1987; Lee *et al.*, 1987). The involvement of these sequences in the protein binding to the -52/+118 region has been further investigated and the results are shown in figure 7.2. Competition with the unlabelled PARAP1 oligonucleotide results in a complete loss of five of the six specific protein-DNA complexes bound to the -52/+118 probe. Interestingly, a consensus AP1 oligonucleotide has no effect on any protein binding to the -52/+118 fragment. The AP2 oligonucleotide and an oligonucleotide containing a cAMP responsive element (Borrelli *et al.*, 1992) also do not display any competition. Thus, the PARAP1 sequence appears to be the target element involved in most of the RA-induced changes in

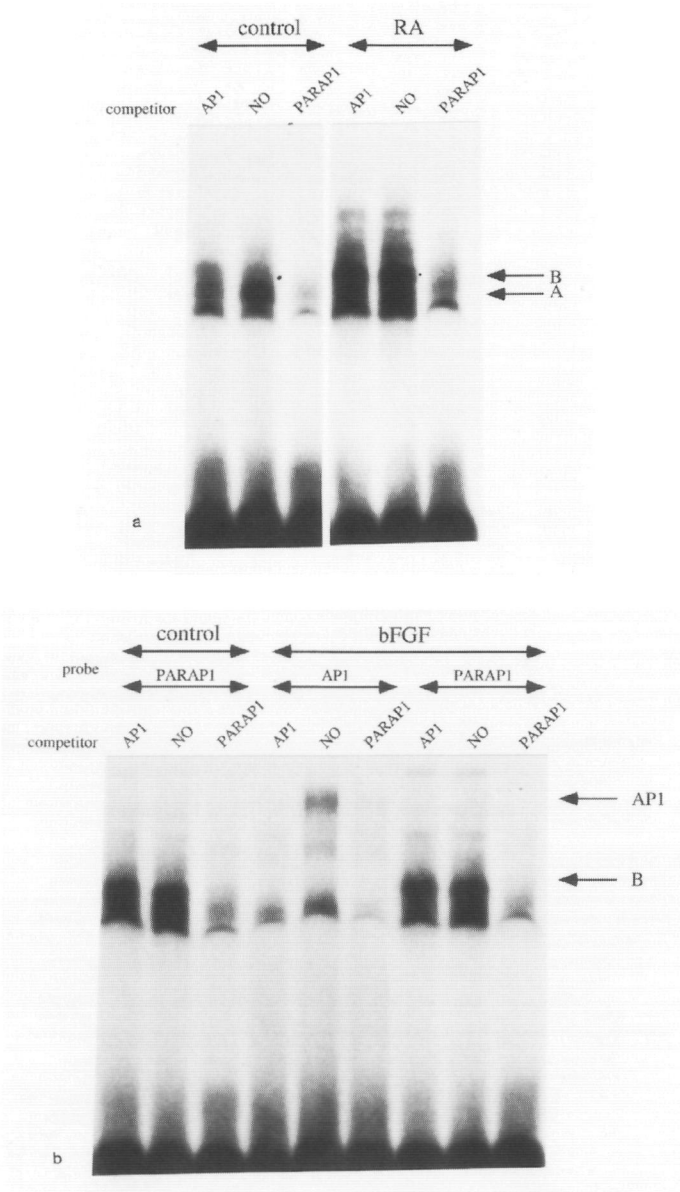

figure 7.3: Effect of RA on protein binding to the PARAP1 element.
Gel retardation analysis was performed with the PARAP1 oligonucleotide as a probe. Nuclear extracts in **A)** were derived from untreated (CONTROL) or 24 hrs RA-treated Tera-2 EC cells and in **B)** were derived from untreated Tera-2 RA cells (CONTROL) or 24 hrs bFGF-treated Tera-2 RA cells. In addition, the consensus AP1 oligonucleotide was used as a probe, and approximately 300-fold excess of unlabelled competitor DNA was added to the reaction mixture where indicated. At least two specific protein complexes could be separated, indicated as A and B.

protein binding to the -52/+118 region

From these competition data it was also concluded that although homologous in sequence, the PARAP1 seems to have different binding characteristics than the consensus AP1 sequence This notion is further supported by the data from gel retardation experiments with the PARAP1 oligonucleotide as probe As shown in figure 7 3A, the PARAP1 oligonucleotide binds a protein (complex A) in Tera-2 EC cells, which can not be competed by an excess of unlabelled non-specific (not shown) or consensus AP1 DNA Upon 24 hrs RA treatment, at least one additional specific protein complex (complex B) can be identified, which again shows no obvious affinity for the consensus AP1 sequence, as determined by competition analysis At the same time, RA treatment reduces complex A Neither of these two PARAP1 binding proteins resembles AP1, which has a much slower mobility than complex A and B (data not shown, and compare with figure 7 3B)

Tera-2 RA cells (treated for 6 days with RA), which have high level *PDGFαR* gene expression and promoter activity, display the same protein-DNA complexes as the 24 hrs RA-treated Tera-2 EC cells (figure 7 3B) Treatment of these Tera-2 RA cells with bFGF, which results in an additional 3-5 fold increase in promoter activity (unpublished observation), induces the binding of AP1 protein to the consensus AP1 oligonucleotide Although PARAP1 still prefers to form the complex B under these conditions, excess of unlabelled PARAP1 is very well able to compete with the consensus AP1 probe for AP1 protein binding

Together these data indicate that the PARAP1 is an AP1-like element, but within the context of the *PDGFαR* promoter it does not bind the fos/jun dimer Instead, some other, as-yet-unidentified proteins bind to this regulatory element

Expression of c-jun induces the activity of the PDGFαR gene promoter

The AP1 protein is apparently not involved in binding to the PARAP1 element, but it is very well conceivable that an AP1-like protein is involved As shown in figure 7 4, co-transfection with a c-*jun* expression vector indeed results in a dramatic increase of the *PDGFαR* promoter activity This result could not be obtained with either c-fos or fra1 expression vectors A similar result was observed in Tera 2 RA cells (data not shown) In addition, the cAMP-inducible transcriptional activators CREB and CRFMτ, which also bind to sequences closely related to the PARAP1/AP1 sequence (Borrelli *et al* , 1992), do not increase the activity of the *PDGFαR* promoter From these data it appears that c-jun could be a limiting component of the proteins binding to the PARAP1 element and thus be involved in the activation of *PDGFαR* gene transcription

A mutation of the *T* nucleotide in the 3' half of the AP1 consensus sequence results in a dramatic loss of fos/jun binding (Risse *et al* , 1989) An identical mutation in the

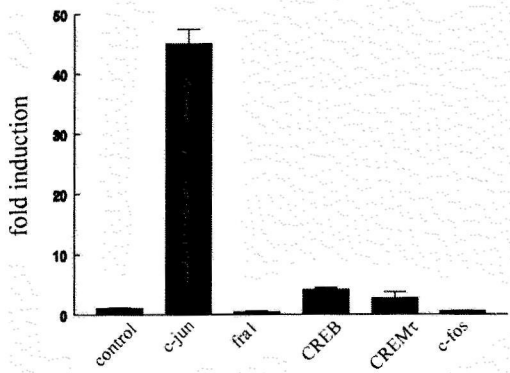

figure 7.4: c-jun activates the *PDGFαR* promoter.The -441/+118LUC promoter construct was transfected into Tera-2 EC cells together with an expression vector containing c-jun, c-fos, fra1, CREB or CREMτ. 48 hrs post-transfection, luciferase levels within the cells were determined and corrected for transfection efficiency. Values represent the fold induction of promoter activity, relative to the activity of -441/+118LUC cotransfected with the empty expression vector (pSG5, Stratagene). Error bars indicate sample standard deviation of duplicate determinations.

PARAP1 sequence (denoted as mPARAP1) also results in a strongly decreased affinity for the AP1 protein, as determined in A431 cells (figure 7.5A), but it does not compete for the proteins bound to the PARAP1 element (data not shown). However, when this mutation was introduced into a promoter-luciferase construct, no decrease of the RA-, RACT- (figure 7.5B) or cAMP- (data not shown) induced promoter activity could be observed. Thus, when c-jun is indeed a component of the proteins binding to the PARAP1, it displays different binding requirements than as being a component of AP1.

DISCUSSION

Transcription of the *PDGFαR* gene in embryonal carcinoma cells is strongly induced upon differentiation of the cells with RA or RACT (Wang *et al.*, 1990; Mosselman *et al.*, 1994). The mechanism by which RA and cAMP induce this *PDGFαR* gene transcription in these cells is not known, but it apparently does not involve direct binding of RA receptors to the *PDGFαR* promoter region (Wang and Stiles, 1994; Afink *et al.*,1995). The experiments in this study were designed to elucidate the molecular mechanisms involved in the RA- and cAMP-stimulated transcription of the *PDGFαR* gene in embryonal carcinoma cells. Deletion mapping studies showed that both the induction and enhancement of *PDGFαR* promoter activity by RA and cAMP are mediated through element(s) located within the -52/+118 region of the gene. A more detailed analysis indicated the involvement of an AP1-like element (denoted as PARAP1) in this process. In addition, the RA- and cAMP-stimulated promoter activity can be mimicked by expression of c-*jun*.

RA and cAMP affect *PDGFαR* promoter activity in both Tera-2 EC and RA-differentiated Tera-2 RA cells, and in both cell types they mediate their effects through

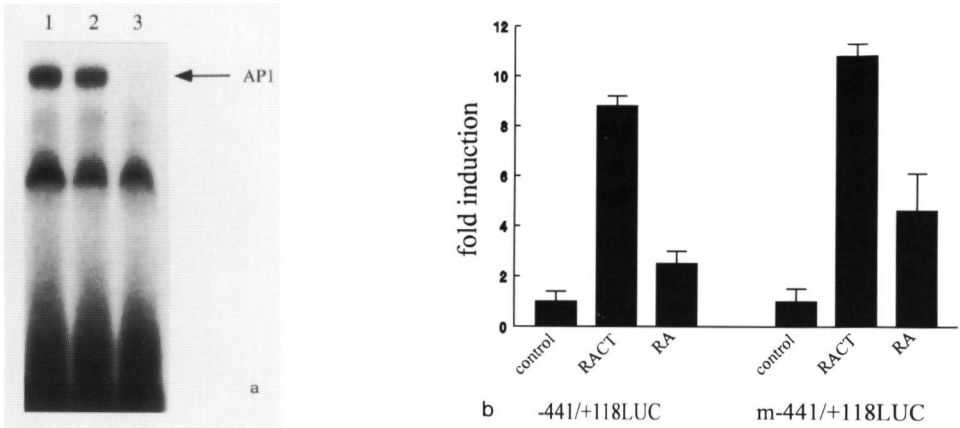

figure 7.5: Mutation analysis of the PARAP1 element.
A) Gel retardation analysis with the consensus AP1 oligonucleotide as a probe and nuclear extracts from TPA-treated A431 cells (lane 1). An 300-fold competition with either unlabelled mPARAP1 (lane 2) or PARAP1 (lane 3) DNA was performed where indicated.
B) The -441/+118LUC and m-441/+118LUC were transfected seperately into Tera-2 EC cells. The cells were subsequently treated for 48 hrs with either RA or RACT, after which the luciferase levels within the cells was determined and corrected for transfection efficiency. The values represent the fold induction of promoter activity, relative to the unstimulated situation for either construct. Error bars indicate sample standard deviation of duplicate determinations.

the same -52/+118 region. This indicates that the RA- and cAMP-induced *PDGFαR* gene transcription in Tera-2 EC cells is not necessarily coupled to differentiation of the cells. This notion is further supported by our previous observation that elements outside the -52/+118 region are involved in the establishment of high basal promoter activity in *PDGFαR* gene expressing cells (Afink *et al.*, 1995).

The target regulatory element for the RA- and cAMP-induced transcriptional activation of the *PDGFαR* gene seems to be the PARAP1 sequence. This conclusion is based on the observation that a PARAP1 oligonucleotide competes five of the six typical protein-DNA complexes identified with the -52/+118 promoter fragment in gel retardation assays (figure 7.2). However, subsequent experiments showed only the appearance of two protein-DNA complexes with the PARAP1 oligonucleotide as a probe. Therefore, additional mechanisms other than exclusive protein binding to the PARAP1 element must occur. One interesting option is that PARAP1 facilitates the binding of several proteins to other parts of the -52/+118 region. This would explain the difference in the number of complexes bound to, and competed by the PARAP1 oligonucleotide. However, additional experiments have to be performed to support this hypothesis. In addition, not all the

typical protein binding to the -52/+118 region can be competed with PARAP1 DNA. Thus the involvement of additional regulatory elements can not be excluded.

Together with its surrounding nucleotides, this PARAP1 forms an 11 nucleotides palindromic element. Although capable of binding AP1, this PARAP1 element diplays a higher affinity for other proteins, and the important question remains, what proteins bind to the PARAP1 site and thus probably mediate the RA- and cAMP-induced transcription of the *PDGFαR* gene? The product of the c-*jun* gene is a good candidate to be involved in this process, as concluded from several observations. Upon RA-induced differentiation of F9 and P19 murine embryonal carcinoma cells, c-*jun* gene expression is strongly increased (De Groot *et al.*, 1990a; Yang-Yen *et al.*, 1990) as a result of an enhanced c-*jun* transcription (Yang-Yen *et al.*, 1990; De Groot *et al.*, 1991; Kitabayashi *et al.*, 1992), and ectopic expression of c-*jun* in these cells leads to differentiation independently of RA (De Groot *et al.*, 1990; Yamaguchi-Iwai *et al.*, 1990). Furthermore, c-jun can also be activated by the cAMP pathway (de Groot and Sassone-Corsi, 1992) and displays affinity for several AP1-like sequences, which is dependent on its partner proteins (Sassone-Corsi *et al.*, 1990; Hai and Curran, 1991; Chatton *et al.*, 1994; De Cesare *et al.*, 1995). Together with the observation that c-*jun* expression activates the *PDGFαR* promoter, these data strongly argue for a role for c-*jun* in the RA- and cAMP-induced *PDGFαR* gene transcription. However, it may also be possible that other, most likely c-jun-related transcription factors are involved in the activation of the *PDGFαR* promoter. At least in Tera-2 RA cells, both junB and junD can also stimulate *PDGFαR* promoter activity (unpublished observations). Current studies are underway to investigate the role of c-jun and possible other, c-jun-related transcription factors in this process.

Together, the current data demonstrate the involvement of an AP1-like regulatory element within the *PDGFαR* gene promoter in the RA- and cAMP-induced expression of the *PDGFαR* gene in embryonal carcinoma cells. However, not the fos/jun dimer, but more likely a complex of jun-related proteins binds to this regulatory element and mediates the RA and cAMP effects.

MATERIALS AND METHODS

Vectors

The construction of the promoter-luciferase reporter gene vectors -2120/+118LUC, -441/+118LUC, -275/+118LUC, -197/+118LUC, -175/+118LUC and -52/+118LUC has been described previously (Afink *et al.*, 1995). The promoter-luciferase construct M-441/+118LUC, which contains a *T* to *G* mutation at position +10, was generated with the Altered Sites Mutagenesis system (Promega). The eukaryotic expression vectors for c-fos, c-jun, CREB, CREMτ and fra-1 were kindly

provided by Dr R de Groot, University of Utrecht, The Netherlands

Cell culture and transfections

The human embryonal carcinoma cell line Tera-2 clone 13 (Thompson *et al* , 1984) was maintained in α-modification of minimal essential medium lacking nucleosides and deoxynucleosides, supplemented with 10% fetal calf serum (Gibco BRL) One day prior to transfections, 25 10^3 cells/cm^2 were seeded in a 0 1% gelatin-coated 6-well tissue culture dish Transfections using the calcium-phosphate technique (Sambrook *et al* , 1989) were carried out with 5 μg/well promoter-luciferase vector and 5 μg/well of expression vector (where necessary) In addition, 2 μg/well of the *lacZ*-expressing vector pCH110 (Pharmacia) was co-transfected to correct for differences in transfection efficiency After the appropriate incubation times, cells were lysed in reporter lysis buffer (Promega) Luciferase activity in the lysate was determined by a luciferase assay kit (Promega), according to the manufacturer's protocol ß-Galactosidase was assayed as described by Jain and Magrath (1991) using Galacton (Tropix) as the substrate

Gel-retardation assays

Nuclear extracts from Tera-2 cells were prepared as described by Schreiber *et al* (1989) These extracts (10-15 μg) were incubated with 30 10^3 cpm ^{32}P-labelled DNA, 2 μg dI-dC, and where necessary unlabelled competitor DNA, in a reaction buffer (20 mM Hepes, pH 7 9, 50 mM NaCl, 1 mM EDTA, 0 15 mM EGTA, 1 mM DTT, 0 15 mM PMSF, 4% (w/v) Ficoll and 8 mM KCl) at room temperature for 10 minutes The resulting protein-DNA complexes were separated on a 4% polyacrylamide gel using 0 5xTBE as running buffer (Sambrook *et al* , 1989), and visualized on X-ray film (Kodak X-Omat, Fuji RX)

Oligonucleotides

The synthetic oligonucleotides PARAP1 (5'-*CTATAACATTGAATCAATTACAA*-3'), mPARAP1 (5'-*CTATAACATTGAAGCAATTACAA*-3'), consensus CRE (5'-*AGAGATTGCCTGACGTCAGAGAGCTAG*-3') and consensus AP1 (5'-*CGCTTGATGACTCAGCCGGAA*-3') were obtained as single-stranded oligonucleotides in both the sense and anti-sense conformation Equal amounts of the sense and anti-sense oligonucleotide were mixed in distilled water, heated to boiling temperature and slowly cooled to room temperature The resulting double stranded product was subsequently purified by polyacrylamide gel electroforesis if necessary (Sambrook *et al* , 1989) The double-stranded consensus AP2 (5'-*GATCGAACTGACCGCCCGCGGCCCGT*-3') oligonucleotide was obtained from Promega

CHAPTER 8

Specific expression in mesoderm- and neural crest-derived tissues of a human PDGFα receptor promoter/*lacZ* gene in transgenic mice

ABSTRACT

Platelet-derived growth factor α receptor (PDGFαR) has previously been shown to be required for normal development of mesoderm and cephalic neural crest derivatives. The purpose of the present study was to demonstrate the *in vivo* promoter function of 2.2 kb and 0.9 kb genomic DNA fragments representing the 5'-flanking part of the human *PDGFαR* gene. The fragments, ligated to a *lacZ* reporter gene, were microinjected into fertilized mouse eggs. Several transgenic mouse lines were established and nearly half of those which had incorporated the construct, expressed the *LacZ* gene. With the exception of the central nervous system, the expression patterns were basically similar in these lines and overlapped grossly the *PDGFαR* gene expression pattern. The results demonstrate that 2.2 kb and most likely even 0.9 kb upstream regulatory sequences are sufficient to drive the activity of the *PDGFαR* gene properly in most tissues *in vivo*. The transgenic line with the highest expression level was shown to express *LacZ* in paraxial mesoderm and branchial arches from embryonic day 8.5. The gene continued to be expressed during the remaining embryonic period, when *LacZ* expression was mainly confined to tissues of mesodermal and neural crest origin. These data are consistent with previously reported PDGFαR mRNA expression patterns. The gene was also expressed however, in specific areas of the developing neural tube. In addition, *lacZ* expression in brain was seen after birth and was strong in cerebellum and in some restricted areas of cerebral cortex, thalamus, hypothalamus and hippocampus. The location and morphology of the *lacZ*-expressing cells in brain and spinal cord indicate that they are mostly neuronal, while PDGFαR mRNA in postnatal central nervous system has been described as confined mainly to glial derivatives. In conclusion, the isolated human *PDGFαR* promoter contains most of the necessary regulatory elements to establish tissue specific reporter gene expression in transgenic mice. The resulting *lacZ* expression pattern during development points to an important role of the PDGFαR in the formation of skeletal, central and peripheral nervous system, lung and facial structures.

with: Xiao-Qun Zhang, Kristian Svensson, Erik Bongcam-Rudloff, Inga Hansson, Karin Forsberg-Nilsson, Everardus J.J. van Zoelen, Bengt Westermark and Monica Nistér

INTRODUCTION

Platelet-derived growth factor (PDGF) is produced by a wide variety of both normal and tumor cells (reviewed by Raines *et al.*, 1991; Westermark, 1993). The PDGF molecule is a dimer composed of two different, but highly homologous PDGF chains, denoted A and B. The A and B chains can either form homo- or hetero-dimers by disulfide bonds, which results in three PDGF isoforms: AA, AB and BB. Although PDGF is the best known for its mitogenic effect on mesenchymal cells, it is able to elicit many different effects ranging from vasoconstriction to chemotaxis (see Ross *et al.*, 1990; Raines *et al.*, 1991). As a result of this widely distributed expression and pleiotropy of effects, PDGF has been postulated to play an important role in many different biological processes, including embryogenesis, wound-healing, atherosclerosis and neoplasia (Ross *et al.*, 1986; Bowen-Pope *et al.*, 1991; Raines *et al.*, 1991; Raines and Ross, 1993).

Two different PDGF receptors have been characterized, denoted α (PDGFαR) and ß (PDGFßR), which belong to the superfamily of tyrosine kinase receptors. Like their ligands, the PDGF receptors are highly homologous molecules, and they form homo- and hetero-dimers upon ligand binding. The PDGFαR displays high affinity for all three PDGF isoforms, whereas the PDGFßR binds only the BB-isoform with high affinity. As a result, $\alpha\alpha$, αß and ßß receptor dimers will be formed depending on the PDGF isoform (Claesson-Welsh, 1994a).

Although it is difficult to discriminate between the contribution of either receptor subunit in the different biological responses to PDGF, several studies have indicated a specific role for PDGF-A/PDGFαR in embryonic development. Most of this information is derived from expression studies in mouse and Xenopus embryos. In the mouse pre-implantation embryo, both PDGF-A and the PDGFαR are co-expressed in all cells from the two-cell stage onwards (Rappolee *et al.*, 1988; Palmieri *et al.*, 1992). Following implantation however, the expression patterns of PDGF-A and the PDGFαR become separated. Expression of PDGF-A becomes limited to the ectoderm, whereas PDGFαR expression becomes restricted to the mesoderm (Orr-Urtreger and Lonai, 1992; Palmieri *et al.*, 1992). A similar observation has been made in Xenopus embryos, where mesoderm induction establishes this lineage-specific pattern of ligand and receptor gene expression (Jones *et al.*, 1993). Expression of PDGF-B and the PDGFßR have not been detected in pre-implantation embryos. However, the ß-receptor subunit is expressed shortly after implantation, but to a lesser extent than the PDGFαR (Mercola *et al.*, 1990). During later development PDGFαR expression remains characteristic for many mesoderm-derived structures, including mesenchyme and connective tissue, intervertebral discs and dermis (Orr-Urtreger *et al.*, 1992; Orr-Urtreger and Lonai, 1992; Schatteman *et al.*, 1992). In

addition, the PDGFαR is expressed in a non-neural subset of neural crest-derived cells (Morrison-Graham *et al.*, 1992; Ho *et al.*, 1994). The functionality of this PDGFαR expression in the formation of these tissues is clearly demonstrated in *Patch* mice, which carry a naturally occuring deletion of the *PDGFαR* gene. Mice homozygous for this mutation display severe developmental defects in mesoderm- and neural crest-derived structures, eventually resulting in the death of the embryo (Orr-Urtreger *et al.*, 1992; Morrison-Graham *et al.*, 1992; Schatteman *et al.*, 1992).

The mechanisms that control this tissue specific expression of the PDGFαR are currently unknown. Several growth modulating factors, which are also expressed in the developing embryo, transmodulate PDGFαR expression *in vitro*. This includes transforming growth factor ß (Gronwald *et al.*, 1989; Battegay *et al.*, 1990; Yamakage *et al.*, 1992; Paulsson *et al.*, 1993; Yeh *et al.*, 1993a), fibroblast growth factor (McKinnon *et al.*, 1990; Tsukamoto *et al.*, 1991; Schöllmann *et al.*, 1992) and retinoic acid (Mercola *et al.*, 1990; Wang *et al.*, 1990; Mosselman *et al.*, 1994; Tsukamoto *et al.*, 1994). With respect to embryonic development, the retinoic acid-induced expression of the *PDGFαR* gene in embryonal carcinoma cells is of particular interest (Mercola *et al.*, 1990; Wang *et al.*, 1990; Mosselman *et al.*, 1994). These embryonal carcinoma stem cells resemble in many aspects the pluripotent inner mass cells of the early embryo. Thus, the regulatory mechanisms that control the *PDGFαR* gene expression in these cells may also occur in the developing embryo.

We have recently cloned and characterized the promoter region of the human *PDGFαR* gene and shown that retinoic acid strongly induces the promoter activity in human embryonal carcinoma cells (Afink *et al.*, 1995). A similar observation has been made for the mouse *PDGFαR* promoter (Wang and Stiles, 1994). Although these studies have established a role for the *PDGFαR* promoter in some regulatory aspects of PDGFαR expression *in vitro*, it is still unclear whether it contributes to the *in vivo* regulation.

In this paper we assess the functionality of the isolated human *PDGFαR* promoter *in vivo*, using a transgenic mouse model. A 2.2 kb human *PDGFαR* promoter fragment cloned in front of a *lacZ* reporter gene drives the tissue specific ß-galactosidase expression in the developing mouse. Virtually similar results can be obtained with a shorter 0.9 kb promoter fragment. These results indicate that approximately 0.9 kb upstream regulatory sequences of the human *PDGFαR* gene contain most of the cis-acting elements necessary to establish tissue specific expression of the PDGFαR.

RESULTS

Generation of transgenic mouse lines

A total of ten founders of 2.2 kb and 0.9 kb PDGFαR-*lacZ* transgenic mouse lines were established (see materials and methods). The expression of *lacZ* was investigated in brains at 5-10 days postnatal stage (P), and in embryonic day (E) 9 to E15 embryos using X-gal staining of whole mount tissues or embryos (table 1). The expression patterns differ somewhat from line to line but are mainly overlapping irrespective which of the two different constructs was used. Two 2.2 kb lines and three 0.9 kb lines showed similar expression patterns in mesoderm-derived and facial structures of the developing embryo, i.e. expression in sclerotome, limb buds and branchial arches. Only two lines, however, the 2.2-07 and 0.9-04 expressed *lacZ* in postnatal cerebellum, and the 2.2-0.7 line also in specific structures of the cerebrum. The levels of expression varied considerably between lines, and the strongest expression was found in the 2.2-07 line which therefore was chosen for a more detailed analysis.

transgenic line	*LacZ* brain	*LacZ* embryo
2.2-03	-	
2.2-07	+*	+**
2.2-11	-	-
2.2-12	-	-
2.2-14	-	+**
2.2-19	-	
0.9-02	-	
0.9-04	+*	+
0.9-10	+	+
0.9-14	+	

table 1: Transgenic lines generated for both promoter constructs
Ten independent transgenic founder lines were established and *lacZ* gene expression in brain of P5 to P10 mice and/or in whole mount E9 to E15 embryos was determined (+ expression, -: no expression) The different transgenic lines are identified by promoter construct used (either 2.2 or 0 9) and a number.

* *LacZ* expression in cerebellum

** *LacZ* expression in somites and branchial arches

Expression of LacZ in the developing 2.2-07 transgenic mouse

The *lacZ* expression was examined between E7.5 and E15.5 by whole mount X-gal staining. The stained E7.5, E9.5, E12.5 and E15.5 embryos were also paraffin embedded and sectioned. Sagittal or/and transverse sections were analysed. There were no *lacZ* positive cells at E7.5, neither in embryonic nor in extraembryonic parts. During mouse

development, the first somite arises in the cervical region at E8 after which pairs of new somites appear in craniocaudal direction. The *lacZ* expression was found to start in somites and the first branchial arch of E8.5 embryos, and subsequently followed the somite formation pattern, starting from the cervical region and spreading craniocaudally. In sagittal and transverse sections of E9.5 embryo, strong *lacZ* expression was thus detected in developing somites, first, second and third branchial arches (figure 8.1A, C). Around E12.5, the somite-derived segmentally arranged sclerotome, dermatome and myotome appear. *LacZ* expression in this stage was mainly seen in the sclerotome and in the mesenchyme of the developing dermis (figure 8.1B, D, E).

Whole mount X-gal staining also revealed *lacZ* activity in developing limb buds (figure 8.1B), and was found in the developing chondrocytes of the primordia of vertebral bodies, ribs, and skeleton of limbs. Expression was especially strong in the condensed perichondrial mesenchyme during formation of these structures at E12.5 and E15.5 (figure 8.1B, E, figure 2A, B). There was no expression in the ossified areas, however. Figure 8.2C and D shows the *lacZ* expression in the cartilagineous skeleton of a P8 transgenic mouse. *LacZ* was expressed in the cartilaginous epiphyseal areas of the long bones, carpal and metacarpal bones, but was absent from the diaphyseal regions that had already ossified. Likewise, expression was absent in the ossified centers of the vertebrae but present in the cartilaginous laminae. The centers of the intervertebral discs, which are supposed to develop from the notochord, were negative. The cartilage of ribs and sternum continuously expressed *lacZ* during adulthood.

In mouse development, the mesenchymal tissue of the first and second branchial arches are of neural crest origin and later differentiate into facial and neck muscles and cartilages. *LacZ* was expressed in mesenchymal tissue of the first branchial arch at E8.5 and in the maxillary and the mandibular processes, with Meckel's cartilage, at E12.5 (figure 8.1A, C). Strong expression was found in well demarcated areas of the mesenchymal tissue of the developing upper and lower lips (figure 8.3A), eyelids and nostrils (not shown) at E15.5. The mesenchymal tissue of the future dermal hair papilla (figure 8.3C) also stained blue during the formation of the hair follicle, as seen at E15.5. The epithelial structures of the otic vesicle, and the lateral and posterior semicircular canal, had strong *lacZ* expression at E9.5 and E12.5 respectively (figure 8.3B, figure 8.4A). Although previous *in situ* studies have shown PDGFαR expressed in the retina, lens and optic nerve (Schatteman, *et al.* 1992, Mudhar, *et al.* 1993), we did not find *lacZ* expressed in the eye, nor in the optic nerves at E12.5, E15.5 and postnatal stages of the 2.2-07 line (not shown).

Mesenchymal tissue of lung, gut, kidney, pancreatic primordium and urogenital sinus expressed *lacZ* during organogenesis stage of E9.5 to E15.5 as exemplified in the

lung (figure 8.3D, figure 8.4B). Mesenchymal tissue of trachea and lung buds had detectable *lacZ* expression at E9.5 and E12.5. At E15.5, an abundance of *lacZ* positive cells were found in the interstitial mesenchymal tissue surrounding epithelium of the bronchioli (figure 8.3D, E). *LacZ* expression in lung mesenchymal tissue declined after one postnatal week and expression had disappeared around P20 (data not shown). Tracheal cartilage expressed *lacZ* for a longer period than lung mesenchymal tissue and the blue staining appeared in whole mounts of this tissue during more than 200 days postnatally. Gut and kidney have endogeneous ß-galactosidase, therefore it is difficult to evaluate the *lacZ* expression in these tissues in the postnatal period. Urinary bladder mesenchyme had strong *lacZ* expression from embryonic stage until adulthood in the 2.2-07 line but not in non-transgenic mice (data not shown). Uterus, ovary and testis did not show *lacZ* expression in postnatal stage.

LacZ expression in the central nervous system (CNS)

At E12.5 *lacZ* expression was found in areas of the developing neural tube (figure 8.4A, B, C, D, figure 8.1B, D), i.e. the neuroepithelial cells at the dorsal aspect of the central canal, as well as in small well demarcated ventro-lateral regions of the spinal cord, in the roof of the third ventricle, in the epithalamus, hypothalamus, and rhombencephalon. Strong expression was seen in the rhombic lip of the fourth ventricle which is the future cerebellum. The thin roof of the fourth ventricle, which develops the choroid plexus, however, was not stained with blue color, neither was staining found in the choroid plexus of the fourth ventricle at embryonic and postnatal stages. The *lacZ* positive cells in the spinal cord were large and closely packed in small groups which array along the longitudinal axis in a dorsal as well as a ventro-lateral portion of the spinal cord at E12.5. The location and morphology of these cells indicate that they are neuronal cells. *LacZ* expression in the posterior aspect of the spinal cord was transient and disappeared at E15.5. *LacZ* stained cells were found at the ventral horn of the spinal cord, but were no longer detectable in the lateral portion at E15.5 and postnatally. Facial ganglion cells, which are of neural crest origin, expressed *lacZ* at E15.5 and in P2 mice (figure 8.4E).

Strong *lacZ* expression in the brain of line 2.2-0.7 mice starts at the end of the embryonic stage and is maintained for about two weeks postnatally. Thereafter the blue color becomes faint when compared to newborn mice, but still continues into adulthood. A sheet and/or dot shaped blue color appeared in several specific areas of the brain, e.g. cerebral parietal cortex, olfactory bulb, hypothalamus, thalamus, pontine nuclei and hippocampus (figure 8.4F, G). The shape and the size of the *lacZ* positive cells of the cerebral cortex suggest that they are neuronal cells. The strongest expression was found in the cerebellum where abundant *lacZ* positive cells covered the molecular and granular

layers but were absent from the white matter (figure 8.4 F, G and not shown). This localization indicates that the *lacZ* positive cells in the cerebellum are probably not glial cells since glial cells are known to be abundant in the white matter.

The same result was noticed from the combined *lacZ* and immunohistochemistry staining with the glial fibrillary acidic protein (GFAP) antibody. The Purkinje cells as well as probably the granular neurons themselves were, however, *lacZ* negative. Microtubule associated protein (MAP) 2 and GFAP immunohistochemistry staining combined with whole mount brain *lacZ* staining was followed by paraffin embedding and immunostaining. The coronal sections of both the cerebral cortex and cerebellum contained different cell populations which were immuno-reactive with the anti-MAP2 and the anti-GFAP antibodies respectively. MAP2 has been proven to be a useful dendritic marker and is present in most dendrites of neurons in the grey matter. GFAP is an astrocyte specific marker. As expected, it was found that MAP2 antibodies stained the neurons in cerebral cortex. The *lacZ* positive cells co-localized with the MAP2 staining (Figure 8.5), but not with GFAP staining (not shown). This result clearly demonstrated that the *lacZ* positive cells in the cerebral cortex are neurons. In cerebellum, MAP2 stained mainly cells in the granular layer. Here it was difficult to identify single double-labeled cells because of overlapping cell bodies. The blue cells in the molecular layer were, however, negative for MAP2, and GFAP positive cells were located in the white matter of cerebellum (not shown) where *lacZ* expression was never detected. This clearly different expression pattern of *lacZ* and GFAP proved that the *lacZ* cells in cerebellum are non-astrocytic cells. Possibly, the *lacZ* positive cells represent a subset of cerebellar interneurons, but this remains to be proven.

PDGFαR in situ hybridization

In order to compare the expression pattern of *lacZ* and PDGFαR mRNA, we examined E12.5 sagittal sections of normal mice with a digoxigenin-labelled PDGFαR probe. A similar expression pattern as *lacZ* was found at E12.5 in that the PDGFαR signals were strongly detected in the sclerotome and the branchial arches (figure 8.6A). A different expression pattern was, however, found in cerebellum and cerebral cortex of P5 transgenic mice (figure 8.6B-D). In cerebellum most of the PDGFαR positive cells were located in the white matter and in meninges, and only scattered signals were found in the molecular and granular layers. Abundant signals also covered the brain stem. None of these structures expressed *lacZ*. In the cerebral cortex the PDGFαR positive cells were negative for *lacZ* expression, and were found scattered throughout the whole of the brain tissue. The mRNA positive cells had small round cell bodies indicating that they probably are glial cells or their precursors but not neurons.

DISCUSSION

The aim of the present study was to demonstrate the functionality of the human *PDGFαR* promoter *in vivo*. For this purpose, transgenic mice were generated containing either 2.2 kb or 0.9 kb of human *PDGFαR* promoter sequences fused to the E.coli *lacZ* reporter gene. The resulting *lacZ* expression pattern, as determined by whole mount X-gal staining, grossly overlapped the *PDGFαR* expression pattern. Although not analyzed in detail, both the 2.2 kb and 0.9 kb promoter-*lacZ* constructs display a similar pattern of expression. These data demonstrate that 2.2 kb, and probably even 0.9 kb, of promoter sequence is sufficient to realize most of the tissue specific expression characteristics of the *PDGFαR* gene.

A more detailed analysis of transgenic line 2.2-07 showed that the first *lacZ* expression in the post-implantation embryos could be detected in the developing somites and branchial arches. Upon further differentiation, *lacZ* expression becomes localized to the subsequently derived axial skeleton and dermis. This pattern of expression correlates very well with the PDGFαR *in situ* hybridization signals reported for these structures by others (Orr-Urtreger *et al.*, 1992; Orr-Urtreger and Lonai, 1992; Palmieri *et al.*,1992), and that was confirmed in this study. In addition to the paraxial mesoderm and its derivatives, *lacZ* expression overlaps rather well with the PDGFαR expression pattern in most other mesoderm- and neural crest-derived structures, i.e. cartilage primordia, mesenchymal tissues of developing internal organs, branchial arch and facial neural crest-derived mesenchyme (Morrison-Graham *et al.*, 1992; Orr- Urtreger *et al.*, 1992; Orr-Urtreger and Lonai, 1992; Schatteman *et al.*, 1992). Very strong *lacZ* expression was detected in the cartilagineous parts of the skeleton and this expression even continues after birth, but decreases upon ossification. It has been shown previously that the PDGFαR is expressed in the perichondrium/periosteum of the developing bones (Schatteman *et al.*,1992). This expression appears to be important for at least the formation of the axial skeleton, since *Patch* embryos have severe vertebral defects (Grüneberg and Truslove, 1960). The current data therefore indicate that the PDGFαR expression correlates very well with the development of all skeletal elements in both embryonic and adult stages and therefore may play an important role in bone (re)modeling in general.

Our results also support the theory that the PDGFαR is involved in facial development probably by controlling differentiation, migration and proliferation of the neural-crest derived cells. The homozygous *Patch* mutant mice also have a deformed, split face, which provides more evidence for the role of PDGFαR during facial formation. It is notable that heavy *lacZ* expression is found in condensed mesenchyme immediately adjacent to epithelium of newly formed facial structures. Likewise, the presence of

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figure 8.1: Expression of *lacZ* in somites, branchial arches and limb buds.
LacZ staining of whole mount embryos cleared in BABB to reveal internal staining **A)** Lateral view of a whole mount embryo of E10 5 shows *lacZ* mainly expressed in somites (***S***) and branchial arches (***BA***) **B)** A dorsal view at E12 5 *LacZ* was expressed in the neural tube, particularly in the rhombencephalon and rhombic lip in the area of the fourth ventricle (***FV***), in primodium of ribs (***Ri***) and in developing limbs (***L***). **C)** Transverse section of an E9.5 embryo double stained with X-Gal and H&E *LacZ* was expressed in the sclerotome (***ST***). **D)** Parasagittal section of an E12 5 embryo with X-Gal staining shows *LacZ* expressed in the neuroepithelium of the fourth ventricle (***FV***), lung buds (***LB***), urogenital sinus (***US***), maxillary (***MX***) and mandibular components (***MC***) **E)** A parasagittal section shows *lacZ* expressed in sclerotome (***ST***) and dermis (***D***) of an E12 5 embryo Root ganglion (***RG***) is negative.

figure 8.2: Expression of *lacZ* in chondroid tissue.
A) Parasagittal section of E12 5 embryo shows heavily stained condensed mesenchyme of developing ribs (***Ri***) **B)** Section of palm of a E12 5 embryo shows *lacZ* expression in cartilage primordium of phalangeal bones (***P***) and surrounding mesenchyme (***M***) **C-D)** *LacZ* expressed in the cartilagineous skeleton in a P8 mouse Notice that the costo-chondral junction and the epiphysis (***Ep***) of the long bones are heavily stained, while ossified areas are negative (***V***) vertebra, (***ID***) intervertebral disc

figure 8.3: Expression of *lacZ* in newly formed tissues
A) Sagittal section of lips and tongue (***T***) of an E15.5 embryo shows strong blue colour in an area of the mesenchymal tissue (***M***) adjacent to newly forming epithelial structures of the lower lip (***LP***) **B)** Parasagittal section of an E12 5 embryo shows *lacZ* expressed in neuroepithelium of otic vesicle (***OV***) **C)** High magnification of the skin of the lip with the primordium of a hairfollicle of an E15.5 embryo shows *lacZ* only expressed in the condensed mesenchyme (***M***) apposing the epithelial bud (***E***) **D)** Sagittal section of an E15 5 embryo shows that abundant *lacZ* positive cells are present in the lung mesenchymal tissue (***M***), while the epithelium (***E***) is negative **E)** *LacZ* expressed in whole lung lobes (***LL***) and in cartilage of trachea (***T***) and bronchi in whole mount staining of a P8 mouse

PDGFαR promoter activity in the interstitial mesenchyme of internal organs, like the lung, indicates that PDGF paracrine stimulatory mechanisms might play an important role during the proper development of these organs.

The *lacZ* expression pattern in the postnatal CNS, however, deviates from that of the PDGFαR *in situ* hybridization signal PDGFαR mRNA *in situ* hybridization data reported by others (Pringle *et al.*, 1992; Yeh *et al* ,1993) and in this study clearly demonstrate that PDGFαR expression in the postnatal CNS is restricted to glial cells and their precursors Although the exact nature of the *lacZ* expressing cells in the 2.2-0.7 line has not been determined, they appear to be neurons This is indicated by cell morphology and MAP2 staining, but also by their spatial expression pattern. The *lacZ* expression is confined to well demarcated, sometimes lamellated, structures. Likewise, the expression is highest before and around birth and starts to decline shortly thereafter, whereas the majority of

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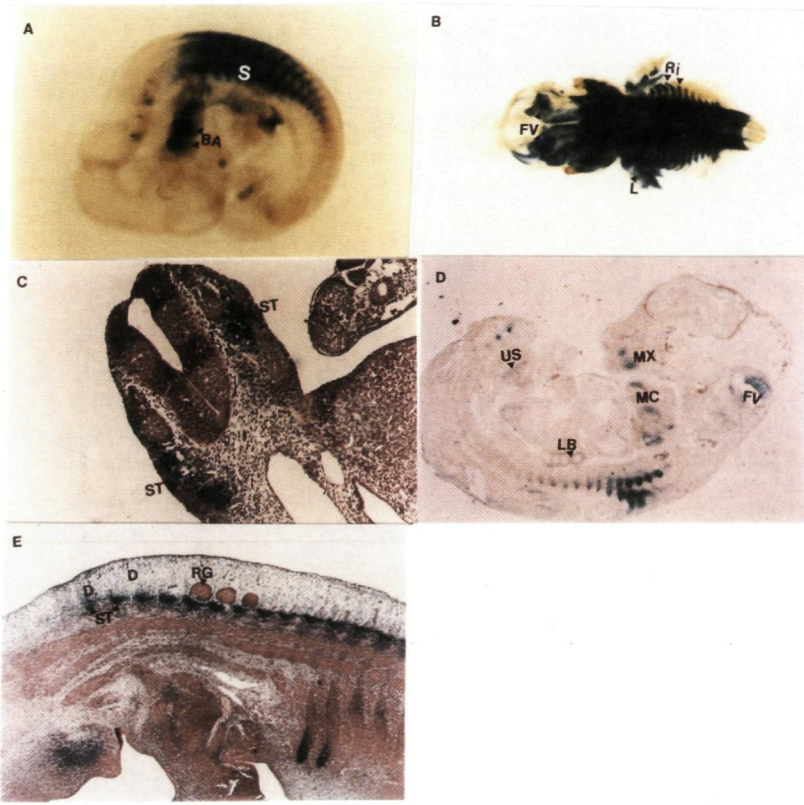
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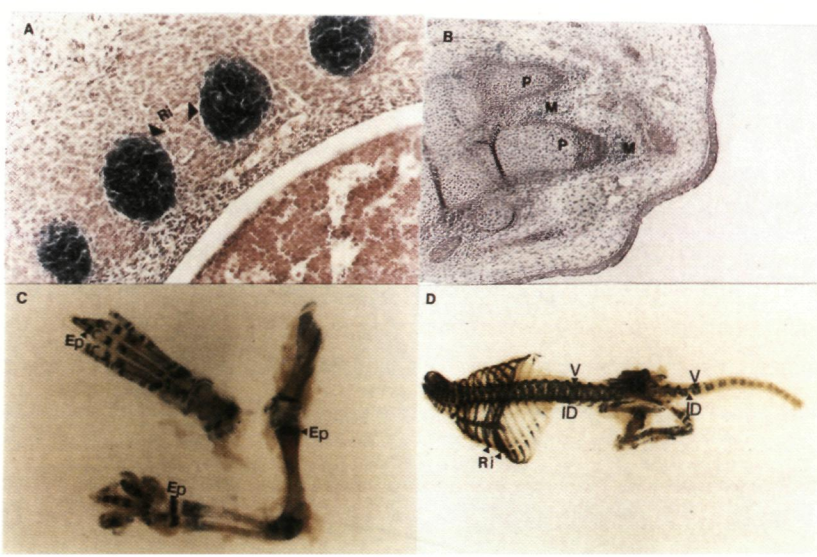
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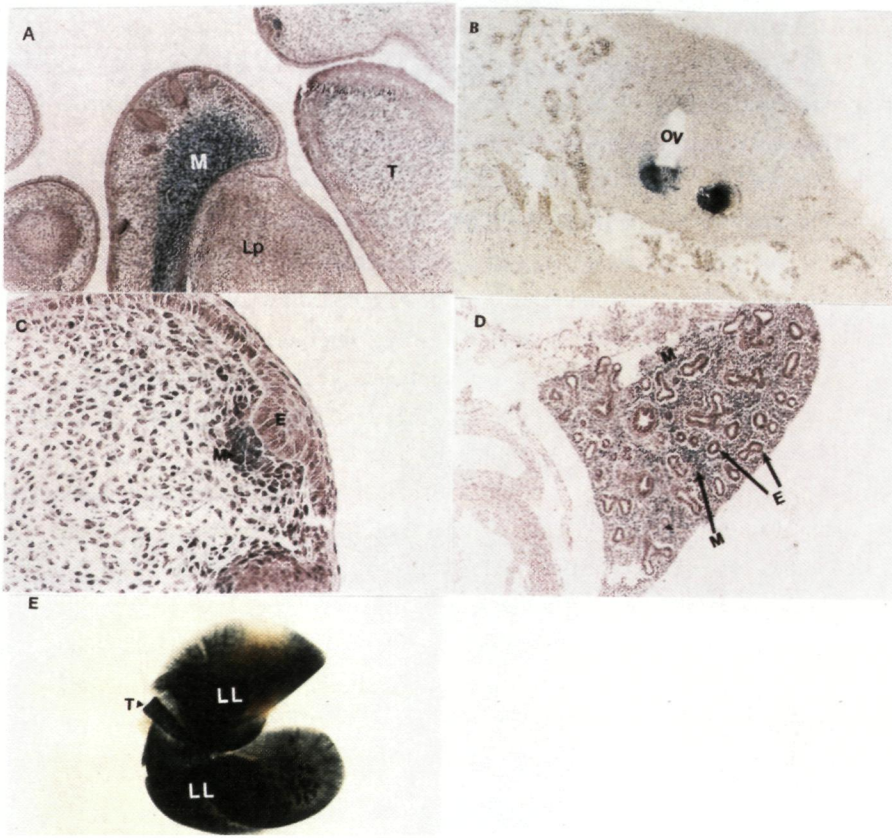

PDGFαR-expressing oligodendrocyte-astrocyte precursors develop postnatally. Another discrepancy is the lack of *lacZ* expression in the developing eye and optic nerve, where oligodendrocyte-astrocyte precursors, which contain the PDGFαR messenger, have been described extensively (Schatteman *et al.*, 1992; Mudhar *et al.*, 1993). The cause of these discrepancies in *lacZ* and PDGFαR mRNA expression is currently not known. It may reflect a positional effect of integration of the transgene, since the *lacZ* expression pattern in the CNS differs among the lines. However, the dominant *lacZ* expression in the cerebellum has been found in two independent transgenic lines with two different promoter constructs, which indicates that the ectopic expression of *lacZ* is also partly a result of some regulatory element that is missing within the transgene.

LacZ was heavily expressed in neuroepithelial cells of the rhombencephalon and of the rhombic lip. These areas constitute the presursors of cephalic neural crest and cerebellar cells respectively. Both cell populations contain multipotent stem cells that can give rise to multiple differentiated cell types. Such E13 mouse neuroepithelial cells of the cerebellar external granular layer that forms from the rhombic lip have the capacity to differentiate into both neuronal and glial cell types (Gao and Hatten, 1994). Human medulloblastoma cells, which are tumor cells supposed to represent multipotent neuroepithelial stem cells of the cerebellum, constantly express PDGFαR (Smits *et al.*, 1995). Also in other experimental systems the existence of a common neuron and oligodendrocyte precursor cell has been indicated (Williams *et al.* 1991). It can therefore be postulated that components which specify the receptor down-regulation in developing neuronal cell types and upregulation or persistence in oligodendrocytes, are missing in the transgene. In addition, there may be other regulatory mechanisms involved than transcription regulation, like alternative promoter use or posttranscriptional mechanisms, with the consequence that levels of mRNA and levels of transcriptional activity do not directly parallel each other. However, the current experimental approach does not allow to discriminate between all these various possibilities. The use of a heterologous system may also have contributed to the observed differences in *lacZ* and *PDGFαR* gene expression. Although the mouse and human *PDGFαR* gene and promoter share many characteristics in embryonal carcinoma cells (Wang *et al.*, 1990; Mosselman *et al.*, 1994; Wang and Stiles, 1994; Afink *et al.*, 1995), it cannot be ruled out that important differences exist between the regulation of *PDGFαR* gene expression in humans and mice. Additional experiments are currently undertaken to investigate the details of the *PDGFαR* promoter activity in the CNS.

The data presented in this paper clearly demonstrate the *in vivo* functionality of the isolated human *PDGFαR* gene promoter to establish the spatio-temporal correct reporter gene expression in most mesoderm- and neural crest-derived structures. The results also

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Figure 8.4: Expression of *lacZ* in the CNS.
A) Transverse section of an E12 5 embryo through the fourth (***FV***) and third (***TV***) ventricles shows *lacZ* expressed in the rhombic lip (***RL***) of the fourth ventricle and the top wall of the third ventricle (***WTV***) **B)** A transverse section shows spotted *lacZ* staining along the posterior and lateral aspect of the developing spinal cord (***SC***), and heavy staining in the sclerotome (***ST***) and the mesenchyme at the ventral aspect of trachea (***TC***) **C)** A longitudinal section through the spinal cord of an E12 5 embryo Notice that *lacZ* is expressed in longitudinally arranged spots of the dorsal and ventral parts of the spinal cord **D)** Lateral view of a whole mount E12 5 embryo shows the same linear array of blue dots in the cervical part of the spinal cord **E)** *LacZ* positivity in the facial ganglion cells of a P2 mouse **F)** Whole brain and **G)** coronal section of forebrain of a P8 mouse shows *lacZ* expressed in olfactory bulb (***OB***), cerebral parietal cortex(***CPC***), thalamus (***TM***), hypothalamus (***HTM***) and pontine nuclei (***PN***)

figure 8.5: Immunohistochemistry staining of brain with MAP2 antibody using paraffin embedded sections of *lacZ* stained whole mounts.
LacZ and MAP2 double stained neuronal cells are shown in an area of parietal cerebral cortex in postnatal mouse brain.

figure 8.6: PDGFαR *in situ* hybridization
A), B) Combined *lacZ* and PDGFαR *in situ* hybridization of a coronal section of the cerebral cortex shows that *lacZ* positive cells (blue-green) are PDGFαR negative PDGFαR positive cells (dark brown-blue) are found scattered over the brain substance while *lacZ* positive cells are confined to a laminar region **C)** Coronal section of cerebellum (***CB***) and brain stem (***BS***) of a P5 mouse The PDGFαR positive cells (dark brown-blue colour) were scattered over the whole brain stem and in the cerebellum mainly located in the white matter and sparsely scatteredin the granular and molecular layers **D)** A high magnification of the cerebellum of C. (***WM***) white matter,(***EGL***) external granular layer; (***ML***) molecular layer

indicate that transcriptional control is the important mechanism in both induction and repression of *PDGFαR* gene expression during embryonic development. However, the isolated promoter does not confer specific reporter gene expression to all tissues, in particular not in the CNS. Despite some discrepancies with the *PDGFαR* gene expression pattern, these PDGFαR promoter-*lacZ* transgenic mice provide a powerful tool to study the regulation and function of *PDGFαR* gene expression under normal and pathological conditions. Since expression of the *PDGFαR* gene is indispensible for the formation of many mesoderm- and neural crest- derived structures (Morrison-Graham *et al.*, 1992; Orr-Urtreger *et al.*, 1992; Schatteman *et al* , 1992), elucidation of the gene expression control mechanisms will provide relevant information about the molecular mechanisms involved in the formation of these embryonic structures.

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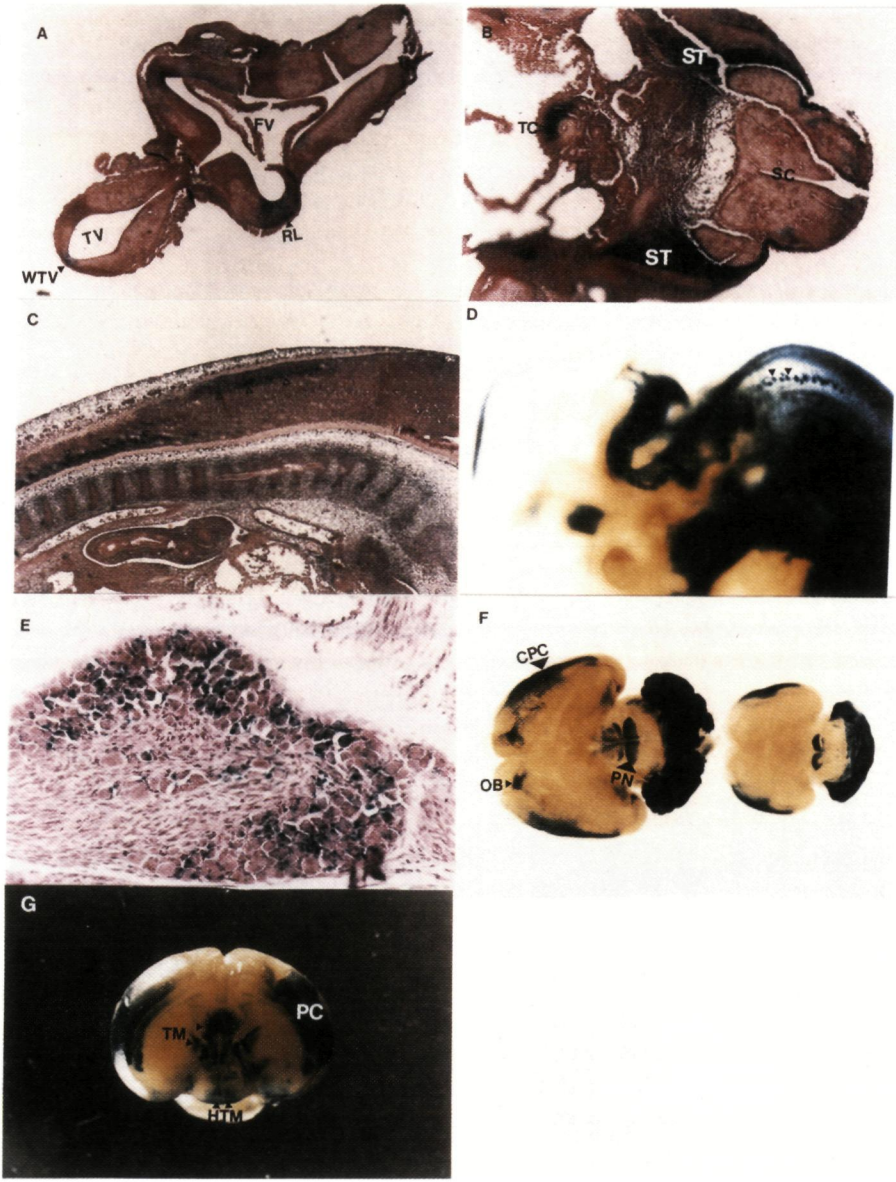

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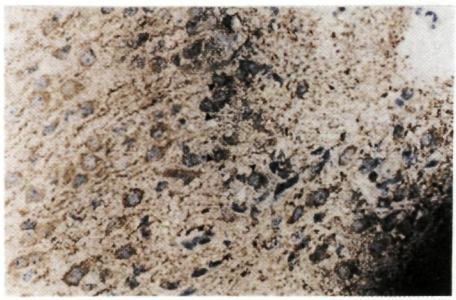

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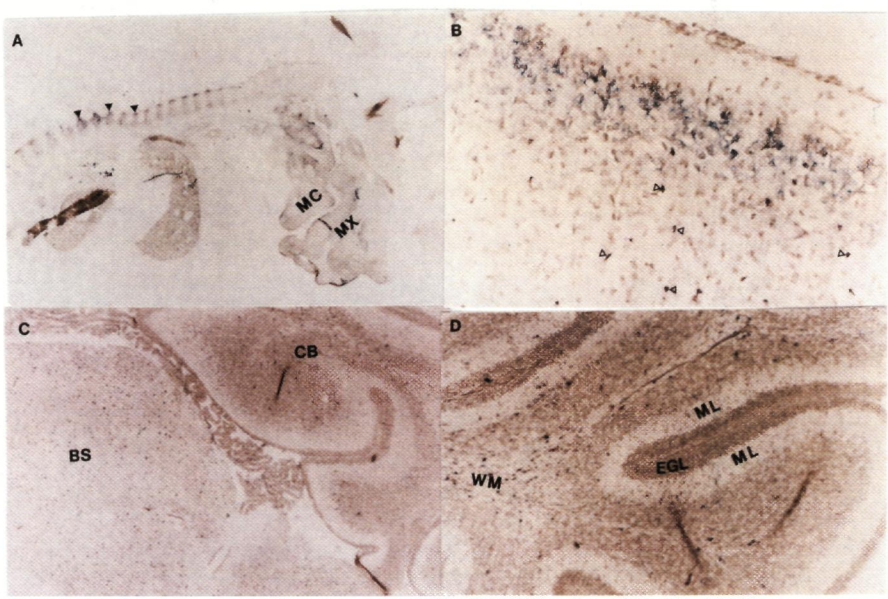

MATERIALS AND METHODS

Design of transgenic mice

An approximately 2 2 kb and a 0 9 kb genomic DNA fragment encompassing nucleotides -2120 to +129 (2 2 kb) and -825 to +129 (0 9 kb) of the human *PDGFαR* gene 5'-flanking part (Afink *et al* , 1995) were cloned into the HindIII restriction site of the lacZ reporter vector pCH110 (Pharmacia) using standard molecular biological techniques (Sambrook *et al* , 1989) Before injection, the *PDGFαR* gene-*lacZ* fragments were released from the rest of the pCH110 vector by digestion with AvrII and BamHI (Promega) and purified by gel electrophoresis The resulting 6kb (2 2-*lacZ*) and 5kb (0 9-*lacZ*) DNA fragments, at a concentration of 2 mg/ml, were microinjected into pronuclei of fertilized eggs Injected eggs were cultured to two cell stage and transferred into the oviduct of pseudo-pregnant female mice Tail DNA of P20 pups was isolated with proteinase K digestion in a buffer containing 50 mM Tris-HCl (pH 8 0), 100 mM EDTA, 100 mM NaCl and 1% SDS at 37 °C overnight followed by phenol and chloroform isoamylalcohol (24 1) extractions Transgenic founder mice were identified by PCR using one primer (5'-*TAAGCCGTAGATAAACAGGC*-3') corresponding to a sequence in the *lacZ* gene, and the second primer (5'-*CACCCAGTCTTGTACACACA*-3') corresponding to a sequence in the PDGFαR promoter Positive results were confirmed by Southern blot analysis using a ^{32}P-labelled *lacZ* DNA probe A total of ten transgenic founders were established (listed in table 1) *LacZ* expression was determined by whole mount X-Gal staining of postnatal brains or/and of embryos There were no obvious differences detected between the growth of transgenic and normal mice

ß-Galactosidase assay of whole mount embryos or tissues

The methods used for the analysis of ß-galactosidase activity in whole mount tissues and embryos have been described previously (Jegalian *et al* , 1992) Embryos were dissected out free of extracellular membranes and fixed in 2% paraformaldehyde in PBS (phosphate buffered saline) for 1hr Embryos and tissues were then rinsed three times in PBS and permeabilized in 2 mM $MgCl_2$, 0 02% NP40, 0 01% Na-deoxycholate in PBS three times 20 min at room temperature The ß-galactosidase was detected with 5-bromo-4 chloro-3-indolyl-ß-D-galactopyranosidase (X-Gal, Promega) as a substrate X-Gal staining was performed with a substrate concentration of 1 mg/ml in a buffer containing 2 mM $MgCl_2$, 0 02% NP40, 0 01% Na-deoxycholate, 5 mM $K_4Fe(N)_6$, 5 mM $K_3Fe(N)_6$ in PBS at 37 °C overnight in the dark The embryos and tissues were subsequently washed three times in PBS, and could either be dehydrated in methanol and cleared in BABB (benzyl alcohol benzyl benzoate 1 2) for photos of whole mounts or embedded in paraffin for histological studies

Histology

Tissues and embryos were dissected and fixed in freshly prepared 2% paraformaldehyde in PBS for one hour For dissected tissues, the animal was first fixed by whole body perfusion with 2% paraformaldehyde Paraffine embedding was performed either directly after fixation or after X Gal staining with the method described above For paraffin embedded embryos and tissues, five to ten micron sections were cut and mounted on gelatin-coated or Superfrost Plus (Fisher) slides Sections were air dried for 24 hours After deparaffination, part of the sections were counterstained with hematoxylin and eosin (H&E)

In situ hybridization

Embedding, cryosectioning, postfixation, hybridization and washing was as previously described by Pringle *et al* (1989, 1992) The PDGFαR sense and antisense RNA probe were *in vitro* transcribed from a 0 7 kb HindIII fragment corresponding to the mouse PDGFαR cDNA, encoding most of the extracellular domain

(obtained from M Mercola, Boston, USA and B Richardson, London, UK) The probes were labelled either with digoxigenin or with ^{35}S

Immunohistochemistry

Tissue dissection, fixation, paraffin embedding and sectioning was as described for the histological analysis The immunohistochemistry protocol was based on that of Li *et al* (1995) Monoclonal anti-microtubule associated protein (MAP2) antibody (Amersham) was used at a 1 1000 dilution in blocking solution (1% BSA in PBS) and the monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (Labsystems Oy, Helsinki, Finland) at a 1 200 dilution The secondary antibody, biotinylated anti-mouse IgG (Vector laboratories), was used at a 1 200 dilution

CHAPTER 9

Discussion & references

Expression of the *PDGFαR* gene is strictly regulated during embryonic development and appears to be deregulated under certain pathological conditions. The mechanisms behind this specific expression are not known, but several external stimuli have been shown to affect *PDGFαR* gene expression *in vitro*. In order to investigate these regulatory mechanisms, the promoter region of the human *PDGFαR* gene was cloned and its functionality was demonstrated both *in vitro* and *in vivo*. Although most aspects of this work has already been discussed in the individual chapters, some points deserve some additional attention.

Interspecies conservation of PDGFαR gene regulation

Several observations have indicated that regulation of *PDGFαR* gene expression is a rather conserved process among the different species studied. Both in mouse and Xenopus, induction of *PDGFαR* gene expression appears to be tightly correlated with the induction of mesoderm (Orr-Urtreger and Lonai,1992; Orr-Urtreger *et al.*, 1992; Palmieri *et al.*, 1992; Schatteman *et al.*, 1992; Jones *et al.*, 1992), and with the migration of neural crest cells (Morrison-Graham *et al.*, 1992; Ho *et al.*, 1992). In addition, RA induces expression of the *PDGFαR* gene in a similar fashion in both human and mouse embryonal carcinoma cells (Mercola *et al.*, 1990; Wang *et al.*, 1990; Mosselman *et al.*, 1994).

This conservation of *PDGFαR* gene regulation is also rather obvious in the described promoter studies. Both the human (chapter 6) and the mouse (Wang and Stiles, 1994) promoter activity are strongly increased upon RA and cAMP treatment of embryonal carcinoma cells. In both cases, this effect is apparently not mediated via a direct binding of RA receptors to the *PDGFαR* promoter. As shown in chapter 7, this RA- and cAMP-stimulated human promoter activity appears to be mediated through an AP1-like regulatory element. Whether a similar mechanism occurs in the mouse is currently unknown. Although a RACT-responsive element in the mouse promoter has been confined to an approximately 450 bp fragment (which is located at least 500 bp upstream of the transcription initiation site), no additional information concerning this regulatory element has been reported yet. Since the nucleotide sequence of the mouse promoter region has not been disclosed, it is not possible to align the mouse and human promoter sequences and search for conserved elements within the two promoters. Almost upon completion of this thesis, the sequence of the rat *PDGFαR* promoter was published (Kitami *et al.*, 1995).

Interestingly, the rat promoter sequence shares a high degree of homology with the human promoter sequence, including the -52/+118 region, and the PARAP1 element is completely conserved in both species The elements involved in the basal transcription seem also to be conserved As shown in chapter 6, the activity pattern of the promoter deletion mutants is the same in both human and mouse cells However, the best example of the conserved regulation of *PDGFαR* gene expression is described in chapter 8 The human *PDGFαR* promoter is capable to drive tissue-specific reporter gene expression in transgenic mice The expression pattern of the reporter gene overlaps the endogeneous mouse PDGFαR pattern in most tissues

From these data it seems appropriate to conclude that both the *in vitro* and *in vivo* regulation of the *PDGFαR* gene expression in human and mouse share many characteristics This conclusion has certainly an important impact on the experimental approach that can be undertaken to investigate the mechanisms behind this regulation of gene expression The combined use of human, mouse and even Xenopus systems may provide the relevant information about the molecular mechanisms behind the regulation of *PDGFαR* gene expression in normal and disease conditions In addition, the described transgenic mouse model will allow a rather simple detection of *PDGFαR* promoter activity, and thus *PDGFαR* gene expression, during most stages of embryonic development and in adult life Therefore, these mice may serve as a powerful tool to investigate the possible role of PDGFαR expression in the development and maintenance of certain tissues

Molecular mechanisms involved in the regulation of the PDGFαR promoter

The data presented in chapter 8 strongly indicate that transcription regulation is the most important determinant of the correct spatio-temporal *PDGFαR* gene expression *in vivo* In addition, *in vitro* studies have clearly demonstrated that transmodulation of PDGFαR expression by RA in embryonal carcinoma cells (Wang *et al* , 1990, Wang and Stiles, 1994, chapter 6), and Il-1 in osteosarcoma cells (Xie *et al* , 1994) is also controlled at the level of transcriptional regulation Therefore, identification of the transcription factors that affect *PDGFαR* promoter activity is an important step in the understanding of the molecular mechanisms behind this regulation of PDGFαR expression

A first attempt to establish the identity of these transcription factors has been described in chapter 7 and has resulted in the identification of AP1-like transcription factors as candidates to mediate the RA- and cAMP-induced transcription of the *PDGFαR* gene in embryonal carcinoma cells It should be stressed however, that this is a preliminary conclusion The work described in chapter 7 is not completely finished and additional experiments have to be performed to sustain the conclusions In particular the

role of the PARAP1 element should be confirmed by extended mutational analysis and the possible involvement of c-jun in PARAP1 binding should be analyzed with specific antibodies. Preliminary data from *in vivo* footprint analysis on the -52/+118 region did so far not provide additional support for the involvement of the PARAP1 element in the RA-induced *PDGFαR* promoter activity (HJ Kraft *et al.*, unpublished). In addition, the described RA and cAMP effects are specific for embryonal carcinoma cells. In other cell types, for instance mouse Swiss 3T3 fibroblasts, RA appears to affect *PDGFαR* promoter activity through inhibitory elements, while cAMP is without any effect in these cells (in collaboration with B Stassen and E Lammerts, unpublished).

As already mentioned in the prologue, the TGFß-mediated downregulation of PDGFαR expression in fibroblasts and the resulting decrease in mitogenic response to PDGF (Gronwald *et al.*, 1989; Paulsson *et al.*, 1993) was the initiative for the current work on the transcriptional regulation of the *PDGFαR* gene. However, preliminary studies in mouse Swiss 3T3 and human AG1523 fibroblasts never indicated a decrease in *PDGFαR* promoter activity upon TGFß treatment (in collaboration with E. Lammerts, B Stassen, unpublished). Therefore, it had to be concluded that this downregulation process in these cells was not controlled at the transcriptional level or was mediated through regulatory elements outside the investigated -2120/+118 promoter region. In MG63 osteosarcoma cells, both TGFß and Il-1 have been shown to decrease PDGFαR mRNA levels, which resulted in an attenuated biological response to PDGF (Yeh *et al.*, 1993a). Subsequent studies have demonstrated that at least the IL-1 effect is mediated at the level of gene transcription (Xie *et al.*, 1994), but these studies do not provide information about the working mechanism of TGFß. However, recent experiments indicate that both IL-1ß and TGFß are indeed inhibitors of *PDGFαR* promoter activity (in collaboration with E Lammerts, unpublished). This effect is confined to the -441/+118 region of the human promoter and additional experiments are being performed to exactly locate the regulatory elements and transcription factors involved in this inhibitory process. Together these data indicate however, that inhibition of PDGFαR numbers by TGFß is mediated through different, cell type specific mechanisms.

Another topic of interest, which is currently being investigated, is the elucidation of the molecular mechanisms that regulate the tissue-specific expression of the *PDGFαR* gene in embryonic development. The embryonic expression pattern of many transcription factors has been described in detail, and some of these patterns display a striking overlap with that of the *PDGFαR* gene. One important candidate transcription factor to regulate the *PDGFαR* gene expression is GLI. The GLI gene was originally identified by its amplified copy number in a human glioma (Kinzler *et al.*, 1987), a tumor type which also frequently displays high PDGFαR levels (Hermanson *et al.*, 1995). The GLI protein is

presumably a transcription factor which binds to the DNA sequence 5'-*GACCACCCA*-3' (Kinzler and Vogelstein, 1990) This sequence motif is also present within the *PDGFαR* promoter (nucleotide -660 to -651, figure 2, chapter 6) In addition, GLI expression in the developing embryo overlaps that of the PDGFαR in many tissues, including limbs, vertebra, lung, bones and facial structures (Walterhouse *et al* , 1993, Hui *et al* , 1994) The final observation that supports a possible role for GLI in regulation of *PDGFαR* gene expression is the recent demonstration that transfection of a GLI expression vector, together with a promoter-luciferase construct, into glioma cells results in an increase of *PDGFαR* promoter activity (in collaboration with J Jacobs, unpublished) Another candidate transcription factor is PAX1 PAX1 expression (Balling *et al* , 1988, Wallin *et al* , 1994) correrates well with the PDGFαR expression in the developing axial skeleton, and recently Helwig *et al* (1995) reported an functional interaction between PAX1 and the PDGFαR in the correct formation of this structure In addition, expression of PAX1 together with a promoter-luciferase construct also leads to an increase in *PDGFαR* promoter activity in several cell lines tested (in collaboration with J Jacobs, unpublished) Thus, at least in the somites and its derivatives PAX1 could be a determinant of correct *PDGFαR* gene expression

There are of course many other candidate transcription factors that may play a role in regulation of tissue specific *PDGFαR* promoter activity Ho *et al* (1994) already indicated the possible involvement of *distall-less*, *twist* and *snail* in *PDGFαR* expression during the migration of Xenopus cephalic neural crest Future research may reveal the possible involvement of these and other transcription factors in the regulation of *PDGFαR* gene expression during embryonic development

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SUMMARY

Growth factors play a prominent role in the intercellular communication between cells in multicellular organisms. In this way they participate in the coordinated growth and maintenance of the organism. Growth factors are released from cells and achieve their effects on the same (autocrine stimulation), on nearby (paracrine stimulation) or distantly located (endocrine stimulation) cells. This effect on cells is mediated through specific receptors, which are located within the cells' plasmamembrane. Upon binding of a growth factor to its cognate receptor, the receptor becomes "activated", and transduces a signal to the inside of the cell. This last process is a highly complex system of biochemical reactions, which finally results in a "growth response". In this thesis, two studies have been carried out which deal with this growth factor signal transduction process.

In part A, the signal transduction mechanisms of growth factors that affect NRK cell proliferation were investigated. NRK cells are a model system for a process called density-dependent growth arrest or contact-inhibition. This process restricts cell growth of most normal cells when they reach a critical cell density. However, tumor cell have lost this inhibitory mechanism, and as a result they can grow without limitation. In NRK cells, several growth factors (e.g. TGFß) are able to overcome this density-dependent growth arrest and thus phenotypically transform these cells to a "tumor-like" cell type. From the data in chapter 2 it has become clear that the induction of a receptor for another growth factor (EGF) is the crucial signal transduction step by which TGFß, but also the vitamin A derivative retinoic acid exert their action. On the other hand, the lipid LPA does not seem to transform NRK cells through this indirect mechanism, but by an as-yet unknown mechanism. In addition, the small peptide bradykinin inhibits the transformation process elicited by TGFß, retinoic acid and LPA. This inhibitory effect is presumably mediated through the production of a growth inhibitory prostaglandin as described in chapter 3. The exact nature of this prostaglandin and its intracellular targets are currently being investigated.

In part B the regulation of the human PDGFα receptor (PDGFαR) expression has been studied. The first requirement for a cell to respond to a growth factor is obviously the expression of its cognate receptor. One receptor for PDGF is the PDGFαR. The expression of this receptor is strictly regulated during embryonic development. Only certain cell types at very specific time points express the PDGFαR and hence are responsive to PDGF. Also in adult life, the PDGFαR expression is restricted to specific cell types. Disregulation of this tightly regulated expression can result to severe embryonic defects and diseases such as cancer. To investigate the mechanisms involved in this

specific regulation of PDGFαR expression, the regulatory elements of the gene encoding the human PDGFαR were characterized. As described in chapter 6, the region that controls the expression of the PDGFαR gene (PDGFαR promoter) was cloned and its functionality was assessed. In addition, the mechanism by which retinoic acid, a potent inducer of PDGFαR expression, increases the activity of the promoter and thereby the expression of the PDGFαR gene was analysed. This work has been extended in more detail in chapter 7. From these data it was concluded that a small, but specific sequence of DNA within the human PDGFαR promoter mediates the activating effect of retinoic acid. In addition, in chapter 8 the functionality of the cloned promoter was examined *in vivo*, using a transgenic mouse model. For this purpose, the human PDGFαR promoter was attached to a reporter gene and incorporated into the mouse genome. The activity of the promoter in these transgenic mice can be easily detected by means of the attached reporter gene. The reporter gene produces a typical blue color in cells where the promoter is active. In this way, the activity of the promoter, and the regulation of this activity could be monitored during embryonic development. This approach has lead to more insight in both the function of the PDGFαR in embryonic development, and the *in vivo* functionality of the PDGFαR promoter.

SAMENVATTING

Groeifactoren spelen een belangrijke rol in de onderlinge communicatie tussen verschillende cellen in een multicellulair organisme. Op deze wijze dragen zij bij aan de gecoördineerde groei van het organisme. Groeifactoren worden geproduceerd door cellen en hebben vervolgens effect op diezelfde cellen (autocriene stimulatie), naburige cellen (paracriene stimulatie) of cellen in andere weefsels binnen het lichaam (endocriene stimulatie). Dit effect van een groeifactor op een cel wordt gemediëerd door specifieke receptoren, die aanwezig zijn in de plasmamembraan van de cel. Nadat binding van de groeifactor aan zijn bijbehorende receptor heeft plaatsgevonden, wordt deze "geactiveerd" en geeft een signaal door aan de binnenkant van de cel. Dit laatste proces is een zeer complex systeem van verschillende biochemische reacties, die uiteindelijk uitmonden in een "groeirespons". In dit proefschrift worden twee onderzoeken beschreven die betrekking hebben op deze groeifactor signaalgeleiding.

In deel A worden de signaalgeleidingmechanismen onderzocht van groeifactoren die een rol spelen in de proliferatie van NRK cellen. NRK cellen vormen een modelsysteem voor contact-inhibitie. Contact-inhibitie is een proces dat normaliter cellen remt in hun groei als ze een bepaalde kritische dichtheid bereiken. Tumorcellen hebben echter deze groeiremmende eigenschap verloren en als gevolg daarvan kunnen ze dan ook ongehinderd door blijven groeien. Verschillende groeifactoren (b.v. TGFß) zijn echter in staat om deze contact-inhibitie in NRK cellen op te heffen en op die manier deze cellen fenotypisch te transformeren naar een "tumor-achtig" cel type. Uit de gegevens beschreven in hoofdstuk 2 is daarbij duidelijk geworden dat in geval van fenotypische transformatie de inductie van een receptor voor een andere groeifactor (EGF) de essentiële stap is in de signaalgeleiding van TGFß, maar ook in die van het vitamine A derivaat retinoic acid (RA). Het vetzuur LPA daarentegen lijkt echter een andere manier van signaalgeleiding te gebruiken in zijn transformerend effect, maar het is op dit moment nog onduidelijk welke. Het eiwit bradykinine is echter in staat om zowel de TGFß-, RA- als LPA-geïnduceerde transformatie te remmen. Deze remming vindt waarschijnlijk plaats door een specifieke inductie van een groeiremmend prostaglandine, zoals beschreven staat in hoofdstuk 3. Verder onderzoek naar de preciese karakterisering van dit prostaglandine en zijn remmende werking is op dit moment gaande.

Deel B beschrijft het onderzoek naar de regulatie van de humane PDGFα receptor (PDGFαR) expressie. De eerste vereiste voor een cel om te kunnen reageren op een groeifactor is natuurlijk de expressie van de bijbehorende receptor. Een van de receptoren voor PDGF is de PDGFαR. De expressie van deze receptor wordt zeer nauwgezet gereguleerd tijdens de embryonale ontwikkeling. Expressie van de PDGFαR, en dus ook

gevoeligheid voor PDGF, vindt alleen plaats in bepaalde celtypen en op specifieke tijdstippen. Ook in het volwassen organisme is de PDGFαR expressie beperkt tot specifieke celtypes. Verstoringen in deze zeer nauwgezet gereguleerde expressie kan ernstige embryonale afwijkingen of ziekten zoals kanker tot gevolg hebben. Voor de bestudering van de mechanismen die betrokken zijn bij deze specifieke regulatie van PDGFαR expressie zijn de regulatoire elementen van het humane PDGFαR gen gekarakteriseerd. Hiertoe werd de genregio verantwoordelijk voor de controle van de expressie van het PDGFαR gen (PDGFαR promoter) gekloneerd en werd de functionaliteit van deze promoter bepaald, zoals beschreven in hoofdstuk 6. Tevens werd het mechanisme waardoor RA, een krachtige stimulus voor PDGFαR expressie, de PDGFαR promoter activeerd en op die manier de PDGFαR expressie stimuleert. Dit werk wordt vervolgens in meer detail voortgezet en de resultaten van dit onderzoek staan in hoofdstuk 7. Als conclusie van dit werk kon worden vastgesteld dat een klein specifiek stuk DNA binnen de humane PDGFαR promoter regio de RA activatie mediëerd. Tenslotte in hoofdstuk 8 werd de *in vivo* functionaliteit van de promoter aangetoond door gebruik te maken van een transgene muis systeem. Hiertoe werd de humane PDGFαR promoter gekoppeld aan een reportergen en vervolgens geïncorporeerd binnen het genoom van de muis. Op die wijze kon de activiteit van de promoter in de transgene muis relatief gemakkelijk worden vastgesteld, doordat het gekoppelde reportergen een blauwkleuring geeft in die cellen waar de promoter actief is. Deze opzet heeft ertoe geleidt dat er meer inzicht werd verkregen in zowel de functie van de PDGFαR in embryonale ontwikkeling, alsook de *in vivo* functionaliteit van de PDGFαR promoter.

DANKWOORD

Alhoewel dit waarschijnlijk het meestgelezen deel van mijn proefschrift zal worden, zal ik het kort houden.

De eerste persoon die hier natuurlijk genoemd moet worden is **Joop van Zoelen(t)**, die mij de mogelijkheid en de begeleiding heeft gegeven om het in dit proefschrift beschreven onderzoek uit te voeren. Weliswaar lagen onze persoonlijkheden ver uit elkaar, maar wetenschappelijk gezien was het nooit een probleem om op één lijn te komen. Vervolgens dient hier **Monica Nistér** te worden vermeld. Naast haar wetenschappelijke bijdrage aan dit proefschrift wil haar bedanken voor haar "moederinstinct", wat vele malen bij haar boven kwam drijven gedurende de toch wel wat moeilijke periode van onderzoek in Uppsala (translation will be provided on request, but due to the similarities between the Swedish and Dutch languages, I do not think it will be necessary). Verder wil ik natuurlijk alle mensen bedanken die ofwel een grote of een kleine bijdrage hebben geleverd aan de totstandkoming van dit proefschrift, waarbij een aantal personen hier toch wel met name genoemd dient te worden. Allereerst **Sietse Mosselman**: grondlegger van het PDGFαR promoter onderzoek en nooit te beroerd om mij op het juiste moment een schop onder de kont te verkopen. Verder **Xiao-Qun Zhang** en **Peter Peters**, die het grootste deel van de experimenten beschreven in respectievelijk hoofdstuk 8 en 2 hebben uitgevoerd. **Danielle Lahaye, Dirk van Alewijk, Bianca Stassen, Pascale Rademakers, Paul Joosten, Marcel van Lith, Ellen Lammerts** en **Jacqueline Jacobs**: zonder uitzondering hebben deze studenten zich enorm ingezet voor mijn onderzoek en veel van hun resultaten zijn dan ook verwerkt in dit proefschrift. **Albert de Roos**: hoort ook in het rijtje van studenten, maar heeft later ook als OIO nog resultaten geproduceerd die zijn opgenomen in deel A van het proefschrift. **Erik Bongcam-Rudloff** en **Jia-Lun Wang**: mijn "gidsen" in Uppsala, zowel binnen als buiten het lab. Natuurlijk wil ik alle medewerkers van de afdeling Celbiologie bedanken voor de leuke werk- en niet-werktijd gedurende de afgelopen vijf jaren. Als laatste nog een zin voor **Diet Gröneveld** en **Ester Piek**. Ik heb het nooit echt duidelijk laten blijken, maar bij deze mijn waardering voor de tijd die jullie in mij gestoken hebben en daarmee toch de randvoorwarden hebben geschapen voor de totstandkoming van dit proefschrift

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 26 november 1965 te Enschede. In deze zelfde stad begon hij aan de eerste 14 jaren van zijn onderwijscarriere, die in 1984 werd afgesloten met het behalen van het VWO diploma aan de Gemeentelijke Scholengemeenschap Zuid. Datzelfde jaar verhuisde hij naar Nijmegen om met de studie biologie te beginnen aan de Katholieke Universiteit Nijmegen. Na de propedeuse in juni 1985, werd het doctoraal examen in de fysiologische/biochemische richting behaald in juni 1990, met als hoofdvak Dierfysiologie (Prof. Dr. C. van Os en Dr. E. van Corven, afdeling Celfysiologie MF) en als bijvakken Celbiologie (Dr. R. Bridges, Department of Physiology and Biophysics, University of Alabama at Birmingham) en Botanie (Dr. J. Derksen en Dr. A. Rutten).

Van augustus 1990 tot augustus 1994 was hij werkzaam als onderzoeker in opleiding (OIO), in dienst van het NWO, bij de vakgroep Moleculaire Biologie en Celbiologie aan de Katholieke Universiteit Nijmegen. Tijdens deze periode werd door hem onder leiding van Prof. Dr. E.J.J. van Zoelen het in dit proefschrift beschreven onderzoek verricht. Een deel van dit onderzoek (januari-juli 1993) werd uitgevoerd in het laboratorium van Dr. M. Nistér binnen de afdeling Pathologie aan de Universiteit van Uppsala. Ditzelfde laboratorium is tevens de huidige werkplek van de auteur.