PRODUCTION OF ACIDIC AND BASIC FIBROBLAST GROWTH FACTORS AND THEIR RECEPTORS BY NORMAL HUMAN FIBROBLASTS

by

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LIST OF ABBREVIATIONS

FGF	fibroblast growth factor
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
KGF	keratinocyte growth factor
cDNA	complementary deoxyribonucleic acid
IL-1	interleukin-1
PDGF	platelet derived growth factor
EGF	epidermal growth factor
IGF-1	insulin-like growth factor 1
PLCg	phospholipase C gamma
DAG	diacyl glycerol
pM	picomolar
FGFR	fibroblast growth factor receptor
kD	kilodaltons
Ig	immunoglobulin
PCR	polymerase chain reaction
kb	kilobase
bp	basepair
Arg	arginine

Met	methionine
Thr	threonine
Tyr	tyrosine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
K_d	dissociation constant
mRNA	messenger ribonucleic acid
GAP	GTP-ase activating protein
nM	nanomolar
HSPG	heparan sulfate proteoglycan
CHO	chinese hamster ovary cell
pA	polyadenylation
FBS	fetal bovine serum
BSA	bovine serum albumin
BPE	bovine pituitary extract
ng	nanogram
ml	milliliter
mM	millimolar
mg	milligram
mAb	monoclonal antibody
TGFa	transforming growth factor alpha

TCA	trichloroacetic acid
µl	microliter
w/v	weight per volume
v/v	volume per volume
M	molar (moles/liter)
cm	centimeter
V	volts
TBS	tris buffered saline
IgG	immunoglobulin G
cRNA	complementary ribonucleic acid
polyA+	polyadenylated mRNA
ED	effective dose
TTBS	tween-tris buffered saline
Taq	thermus-aquaticus DNA polymerase
dATP	deoxy-adenosine triphosphate
dGTP	deoxy-guanosine triphosphate
dCTP	deoxy-cytidine triphosphate
dTTP	deoxy-thymidine triphosphate
dT	deoxy-thymidine repeats

ABSTRACT

Wound healing is an essential process for the maintenance and survival of an organism. It is a complex process involving both migration and proliferation of skin cells under the influence of paracrine and autocrine signals. These signals include growth factors and extracellular matrix proteins which are present in serum or may be secreted locally by inflammatory cells, immune system cells, or by the skin cells themselves, in particular dermal fibroblasts. Although the precise interactions of the components comprising normal wound healing are not understood, a number of conditions such as immunodeficiency, chemotherapy, venous stasis, and diabetes are known to result in delayed wound healing [48].

Fibroblast growth factors (FGFs) are thought to participate in various phases of wound healing. The fibroblast growth factor family, also known as the heparin-binding growth factor family, consists of nine related heparin binding proteins: acidic FGF (aFGF), basic FGF (bFGF), int-2, hst/kFGF, FGF-5, FGF-6, keratinocyte growth factor (KGF), FGF-8, and FGF-9 [12-19]. FGFs are mitogens for a wide variety of cell types and are involved in other processes such as angiogenesis and development [9, 20].

Cells derived from human skin (keratinocytes, fibroblasts, and melanocytes) proliferate in response to FGFs *in vitro* [41, 6]. aFGF and bFGF differ in their potency for mitogenic responsiveness for each skin cell type, and the addition of heparin along with either aFGF or bFGF can alter the mitogenic potency of the factor for an individual cell type. The response of cells to FGF is mediated by binding and activation of specific cell surface receptors possessing intrinsic tyrosine kinase activity [118].

Currently, there are five known human fibroblast growth factor receptor (FGFR) genes. All of the FGFRs tested thus far are capable of binding multiple members of the FGF family. In addition, multiple forms of some of the FGFR genes have been shown to be generated by alternative mRNA splicing. By combining different coding exons within a

particular FGFR gene, the ligand specificity of the receptor can be altered. FGFs also bind another class of receptors, the so-called low-affinity FGF receptors located in the extracellular matrix and on the cell surface, with a much reduced affinity. These low-affinity receptors are heparan sulfate proteoglycans.

The hypothesis for this thesis is that FGFs and their receptors are involved in the growth regulation of human dermal fibroblasts potentially via an autocrine/paracrine mechanism. In the first part of this thesis, the production of bFGF and aFGF in cultures of normal human dermal fibroblasts grown in a defined, serum-free medium was investigated. Under these conditions multiple molecular weight forms of aFGF and bFGF proteins were detected, and the production of bFGF proteins was shown to be regulated by serum treatment of the cells. In contrast, serum treatment of the cultures had no effect on the level of aFGF proteins. The abundance of bFGF mRNA species increased dramatically after serum treatment of the cells, while the abundance of aFGF mRNA species increased only slightly. Theses observations demonstrate that factor(s) present in serum elevate the levels of bFGF mRNA and protein beyond the levels already present in the cultures grown in serum-free medium. The cells used for this study are also shown to proliferate in response to bFGF and aFGF, suggesting that the growth of these cells may be subject to autocrine/paracrine control in certain conditions.

Following these studies, the mechanism(s) by which aFGF and bFGF mediated mitogenic signal transduction in human dermal fibroblasts was addressed. In the second part of this thesis the FGFR gene expressed in dermal fibroblasts which may be responsible for aFGF- and bFGF-induced mitogenesis is described in detail. Also, the FGFR complement of human skin-derived keratinocytes and melanocytes is described. The results show that dermal fibroblasts produce two forms of FGFR-1 protein, one of which is secreted. The secreted FGFR-1 form appears to be down-regulated by serum treatment of the cells. Finally, sulfation of cell surface molecules is critical in FGF-induced mitogenesis in dermal fibroblasts.

Chapter 1: Introduction

Skin

The skin forms a continuous external surface of the body. In different regions of the body the skin varies in thickness, color, and in the presence of hairs, glands and nails. Despite these variations, which reflect different functional demands, all types of skin have the same basic, two-layer structure. The first layer is an ectoderm-derived layer of stratified, squamous keratinizing epithelium, known as the *epidermis*, which rests on a basement membrane. The second layer is an underlying mesoderm-derived layer of connective tissue, known as the *dermis* [1]. The epidermis is composed mainly of keratinocytes and a small number of pigment-containing melanocytes. The dermis is composed mainly of fibroblasts.

Wound Healing

Tissue injury and blood vessel disruption play a major role in the initial stages of wound repair. Extravasation of blood constituents into a wounded area leads to platelet aggregation, blood coagulation, clot formation, and the generation of bradykinin and complement-derived anaphylatoxins. Bradykinin and complement-derived anaphylatoxins release, in turn, leads to an increase in the permeability of undamaged vessels adjacent to the injured area [2]. These "leaky" vessels release plasma proteins into the wounded area. Activated platelets affect blood coagulation and release a multitude of biologically active substances that promote cell migration and ingrowth to the site of injury [3].

A wound alters cell growth control in skin. In the non-wounded state, keratinocytes in the epidermis are progressively more differentiated the further they are

situated from the basement membrane, and the rate of mitosis of the cells lying on the basement membrane approximates the rate of surface loss. In contrast, the dermis and the melanocytes in the epidermis proliferate at a very low level in the non-wounded state. Upon wounding, however, these parameters change. Normal wound healing is thought to occur in three overlapping phases. First, there is migration of inflammatory cells and dermal fibroblasts into the wound over the first several days. Reepithelialization occurs simultaneously with this first stage. Within hours of a cutaneous injury epithelial cells from the free edge of the tissue begin to cross the defect and the epithelial cells remaining at the original edge of the wound begin to proliferate in order to generate an additional population of migrating cells. Second, over the next 2-3 weeks, there is activation of wound macrophages and fibroblasts, resulting in *de novo* synthesis of growth factors and other cytokines, synthesis of extracellular matrix proteins, and proliferation of fibroblasts. Epithelial cells, after completion of migration, begin to secrete a new basement membrane during the second phase of cutaneous wound repair. Third, remodeling occurs with active collagen turnover and crosslinking from two weeks to one year postwounding [4]. It is clear that dermal fibroblasts play a major role in the process of wound healing.

Skin-derived fibroblasts and keratinocytes in culture mimic the conditions present in a wound. Keratinocytes in a culture dish resemble a basal keratinocyte. A basal keratinocyte is the most immature form of a keratinocyte and is similar to the cell that lies adjacent to the basement membrane *in vivo*. Basal keratinocytes in a culture dish undergo multiple rounds of replication and are able to secrete factors which stimulate their own proliferation until a continuous monolayer is formed [5, 6]. Dermal fibroblasts in a culture dish resemble a wound state in that proliferation occurs until a continuous monolayer is formed, although fibroblasts in culture require exogenous growth factors in their medium because they do not proliferate in an autocrine fashion [6]. As mentioned

previously, dermal fibroblasts in the non-wounded state proliferate and/or migrate at very low levels. However, upon wounding, dermal fibroblasts begin to proliferate and migrate into the wounded region, which is similar to the behavior of fibroblasts in culture. The study of skin-derived cells in culture, therefore offers an excellent *in vitro* model through which to examine mechanisms that may be involved in wound healing.

Fibroblast Growth Factors

FGF Family

The fibroblast growth factors (FGFs) constitute a family of closely related polypeptide mitogens. Currently, nine members of this family have been identified on the basis of amino acid sequence homologies. The FGF family has distinguished itself from other growth factor families by virtue of the pleotrophic actions of its members. FGFs stimulate proliferation in a wide variety of cell types [7]. FGFs also have the capacity to induce differentiation, inhibit differentiation, or maintain a differentiated phenotype of cells in culture, depending on the particular cell type involved (for review see [8]). In addition, these molecules are thought to play an important role during development. Of particular interest has been the discovery that FGFs can function as angiogenic and neurotrophic molecules (reviewed in [9]). These characteristics suggest that FGFs may play a role in wound healing, where ingrowth of blood vessels and regrowth of nerve axons to the lesioned area is critical for complete recovery. Hence, FGFs may have potential therapeutic applications [10, 11]. Conversely, inappropriate FGF stimulation may lead to pathological states.

The FGFs vary in size from 155 to 268 amino acids, share 33-65% amino acid sequence identity, and bind heparin with high affinity. The first members of the FGF family isolated were acidic fibroblast growth factor (aFGF) and basic fibroblast growth

factor (bFGF) [12, 13]. Both factors were purified on the basis of their mitogenicity toward fibroblasts. Since that time, seven additional members have been described, some of which stimulate the proliferation of fibroblasts. Hst/KFGF, int-2 and FGF-5 were identified by their ability to induce cellular transformation [14, 15]. Subsequently, FGF-6 was isolated using low stringency hybridization of cDNA with hst/KFGF [16]. FGF-7 or keratinocyte growth factor (KGF) is mitogenic for fibroblasts only at very high concentrations but was purified and cloned on the basis of its mitogenic activity on keratinocytes [17]. FGF-8 was purified and cloned from an androgen-stimulated mammary carcinoma cell line [18]. Finally, FGF-9 was purified and cloned from glial cells [19]. The complementary deoxyribonucleic acids (cDNAs) encoding FGFs are derived from distinct, single-copy genes, and despite their different chromosomal loci, the FGF genes have similar structural organization: three exons and two large introns (for review see [8]).

Mechanism of Action and Regulation of FGFs

FGFs are found in a wide variety of cultured cells and tissues, both adult and embryonic. aFGF and bFGF were originally purified from bovine brain and have now been isolated in a number of tissues including breast, kidney, reproductive organs, inflammatory tissues, skin and retina (for review see [20]). FGFs as well as other types of growth factors (epidermal growth factor, transforming growth factor, platelet-derived growth factor), have differing cell type specificities, such that some growth factors are mitogens for relatively few cell types while others stimulate a wide variety of cell types [11]. In addition, it has been shown that multiple growth factors are required for maximal stimulation of the majority of non-transformed cells [21]. Moreover, the availability of growth factors at so many sites supports the hypothesis that specific combinations of these factors are intimately involved in tissue homeostasis and repair. In contrast, transformed cells can often be maintained in media supplemented with only a

single growth factor. Although it is still not known whether growth factors induce genotypic malignant transformation *in vivo*, analysis of these data suggest that their inappropriate expression at the site of a tumor might contribute to the generation and maintanence of the transformed phenotype. Unlike the long range, endocrine, actions of hormones like insulin, the effects of FGFs *in vivo* are thought to delivered in a paracrine, or short-range, fashion where the FGF diffuses through cellular spaces and acts on adjacent cells [11]. It has been hypothesized that transformed cells that produce FGF could maintain their own growth via autocrine stimulation [9].

While the predicted amino acid sequences of most FGF family members contain signal peptide sequences, aFGF, bFGF, and FGF-9 do not. In this respect, aFGF, bFGF, and FGF-9 resemble the interleukin-1 (IL-1) related proteins, which also lack signal peptide sequences. Despite a tremendous amount of attention to the mechanism by which aFGF and bFGF may be released from a cell, it remains unclear how aFGF and bFGF are secreted from cells. A small number of cell types have been shown to secrete bFGF [22, 23]. In addition, bFGF has been found in the conditioned media of certain cell types, including human dermal fibroblasts [24, 25]. FGF-9 has been shown to exist exclusively in the conditioned medium of cDNA-transfected COS cells [19]. Presumably, the released FGF is utilizing a secretory pathway independent of the endoplasmic reticulum-Golgi complex. Another possibility to explain the existence of bFGF in conditioned medium of certain cells is that cellular contents are released into the media upon cell wounding and/or cell death.

One would predict that members of the FGF family whose activities are so diverse should maintain a very complex and tight form of regulation. Indeed, the regulation of FGF gene expression occurs on a variety of levels, including transcription, mRNA half-life, post-transcriptional processing, translation, post-translational

processing, and secretion (for review see [26]). Many agents are also known that affect FGF gene expression: serum, active phorbol esters, tumor necrosis factor, IL-1, platelet derived growth factor (PDGF) and cycloheximide have all been shown to cause FGF mRNA accumulation in a variety of cell types [27-29]. Other factors have been shown to reduce FGF mRNA accumulation, including dexamethasone and interferon-gamma [30]. A variety of agents are capable of affecting FGF levels *in vitro*, and, undoubtedly, these agents are utilizing multiple second messenger pathways to obtain such results. It still remains unclear how FGF production, excretion/secretion, and activity is regulated *in vivo*.

Multiple protein forms of aFGF and bFGF have been identified. bFGF has been isolated in forms that are larger than its predicted size [31-33]. Evidence from site-directed mutagenesis studies suggests that these higher molecular weight forms of bFGF arise from non-AUG start codons 5' to the predicted AUG start codon [34]. These non-AUG start codons are capable of producing three additional amino-terminal extensions of the parent 155 amino acid bFGF form. Further, Renko et al. have shown that there is an unequal distribution of bFGF forms in subcellular fractions [35]. Specifically, the larger forms of bFGF were localized to the nucleus and the 155 amino acid form was isolated in the cytosol. In this study, bFGF was not localized to secretory vesicles. The amino-terminal extended forms of bFGF contain nuclear translocation sequences [36]. A nuclear translocation sequence is also present in the aFGF gene at residues +21 to +27, downstream of the initiating methionine [37]. Specific functional roles of these higher molecular weight forms of bFGF have not been fully revealed, but it has now been shown that cells transfected with the higher molecular weight forms of bFGF had impaired growth [38]. In addition to larger molecular weight forms of bFGF, truncated forms of both aFGF and bFGF have been described [8, 39]. Several authors have shown that amino-terminal truncated forms of aFGF and bFGF are the result of specific

proteases such that the 155 amino acid primary translation products of aFGF and bFGF are cleaved in two possible locations [40]. For bFGF, the resultant proteins are 146 and 131 amino acids. For aFGF, the resultant proteins are 140 and 134 amino acids. All amino-terminal truncated forms of aFGF and bFGF have been shown to be active in mitogenic assays [40].

FGFs in Skin-Derived Cells

The proliferation *in vitro* of skin-derived fibroblasts, keratinocytes and melanocytes can be influenced by aFGF and bFGF [6, 41]. Cell-type differences in the mitogenic responsiveness of fibroblasts, keratinocytes, and melanocytes to aFGF and bFGF exist. In addition, the presence of heparan sulfate in combination with either aFGF or bFGF in the culture medium can alter the mitogenic potency of the growth factors on certain cell types. For example, while heparin enhances the mitogenic activity of aFGF on fibroblasts [6], it has no effect (either stimulatory or inhibitory) on the ability of aFGF to stimulate keratinocyte mitogenesis [6, 41]. In contrast ,while bFGF causes proliferation of fibroblasts and melanocytes regardless of the presence of heparin, its proliferative effect on keratinocytes is strongly inhibited by the addition of heparin. It has been hypothesized that heparin modulates aFGF and bFGF activity by facilitating binding to high-affinity receptors, a topic that will be discussed below. While these observations demonstrate the effects of exogenously added FGFs, however, the effects of endogenous FGFs are not clear.

mRNA coding for aFGF and bFGF has been shown to be differentially expressed and regulated in individual cultures of keratinocytes, fibroblasts, and melanocytes [42]. In particular, fibroblasts are shown to express aFGF and bFGF mRNA, but keratinocytes do not. In addition, fibroblast expression of bFGF mRNA is induced by serum treatment of the cultures [28]. Because fibroblasts [6], keratinocytes [6] and melanocytes [43]

proliferate in response to bFGF, and the expression of bFGF in fibroblasts is induced by serum [28], it can be hypothesized that the production of bFGF by dermal fibroblasts may regulate the growth of keratinocytes and melanocytes in a paracrine fashion in a wounded state. Analysis of these data suggest that FGFs play an important role in the growth regulation of human skin, and that they may also contribute to the processes that underlie wound healing.

Therapeutic and Pathological Effects of FGFs

While FGFs are just beginning to be evaluated in various systems, many potential clinical uses are already apparent. One diagnostic use of FGFs may be to predict certain pathologies. For example, it has been shown that the urine of mice with bladder carcinoma contains FGFs, suggesting that detection of FGFs in urine may be of value in diagnosis and prognosis of bladder cancer [44]. FGFs are also of therapeutic interest, based in part on their broad range of target cells. Possible targets of FGF based therapy include tissue repair, immunomodulation, and retarding progression of degenerative and proliferative diseases (for review see [10]).

In particular, bFGF may provide a valuable therapeutic aid to the process of wound healing. Evidence of accelerated wound healing can be demonstrated by an increase in fresh wound tensile strength, wound breaking energy, and evidence of histologic wound maturation [45]. Several studies have shown that direct addition of bFGF to incisional wounds accelerates the wound healing process [46, 47]. Moreover, the addition of bFGF to wounds of diabetic mice has been shown to enhance significantly the degree of dermal healing in diabetic mice when compared to non-healing, impaired mice [48].

The ability of FGFs to promote angiogenesis has both therapeutic and

pathological implications as well. For example, conditions such as ischemic heart disease and diabetes could potentially benefit from FGF-induced neovascularization. Recently, Yanagisawa et al. documented improvement in the myocardial function of rats with acute coronary artery occlusion following injections of bFGF when compared to untreated rats [49]. Some regions of the vasculature in diabetic patients has been shown to contain alterations in basement membrane glycosaminoglycans, heparin-like molecules. These alterations in the basement membranes, which may affect bFGF binding and activity, are believed to be due to the effects of long-term hyperglycemia [50]. Conversely, the location of FGFs in certain tumors suggest that FGFs may influence tumor growth via angiogenesis [8, 11]. The observation that overexpression of some FGFs is associated with transformation further suggests that they have oncogenic potential [51, 52]. In this regard, FGF antagonists may prevent angiogenesis and thereby control tumor development, and may also, retard the progression of benign proliferative disorders like psoriasis. bFGF coupled to the cellular toxin, saporin, has been shown to decrease the expression of this growth factor in certain tumors [53]. FGFs also exhibit neurotrophic activities, and therefore, are candidates for use in treatment of neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases [54]. bFGF has been localized to senile plaques in Alzheimer's disease [55]. Lastly, the fact that bFGF injections can rescue photoreceptors destined to degenerate as a result of inherited retinal dystrophy points to the potential therapeutic use of the protein in this condition [56].

Tyrosine Kinase Receptors

Certain growth factors including; FGF, EGF, insulin, PDGF, and CSF mediate their pleotrophic actions by binding and activating cell surface receptors with tyrosine kinase activity. The tyrosine kinase receptor family contains four subclasses: (1) EGF-

like receptors; (2) insulin-like receptors; (3) PDGF-like receptors; and (4) FGF-like receptors. All possess large extracellular ligand binding domains, a single hydrophobic transmembrane domain, and a intracellular tyrosine kinase catalytic domain. Previous work with the EGF receptor, PDGF receptor, insulin receptor, and the CSF-1 receptor (the CSF-1 receptor belongs to the class of FGFR in the tyrosine kinase receptor family) has shown that kinase activity is critical for signal transduction, proper ligand-induced intracellular trafficking of the receptor, mitogenesis, and cellular transformation (for review see [57, 58]). In addition, tyrosine kinase receptors undergo ligand-induced dimerization which enhances kinase activity and ligand binding affinity [59].

Ligand activation of tyrosine kinase receptors triggers a variety of cellular responses which include; Ca^{+2} influx, activation of phospholipase C-gamma 1 (PLC-γ1), increased intracellular pH, and activation of glucose and amino acid transporters (for review see [58]). The phosphatidylinositol signaling pathway has been implicated as one the potential second messenger systems utilized by tyrosine kinase receptors [60]. A summary of a potential mechanism by which tyrosine kinase receptors may activate the phosphatidylinositol signaling pathway is as follows, first there is stimulation of PLC-γ1 leads to phosphatidylinositol metabolites, second, there is a release of Ca^{+2} from intracellular compartments and the generation of diacylglycerol (DAG), third, DAG activates protein kinase C which leads to the phosphorylation of a multiple cytoplasmic substrates. It is postulated that the activation/deactivation of these phosphorylated cytoplasmic substrates in combination with alterations in cellular ion concentrations, provides the stimulus for cell growth. However, the exact mechanism(s) by which a cell proliferates is unknown.

In addition to the phosphatidylinositol signaling pathway, some tyrosine kinase receptors have been shown to be linked to of other signaling systems including;

phosphatidylinositol 3-kinase , *ras* GTPase-activating protein, and *raf* (for review see [58, 61]). The subsequent activation of MAP kinase by the later two substrates mentioned above, has been shown to lead to increased levels of certain transcription factors [61], which may lead to a particular cellular response.

Fibroblast Growth Factor Receptors

Cellular responses to FGFs are mediated by binding and activation of specific cell surface receptors that possess intrinsic tyrosine kinase activity [58]. Early characterization of FGF receptors focused on binding and cross-linking studies using radiolabeled aFGF and bFGF. These studies showed that many cell types express saturable high-affinity receptors with binding constants of 50-500 pM for aFGF [20] and 10-200 pM for bFGF [62, 63]. Competition studies revealed that either ligand was able to compete for high-affinity binding of the other ligand [63, 64]. In addition, either ligand was shown to block cross-linking of the same or different radiolabeled ligand to receptor proteins. A synthesis of these early results strongly supports the hypothesis that different FGF family members share a common receptor.

Nomenclature

Since the isolation of the first complete FGF receptor cDNA in 1989 [65], the complexity of the fibroblast growth factor receptor family (FGFR) has increased dramatically. Five distinct FGFR cDNAs have been cloned to date, and are all members of the tyrosine kinase receptor family. Furthermore, in the case of at least two of these genes it is clear that alternative splicing gives rise to multiple forms of the receptor [66, 67]. A variety of names have been assigned to the individual FGFRs. Table 1 was constructed in an effort to simplify this nomenclature. Throughout the remainder of this dissertation the different FGFR genes will be referred to as FGFR-1, FGFR-2, FGFR-3,

FGFR-4 and FGFR-5.

FGF RECEPTORS

NAME	OTHER NAMES	LIGANDS
FGFR-1	*Flg* cek-1	bFGF aFGF K-FGF/hst
FGFR-2 FGFR-2b	KGF Receptor K-Sam	aFGF KGF
FGFR-2c	*Bek* cek-3 TK14	bFGF aFGF K-FGF/hst
FGFR-3	cek-2 JTK4	bFGF aFGF
FGFR-4	JTK2	aFGF bFGF
FGFR-5	*flg-2*	?

Table 1: FGFR nomenclature and ligands. The table shows some of the names that have been used to describe the different FGFR genes and cDNAs from alternatively spliced FGFR mRNA transcripts [65, 67, 68, 71, 77, 78, 79, 81, 82]. FGFR-2b and FGFR-2 indicate specific splice variants of this gene (see text).

Characterization of FGFRs

The first full-length FGFR to be characterized in detail, FGFR-1, was purified from chicken embryos [65]. Sequence information from this cDNA revealed a high similarity with two previous partial cDNA clones: human *flg* [68] and mouse *bek* [69].

The human *flg* cDNA encoding a fms-like gene was originally isolated by low-stringency screening of a human endothelial cell cDNA library with a probe consisting of the kinase domain of *v-fms*. The mouse *bek* (bacterially expressed kinase) cDNA has been isolated from a cDNA expression library by probing with antiphosphotyrosine antibodies. Although the function of *flg* and *bek* was not known at the time, it has since been shown that the full-length *flg* and *bek* cDNA clones represent specific splice variants of the FGFR-1 and FGFR-2 genes, respectively.

The chicken FGFR-1 encodes a protein which has a core protein size of 92 kiloDaltons (kD) and contains several features commonly found in growth factor receptors (see Figure 1). The protein is characterized by an amino-terminal signal sequence, three extracellular immunoglobulin domains, a single membrane-spanning region, and an intracellular split tyrosine kinase domain. Between the first (I) and second (II) immunoglobulin (Ig) domains there is a unique domain that is not seen in other growth factor receptors called the "acidic box". In FGFR-1, this domain consists of eight consecutive acidic amino acids.

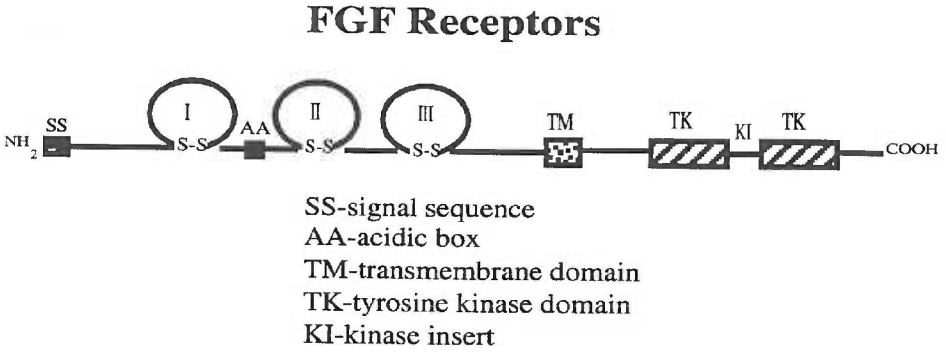


Figure 1

FGFR-1 cDNAs were subsequently isolated from a variety of species including human [67, 70, 71], mouse [72-74], and Xenopus [75]. The human FGFR-1 gene has

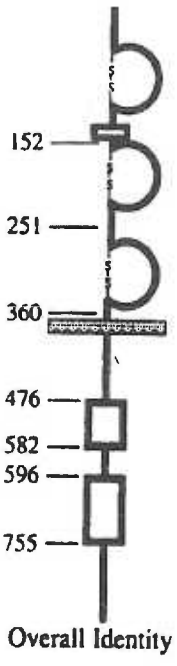
been mapped to chromosome 8p12 [68]. In general, when compared to the human FGFR-1 protein, the mouse, chicken, and Xenopus FGFR-1 proteins are 98, 91 and 78% identical, respectively. The most highly conserved regions of the receptor molecule are the tyrosine kinase domains, kinase I and kinase 2. The least conserved areas are the the signal peptide region, Ig domain I, the membrane proximal region, the transmembrane region, and the kinase insert domain.

Following the isolation of the chicken FGFR-1 cDNA, cDNAs derived from four additional FGFR genes were isolated and characterized by several laboratories. The proteins encoded by these genes are structurally similar to FGFR-1 and are highly conserved at the amino acid level. Comparison of the FGFR-1 sequence and the partial sequence of mouse *bek* revealed several differences at the amino acid and nucleotide levels. Oligonucleotides based on the mouse partial *bek* sequence were used to isolate full-length clones of human *bek* from a brainstem cDNA library [71] which led to the discovery that *bek* was in fact the product of a different gene, referred to as FGFR-2. cDNA clones of FGFR-2 have now been described from a variety of human sources [66, 76-78] , as well as from mouse [69] and chicken [79]. The human FGFR-2 gene has been mapped to the long arm of chromosome 10 [80].

FGFR-3 was obtained by low stringency screening of a human K-562 (chronic myelogenous leukemia cell line) cDNA library with a *v-sea* oncogene probe [81]. Like FGFR-1 and FGFR-2, FGFR-3 cDNAs have also been isolated in chicken [79]. Human cDNA clones derived from a fourth FGFR gene, FGFR-4, were isolated using the polymerase chain reaction (PCR) with tyrosine kinase specific primers followed by library screening with the amplified fragments [82]. The cDNA library used for screening was derived from K-562 cells. To date, FGFR-4 has not been identified in other species. Human FGFR-5, *flg*-2, was cloned from a human keratinocyte cDNA

library by screening with a mouse *flg* probe [83]. As with FGFR-4, FGFR-5 has not yet been identified in other species. FGFR-5 is highly homologous to FGFR-3, sharing 92% overall amino acid sequence identity.

In general, the proteins encoded by the five different FGFR genes are strikingly similar. The most closely related proteins are FGFR-1 and FGFR-2 (72 % amino acid identity), whereas FGFR-1 and FGFR-4 are the least related (55% amino acid identity). A comparison of the domains of different human FGFRs (see Figure 2) reveals a pattern of conservation similar to that observed when comparing FGFR-1 domains across species. The most highly conserved regions of different human proteins are the tyrosine kinase 1 and tyrosine kinase 2 domains, 75% and 84% identity, respectively between FGFR-1 and FGFR-2. The least conserved regions are the signal peptide, immunoglobulin domain I, the membrane proximal region, the transmembrane domain, and the kinase insert domain.



Flg vs Bek	Flg vs FGFR3	Flg vs FGFR4	Flg vs Flg-2	Bek vs FGFR3	Bek vs FGFR4	Bek vs Flg-2	FGFR3 vs FGFR4	FGFR3 vs Flg-2	FGFR4 vs Flg-2
38	21	22	22	27	21	26	26	78	24
81	66	63	67	72	70	71	78	98	77
79	82	74	81	81	72	80	74	98	72
66	41	32	41	45	31	46	34	92	32
88	83	75	82	87	78	88	78	97	78
50	43	14	43	50	29	57	36	79	43
92	91	86	90	92	84	91	86	99	85
60	44	38	45	56	48	56	46	90	73
71	61	56	61	65	57	65	60	92	60

Figure 2: A schematic diagram of the three Ig domain form of FGFR is shown with regional comparisons to the individual FGFR genes. The numbers at the left of the diagram represent the FGFR-1 amino acids used to demarcate boundaries between domains. The numbers at the right of the diagram represent percent amino acid seqeunce identity in the specific regions [92].

Multiple Forms of the FGFRs Arise by Alternative Splicing

An important development in the FGFR field has been the identification of different cDNAs encoding multiple forms of FGFR-1 and FGFR-2 [66, 71, 74, 76, 84]. The genomic structure of human FGFR-1 has been determined [84] and has revealed the basis for the expression of many of the variant isoforms of FGFR-1. This gene can be spliced in a variety of ways, including: (1) choosing between two homologous exons (*mutually exclusive splicing*); (2) inclusion/exclusion of entire coding exons (*cassette splicing*); (3) alternative use of multiple *polyadenylation sites*; and (4) splice site

"skidding" where multiple intron/exon splice consensus sequences are present at a particular site resulting in inclusion/exclusion of a small number of amino acids (see [85, 86] for review). The resultant proteins are structurally distinct.

Analysis of preliminary data has recently indicated that the human FGFR-2 gene is similarly organized to the FGFR-1 gene [66], suggesting that similar patterns of alternative splicing may also contribute to the diversity of FGFR-2. Although the 5' region has not been fully characterized, the FGFR-1 gene consists of 19 exons that span approximately 20 kilobases (kb). Specific domains within the FGFR-1 protein are contained within individual exons (see Figure 3). For example the signal peptide, the acidic box, and the transmembrane domain are each encoded by a separate exon. The extracellular portion is encoded by nine exons. Ig domain I is encoded by a single exon (#3) while Ig domains II and III are each encoded by two exons. Interestingly, the amino-terminal half of Ig domain III is encoded by one exon, the "constant" exon, while the exon encoding the carboxy-terminal half of Ig domain III is triplicated (exons IIIa, IIIb and IIIc) and diversified, the "variable" exon. As seen in Figure 4, FGFR-1 exon IIIa encodes a putative secreted receptor (Figure 4-ii). FGFR-1 exon IIIb and FGFR-1 exon IIIc encode membrane-bound receptors (Figure 4-iii, iv, v, vi). When this analysis was extended to FGFR-2, the same exon arrangement was identified in this region of the gene.

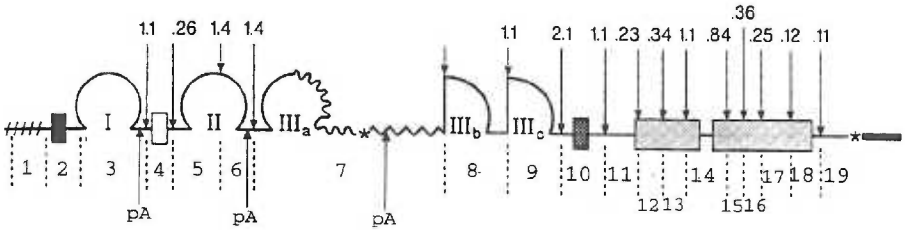


Figure 3: Structure and organization of the human FGFR-1 gene. Arrows and dashed lines indicate the positions of intron sequences. Numbers above the arrows indicate the size of the intron in kilobases. Numbers between the dashed lines indicate the assigned exon numbers. The following structural domains are identified: 5' nontranslated sequence (cross-hatched line), signal sequence (solid square), acidic box (open square), sequences encoding the divergent amino acid sequence which is specific for the secreted receptor (wavy line), transcribed sequences which become the 3' nontranslated sequence unique to mRNA transcripts encoding the secreted receptor form (zigzag line), stop codons (*), and transcribed sequences which become the 3' nontranslated sequence specific for transcripts encoding membrane-bound receptor forms (thick black line). The three alternative coding sequences for the carboxy-half of Ig domain III are indicated as IIIa, IIIb, and IIIc. Potential polyadenylation sites are indicated (pA) (modified [84]).

The use of *mutually exclusive* exons generates multiple forms of FGFR-1. The choice between Ig domain exon IIIa, exon IIIb, or exon IIIc in the production of a mature FGFR molecule has been shown to occur for both the FGFR-1 gene and the FGFR-2 gene [76, 84]. It was subsequently found that the exon that encodes the carboxy-terminal half of Ig domain III dictates ligand binding specificity [87]. This form of mutually exclusive alternative mRNA splicing represents a novel mechanism for generating receptor diversity and ligand-binding specificity by alternative splicing of homologous exons, encoding a confined amino acid sequence at the carboxy-terminal half of Ig domain III. Another form of FGFR-1 cDNA is generated by a similar mechanism, has now been isolated from human hepatoma cells [70]. This form of

FGFR-1 contains an alternative exon in the position of Ig domain I. The alternative exon comprises a unique 144 basepairs (bp) substitution in place of the 267 bp sequence that encodes Ig domain I. The 144 bp insertion encodes a translational stop sequence. As a consequence, another (internal) ATG can be utilized as a translational initiation site which is downstream from the acidic box but precedes Ig domain II. This putative form of FGFR-1 exhibits no apparent signal sequence for membrane translocation, and therefore suggests an intracellular form of the receptor (Figure 4-viii). Thus, the complexity of the FGFR family is increased by the possibility of the generation of multiple distinct receptors, through the use of mutually exclusive exons.

Cassette splicing of mRNA occurs in the FGFR genes. In addition to the alternative splicing mechanism that operates in the choice between Ig domain III exons IIIa, IIIb and IIIc, another event of alternative splicing leads to the expression of FGFR-1 molecules that are missing Ig domain I [74, 88] (Figure 4-iv, vi, xi, xii). In cross-linking studies, the FGFR was initially described as two protein bands migrating at 125 and 146 kD [63, 64, 89]. More recently, the identification of FGFR protein isoforms that contain either two or three Ig domains has clarified the significance of these early results. Using cell lines transfected with cDNAs that encode either two or three Ig domain forms of FGFR-1, it has been shown that when FGFR-1 containing two Ig domains is cross-linked with ligand, a 125 kD protein is obtained, and when FGFR-1 containing three Ig domains is cross-linked to ligand, a 145 kD protein is obtained [71, 73]. The function of Ig domain I is not known, as it has been found that that the exclusion of this domain does not effect the binding specificity of the receptor [67]. Nevertheless these results demonstrate that cassette splicing, by which the exon that encodes Ig domain I is excluded, contributes to the molecular diversity of FGFR-1.

An additional genetic mechanism that was found to generate variants of FGFR-1

is the *alternative use of different polyadenylation sites*. As seen in Figure 3, the FGFR-1 gene contains three internal polyadenylation sites. Johnson et al. described the cloning of a FGFR-1 cDNA from human astrocytoma cells which encodes a putative secreted FGFR-1 protein (there is no actual protein data) that would contain the entire extracellular portion of the receptor molecule extending through Ig domain II, containing only a small area resembling Ig domain III and stopping prior to the transmembrane region [84] . This cDNA was generated by the inclusion of exon IIIa described above, which encodes 79 amino acids followed by a stop codon and polyadenylation site (Figure 4-ii) [84]. Another potentially secreted form of FGFR-1 (no protein data shown) was isolated from a human lung fibroblast cell line that encodes only the signal peptide and Ig domain I followed by 32 unique amino acids, a stop codon and polyadenylation site [90] (Figure 4-i). The 32 unique amino acids in this putative molecule are derived from a separate exon located between the exon encoding Ig domain I and the exon encoding the acidic box.

Splice site "*skidding*" also generates variant forms of FGFR-1. In the FGFR-1 gene, this process generates a cDNA molecule that would encode a normal FGFR-1 amino acid sequence up to the kinase 2 domain, followed by 44 unusual amino acids, presumably derived from intronic sequences [70] (Figure 4-vii). Although this putative FGFR-1 form contains sufficient structural information for ligand binding, the altered cytoplasmic domain would not possess tyrosine kinase activity. While this putative FGFR-1 forms is apparently incapable of transducing signals in response to ligand binding, it has recently been shown to decrease signalling by participating in receptor oligomerization with kinase-competent FGFRs [91]. Many investigators have also described splice site skidding to occur at the 3' end of the exon encoding the acidic box. The resultant receptor either includes or excludes two residues, arginine-methionine (Arg-Met) [84, 90]. The functional significance of this subtle variation in receptor

structure is not known. Lastly, splice site skidding in the FGFR-1 gene at the 3' end of the exon (encoding the transmembrane domain) generates a receptor molecule that includes or excludes two residues, threonine-valine (Thr-Val) [66, 77, 78]. This deletion may also have consequences for signal transduction, should either ligand affinity or tyrosine kinase activity be regulated by phosphorylation of this site by a serine-threonine kinase, as has been shown for the EGF receptor [92].

FGFR-2 gene organization is similar to that of FGFR-1, and a variety of forms of FGFR-2 have been described [66, 76-78, 88, 93]. Like FGFR-1, the variant forms of FGFR-2 are generated from the same mechanisms of alternative mRNA splicing. Some of the FGFR-2 variants are analogous to those of FGFR-1. For example, FGFR-2 can include either two or three immunoglobulin domains [66] (Figure 4-x, xi, xii, xiv). The FGFR-2 gene also encodes three alternative exons for the carboxy-terminal half of Ig domain III. Thus, FGFR-2 IIIa (denotes a FGFR-2 molecule encoding the IIIa exon of the carboxy-half of Ig domain III) encodes a putative secreted receptor consisting only of the extracellular domain (Figure 4-ix); FGFR-2 IIIb and FGFR-2 IIIc encode membrane-bound receptors (Figure 4-x, xi, xii, xiii). In addition, FGFR-2 cDNAs have been isolated that contain the Thr-Val deletion at the 3' end of the exon encoding the transmembrane region, as has been described with FGFR-1 [66, 77, 78].

However, some of the variant forms of FGFR-2 do not have FGFR-1 counterparts. For example, a putative FGFR-2 variant exists with a truncation in the carboxy-terminus which is 57 residues shorter than the intact FGFR-2 (Figure 4-xiv). The truncated form still contains tyrosine #766 (Tyr 766) and Tyr 776, which are candidates for self-phosphorylation [66]. A FGFR-2 variant that lacks only the exon encoding the acidic box has also been described [76] (Figure 4-xi). This variant has been shown to bind ligands, suggesting the acidic box is not necessary for ligand binding.

Three additional forms of FGFR-2, that are putatively secreted, have been reported that do not have FGFR-1 counterparts. One of these potentially secreted forms encodes only a signal sequence, Ig domain I, and acidic box [94] (Figure 4-xv). A second putative secreted FGFR-2 variant has been described that also contains Ig domain II [93] (Figure 4-xvi). This form is generated utilizing a polyadenylation signal located in intronic sequences between Ig domains II and III. The final putative secreted form of FGFR-2 that does not have an FGFR-1 counterpart was described as containing the full-length receptor with a deletion in the carboxy-terminal half of Ig domain III and the transmembrane region; however the data for this cDNA isolate or protein was not shown [93] (Figure 4-xvii). No intracellular form of FGFR-2 has been described.

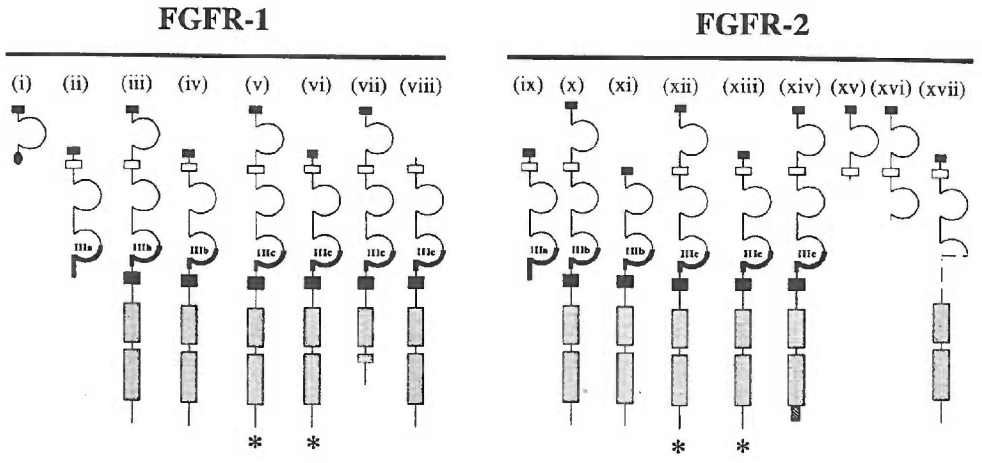


Figure 4: Schematic diagram of FGFR protein structures. The figure shows the structure of variant receptor forms predicted by published cDNAs. The following structural features are identified in the figure: the 32 unique amino acids at the carboxy-terminus of the FGFR-1 IIIa secreted form (solid oval), acidic box (open square), alternative sequences for the carboxy-half of Ig domain III labeled IIIa, IIIb, or IIIc (thick black line), transmembrane domain (solid square), kinase 1 and kinase 2 domains (stippled boxes), and the unique carboxy-terminus domain of FGFR-2 (striped box) (modified [105]). The * indicates forms in which naturally occurring protein has been isolated. References for the individual forms are noted in the text.

The FGFR-3 gene has been recently shown to encode alternative exons in the region encoding Ig domain III, analogous to the FGFR-1 and FGFR-2 genes [95]. Currently, only single cDNAs have been reported for FGFR-4 and FGFR-5. In each case, the cloned cDNAs encode three Ig domain forms containing exon IIIc type sequences in the third Ig domain.

Besides the role of alternative splicing in the generation of variant forms of FGFR, numerous molecular sizes for three Ig domain forms of FGFR-1 and FGFR-2 have been reported in a variety of cell types. The predicted core protein size of the three Ig domain form of either FGFR-1 or FGFR-2 is approximately 92 kD, yet these molecules have been shown to migrate at a variety of positions between 100-150 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two studies utilizing various deglycosylation techniques have attributed this variation in size to cellular glycosylations of a single form of FGFR [71, 96]. For example, full-length FGFR-1 contains seven potential N-linked glycosylation sites. Glycosylation of each site can add 1-7 kD of carbohydrate to the mature FGFR [92].

In summary, Figure 4 illustrates the putative protein structures FGFR-1 and FGFR-2 variants thus described in the literature. It is important to note that while all of the cDNAs listed in Figure 4 may give rise to protein forms in transfected cell lines, only the full-length receptors containing two- and three- Ig domains have been shown to generate naturally occurring proteins (denoted by * on Figure 4). To date, no evidence for naturally occurring secreted and intracellular forms of either receptor have been found.

Binding

Prior to cloning the FGFR cDNAS, it was unclear whether multiple forms of the

receptor, if they existed, would exhibit different ligand binding specificities. Binding studies on cells transfected with cloned FGFR cDNAs have now provided answers to this question. Initial studies demonstrated that three Ig and two Ig domain FGFR-1 forms containing exon IIIc bind either aFGF (K_d=20-80 pM) or bFGF (K_d=50-150 pM) with high affinity [71]. Additional experiments have shown that both receptor forms also bind hst/KFGF with reduced affinity, compared to the binding with aFGF and bFGF [71, 73]. Taken together these experiments make two important points: (1) multiple members of the FGF family can bind to the same receptor; and (2) Ig domain I is not essential for binding. As with FGFR-1, FGFR-2 has shown a similar binding pattern with aFGF (K_d=40-100 pM), bFGF (K_d=80-150 pM) and hst/KFGF [71, 77, 87]. Unlike FGFR-1 and FGFR-2, which bind both aFGF and bFGF, FGFR-3 binds preferentially aFGF and showed approximately 20-fold lower affinity for bFGF [97]. FGFR-4, like FGFR-1 and FGFR-2, binds aFGF (K_d=10-15 pM) and bFGF (K_d=120 pM) [98]. FGFR-4, which exists in only a single cDNA so far, does not bind KGF [98]. The ligand binding characteristics of FGFR-5 are presently unknown but may underlie the sensitivity of keratinocytes to aFGF and KGF but not bFGF [17].

These results suggest a complex picture of overlapping binding specificities of various FGFs to their receptors and raise interest in the structure of the ligand-binding site of FGFR. This issue was addressed by the recent cloning of FGFR-2 IIIb (KGF receptor). The receptor encoding the FGFR-2 IIIb bound KGF and aFGF with equally high affinity [87]. However, bFGF showed a 20-fold reduction in affinity to this receptor [76, 87]. Comparison of the cDNA sequences of FGFR-2 IIIc and FGFR-2 IIIb (KGF receptor) revealed that these receptors arise from the same gene. FGFR-2 IIIb, however, differed from FGFR-2 IIIc in the region encoding the carboxy-terminal half of Ig domain III. FGFR-2 IIIb encoded the IIIb exon in this region and FGFR-2 encoded the IIIc exon in this region. Yayon et al. [99] showed conclusively that the carboxy-terminal half of Ig

domain III is a major element in determining FGFR binding specificity by generating chimeric FGFR molecules. By replacing the region of FGFR-2 IIIc with the sequence of the KGF receptor, exon IIIb, he demonstrated that the resulting chimera gained the binding profile of FGFR-2 IIIb. This chimera was not altered in its ability to bind aFGF. Thus, the exon encoding the carboxy-terminal half of Ig domain III determines ligand specificity.

Further binding studies utilizing FGFR-1 and FGFR-2 proteins led to the following summary: (1) FGFRs containing sequences encoding exon IIIb bind aFGF and KGF with high affinity, and bind bFGF with much reduced affinity [72, 87]; (2) FGFRs containing sequences encoding exon IIIc bind aFGF and bFGF with high affinity but bind KGF with a much reduced affinity [71, 72, 84]; (3) FGFR-1 containing sequences encoding exon IIIa, a putative secreted receptor form, binds both aFGF and bFGF with high affinity [72, 100]. This has not yet been shown for FGFR-2 IIIa. (4) Both Ig domain I and the acidic box are dispensable for high affinity binding of ligand [67, 101]. Although these results suggest that the information encoded in the latter half of Ig domain III dictate ligand binding, other regions of the receptor molecule have been shown to contribute to the formation of an intact ligand binding site. Most notably, Hou et al. have shown that mutation of Ig domain II can disrupt binding to an otherwise intact receptor, suggesting that this region of the receptor molecule is important in ligand binding, as well [102].

Differential Cell and Tissue Expression of FGFRs

Several studies have documented the differential expression of specific FGFR genes in a variety of tissues. These studies have utilized the techniques of northern blot, RNase protection assays, and *in situ* hybridizations. In general, FGFR-1 and FGFR-2 genes exhibit broad but distinct patterns of expression during development and in adult

animals. However, FGFR-3 and FGFR-4 genes appear to have more restricted patterns of expression. For example, the expression of FGFR-1 as determined in human fetal tissue was found to be present in brain, skin and bone growth plates [82]. FGFR-2 expression in the same study was seen in choroid plexus, skin, lung, kidney, and temporal lobe. In contrast, FGFR-3 expression was detected in intestine, lung, kidney, and bone growth plates, while FGFR-4 expression was highest in adrenal glands [82]. Although both FGFR-1 and FGFR-2 are expressed in the brain, FGFR-1 was found to be preferentially expressed in neuronal populations, whereas FGFR-2 was expressed mainly in glia [103]. No information exists on the differential expression of FGFR-5.

The FGFRs also appear to be developmentally regulated. Two studies in mouse embryos using *in situ* hybridization clearly demonstrated the differential expression of FGFR-1 and FGFR-2 genes. FGFR-1 was expressed in the mesenchyme of limb buds, somites and organ rudiments. FGFR-2 was expressed predominantly in epithelial cells of skin and internal developing organs [103, 104]. Thus, it appears that FGFR-1 is the preferred FGFR of mesenchymal cells and FGFR-2 is the preferred FGFR of epithelial cells.

It is important to note that although the studies described above delineate expression patterns of the different FGFR genes, they do not provide information on expression of specific alternatively spliced messenger ribonucleic acids (mRNAs). A variety of studies in cell culture have been performed to address cell- and tissue-specific alternative splicing of FGFR mRNAs. Johnson et al. have shown that several human cell lines simultaneously express transcripts containing of FGFR-1 IIIa, FGFR-1 IIIb, and FGFR-1 IIIc [67]. Some cells lines examined in this study, however, expressed only the IIIc exon form of FGFR-1. Werner et al. have demonstrated differential expression of the IIIa, IIIb and IIIc exons of FGFR-1 in mouse tissues [105]. The IIIc exon was shown

to be expressed in all tissues examined with the exception of the liver, the IIIa and IIIb exons exhibited more restricted patterns of expression. In contrast to FGFR-1, the incorporation of either exon IIIb or exon IIIc in FGFR-2 appears to be mutually exclusive. Exon IIIb of FGFR-2 appears to be exclusively utilized in a variety of epithelial-derived cells, consistent with the sensitivity of these cells to aFGF and KGF [106]. Another example of tissue-specific alternative splicing involves Ig domain I. Analysis of RNase protection assays shows that the three Ig domain form of FGFR-1 is the predominant receptor form expressed during mouse embryogenesis [74]. The two Ig domain form of FGFR-1 is not detected until after birth when both two and three Ig domain forms of FGFR-1 coexist in a number of different tissues.

Signal Transduction and the FGFRs

Binding of ligand to the extracellular domain of receptor tyrosine kinases, such as the FGFRs, induces oligomerization of receptors. This results in increased interaction of cytoplasmic kinase domains and receptor autophosphorylation [58]. Generally, treatment of cells with FGF leads to a variety of events, including increased intracellular pH [107], increased intracellular Ca^{2+} levels [107], increased phosphorylation of cellular proteins [108], and increased transcription of some cellular genes like c-myc and c-fos [109]. Depending on the cell type involved, these events can result in cell proliferation, chemotaxis, differentiation, inhibition of differentiation, or maintenance of a differentiated phenotype. However, the specific signaling mechanisms that give rise to these results are largely unknown.

Receptor oligomerization is thought to be a key point in ligand-induced signal transduction. Homodimeric and heterodimeric complexes between FGFR-1, FGFR-2 and FGFR-3 proteins can be formed following exposure to ligand [57, 110]. These dimeric species are then capable of transducing a signal within the cell. Evidence for

receptor oligomerization is derived from two separate types of experiments. First, cells that were cotransfected with kinase-competent FGFR-1 and kinase- defective FGFR-2 genes were shown to contain tyrosine phosphorylated FGFR-1 and FGFR-2 proteins in a ligand-dependent fashion. Tyrosine phosphorylation of the kinase-defective FGFR was dependent upon the coexpression of the kinase-competent FGFR, as no tyrosine phosphorylation of either kinase-defective receptor was observed in singly transfected cells [57]. Second, when a non-functional mutant of Xenopus FGFR-1 was created by deletion of the cytoplasmic domain and co-injected into oocytes with wild-type Xenopus FGFR-1, the functional response of the normal FGFR-1 was greatly reduced [110, 111]. The results of these types of experiments help illustrate the dominant-negative affect of the coexpression of a non-functional receptor with a wild-type receptor. Analysis of these results suggest that FGFRs are phosphorylated by a trans-mechanism, and provide strong evidence for the possibility that receptor heterodimers from different FGFR genes may form in response to a single ligand which binds both receptors.

As mentioned above, increased tyrosine phosphorylation of a number of cellular proteins occurs following FGF stimulation of cells. A variety of substrates are known to associate with receptor tyrosine kinases. These include phospholipase C-gamma 1 (PLC-γ1), $p21^{ras}$ GTPase-activating protein (GAP), and the putative regulatory subunit of phosphatidylinositol (PI) 3'-kinase (p85) (for review see [92]). Currently, only PLC-γ1 has been identified as a candidate substrate of an FGFR [91, 112]. PLC-γ1 is phosphorylated on tyrosine residues following FGF stimulation, and direct association with FGFR has been demonstrated. A 28 amino acid peptide derived from the most 3' region of FGFR-1 has been shown to bind to the SH2 domain of PLC-γ1 [113]. This region of FGFR-1 contains two potentially phosphorylated tyrosines (positions #653 and #766) [91, 114]. Mutation of Tyr #766 to phenylalanine (Phe) (Tyr/Phe^{766} FGFR-1) generates a receptor protein that does not associate with or phosphorylate PLC-γ1 [114].

It also does not mediate FGF-dependent phosphatidylinositol turnover [114]. Interestingly, cells expressing the Tyr/Phe^{766} FGFR-1 mutant proliferate in response to FGF, suggesting that PLC-γ1 may not be important for pathways leading to FGF-induced mitogenesis. As mentioned previously, FGFR-1 variants exist that contain a C-terminus truncation and do not encode Tyr #653 or Tyr #766 (see Figure 4-vii). This truncated FGFR-1 was recently shown to act as a dominant-negative suppressor of ligand-induced stimulated proliferation in a prostate carcinoma cell line [91]. The potential involvement of other signaling molecules remains untested.

Low-Affinity FGF Binding Sites

A unique feature of FGFs is their strong affinity for the glycosaminoglycan heparin [7]. Glycosaminoglycans are large carbohydrates that are composed of repeating disaccharide units and exist in four main forms: (1) heparin and heparan sulfate; (2) dermatan sulfate and chondroitin sulfate; (3) keratin sulfate and (4) hyaluronic acid (for review see[115]). As detailed above, the FGFR family binds ligand with affinities in the range of 10-150 pM, thus representing high-affinity binding sites for FGF. A lower affinity (K_d = 2 nM), large capacity class of binding sites for FGF are made up of heparan sulfate proteoglycans, HSPGs [62].

The strong affinity of aFGF and bFGF for heparin, along with the observation that many cell types possess heparin-like molecules on their cell surfaces and in their extracellular matrix, suggests a physiological role for heparin-like, low-affinity FGF binding sites. Evidence for this role was obtained by the generation of mutant cells that do not express HSPGs. Yayon et al. showed that CHO cells expressing cell surface HSPGs and high-affinity FGFR bound bFGF. However, CHO cells deficient in HSPGs but expressing high-affinity FGFR did not bind bFGF [116]. The authors concluded that the low-affinity receptor, HSPG, is an accessory molecule required for binding of bFGF

to the high-affinity site. An "induced fit" model of the different possible cooperative interactions of low-affinity HSPGs and high-affinity FGFRs for bFGF is presented in Figure 5 [116]. The same authors proposed a model of FGF-FGFR binding as a trimolecular complex between ligand, receptor, and heparin. Most recently, Kan et al. showed that heparin interacts independently of FGF with a specific sequence, a "heparin-binding" sequence, on FGFR-1 located in the 5' end of Ig domain II [117]. Subsequent analysis of the conditions required for high-affinity binding of bFGF to soluble recombinant receptors showed soluble FGFR bind ligand only in the presence of heparin [118]. Similarly, the ability to chemically crosslink labeled aFGF and bFGF to FGFR-1 transfected FDC-P1 cells, which produce no extracellular matrix, requires the addition of heparin [119]. Finally, when low-affinity sites were biochemically altered in Swiss 3T3 cells by treatment with chlorate, an inhibitor of the enzyme adenyltransferase (which is required for the sulfation of glycosaminoglycans), both an inhibition of FGF binding and a decrease in bFGF-mediated mitogenesis were noted [120].

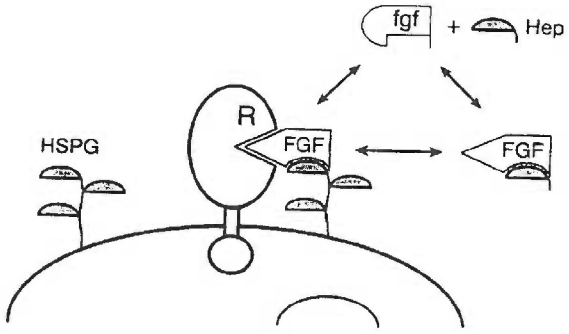


Figure 5: An induced-fit model for the heparin-dependent high affinity receptor binding of FGF. Hep, heparin: R, high affinity receptor; HSPG, heparan sulfate proteoglycan [116].

All these studies indicate that the binding and mitogenic activity of FGF are dependent to some degree on cell surface HSPGs or, in their absence, exogenously added

heparin. Two potential cell surface proteoglycans that may interact with FGF are syndecan and glypican. Syndecan is a developmentally regulated, membrane bound HSPG which binds to bFGF with nanomolar affinity (for review see [115] and [121]). Glypican has been shown to bind FGFs [122], and is expressed abundantly in the central nervous system [123]. Alternatively, the heparan sulfate moiety necessary for high-affinity FGF binding could be present on the high-affinity receptor itself. This has recently been proposed by Sakaguchi et al., who isolated a form of FGFR that binds aFGF with very high affinity (4 pM) from a rat parathyroid cell line [124]. Incubation of this receptor with heparitinase, an enzyme that removes heparin-like side chains from proteins, abolished aFGF binding. The presence of carbohydrate residues on FGFR has been previously reported to be required for receptor function [125].

Still to be determined is whether specific HSPGs alter FGFR complex specificity for different members of the FGF family. Givol et al. have proposed that a balance may exist between two classes of HSPGs; stimulatory and inhibitory classes. Inhibitory HSPG classes may bind FGF and function as local suppressors through direct inhibition of FGFR interactions [106]. Stimulatory HSPGs would promote ligand high-affinity binding. The balance between these two classes of HSPG may therefore determine the degree and extent of FGF-induced cellular responses.

FGFRs and Pathology

As with FGFs, FGFRs may have implications in a variety of pathological states. Given the multitude of alternative transcripts, which possess different binding specificities, it is tempting to wonder whether changing the pattern of FGFR gene splicing by a particular cell could confer on that cell the capacity to respond differently to its environment and lead to tumor progression. Interestingly, it has been noted that surgically excised nevi contain more FGFR-2 IIIb than FGFR-2 IIIc mRNA, while the

opposite is true for surgically excised melanoma [66]. Further, inhibition of the FGFR-1 gene in human melanocytes and malignant melanomas by utilization of antisense oligonucleotides leads to inhibition of proliferation and some evidence of induced differentiation suggesting that FGFR-1 gene expression represents an important mediator in melanocyte growth regulation [126]. It has also been demonstrated that when transgenic mice are prepared expressing a truncated FGFR-1, that the expression of the mutant FGFR-1 induces phenotypic abnormalities in the epidermis of these mice [127]. For example, expression of the mutant FGFR-1 was shown to disrupt the organization of epidermal keratinocytes, induce epidermal hyperthickening, and result in an aberrant expression of certain types of keratin. This suggests that FGFR is essential for the morphogenesis of keratinocytes and for the establishment of the normal program of keratinocyte differentiation. Several studies have indicated that FGFRs may be involved in carcinogenesis. FGFR-1 and FGFR-2 genes have been shown to be amplified in certain types of breast cancer [128]. A particular splice variant of FGFR-1 (Figure 4-viii), has shown increased expression in rat prostate and liver tumors [129].

In order to fully understand how FGFs exert such a wide variety of effects in responsive cell types a number of questions must be answered, including; (1) How is FGF expression/production regulated in a particular cell type? (2) What are the signaling pathways used by FGFRs? (3) Are there different signaling pathways that control FGF-dependent cellular responses like mitogenesis, chemotaxis, neurite outgrowth, and inhibition of differentiation? (4) Are specific FGFRs responsible for inducing the many different FGF-dependent cellular events? (5) Are FGFRs involved in a novel FGF secretory pathway? (6) Can the FGFR expression complement of a particular tissue or cell account for the FGF-mediated behaviors of that tissue or cell? and, (7) What FGFR(s) is involved in aFGF and bFGF induce mitogenesis in dermal fibroblasts. This thesis directly addresses the production of aFGF and bFGF, as well as, the FGFR that is

involved with FGF mediated mitogenesis in dermal fibroblasts.

Chapter 2: Dermal fibroblasts produce and respond to aFGF and bFGF in serum-free medium

[1]Results

Both bFGF and aFGF mRNAs are Expressed in Dermal Fibroblasts Grown in Serum-Free Medium

Previous studies have demonstrated the presence of multiple species of bFGF and aFGF mRNA in human neonatal dermal fibroblasts grown in serum-free medium containing bFGF, or in medium containing FBS [28, 130]. To facilitate the examination of FGF proteins, human neonatal dermal fibroblasts were grown in medium lacking serum and FGF. Fibroblasts were harvested from stock culture flasks, plated and grown in medium MCDB 202a supplemented with EGF, insulin, and BSA as described in the Materials and Methods. When the cultures reached high density (see Materials and Methods), RNA was harvested from serum-free or acutely serum-treated cultures and examined the expression of aFGF and bFGF mRNA by northern blot analysis (Figure 6). Because mRNA collected from normal human keratinocytes has been shown to include very little bFGF and aFGF message [28], it was used as a negative control on the northern blots. As a control for variation in loading the gels and capillary transfer blots were subsequently probed with cRNA encoding a constitutively expressed gene, cyclophilin (1B15) (Figure 6, bottom panel). As shown in Figure 6; panel A, fibroblasts express three predominant bFGF mRNA species with molecular sizes of 7.1, 4.1, and 1.8 kilobases (lanes B and C). Two additional bFGF mRNA species were evident in the serum-stimulated cultures with sizes of 3.2 and 1.4 kilobases. As previously reported [28], the relative abundance of the bFGF mRNAs increased with serum treatment. No bFGF mRNA transcripts were detected in RNA from human keratinocytes grown in serum-free medium (Figure 6; panel A, lanes D-F) also as previously reported [28].

[1] The results detailed in this section have been previously published [139].

A similar blot was probed with an aFGF cRNA to determine if aFGF mRNA could be detected in fibroblasts grown under the same conditions. As shown in Figure 6; panel B, multiple species of aFGF mRNA with sizes of 3.9, 2.8, 2.2, 0.8, and 0.5 kb were detected in these cells (Figure 6; panel A, lane G-I). The abundance of all aFGF mRNA species did not increase after 4 hours of FBS treatment (Figure 6; panel A, lane H). The level of the major aFGF transcript at 3.9 kb decreased with 24 hours of serum treatment; however, levels of aFGF mRNA transcripts at 0.8 and 0.5 kb increased (Figure 6; panel A, lane I). The sizes of the aFGF and bFGF mRNA species detected under these conditions are similar if not identical to those previously reported by us and others [28, 130]. For comparison, keratinocytes showed very little hybridization with the aFGF probe (Figure 6; panel A, lanes J-K). Thus, fibroblasts grown continuously in serum-free medium lacking FGF produce multiple species of both aFGF and bFGF mRNA, and under these conditions, the abundance of bFGF mRNA abundance can be elevated by serum treatment.

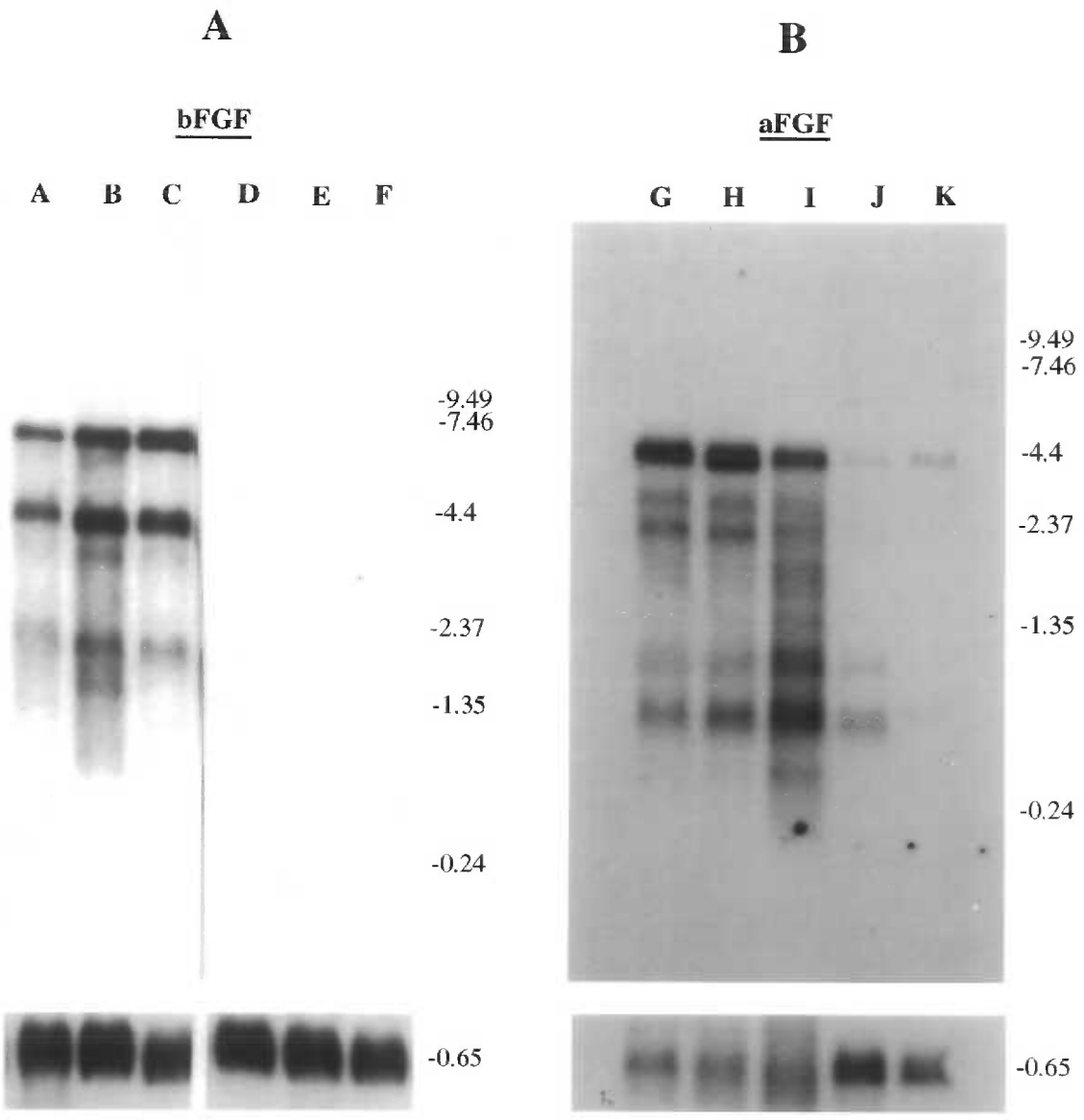

A
bFGF
A B C D E F
-9.49
-7.46
-4.4
-2.37
-1.35
-0.24
-0.65
B
aFGF
G H I J K
-9.49
-7.46
-4.4
-2.37
-1.35
-0.24
-0.65

Figure 6: Northern blot analysis of poly-A+ RNA from normal human dermal fibroblasts and human keratinocytes. 3.0 μg of poly-A+ RNA from normal human dermal fibroblasts or 5.0 μg of poly-A+ RNA from normal human keratinocytes was loaded into each lane. **Panel A:** Northern blot hybridized with the probe specific for bFGF. Lane A, fibroblasts grown in serum-free medium (control); lane B, fibroblasts grown in serum-free medium + 5% FBS for 4 hours; lane C, fibroblasts grown in serum-free medium + 5% FBS for 24 hours; lane D, keratinocytes grown in serum-free medium; lane E, keratinocytes grown in serum-free medium + TGFα (10 ng); lane F, keratinocytes grown in serum-free medium + anti-EGF receptor antibody (10nM). **Panel B:** Northern blot hybridized with the probe specific for aFGF. Lane G, fibroblasts grown in serum-free medium (control); lane H, fibroblasts grown in serum-free medium + 5% FBS for 4 hours; lane I, fibroblasts grown in serum-free medium + 5% FBS for 24 hours; lane J, keratinocytes grown in serum-free medium + TGFα (10 ng); lane K, keratinocytes grown in serum-free medium + anti-EGF receptor antibody (10nM). A probe specific for the constituitive gene, cyclophilin (1B15), was hybridized to both blots and appears as the 0.65 kb band (shown at the bottom). Migration of RNA ladder (x 10^{-3}) is on the right.

Fibroblasts Contain Multiple bFGF Proteins that Increase in Abundance with Serum Treatment

To test whether bFGF protein was present in fibroblasts and regulated by serum exposure, bFGF proteins were anlayzed in these cells by western blot analysis using a monoclonal anti-bFGF antibody. Cultures were grown to high density in serum-free medium and treated with 5% (v/v) FBS as in the experiments described above. Cell lysates were collected after 8 hours of serum treatment and incubated with heparin-acrylamide beads. Bound material was eluted with SDS-PAGE sample buffer, electrophoresed on SDS-polyacrylamide gels, and western blot analysis performed as

described in Materials and Methods. Human recombinant bFGF protein (154 amino acid form) was used as a standard. As demonstrated in Figure 7, the monoclonal antibody reacted with three proteins in the fibroblast lysates with apparent molecular weights of 26.6, 23 and 18 kD. An accumulation of all three molecular forms of bFGF (particularly the 26.6 and 23 kD forms) was seen in serum-treated cultures when compared with the serum-free controls.

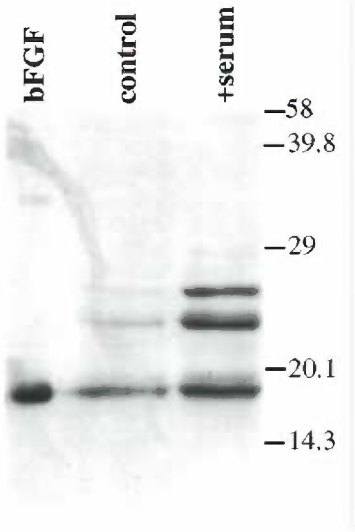


Figure 7: Western blot analysis of neonatal normal human dermal fibroblasts cell lysate. Normal human dermal fibroblasts were grown in serum-free medium (control) or treated for 8 hours with 5% (v/v) FBS (+serum). Cell lysates were collected and western blot analysis performed as described in Materials and Methods. 10 ng of human recombinant bFGF (154 AA form) was loaded in the left lane. Migration of molecular weight markers (x 10^{-3}) is indicated on the right side.

A rabbit anti-bFGF polyclonal antibody raised against a synthetic decapeptide representing amino acids 24-33 of human bFGF was also used in the analysis of my western blots. This antisera also detected 18.0, 23.0 and 26.6 kD proteins in fibroblast lysates. Preincubation of this polyclonal antibody with the decapeptide prior to exposure to the immunoblots blocked the ability of the antibody to recognize any of the forms of bFGF (data not shown). The fact that a different anti-bFGF antibody recognizes the

same proteins confirms that the proteins detected are, indeed, bFGF. Thus, fibroblasts produce three molecular forms of bFGF protein and serum treatment of these cultures causes an accumulation of all three forms.

Increases in general protein synthesis with FBS treatment of the cultures does not explain the accumulation of bFGF proteins. When cell lysates from identical cultures were analyzed in western blots with either the anti-bFGF monoclonal antibody (mAb) or an anti-aFGF polyclonal antibody, an accumulation of all three species of bFGF protein was apparent: however, no accumulation in aFGF protein was seen (see, for example, Figure 9). FBS was also analyzed for the presence of bFGF protein. FBS was incubated with heparin-acrylamide beads and the bound material was eluted with SDS-PAGE sample buffer, electrophoresed on SDS-polyacrylamide gels, and probed in an immunoblot using the anti-bFGF mAb. Under these conditions, no immunologically reactive bFGF bands were detected (data not shown).

To determine if the production of multiple forms of bFGF protein was a phenomenon of this particular strain of fibroblasts or due to the age of the donor, human adult dermal fibroblasts were cultured in the same fashion and similar cell lysates were obtained. Adult fibroblast cell lysates were treated identically to cell lysates from human neonatal fibroblasts. All three forms of bFGF protein, molecular weights of 26.6, 23. and 18 kD, were present in adult fibroblasts, and an accumulation of the three molecular forms was seen in the serum-treated cultures (data not shown).

The effect of varying doses of FBS on the expression of multiple bFGF proteins was examined. Neonatal fibroblasts were grown in serum-free medium as above and then treated with 0.1, 1.0, 2.5, 5.0, and 10.0 percent (v/v) FBS for 8 hours. A western blot analysis was performed to determine the relative abundance of bFGF proteins. As

shown in Figure 8, an accumulation of all three molecular forms of bFGF (18, 23 and 26.6 kD) was seen with serum treatment. The 26.6 and 23 kD forms of bFGF appear to increase more dramatically with serum treatment, compared with the 18 kD form. Treatment of cultures with serum concentration above 2.5% appeared to cause a maximal accumulation of bFGF proteins.

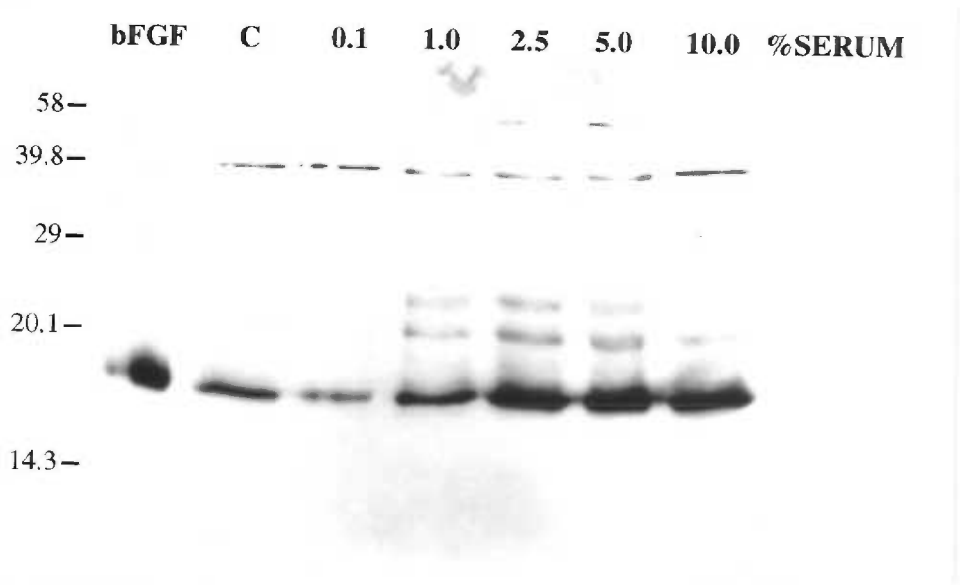


Figure 8: Western blot analysis of normal human dermal fibroblasts cell lysate from cultures grown in varying concentrations of FBS. Cells were grown in serum-free medium and treated with FBS for 8 hours as described in Materials and Methods. **bFGF**: 10 ng human recombinant bFGF (154 AA form). **C**: Cell lysate from cultures grown in serum-free medium. **0.1**: Cell lysate from cultures grown in serum-free medium treated with 0.1% (v/v) FBS for 8 hours. **1.0**: cultures grown in serum-free medium treated with 1.0 % (v/v) FBS for 8 hours. **2.5**: Cell lysate from cultures grown in serum-free medium treated with 2.5% (v/v) FBS for 8 hours. **5.0**: Cell lysate from cultures grown in serum-free medium treated with 5.0% (v/v) for 8 hours. **10**: Cell lysate from cultures grown in serum-free medium treated with 10% (v/v) FBS for 8 hours. Migration of molecular weight markers (x 10^{-3}) is indicated on the left side.

In many of the immunoblot experiments a 40 kD immunoreactive protein was detected in the cultures but did not increase with serum treatment. Multiple washings of the heparin-acrylamide beads did not reduce the immunoreactivity of the 40 kD. This immunoreactive band was not detected in all cultures. In addition, the 40 kD protein does not appear to be an artifact of serum treatment as it is present with the same relative intensity in untreated (control) cultures. However, the identity of the 40 kD protein remains unknown. An additional immunoreactive band is seen migrating with the 58 kD marker in Figures 7 and 8. This band appears to be the result of serum treatment of the cultures. It is likely that this 58 kD band is caused by nonspecific interactions of the protein with the heparin-acrylamide beads, as multiple washings of the heparin-acrylamide beads with 0.15 M NaCl solution prior to elution with sample buffer dramatically decreases the intensity of this band.

Multiple aFGF Immunoreactive Species are Present in Fibroblast Lysates

The question arose as to whether the expression of other members of the FGF family could also be regulated by serum. Fibroblasts produce multiple species of aFGF mRNA (see Figure 6 and [131, 132]). To test whether fibroblasts also produce aFGF protein and if aFGF protein expression could be regulated by serum, neonatal fibroblast cultures were grown to confluency in serum-free medium and stimulated with 5% (v/v) FBS. Cell lysates were collected and western blot analysis was performed as described, except that a rabbit polyclonal antibody raised against aFGF was used for detection of immunoreactive species. In fibroblast cell lysates, two prominent immunoreactive bands with molecular weights of 28.6 and 19.2 kD were detected (Figure 9; panel A). An additional species of 18.4 kD (Figure 9, panel A) was also occasionally detected. No accumulation of these three species was seen in the serum-treated cultures when compared with the control cultures. A 141 amino acid form of human recombinant aFGF was used as an internal standard, which migrated with a molecular weight 16.6 kD. The

antibody did not detect purified human recombinant bFGF (Figure 9; panel A), while preincubating the antibody with excess purified human recombinant aFGF (Figure 9; panel B) blocked the subsequent detection of all of these bands except the highest level of purified aFGF tested (100 ng/lane). Thus, fibroblasts appear to produce three molecular forms of aFGF protein which (like the mRNA for this growth factor) do not appear to increase dramatically after serum treatment.

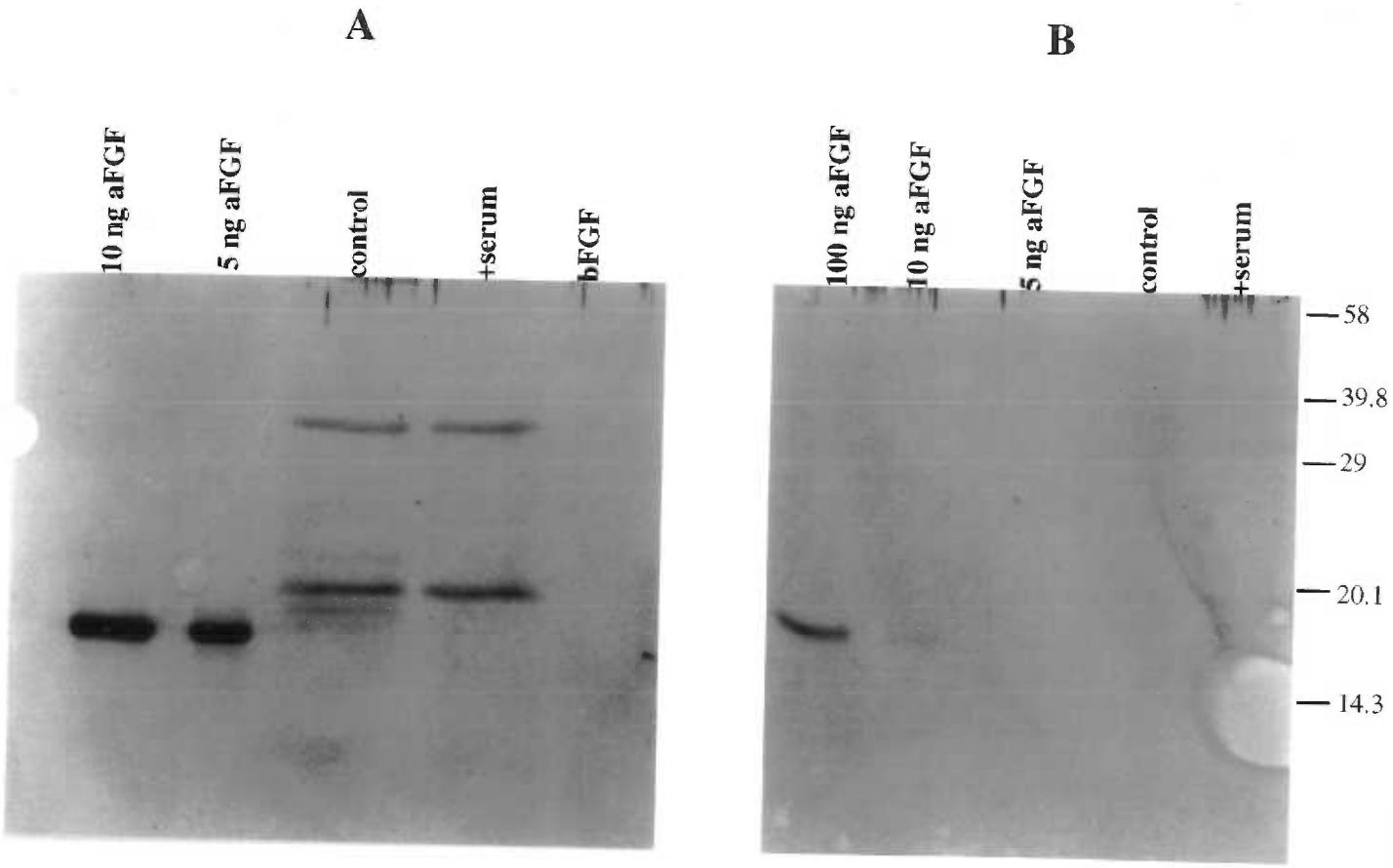


Figure 9: Western blot analysis using an anti-aFGF antibody of normal human dermal fibroblasts cell lysate . Cells were grown as described in figure 7. **Panel A:** Blot treated with the rabbit polyclonal anti-aFGF antibody. 10 and 5 ng of purified human recombinant aFGF (141 amino acid form) was loaded as a positive control. 10 ng of purified human recombinant bFGF (154 AA form) was loaded as a negative control. **Panel B:** Blot treated with the rabbit polyclonal anti-aFGF antibody preincubated with excess purified human recombinant aFGF (see text). 100, 10 and 5 ng purified human recombinant aFGF (141 AA form) were loaded as controls. Migration of molecular weight markers (x 10^{-3}) is indicated on the right side.

Purified aFGF and bFGF Stimulate DNA Synthesis in Human Fibroblasts in Serum-Free Medium

Purified recombinant aFGF and bFGF which served as controls in the western blot experiments were used to examine the mitogenicity of these growth factors on the dermal fibroblasts. Both of the factors wre tested over a broad range of concentrations in the presence and absence of heparin in a serum-free thymidine incorporation assay (see Materials and Methods). Because of variability of this type of mitogenic assay, it is difficult to access statistical differences in the stimulatory effects of the growth factors tested. As shown in Figure 10, these mitogens were capable of stimulating DNA synthesis in the cells in the presence of heparin (bFGF effective dose $(ED)_{50}$ =10 pM; aFGF ED_{50}=40 pM; ED_{50} was determined by the concentration of growth factor that created half-maximal incorporation of 3H-thymidine, as shown in Figure 10). However, when added to the cultures in the absence of heparin, aFGF was much less effective in stimulating DNA synthesis in these cultures (approximately 100-fold less effective), while the mitogenicity of bFGF was essentially unchanged.

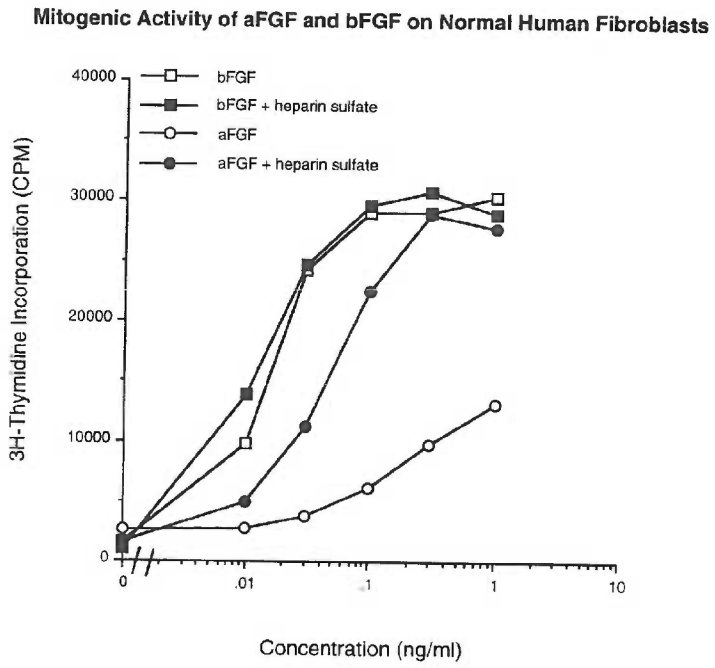


Figure 10: Response of normal human dermal fibroblasts to varying concentrations of bFGF and aFGF in the presence and absence of heparin. Normal human dermal fibroblasts were plated in MCDB 202a supplemented with 1.0 µg/ml insulin and the indicated concentrations of either bFGF or aFGF. Heparin was used at a concentration of 10µg/ml.

Discussion

Multiple species of aFGF and bFGF mRNA are present in normal human fibroblasts grown in a serum-free medium that does not contain FGF. Under these conditions, these cells produce at least three molecular forms of bFGF protein and three molecular forms of aFGF protein. These studies are the first to demonstrate multiple species of these growth factors in normal human fibroblasts. An accumulation of bFGF protein and bFGF mRNA species were detected after fetal bovine serum treatment. The abundance of aFGF protein was not affected by serum exposure.

Serum as a Inducer of bFGF Expression

The serum factor(s) that are responsible for the increase in bFGF mRNA and accumulation of bFGF protein have not been determined. Regulation of bFGF gene expression can manifest at a variety of levels: transcription, mRNA half-life, mRNA processing, translation, post-translational processing, secretion, and protein half-life. Serum may be affecting a single level or multiple levels of regulation in the pathway of expression of this gene. It is interesting that there is no induction of aFGF gene expression with serum treatment of the cells. Analysis of the data presented here suggests that aFGF gene expression is constitutive. It is possible that aFGF is the preferred mitogen for human skin-derived fibroblasts and keratinocytes in the non-injured state, i.e. aFGF is the mitogen for tissue homeostasis. When a injury occurs, serum is present at the site, and thereby increases the expression of bFGF which could be beneficial for tissue repair.

Alternative Open Reading Frames for bFGF and aFGF

Initial analysis of aFGF and bFGF cDNAs suggested that both of these mitogens

are synthesized as 155 amino acid (AA) primary translation products with translation initiated at AUG start codons [13, 133]. bFGF was originally purified as a 146 AA peptide from bovine pituitary glands, although several other forms of both higher and lower molecular weight have been isolated, all of which have been shown to have the same biological activity as the 146 AA form [31, 39, 130, 134]. aFGF protein has been identified in three major forms of 154, 140 and 134 amino acids with corresponding molecular weights ranging from 16 to 18 kD. Proteolytic cleavage during extraction of aFGF from tissue results in a 14 and 20 AA truncation of the 154 AA intact form [135].

Previous studies utilized cDNAs cloned from a human hepatoma cell line to show that CUG (leucine) start codons 5' to the previously identified AUG (methionine) start codon (see Figure 11) can initiate bFGF translation, and these higher molecular weight forms of bFGF have similar mitogenic activity [34, 133]. It is probable that the multiple molecular weight species of bFGF protein that I have demonstrated in normal human fibroblasts occur via translation of CUG codons. As illustrated in Figure 11, two of these CUG (leucine) codons located -122 and -164 basepairs (bp) from the AUG start site are surrounded by the preferred ribosomal scanning sequences predicted by Kozak [136]. A less preferred CUG codon is also present at -137 bp from the AUG start site. Translation initiating from these CUG codons would give rise to peptides of 196 AA, 201 AA and 210 AA. The higher molecular weight forms of bFGF found in this study (23 and 26.6 kD) could be explained by the utilization of the -122 and -164 CUG codons as illustrated in Figure 11. In previous studies, Florkiewicz et al. found four sizes of bFGF protein (17, 22.5, 23.1, and 24.2 kD) when analyzing *in vitro* translation products and protein products of COS 1 cell transfectants. In a similar fashion, Prats et al. found three sizes of bFGF protein; 18, 21, and 22.5 kD. Although the sizes of bFGF protein found in my study were slightly different from the previously reported forms, the differences seem most likely to be due to the use of different gel apparatuses and/or molecular weight

markers. Alternatively, multiple forms of bFGF protein could be explained by 5' sequence heterogeneity among bFGF mRNA species which are the result of differential RNA splicing.

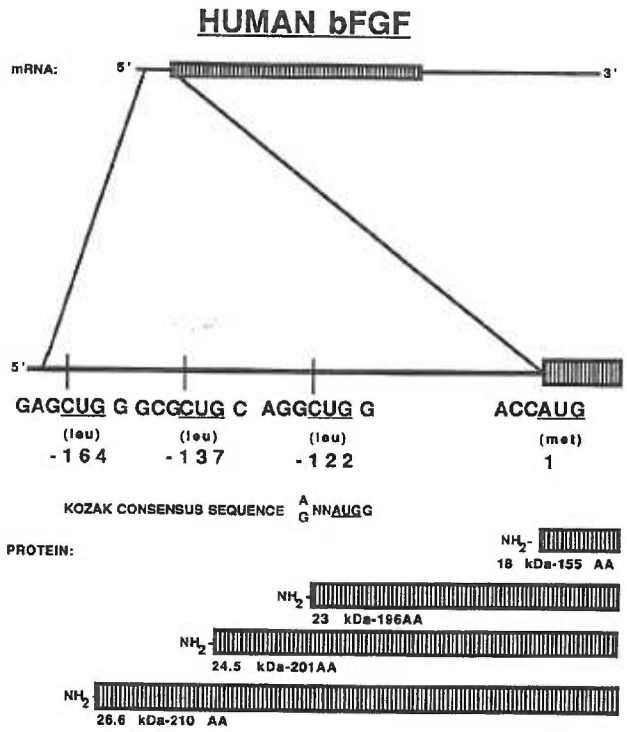


Figure 11: Schemetic drawing of human bFGF mRNA and protein. Potential CUG initiation codons are shown. Numbers refer to the position of bp from AUG start site. The three CUG codons upstream from the AUG start site are shown with their surrounding base sequences. CUG codons located at positions -122 and -164 contain the Kozak consensus sequence (see text). The potential peptides that the AUG and various CUG sites may give rise to are diagrammed with their amino acid number and corresponding molecular weights at the bottom of the figure.

The differential function, if any, of the multiple molecular forms of bFGF remains to be determined. Recently Couderc et al. demonstrated that cells transfected

with the AUG-initiated (18 kD) bFGF form undergo transformation. However, cells transfected with the CUG-initiated bFGF forms have been shown to be immortal [137]. It has been hypothesized that different forms of bFGF protein are produced at different times of the cell cycle and these forms are differentially stored and released in response to stimuli [34]. Some of the higher molecular weight forms of bFGF may preferentially migrate to the nucleus of the cell [35].

My results are the first to show expression of aFGF protein in normal fibroblasts. I detected a 19.2 kD protein which is likely to be the 154 amino acid form of aFGF, and an immunoreactive molecular weight form of aFGF that is larger than the predicted molecular weight of the 154 AA form. It is not known how this putative higher molecular weight form of aFGF protein might arise. Like bFGF mRNA, aFGF mRNA exists as multiple transcripts in human dermal fibroblasts [131]. The definitive transcriptional start sites for the bFGF and aFGF genes have not been identified. Unlike sequences in bFGF cDNA, the aFGF cDNA contains an in frame translational stop codon three bp 5' to the putative AUG translation initiation codon as well as potential splice acceptor site 34 bp 5' to the AUG [131] . In addition to the three previously identified coding exons, two alternative 5' exons which are utilized to synthesize human aFGF mRNA have been identified [88, 138]. Crumley et al. found that one of these 5' exons contains four ATG codons. Thus, it is possible that alternative splicing (3' to the previously identified AUG) coupled with translation initiating in this newly discovered exon could result in the production of larger molecular weight forms of aFGF protein.

Mitogenic Responsiveness of Skin-Derived Cells

Cell-type differences in the mitogenic responsiveness of fibroblasts, keratinocytes, and melanocytes to aFGF and bFGF exist. In addition, the presence of heparan sulfate in combination with either aFGF or bFGF in the culture medium can alter

the mitogenic potency of the growth factor on certain cell types. In this study, I found that heparan sulfate enhances the mitogenic activity of aFGF on fibroblasts. In contrast, aFGF is fully active in the presence or absence of heparin on keratinocytes [6]. While bFGF causes proliferation of fibroblasts and melanocytes regardless of the presence of heparin, the proliferative effect of bFGF on keratinocytes is strongly inhibited by the addition of heparin [6, 43].

It is possible that the aforementioned differences are due to the receptor complement expressed by the individual cell types. The fact that both high- and low-affinity receptors exist increases the complexity of these potential interactions even further. For example, skin-derived cells may express all the same high-affinity FGFRs but express different low- affinity FGFRs. The low-affinity receptors, in this case, would be responsible for the differences in mitogenic responsiveness to FGFs. Alternatively, skin-derived cells could express a variety of high-affinity FGFRs and the same low-affinity receptors. The most likely case is, however, that skin-derived cells express a varied high-affinity and low-affinity receptor complement.

Mechanism of Action of bFGF and aFGF

It has been shown that bFGF and aFGF stimulate multiple rounds of replication in neonatal fibroblasts (see Figure 10 and [28]). In the current study, I show that multiple aFGF and bFGF peptides are synthesized by these cells. Thus, it is possible that under some conditions the regulation of growth in these cells is under autocrine/paracrine control. Because FGFs affect a broad spectrum of target cells and have numerous biological functions, co-expression of FGFs in fibroblasts may function to coordinate mitogenesis and differentiation of multiple cell types during growth, homeostasis and tissue repair. It is also possible that skin-derived fibroblasts are the primary source of these peptide growth factors in human skin. As mentioned previously, keratinocytes and

melanocytes do not express aFGF or bFGF [42, 139]. Neither bFGF nor aFGF have classic signal sequences to target them for secretion; however, several groups have found bFGF in the conditioned media of cultured cells [24, 25]. I have not detected FGF-related biological activity in medium conditioned by human fibroblasts. The question remains as to how these factors may escape from the cell to produce their presumed effects.

One hypothesis is that wounding and/or cell death leads to the release of both mitogens into the extracellular space, such that binding to cell surface receptors of intact cells could cause growth in a paracrine fashion [140]. Another possibility is that FGF could bind a receptor intracellularly and then be transported outside the cell, such that it could interact with neighboring cells, the extracellular matrix, or on the cell itself. Alternatively, autocrine stimulation of cell growth could result from intracellular action of these growth factors via binding to a receptor within the cell. For example, IL-3 has been shown to bind its receptor intracellularly and cause autocrine growth [141]. As mentioned previously, a variety of FGFRs exist (for review see [92, 105]), and alternative splice products for human FGFR-1 and FGFR-2 have been described. In the current study, a cDNA from human skin-derived fibroblasts has recently been isolated encoding a FGFR-1 without a transmembrane domain. It is possible that this receptor form functions intracellularly or is secreted.

Conclusion

In this study, it has been shown that bFGF and aFGF proteins are produced by human dermal fibroblasts grown in serum-free medium, and that an accumulation of bFGF protein and mRNA was seen with serum treatment of the cultures. aFGF protein and mRNA production did not appear to be regulated by serum in these cells. Both

bFGF and aFGF protein were shown to be present in multiple molecular weight forms. Human dermal fibroblasts responded mitogenically to bFGF regardless of the presence of heparin and had an enhanced mitogenic response to aFGF in the presence of heparin.

Normal human dermal fibroblasts produce and respond mitogenically to aFGF and bFGF. The mechanism(s) by which aFGF and bFGF mediate signal transduction in these cells is undetermined. The next chapter of this thesis begins to address the mechanism by which aFGF and bFGF may mediate mitogenic signal transduction by identifying high-affinity and low-affinity FGFRs produced by these cells.

Materials and Methods

Cell Culture

Dermal Fibroblasts: Human neonatal dermal foreskin fibroblasts (strain NFF-5) were isolated from normal tissue by collagenase digestion as previously described [28]. Human adult dermal fibroblasts were obtained from American Type Culture Collection, CRL1505, (Rockville, MD). Stock cultures of fibroblasts were maintained in medium MCDB 202a supplemented with 5% (v/v) fetal bovine serum (FBS) (J.R. Scientific, Woodland, CA). Cultures used for experiments were between population doubling level 8 and 25. Quiescent stock cultures were trypsinized using ice cold 0.05% trypsin and resuspended in medium MCDB 202a supplemented with 5% FBS to neutralize the trypsin. The cells were recovered by centrifugation at 180 x g and the cell pellet was resuspended in medium MCDB 202a supplemented with 5 ng/ml epidermal growth factor (EGF) (Amgen Biologics, Thousand Oaks, CA), 1 µg/ml insulin, and 100 µg/ml bovine serum albumin (BSA). Cells were then plated in 10 cm plates at 2.5 x 10^3 cells/cm^2 and grown for seven days without a medium change. On day seven, the medium was changed to fresh medium as above, and the cultures were incubated for an additional three days. On day 10 when the cultures had formed a complete monolayer on the dish surface without overlapping, FBS was added directly to the medium and the cells were allowed to incubate for 8-24 hours as described in the text and legends to figures.

Keratinocytes: Human neonatal keratinocytes (strain NHEK 239) were obtained from Clonetics Corporation (San Diego, CA) and stock cultures grown in medium MCDB 153 supplemented with 0.2% bovine pituitary extract (BPE) (Clonetics, San Diego, CA), 10 ng/ml EGF, 5.0 µg/ml insulin, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, and 0.5 mM hydrocortisone as previously described (referred to

as complete MCDB 153 in the text) [28, 42]. For isolation of mRNA from keratinocyte cultures, secondary cultures of keratinocytes were trypsinized from stock culture flasks and plated in complete MCDB 153, complete medium supplemented with 4.5 mg/ml monoclonal antibody (mAb) LA1 (an EGF receptor antagonist; Upstate Biotechnology, Inc., Lake Placid, NY) or 10 ng/ml transforming growth factor type-a (TGF-a) (a gift from Dr. Rik Derynck, Genentech, Inc.). These treatments, which regulate the level of TGF-a mRNA in keratinocytes, were used as negative controls for bFGF hybridization.

Melanocytes: Normal human skin-derived melanocytes were maintained in MCDB 153 containing 0.2% BPE, 10 ng/ml EGF, 5 µg/ml insulin, 5 x 10^{-7} hydrocortisone, 10 ng/ml phorbol ester, 2 ng/ml bFGF [42].

DNA Synthesis Assay

For measurement of ^{3}H-thymidine incorporation into DNA, dermal fibroblasts were removed from stock culture flasks, resuspended, and centrifuged as described above. The cells were then resuspended in cold medium MCDB 202a supplemented with 5.0 µg/ml insulin and plated at 1 x 10^{4} cells cm^{2} in 24 well culture dishes. The cells were incubated for 72 hours without a medium change. After the first 48 hours of the incubation, various concentrations of growth factors with or without heparin were added to some wells and the incubation continued. After 68 hours of incubation, 1.0 µCi/ml ^{3}H-thymidine was added to each well in a small volume. At the end of the incubation, the medium was removed and the cells were fixed with cold 10% trichloroacetic acid (TCA) (Sigma Chemical Co., St. Louis, MO). Relative incorporation of ^{3}H-thymidine into TCA-insoluble material was determined as previously described [142].

Preparation of Cell Lysates and Western Blot Analysis

To prepare lysates, the culture medium in the plate was decanted and the cells were washed with cold solution A (10.0 mM glucose; 3.0 mM KCl; 130.0 mM NaCl; 1.0 mM Na_2HPO_4 $7H_2O$; 0.0033 mM phenol red; 30.0 mM HEPES). A small volume of ice cold lysis buffer (50 mM TRIS pH 7.5; 400 mM NaCl; 1 mM MgCl; 1% (v/v) NP40; 1µg/ml leupeptin) was added and the plate was scraped with a rubber spatula. The cell lysate was then transferred to another plate of cells followed by scraping. Cell lysate from a total of two plates was then pipetted into a 1.5 ml microfuge tube and held on ice. Lysates were centrifuged at 4 ^{o}C; 15,000 x g for 10 minutes to pellet nuclei and cellular debris. The supernatants were transferred to clean 1.5 ml microfuge tubes and approximately 10 µl of a suspension of heparin-acrylamide beads were added to each tube. Cell lysate was incubated for at least 2 hours at 4 ^{o}C on rocker table, the beads were removed by centrifugation, washed in 130 mM NaCl; 20 mM HEPES pH 7.4 and resuspended in SDS/PAGE sample buffer (0.125 M TRIS pH 6.8; 4% (w/v) SDS; 20% (v/v) glycerol; 200 mM dithiothreotol). The bead suspensions were boiled for approximately 8 minutes, centrifuged to remove the beads, and the eluants loaded onto SDS-12% polyacrylamide gels. As a result, the equivalent of two plates was loaded onto each lane of the gel. A single 10-cm plate of cells corresponds to approximately 3.6 x 10^6 cells and 4.36 x 10^6 cells for serum-free and 24-hour serum-treated cultures, respectively. Electrophoresis was conducted on the Mini-Protean II System (BioRad) at 200 V for 45 minutes and electrophoretically transferred to nitrocellulose using a dry blotter (LKB) at 15 V for 1 hour. To prepare blots for immunodetection, the membranes were allowed to air dry and then incubated in blocking solution (3% (w/v) gelatin; 0.5 M NaCl; 20 mM TRIS pH 7.4; 0.02% (w/v) sodium azide; 0.1% (v/v) Tween-20) for 20-30 minutes. The blocking solution was removed by brief washing in TRIS-buffered saline (TBS) (150 mM TRIS pH 7.2; 10 mM NaCl). The blots were subsequently incubated with a 1:6,000 dilution in antibody buffer of an ascites fluid containing a mouse mAb

(148.6.1.1) raised against human recombinant bFGF (a gift from Dr. C. Hart, Zymogenetics Inc., Seattle, WA) or a 1:1000 dilution in antibody buffer of a rabbit polyclonal antibody (A2) raised against human recombinant aFGF (a gift from Dr. Ken Thomas, Merck Institute, Rathway, NJ). bFGF polyclonal antibody raised against a synthetic decapeptide representing amino acids 24-33 of human bFGF was the gift of Dr. E. Smith, Children's Hospital Medical Center, Cincinnati, OH.

Antibodies were diluted in antibody buffer (500 mM NaCl; 10 mM TRIS pH 7.2; 0.05% (v/v) horse serum (GIBCO); 0.2% (w/v) sodium azide). Following primary antibody incubation, the membranes were washed briefly in TBS, and incubated with a 1:7,500 dilution of an alkaline phosphatase- conjugated anti-mouse IgG (Promega, Madison, WI) and then developed in BCIP/NBT substrates. Developing solution contained 100 mM TRIS pH 9.5; 100 mM NaCl; 5 mM MgCl; 6.6 mg/ml nitro blue tetrazolium (NBT); 3.3 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Specificity of the anti-bFGF mAb was determined by two methods. First, incubation of western blots containing both purified aFGF (10-100 ng/lane) and bFGF (10 ng/lane) with the bFGF mAb demonstrated that the mAb reacted only with bFGF. Second, I crosslinked human recombinant bFGF to an affinity support column, Affigel 10, (Biorad) that immobilizes proteins via crosslinking to their primary amino groups. Ascites fluid containing the bFGF mAb was passed over the column so as to remove bFGF-specific immunoglobins from the fluid. Analysis of the ascites fluid that passed through the column via western blot showed no immunoreactive bands (data not shown). The column was treated at high pH to release the antibody. The immunopurified antibody detected three molecular weight forms of bFGF. Specificity of the aFGF antibody is demonstrated in the text and figures.

Oligoribonucleotide Probes

A complementary ribonucleic acid (cRNA) bFGF probe corresponding to the first 530 nucleotides of sequence 3' to the translational stop codon were synthesized from plasmid pGb530 [13] which consists of a 530 bp Bal I/Eco RI restriction fragment of human bFGF cDNA cloned into the Sma I site of vector pGEM4Z (Promega). cRNA probes for aFGF corresponding to the first 479 nucleotides of the protein coding sequence from plasmid pJC3-5 which consists of a 479 Nco I/Eco RI [131] restriction fragment of human aFGF cDNA cloned into the Sma I site of vector pGEM4Z. cRNA probes corresponding to the cyclophilin gene (1B15) was synthesized as previously described [143]. cRNA reactions were carried out according to the reaction of Melton et al. [144].

Northern Blots

Preparation of RNA for northern blot analysis was performed as previously described [28]. Poly A+ mRNA was purified by oligo(dT)-cellulose chromatography. Northern blots were prepared as previously described [28]. RNA size markers were obtained from Bethesda Research Laboratories (Bethesda, MD). Hybridization with cRNA probes was conducted for 18 hours at 60-65 ^{o}C using 1-5 x 10^{6} cpm/ml of probe in hybridization solution (50% formamide, 5 x SSC, 0.1% SDS, 10 x Denhardt's solution, 50 µg/ml polyadenosine, and 250 µg/ml herring sperm DNA). Autoradiography was performed using Kodak X-Omat film with an intensifying screen at -80 ^{o}C for 3-24 hours. After the radioactivity on the blot decayed, the same blot was subsequently hybridized with 1B15 cRNA probe under the same conditions.

CHAPTER 3: Dermal fibroblasts express a single high-affinity FGFR gene, FGFR-1.

Results

Human Dermal Fibroblasts Express only FGFR-1

To begin the analysis of which FGFR(s) could be mediating mitogenic signal transduction in human dermal fibroblasts, the expression of FGFR(s) in normal human skin-derived fibroblasts was examined by northern blot. For comparison RNA from cultured keratinocytes and melanocytes were also analyzed. Utilizing cDNA and cRNA probes for FGFR-1, FGFR-2, FGFR-3 and FGFR-4, as described in materials and methods, it was found that dermal fibroblasts express only FGFR-1; keratinocytes express FGFR-2 IIIb (KGF receptor) and FGFR-3; and melanocytes express only FGFR-1. As shown in Figure 12, dermal fibroblasts express two FGFR-1 mRNA species with molecular masses of 5.9 kb and 4.4 kb. The predominant species is at 4.4 kb. When a FGFR-2 IIIb specific probe was used, keratinocytes were found to express two predominant FGFR-2 IIIb mRNA species with molecular masses of 4.8 and 3.9 kb. Keratinocytes also express a single FGFR-3 mRNA species with a size of 5.0 kb. Melanocytes express a single FGFR-1 mRNA species with a size of 4.4 kb. No expression of FGFR-4 was detected in these cell types. These mRNA sizes are all consistent with those published in the literature.

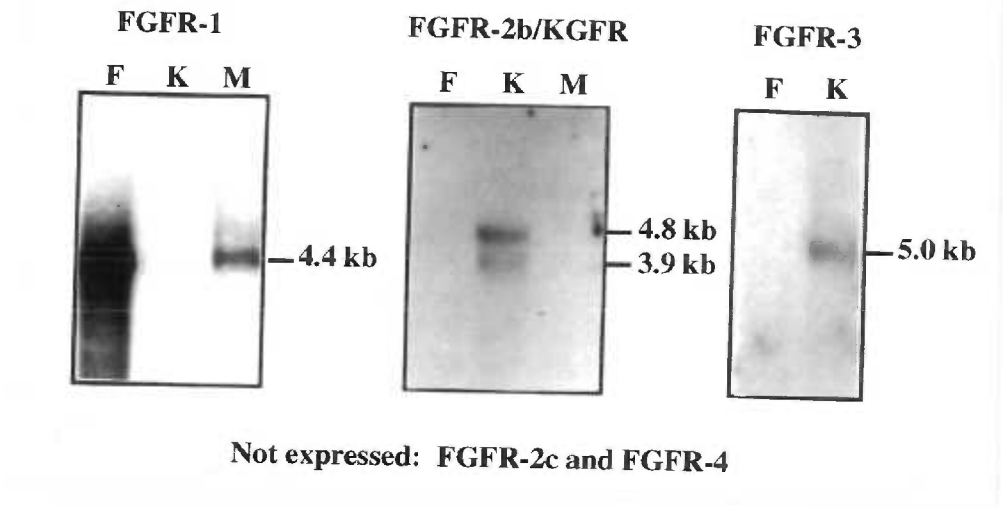


Figure 12: Northern blot analysis of polyA+ mRNA from normal, human skin-derived fibroblasts, melanocytes and keratinocytes. 3.0 μg of polyA+ mRNA was loaded into each land. FGFR-1: northern blot hybridized with a probe specific for FGFR-1. FGFR-2b/KGFR: northern blot hybridized with a probe specific for FGFR-2 IIIb (KGF receptor). FGFR-3: northern blot hybridized with a probe specific for FGFR-3. F=fibroblast; K=keratinocyte; M=melanocyte. FGFR-2b/KGFR=KGF receptor (a splice variant of the FGFR-2 gene). FGFR-2c=FGFR-2 molecule encoding the IIIc exon in the carbxoy-half of Ig domain III. The sizes of the hybridizing mRNA(s) are indicated at the right of each panel.

The pattern of FGFR gene expression in dermal fibroblasts was confirmed by an alternate experimental method. First, PCR was conducted, using degenerate primers, on single stranded cDNA derived from fibroblasts or keratinocytes, generating a 370 bp fragment (see Figure 13). The amplified fragment corresponded to the kinase insert domain of a FGFR. Then the kinase insert domain encodes the region most unique to a particular FGFR gene [92] and, therefore, provides a reliable means of distinguishing among the different receptors. The fragments were gel purified and reacted with several restriction endonucleases, including Ava I, BstE2, Pst I, Nar I and Sca I (data not shown).

Analysis of the restriction patterns supported data obtained from the northern blot experiments: dermal fibroblasts express only FGFR-1 and keratinocytes express FGFR-2 IIIb and FGFR-3 (see Figure 13).

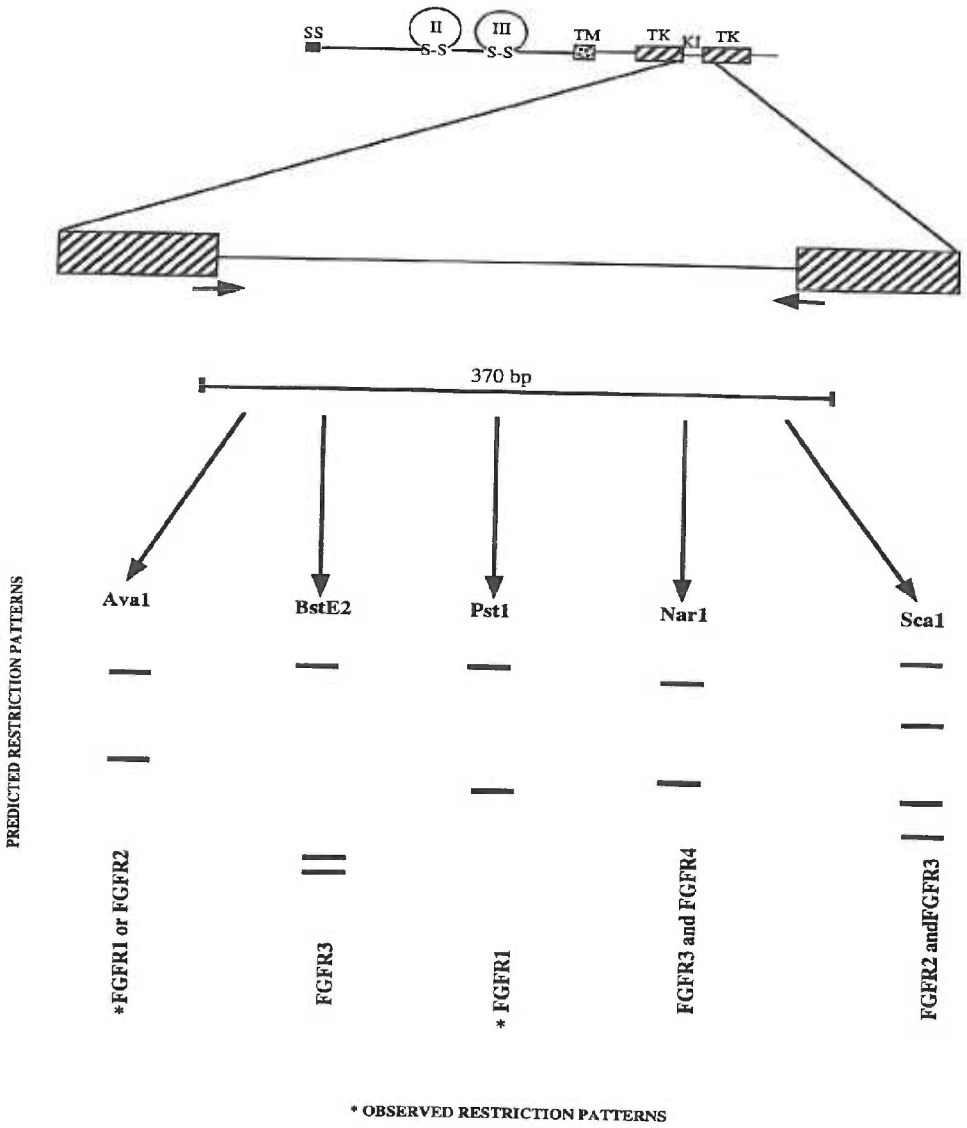


Figure 13: PCR was conducted on cDNA from normal human dermal fibroblasts utilizing primers located just outside of the tyrosine kinase insert region. These primers are common to all FGFRs. Following PCR, the product, which is a 370 bp band, was digested with various restriction endonucleases. The predicted restriction patterns for each enzyme is drawn below. The observed restriction patterns seen with the digestion of a PCR product from normal human dermal fibroblasts is noted by (*). It was necessary to digest the PCR product with two different restriction enzymes to determine FGFR-1 expression.

Dermal Fibroblasts Contain Multiple FGFR-1 Proteins

To determine if FGFR-1 protein was produced by dermal fibroblasts cell lysates wre analyzed for the presence of FGFR-1 protein(s) by western blot. Cells were grown as described in the materials and methods. Proteins in the lysates were immunoprecipitated with either anti-FGFR-1 mAb (UBI), anti-phosphotyrosine mAb (Sigma) or anti-FGFR-1/FGFR-3 polyclonal antibody (SB102) and protein A sepharose. Bound material was eluted with Laemmli buffer, electrophoresed, and immunoblotted. Human recombinant FGFR-1 (two-immunoglobulin domain form) produced in a baculovirus system was used as a standard. As demonstrated in Figure 14; panel A, dermal fibroblasts contain two immunoreactive FGFR-1 species migrating with apparent molecular weights of 97.6 kD and 83.9 kD. The FGFR-1 standard migrated at 100.1 kD. The same results were obtained regardless of which antibody was used (anti-FGFR-1 mAb, anti-FGFR-1/FGFR-3 polyclonal antibody, or anti-phophotyrosine mAb) to immunoprecipitate cell lysates with or as the primary antibody on immunoblots. Importantly, the anti-phosphotyrosine mAb (Sigma) produced the same pattern of bands on western blots as the FGFR-specific antibodies. This implies that these immunoreactive species exist as tyrosyl-phosphorylated molecules in the cell. Thus, dermal fibroblasts produce two molecular weight species of FGFR-1 both of which are phosphorylated on tyrosine residues.

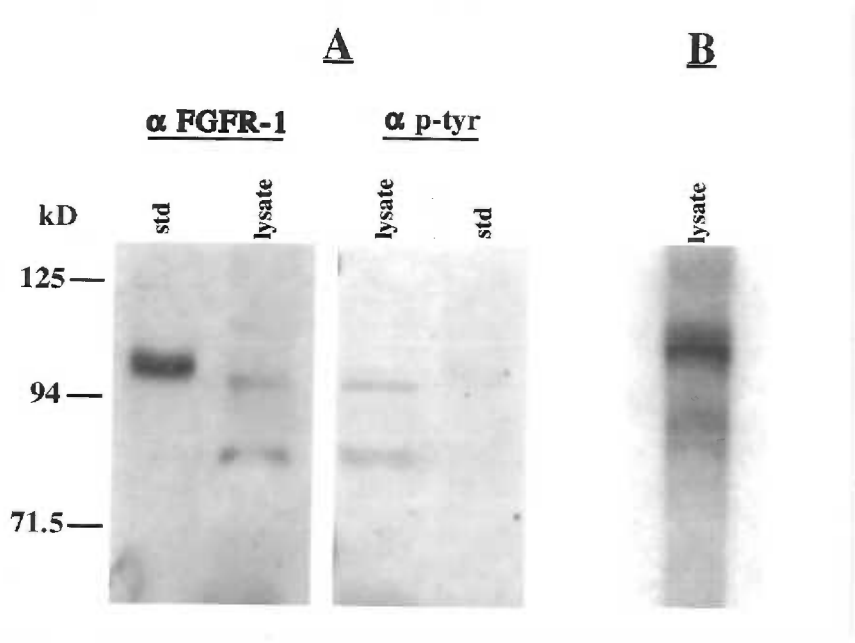


Figure 14: (A) Western blot analysis of dermal fibroblast cell lysate. Cell lysates were collected and immunoprecipitated with the anti-FGFR-1 mAb (UBI) as described in Materials and Methods. α-FGFR-1: indicates the blot was reacted with the anti-FGFR-1 mAb (UBI). α-p-tyr: indicates the blot was reacted with the anti-phosphotyrosine mAb (SIGMA). Std=two-Ig domain form of FGFR-1 standard (see Materials and Methods). Migration of molecular weight markers (x 10^{-3}) is indicated on the left. (B) *In vitro* phosphorylation assay of dermal fibroblast cell lysate. Samples were immunoprecipitated with the anti-FGFR-1 mAb (UBI) and incubated with radiolabeled ATP as described in Materials and Methods.

I*n vitro* phosphorylation assays were conducted to see if these two proteins were active kinase molecules. Dermal fibroblasts were grown in serum-free medium, cell lysates were collected, and the lysates were immunoprecipitated with anti-FGFR-1 mAb (UBI) as described in materials and methods. Bound material was incubated with {γ ^{32}P}-ATP and kinase buffer (see Materials and Methods). Eluted samples were separated on SDS-PAGE and the data was analyzed with autoradiography. Figure 14; panel B

shows immunoprecipitated species migrating with corresponding molecular masses when compared to the pattern seen in Figure 14; panel A. Both immunoreactive species had incorporated radioisotope suggesting that both proteins undergo phosphorylation.

FGFR-1 Proteins Present in Dermal Fibroblasts do not Change Migration Patterns with Tunicamycin Treatment of the Cells

To determine if the FGFR-1 proteins produced by dermal fibroblasts were glycosylated, and, in particular, to see if the protein migrating at 83.9 kD was an under-glycosylated form of the larger, 97.6 kD, protein, dermal fibroblasts were cultured in medium containing tunicamycin. Tunicamycin is an antibiotic which inhibits asparagine-linked glycosylation by preventing the transfer of the first carbohydrate residue to the amino acid linkage site [145]. Within the full length open reading frame of the human FGFR-1 gene, there are nine potential N-linked glycosylation sites [70]. Dermal fibroblasts were grown in serum-containing medium and tunicamycin was added to the medium for 24 hours prior to cell lysate collection. Proteins within the lysates were immunoprecipitated as described above. As a control for tunicamycin treatment of the cells, the transferrin receptor was also immunoprecipitated from dermal fibroblast cell lysates using an anti-human transferrin receptor polyclonal antibody (a gift from Dr. C. Enns). The transferrin receptor has been shown to be N-linked glycosylated in a variety of cell types [146]. No shift in migration was observed with the two FGFR-1 immunoreactive species migrating at 97.6 and 83.9 kD after tunicamycin treatment of the cells (Figure 15). The transferrin receptor from dermal fibroblasts migrated as a doublet at 97.6 and 92 kD. Upon tunicamycin treatment, this doublet shifted to 92 and 86.8 kD (data not shown). Thus, the two FGFR-1 protein species expressed by dermal fibroblasts do not appear to shift their molecular weight with tunicamycin treatment suggesting that the two proteins are not differentially glycosylated forms of the same protein, and that the proteins are not heavily N-linked glycosylated.

The data suggesting that FGFR-1 proteins produced by normal human dermal fibroblasts contained little if any N-linked glycosylations was confirmed by endoglycosidase H studies. Endoglycosidase H (endo H) removes high-mannose uncomplexed N-linked glycosylations from proteins. Cell lysates were collected and immunoprecipitated with anti-FGFR-1 mAb (UBI) and submitted to endo H treatment overnight. The reactions were analyzed on SDS-PAGE gels. No reduction in molecular mass was detected with endo H treatment (data not shown). Thus, it appears that dermal fibroblasts produce two molecular weight species of FGFR-1 that contain few, if any, N-linked glycosylations.

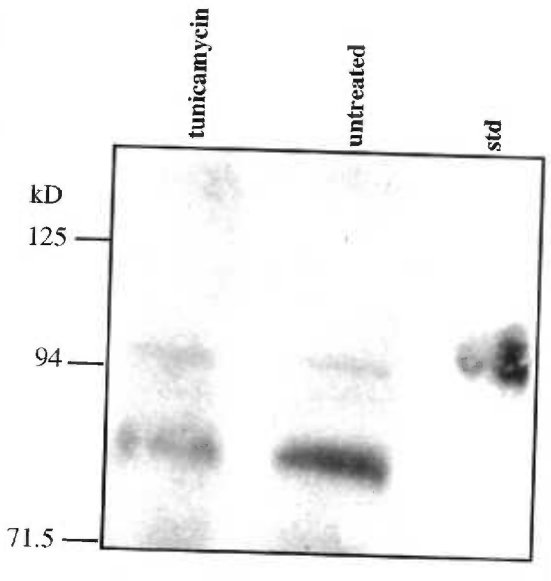


Figure 15: Western blot analysis of dermal fibroblast cell lysate in the presence and absence (untreated) of tunicamycin. Cells were grown as described in Materials and Methods. Std=two-Ig domain form of FGFR-1 standard (see Materials and Methods). Migration of molecular weight markers (x 10^{-3}) is indicated on the left.

The Smaller FGFR-1 Protein Produced by Dermal Fibroblasts Exists in Both the Cell Lysate and the Conditioned Media

To determine if any of the FGFR-1 proteins produced by dermal fibroblasts were

secreted, actively growing cultures were incubated with ^{35}S-methionine for 5 hours and the cell lysates were immunoprecipitated as well as the conditioned medium with anti-FGFR-1 mAb (UBI). Figure 16 shows that both proteins exist in the cell lysate with molecular sizes of approximately 98 and 85 kD. However, the conditioned medium contain only the smaller FGFR-1 protein, 85 kD. Thus dermal fibroblasts apparently produce two FGFR-1 proteins, two can be found cell-associated but only one appears to be secreted.

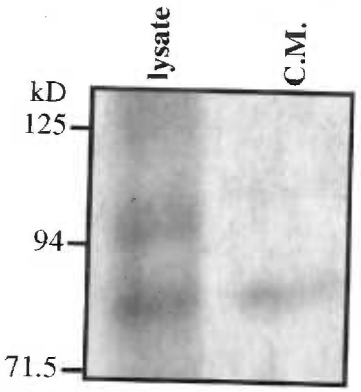


Figure 16: Metabolic labeling of dermal fibroblasts in culture. Cells were exposed to ^{35}S-methionine as described in Materials and Methods. Cell lysate and conditioned medium (C.M.) was immunoprecipitated with anti-FGFR-1 mAb (UBI) and electrophoresed on SDS-PAGE. This figure shows an autoradiogram of the separated material. Migration of molecular weight markers (x 10^{-3}) is indicated on the left.

Isolation and Characterization of Two Distinct cDNA Clones Encoding FGFR-1

As mentioned earlier, many FGFR-1 variants have been reported (for review see Introduction and [92, 105]). These variants involve deletions and/or substitutions in the extracellular, transmembrane and intracellular regions of the molecule. To determine what exons combine to generate the FGFR-1 protein molecules isolated from dermal fibroblasts, PCR cloning techniques were utilized to generate two distinct clones that

encode FGFR-1. Amplification with single- stranded cDNA from dermal fibroblasts using primers that hybridized in the 5' and 3' untranslated region of human FGFR-1 gave rise to two fragments, with molecular sizes of 2300 bp and 2000 bp. The predominant fragment migrated at 2300 bp on an agarose gel and a minor fragment migrated at 2000 bp (data not shown). The 2300 bp fragment was cloned into the vector pCRII (2300 bp FGFR-1). This fragment was cloned multiple times utilizing PCR products from different PCR reactions. The 2000 bp fragment could not be cloned as readily as the 2300 bp fragment. However, a clone was obtained using a different combination of FGFR-1 specific primers. The 2000 bp fragment was cloned into the vector pTZBlue (2000 bp FGFR-1). For unknown reasons, the 2000 bp fragment was extremely difficult to successfully clone and, therefore the positive clone that finally was obtained was not repeated. Both inserts were later subcloned into a mammalian expression vector, pRC/CMV.

The 2300 bp FGFR-1 clone and the 2000 bp FGFR-1 were sequenced by the chain termination method. The 2300 bp FGFR-1 clone, as depicted in Figure 17, encodes a FGFR-1 containing an open reading frame of 730 amino acids. The predicted protein would contain two immunoglobulin domains (domains II and III), a signal sequence, an acidic box, a transmembrane domain, and a split tyrosine kinase domain. The predicted size of the core protein would be approximately 82 kD. This molecule contains seven potential N-linked glycosylation sites [70]. Potential O-linked glycosylation sites are not as easily definable as no consensus pattern has been identified to predict these sites [147]. Nevertheless, some sequence patterns have been shown to be statistically likely to be O-linked glycosylated; in which case, there are 21 potential O-linked glycosylation sites on the 2300 bp FGFR-1 molecule. The carboxy-terminal half of immunoglobulin domain III encodes the IIIc form of FGFR-1 [84]. The sequence of the 2300 bp FGFR-1 clone is an alternatively spliced variant in which two small

deletions were noted: (1) Arg-Met (AA # 61 and 62) deletion at the 3' end of the exon encoding the acidic box; (2) Val-Thr (AA # 336 and 337) deletion at the 3' end of the exon encoding the transmembrane domain. Both of these deletions have been described in the literature and are thought to arise via alternative use of splice donor sites at exon/intron boundaries (see Discussion).

The 2000 bp FGFR-1 clone encodes a yet unreported form of human FGFR-1 mRNA. This molecule encodes a FGFR-1 identical to the 2300 bp FGFR-1 clone with two major deletions: (1) the exon encoding the carboxy half of the third immunoglobulin domain is missing; and (2) the exon encoding the transmembrane domain is missing; (3) as with the 2300 bp FGFR-1 clone described above, the 2000 bp FGFR-1 clone does not contain Arg-Met and the Val-Thr residues as the locations described above (see Figure 17). The predicted core protein size of this molecule is approximately 69.1 kD containing 614 amino acids. There are five potential N-linked glycosylation sites.

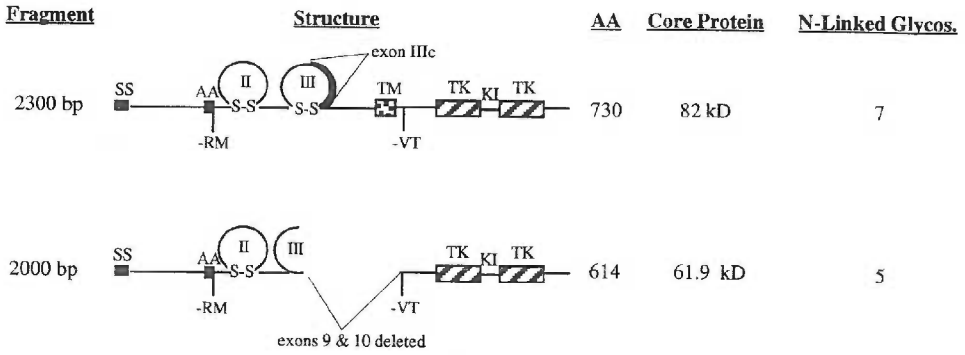


Figure 17: Schematic representation of the predicted protein structures deduced from sequencing the 2300 bp FGFR-1 clone and the 2000 bp FGFR-1 clone. AA: total number of amino acids. Core Protein: predicted size of the primary translation product. N-linked Glycos.: number of potential N-linked glycosylation sites. -RM and -VT below each diagram indicate the Arg-Met and Val-Thr deletions, respectively (see text). Exons #9 and #10 deletion encoding the carboxy-terminal half of Ig domain III and the transmembrane domain, respectively, are indicated below the diagram of the 2000 bp FGFR-1 clone. SS: signal sequence; AA: acidic box; S-S: disulfide bonds forming Ig loops; TM: transmembrane domain; TK: tyrosine kinase domain; KI: kinase insert domain.

These FGFR-1 clones differ in size by approximately 300 bp, which encodes approximately 13 kD of protein. The two dermal fibroblast-derived FGFR-1 proteins present in cell lysates differ by 13.7 kD. If the two dermal fibroblast-derived FGFR-1 proteins are truly unique, then they must be generated from two distinct mRNA molecules. The cloning of two distinct cDNAs from these cells supports this hypothesis.

Specific mRNAs Representing FGFR-1 Exist in Dermal Fibroblasts

To confirm that mRNA molecules which could encode the 2300 and 2000 bp FGFR-1 clones, RNase protection assays were conducted. Poly A+ mRNA from dermal fibroblasts was hybridized with a 377 bp antisense riboprobe, which was generated by Pvu II linearization of pCRII877c2 (see Figure 25). The probe encoded a portion of the extracellular domain of FGFR-1 (see Figure 25). The predicted protected fragments are 303 bp, which would indicate the presence of a mRNA which could encode the 2300 bp FGFR-1 clone, and 199 bp, which would indicate the presence of a mRNA which could encode the 2000 bp FGFR-1 clone. Both poly A+ mRNA from serum-stimulated dermal fibroblasts and serum-free dermal fibroblasts were assayed. Controls for the assays included: (1) antisense riboprobe hybridized to full length sense 2300 bp RNA (Figure 18, lane 4); (2) antisense riboprobe hybridized to 2000 bp sense RNA (Figure 18, lane 5); (3) antisense riboprobe hybridized to yeast tRNA (Figure 18, lane 6); (4) unhybridized riboprobe (Figure 18, lane 7). Figure 18 shows the results of these assays. When the probe and a 2300 bp sense RNA control were hybridized, two protected fragments migrating at approximately 300 bp and 160 bp were seen (Figure 18, lane 4). The same protected fragments were seen when the probe and both serum-treated and serum-free dermal fibroblast poly A+ mRNA samples were hybridized (Figure 18, lanes 2 and 3). It is not clear what the 160 bp protected fragment represents. When the probe and a 2000 bp sense mRNA control sample were hybridized, a single protected fragment was seen migrating at approximately 200 bp, as predicted (Figure 18, lane 5). This 200 bp protected fragment was only seen when dermal fibroblast poly A+ mRNA derived from cells grown in serum-free medium was hybridized with the probe (Figure 18, lane 2). In conclusion, RNAs that could encode the 2300 and 2000 bp FGFR-1 clones exist in dermal fibroblasts. Moreover, the RNA which represents the 2000 bp FGFR-1 clone is down-regulated by serum treatment of the cells.

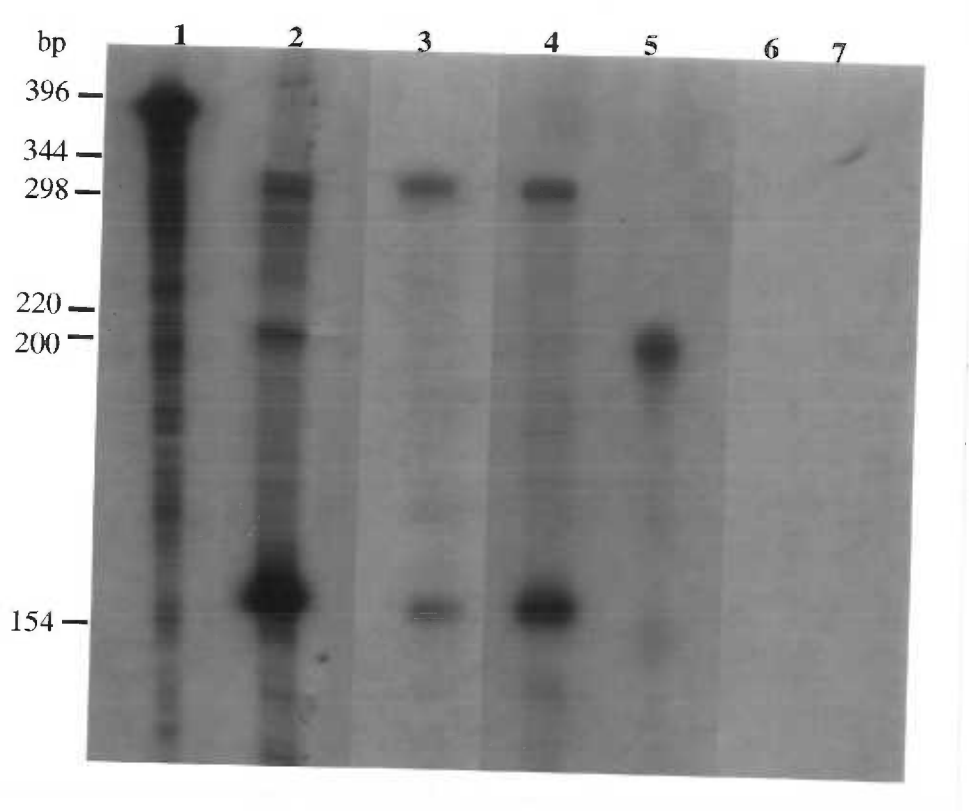


Figure 18: RNase protection assay of polyA+ mRNA from dermal fibroblasts hybridized with a FGFR-1 specific cRNA probe. (1) undigested probe (approx. 370 bp). (2) polyA+ mRNA from dermal fibroblasts grown in serum-free medium (see Materials and Methods). (3) polyA+ mRNA from dermal fibroblasts grown in serum-containing medium. (4) full-length sense RNA derived from the 2300 bp FGFR-1 clone. (5) full-length sense RNA derived from the 2000 bp FGFR-1 clone. (6) yeast tRNA control. (7) unhybridized, digested probe control. Migration of markers are indicated on the left.

In Vitro Translation of Dermal Fibroblast-Derived FGFR-1 Clones Gives Rise to Proteins that Correlate in Size with Those Found in Cultured Cells

To further investigate if the sequences encoding 2300 and 2000 bp FGFR-1 clones could give rise to proteins that correlate in size with those seen from dermal fibroblast cell lysates, *in vitro* translation experiments on the FGFR-1 clones were

performed. Plasmid constructs (see Material and Methods and Figures 14 and 15) were submitted to an *in vitro* transcription/translation reaction in the presence of ^{35}S-methionine. Controls included: (1) translation from empty vector (Figure 19, lane 4); (2) no added DNA (Figure 19, lane 6); (3) and translation from control plasmid DNA obtained from the kit manufacturer encoding the luciferase open reading frame (Figure 19, lane 5). Results of these reactions were analyzed on SDS-PAGE. As seen in Figure 19; lane 1, three translation products were obtained from translation of the 2300 bp FGFR-1 clone. These products migrated at 97.6, 82 and 71.7 kD. Translation from the 2000 bp FGFR-1 clone also gave rise to three translation products (Figure 19; lane 2), migrating at 83.9, 65 and 50 kD. When the two plasmids were combined and translation was allowed to take place from both sequences simultaneously, two predominant translation products were generated migrating at 97.6 kD and 83.9 kD (Figure 19; lane 3). A minor product was seen migrating at 65 kD. It is not clear why translation from a single plasmid construct created multiple products. However, when the plasmids were combined, this phenomenon was nearly eliminated. Although the predicted core protein sizes for the 2300 and 2000 bp FGFR-1 clones are 82 and 69.1 kD, respectively, the *in vitro* translated products migrated at slightly higher molecular weights. The translated products in Figure 19; lane 3 correlate in size with those seen from dermal fibroblast cell lysates.

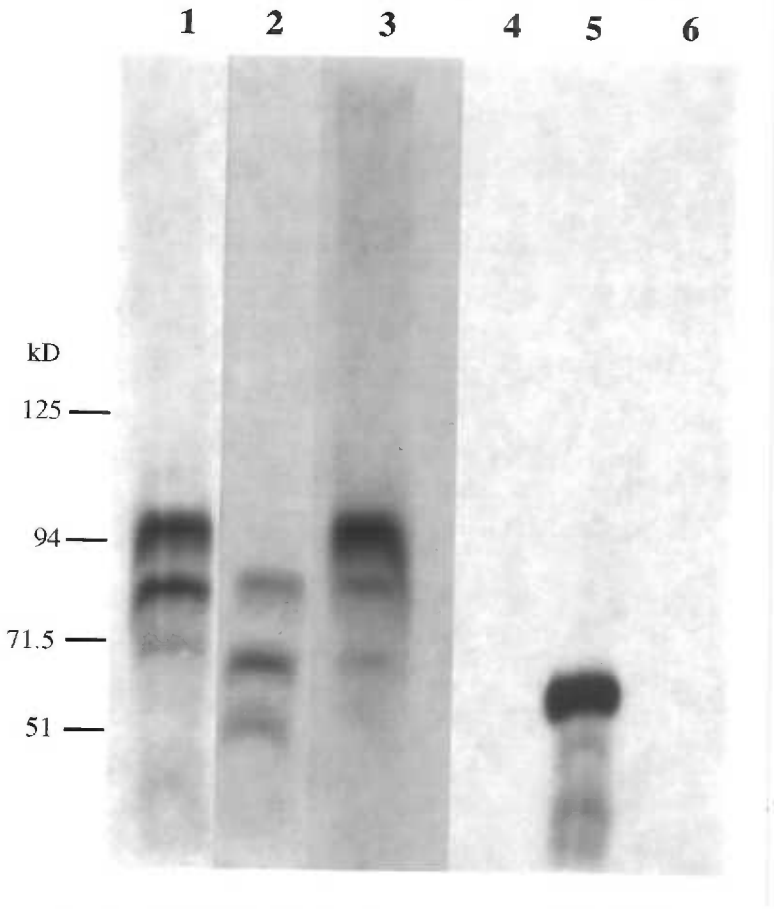


Figure 19: *In vitro* translation products of the 2300 bp FGFR-1 clone and the 2000 bp FGFR-1 clone. The clones were submitted to an in vitro transcription/translation reaction as described in Materials and Methods. (1) translation products of the 2300 bp FGFR-1 clone. (2) translation products of the 2000 bp FGFR-1 clone. (3) translation products of the combination of the 2300 bp FGFR-1 clone and the 2000 bp FGFR-1 clone. (4) translation products from empty vector (control). (5) translation from control plasmid, luciferase, which encodes a 61.9 kD primary translation product. (6) translation products from no added DNA (control). Migration of molecular weight markers (x 10^{-3}) is indicated on the left.

Sulfated Molecules are Important in FGF Mediated Mitogenic Signal Transduction

Sulfation of extracellular associated molecules have been shown to be critical in

FGF signaling in certain immortal cell lines and transfected cells [120]. To determine if sulfated molecules produced by dermal fibroblasts were important for aFGF and bFGF mitogenesis, mitogenic assays were conducted in the presence of sodium chlorate. Sodium chlorate is an inhibitor of the enzyme adenyltransferase (which is required for the sulfation of glycosaminoglycans) [120]. The cells were plated directly in either medium containing 30 mM sodium chlorate or medium containing 30 mM NaCl (a control for osmolarity) and allowed to grow to confluency. The mitogens were added to the culture medium in the presence or absence of soluble heparin and DNA synthesis was assayed. Figure 20 represents a typical mitogenic assay under these conditions. Dermal fibroblasts, in the presence of 30 mM NaCl, responded to aFGF and bFGF as described earlier. bFGF was fully mitogenic regardless of the presence of soluble heparin and aFGF was mitogenic in the absence of soluble heparin but its mitogenic effect was greatly enhanced in the presence of soluble heparin. In contrast, fibroblasts cultured in the presence of chlorate required the addition of soluble heparin in order to respond to either aFGF or bFGF. The addition of either aFGF or bFGF to the culture medium of dermal fibroblasts even at elevated concentrations (>100 ng/ml) did not overcome the effects of chlorate treatment (data not shown). Also, when the cells were cultured in the presence of chlorate their ability to respond mitogenically to EGF was unchanged (data not shown), suggesting that the effect of chlorate was specific for heparin-binding molecules, and demonstrates that the effect of chlorate is not merely toxic Thus, chlorate treatment of dermal fibroblasts in culture reduces the mitogenic response of these cells to aFGF and bFGF.

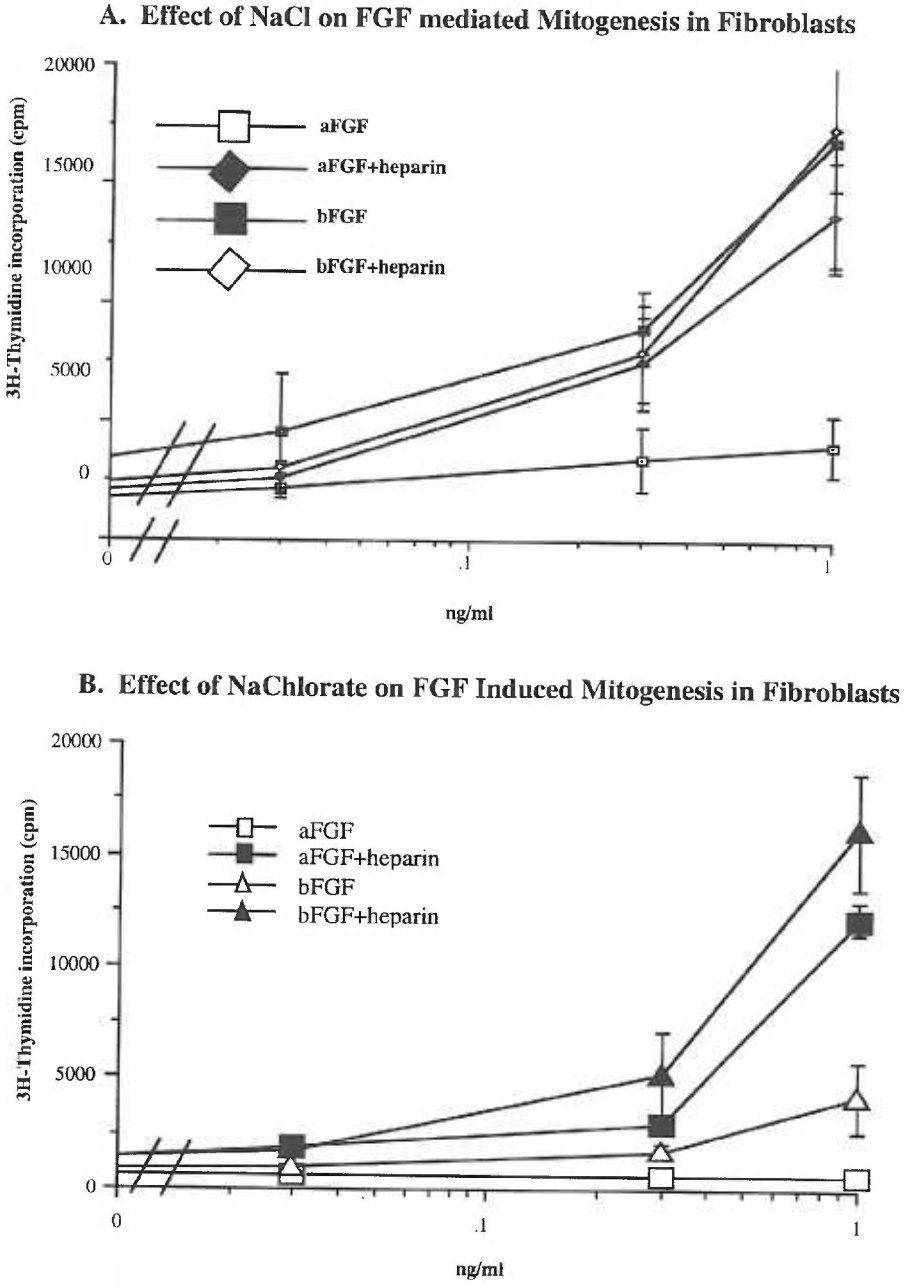


Figure 20: Response of normal human dermal fibroblasts to varying concentrations of bFGF and aFGF in the presence and absence of heparin (10μg/ml). Normal human dermal fibroblasts were plated in MCDB 202a supplemented with 1.0 μg/ml insulin. **(A) CONTROL**-cells were plated in media above containing an additional 30mM NaCl as a

control for osmolarity for the following experiments. **(B) SODIUM CHLORATE**-cells were plated in MCDB 202a + insulin + 30mM NaChlorate. All cultures were treated with the indicated concentrations of either bFGF or aFGF and ^{3}H-thymidine incorporation was determined as described in Materials and Methods.

Discussion

This study has shown that dermal fibroblasts express a single high-affinity FGFR gene which may be responsible for aFGF and bFGF induced mitogenesis. Two alternatively spliced forms of FGFR-1 were described. Dermal fibroblasts express a FGFR-1 IIIc variant. For comparison, other cells that reside in the same tissue as dermal fibroblasts *in vivo* were also examined for FGFR expression. Keratinocytes express FGFR-2 IIIb (KGF receptor) and FGFR-3; and melanocytes express FGFR-1. Dermal fibroblasts produce two FGFR-1 proteins with apparent molecular weights of 97.6 and 83.9 kD. Both proteins are substrates for tyrosine phosphorylation. The two dermal fibroblast-derived FGFR-1 proteins do not shift their molecular weights when treated with reagents to remove glycosylation sites. The larger, 97.6 kD, form of FGFR-1 was found in dermal fibroblast cell lysate, while the smaller, 83.9 kD, form of FGFR-1 was found both in the cell lysate and the conditioned medium. Cloning and sequencing two distinct FGFR-1 cDNAs from dermal fibroblasts revealed the fine structure of these molecules. Sequence analysis of the 2300 bp FGFR-1 clone showed an open reading frame of 730 amino acids. The predicted protein would contain two immunoglobulin domains (domains II and III), a signal sequence, an acidic box, a transmembrane domain, and a split tyrosine kinase domain. The predicted size of the core protein would be approximately 82 kD. Sequence analysis of the 2000 bp FGFR-1 clone showed an open reading frame of 614 amino acids. This molecule encodes a FGFR-1 identical to the 2300 bp FGFR-1 clone with two major deletions: (1) the exon encoding the carboxy half of the third immunoglobulin domain is missing; and (2) the exon encoding the transmembrane domain is missing. The predicted size of the core protein would be approximately 69.1 kD. Distinct mRNAS expressed by dermal fibroblasts were seen in RNase protection assays when using an FGFR-1 specific probe. The expression of the 2000 bp FGFR-1 mRNA was down-regulated by serum-treatment of the cells. *In vitro*

translation of the full length clones isolated from dermal fibroblast cDNA showed that the individual FGFR-1 cDNA clones could encode proteins that correlate in size with those found in dermal fibroblast cell lysates. Mitogenic assays performed in the presence of chlorate suggest that sulfated molecules are critical for aFGF and bFGF mediated mitogenesis in dermal fibroblasts.

Two Alternatively Spliced FGFR-1 Variants are Produced by Dermal Fibroblasts

Two FGFR-1 proteins were identified in dermal fibroblast lysates. These proteins were not found to be differentially glycosylated forms of the same protein. Subsequently, two dermal fibroblast-derived cDNA clones were obtained by PCR amplification with FGFR-1 specific primers. When sense RNA was generated from the two cDNA clones and hybridized to a FGFR-1 specific probe in RNase protection assays, the observed protected fragments were identical to the protected fragments seen when the same probe was hybridized to poly A+ mRNA from dermal fibroblasts. Finally, *in vitro* translation of the two cDNA clones gave rise to proteins that migrated with similar molecular weights when compared with the molecular weights of the dermal fibroblast-derived FGFR-1 proteins. In conclusion, the 2300 bp FGFR-1 clone gives rise to the 97.6 kD dermal fibroblast-derived protein, and the 2000 bp FGFR-1 clone gives rise to the 83.9 kD dermal fibroblast-derived protein.

The predicted sizes of the primary translations products from the clones differ from the *in vitro* translated proteins. The differences in molecular weight between the predicted primary translation products and the *in vitro* translated products are likely relative as the molecular weight determinations were based on the migrations compared with the molecular weight ladder. When using the same molecular weight ladder, the apparent molecular weights of the two dermal fibroblast-derived FGFR-1 proteins correlate nicely with the *in vitro* translated proteins.

The dermal fibroblast-derived FGFR-1 proteins identified in this study do not appear to be heavily N-linked glycosylated. The presence of carbohydrate residues has been previously reported to be required for FGFR function [125]. Several labs have reported large reductions in the molecular weights of FGFRs from a variety of transfected cell lines when treated with reagents to remove glycosylated side chains [71, 96]. The data obtained in my study suggests that dermal fibroblasts glycosylate their FGFR-1s very little or not at all, yet are capable of aFGF and bFGF induced mitogenesis. Recently a soluble non-glycosylated form of FGFR-1 which was manufactured in E.Coli, was shown to bind bFGF with high-affinity and compete in a dose/response fashion in biological assays [148] suggesting that glycosylation of FGFR-1 may not be critical for FGF- mediated signal transduction in certain cell types.

Sequence analysis of the 2300 bp FGFR-1 clone and the 2000 bp FGFR-1 clone reveals the exons which combine to form these clones. The form of FGFR-1 encoded by the 2300 bp FGFR-1 clone has been reported to exist both in human and mouse cells [73, 84]. The Arg-Met deletion at the 3' end of the exon encoding the acidic box found both in the 2300 bp FGFR-1 clone and the 2000 bp FGFR-1 clone has been previously described [67, 149]. This alternative splice variant has only been described in the FGFR-1 gene. The significance of this deletion is not known. A variation of the Val-Thr deletion at the 3' end of the exon encoding the transmembrane domain described for both clones has been previously described in the FGFR-1 and FGFR-2 genes (the generation of these deletions will be discussed further below) [66, 70, 78]. In addition to these small deletions, the 2000 bp FGFR-1 clone was found to encode a yet unreported form of FGFR-1 that is missing both the exon encoding the carboxy-terminal half of Ig domain III and the exon encoding the transmembrane domain. A similar FGFR-2 cDNA was reported to have been isolated from a human placental cDNA library, however, no data (either cDNA or protein) was shown [93]. The sequence analysis from the 2000 bp

FGFR-1 clone suggested that a protein generated from this clone could give rise to a secreted receptor. cDNAs encoding putative secreted FGFRs have been reported [84, 93]. Duan et al. showed that a FGFR-1 IIIa construct, originally cloned from a human placental cDNA library, encoding a putative secreted form of FGFR-1 (see Figure 4-i), when transfected into CHO cells could be found in the conditioned medium [100]. Although, there is no data to suggest that this receptor is actually secreted from the cell from which the cDNA was originally obtained. The isolation of a cDNA from dermal fibroblasts that encodes a potentially secreted receptor supports the possibility that the FGFR-1 I identified in the medium conditioned by these cells is actually secreted.

For unclear reasons, the 2000 bp FGFR-1 clone was extremely difficult to obtain. The 2000 bp PCR fragment could be visualized on an agarose gel, albeit at a much lower concentration than the 2300 bp PCR fragment, when using multiple sets of FGFR-1 specific primers. However, the 2000 bp PCR fragment was successfully cloned when using only the primer pair, P1a and 3'R1XbaI3.1.93 (see Figure 21). The 2300 bp PCR fragment could be cloned successfully regardless of what FGFR-1 specific primer pair was used. It's possible that the 5' primer containing the Hind III cloning site interfered with the cloning of the 2000 bp PCR fragment. It is also possible that the 2000 bp PCR fragment was not cloned easily because of effects due to low concentration.

Both FGFR-1 Proteins are Substrates for Tyrosine Phosphorylation

The FGFR-1 proteins produced by dermal fibroblasts in this study can act as substrates for tyrosine phosphorylation. This is important as recent evidence shows that tyrosine phosphorylation of receptors with tyrosine kinase activity is essential for mediating interactions with signaling molecules (for review see [58]). Also, ligand-induced autophosphorylation of certain tyrosine kinase receptors is thought to cause a conformational change within the receptor itself such that it becomes competent to

interact with and phosphorylate cellular substrates [150]. Recently, Bellot et al. showed that the three Ig domain forms of both FGFR-1 and FGFR-2, when transfected into NIH 3T3 cells, are capable of autophosphorylation *in vitro* [57]. Because of the experimental design utilized in my study, it is not clear if the FGFR-1 proteins produced by dermal fibroblasts are obtaining phospho-tyrosine through an autophosphorylation mechanism or a transphosphorylation mechanism. Regardless of the mechanism by which their tyrosine residues become phosphorylated, the two dermal fibroblast-derived FGFR-1 proteins described in this study can exist in the "activated/phosphorylated" state and may participate in a mitogenic signal pathway within dermal fibroblasts.

FGFR Expression in Human Skin-Derived Cells Correlates with Their FGF Responsiveness

Previous studies have demonstrated FGFR expression in skin tissue [82]. Werner et al. have shown FGFR expression in skin compartments, the dermis and the epidermis [127]. They found FGFR-1, FGFR-2 and FGFR-3 expressed in both dermis and epidermis. This data confirms and extends the data obtained in my study. In the Werner study epidermis was shown to express FGFR-1, FGFR-2, and FGFR-3 simultaneously. Analysis of the data from my study shows that expression of FGFR-1 is likely contributed by the melanocytes that reside in the epidermis, and expression of FGFR-2 and FGFR-3 are likely contributed by keratinocytes that also reside in the epidermis. In the Werner study dermis was shown to express FGFR-1, FGFR-2, and FGFR-3, as well. Analysis of the data from my study suggests that expression of FGFR-1 is likely contributed by the fibroblasts that reside in the dermis. The expression of FGFR-2 and FGFR-3 are probably contributed by vascular endothelial cells and inflammatory cells that also reside in the dermis, although these cells were not specifically analyzed in either study.

The FGFR expression pattern of human skin-derived cells shown in this study fits with what we know about the mitogenic responsiveness to aFGF and bFGF by these individual cell types. Dermal fibroblasts respond to aFGF and bFGF (see Figures 10 and 20), but have a greatly reduced mitogenic response to KGF [17]. Dermal fibroblasts express only FGFR-1 IIIc. Binding studies with FGFR-1 IIIc have shown that aFGF and bFGF bind with similar affinities in the range of 20-100 pM [71]. FGFR-1 IIIc binds KGF with a reduced affinity [72]. Keratinocytes respond to aFGF, bFGF, and KGF [6, 17, 41] and express FGFR-2 IIIb (KGF receptor) and FGFR-3. FGFR-2 IIIb binds KGF and aFGF with similar affinities of approximately 20 pM, but binds bFGF with much reduced affinity [76, 87]. Because FGFR-3 has been shown to be activated equally in the presence of either aFGF or bFGF [81], it may represent the bFGF receptor in keratinocytes. In contrast, melanocytes respond only to bFGF [43] and express FGFR-1 (see Figure 12). Although FGFR-1 is capable of binding aFGF, the fact that melanocytes do not respond to aFGF may be due to a lack in the expression of a low-affinity FGF site that is important for aFGF mitogenic signal transduction.

Low-Affinity FGF Sites are Critical for FGF Induced Mitogenesis

The strong affinity of FGFs for heparin along with the observation that many cell types possess heparin-like molecules on their cell surface has suggested a physiologic role for these moieties as low-affinity FGF binding sites. Heparin is a sulfated glycosaminoglycan with a strong negative charge [115]. This charge makes it possible to bind many substances, including certain growth factors. However, proteins can also bind specifically to heparin-like molecules via recognition of a unique sulfation pattern or polysaccharide sequence [151]. Both aFGF and bFGF possess two potential binding domains for heparin [20, 152], and recently a potential heparin specific binding domain was identified for FGFR-1 [117]. This current study has shown that dermal fibroblasts require sulfation of cell surface molecules for FGF induced signal transduction. This

study is the first to show a requirement for sulfated cell surface molecules in FGF signal transduction in normal cells. Dermal fibroblasts, in the absence of chlorate, respond to bFGF in the absence or presence or heparin and have an enhanced mitogenic response to aFGF in the presence of heparin. With chlorate treatment, the cells still require soluble heparin to respond to aFGF, but also require soluble heparin to respond to bFGF. This suggests that soluble heparin is as efficient as the putative bFGF low-affinity binding site present on dermal fibroblasts at assisting in triggering mitogenic signal transduction. Because aFGF in the absence of chlorate requires soluble heparin it is likely that aFGF does not bind the same low-affinity site as bFGF. Soluble heparin likely acts as a low-affinity site for aFGF. By altering cell surface sulfation with chlorate, dermal fibroblasts loose this low-affinity site and bFGF is no longer active. bFGF's effect can be totally recovered, however, with the addition of soluble heparin supporting the hypothesis that bFGF requires prior binding to some sort of sulfated moiety before high-affinity binding can occur.

Functions of a Secreted Receptor

This is the first report of a naturally occurring secreted FGFR. This is also the first study to show regulation of a particular form of a FGFR. This study has shown that both dermal fibroblast-derived FGFR-1 forms are capable of undergoing tyrosine phosphorylation which suggests they are both active kinase molecules. In addition, the secreted form of FGFR-1 identified in this study contains sequences that may allow it to bind ligand, albeit, with a reduced specificity as the region that confers ligand specificity (the exon encoding the carboxy-terminal half of Ig domain III) is deleted. This assumption is speculative as binding experiments were not performed in this study. Recently, Zimmer et al. showed that multiple receptor elements which are located at both immunoglobulin domains II and III are involved in ligand binding and specificity [153]. Because this receptor retains its tyrosine kinase domain it is conceivable that an

extracellular tyrosine kinase domain could be involved in FGF mediated signal transduction by phosphorylation of cell surface proteins. It is also possible that this secreted receptor could associate with membrane bound FGFRs on fibroblasts themselves or neighboring cells that reside in the same tissue, and by this association could either enhance or inhibit FGF mediated signal transduction. Cotransfection experiments illustrate that heterodimers between different FGFRs can form [57]. The dermal fibroblast-derived secreted FGFR-1 may cause a dominant-negative suppressive effect of FGF-induced proliferation by preventing membrane-bound heterodimers from forming. Interestingly, the secreted FGFR-1 appears to be most abundant when the cells are confluent and in the absence of serum, which suggests that there may be a predominance of the secreted FGFR-1/membrane-bound FGFR-1 complex at the cell surface under these conditions. This may cause a relative inability of the cell to respond to aFGF or bFGF. When serum stimulation occurs and the secreted FGFR-1 is down-regulated, this constraint may be eliminated, thus allowing cells to respond fully to aFGF or bFGF. It is also possible that a secreted FGFR-1 could associate with a membrane-bound FGFR from another gene. This potential effect could amplify the response of cells to various FGFs and their corresponding receptors.

The genes for several transmembrane receptor proteins, including FGFR as well as cytokine receptors, have recently been shown to encode potential secreted alternative proteins [154]. Additional putative functions of a secreted receptor include: (1) competition with ligand for binding to membrane-bound counterparts; and (2) transportation of ligand to aid in the availability of the ligand to membrane-bound counterparts. It is interesting to note that the secreted form of dermal fibroblast-derived FGFR-1 is down-regulated by serum treatment of the cells. The presence of serum may mimic a wound state. A wound may cause down-regulation of the secreted form of FGFR-1 so that this form of the receptor cannot compete for ligand. Thus, allowing all

the available FGF in the wound mileau to participate in mitogenesis for rapid cell growth and healing. Accordingly, soluble FGFR may hold significant potential for therapeutic use in modulating action of FGF *in vivo* as a competitive antagonist, in situations where autocrine/paracrine stimulation by FGF leads to pathological consequences. It is also possible that a secreted FGFR could be used as a competitive antagonist to decrease the number of herpes simplex virus type 1 infected cells as this virus has been shown to utilize a FGFR for cellular entry [155].

Creation of Multiple FGFR Proteins

As shown previously in Figure 3, alternative splicing of FGFR mRNA results in either (a) inclusion/exclusion of additional amino acids or (b) the use of alternate coding exons. In either case, the resultant proteins are structurally different. Sequence analysis of the FGFR-1 clones described in my study contain both types of alterations stated above. Both FGFR-1 variants contain two small deletions at amino acids #61 and #62, Arg-Met, and amino acids #336 and #337, Val-Thr. Analysis of the sequences at the Arg-Met site suggest that this deletion arises from alternative use of the intron/exon splice site located between exons #4 and # 5 (see Figure 3 and [85]). FGFR-1 containing the Arg-Met deletion has been shown to be fully active [70]. The significance of this deletion is not known. The Val-Thr deletion described here is a subtle variation of the Thr-Val deletion previously described in FGFR-1 and FGFR-2 [66, 70, 78]. The amino acid sequence at the exon #10-exon #11 junction of FGFR-1 is . . . Q V T/V S A . . . The previously described Thr-Val deletion arises from splicing the above sequence at . . .Q V/ (T V)/S A . . . The FGFR-1 variant forms described here likely arise from alternate use of the exon #10 splice site. The base sequence at this site contains at least two consensus splice sites. Splice site choice is based on multiple factors including the presence of consensus splicing sequences as well as pre-mRNA secondary structure [85]. Undoubtedly, dermal fibroblasts analyzed in my study have chosen to utilize an alternate

consensus splice site to result in . . . Q/(V T)/V S A . . . This deletion may have consequences on signal transduction should either ligand affinity or tyrosine kinase activity be regulated by phosphorylation of this site by a serine-threonine kinase, as has been shown for the EGFR [58]. The 2000 bp FGFR-1 clone contains an additional large deletion at exons #9 and #10 (see Figure 3). These exons encode the carboxy-terminal half of Ig domain III and the transmembrane domain. The generation of this FGFR-1 variant can also be explained by alternate use of potential exon splice sites. Both boundaries of the deleted region encode consensus splice site sequences.

Conclusions

In this study, the FGFR (FGFR-1) expressed in dermal fibroblasts which may be responsible for aFGF and bFGF induced mitogenesis has been described. Also, the FGFR expression of human skin-derived keratinocytes and melanocytes has been detailed. It was shown that dermal fibroblasts produce two forms of FGFR-1 protein, one of which is secreted. The secreted FGFR-1 form appears to be down-regulated by serum treatment of the cells. In addition, it was shown that sulfation of cell surface molecules is critical in FGF induced mitogenesis in dermal fibroblasts.

Materials and Methods

Cell Culture

For the purpose of cell lysate collection stock cultures of dermal fibroblasts were maintained in McCoy's 5A medium (Gibco, Grand Island, NY) supplemented with 5% FBS. For studies containing sodium chlorate, dermal fibroblasts were plated in a 24-well dish at 5 x 10^4 cell/well in MCDB 202a supplemented with 5 ng/ml EGF, 1 μg/ml insulin, 100 μg/ml BSA and 30 mM sodium chlorate (Sigma Chemical Co., St. Louis, MO). Control cultures for the sodium chlorate experiments were plated in an identical fashion with 30mM NaCl in place of the sodium chlorate as a control for osmolarity.

Glycosylation analysis of FGFR-1 expressed by dermal fibroblasts cells was obtained by culturing the cells in McCoy's 5A medium supplemented with 5% calf serum and 0.5 μg/ml tunicamycin (a gift from Dr. C. Enns). Tunicamycin treatment was conducted for 24 hours, followed by collection of cell lysate and western blot analysis.

Pulse-chase experiments of dermal fibroblasts cells were conducted by incubating the cells in MCDB 202a medium without methionine for 2 hours, followed by addition of fresh medium containing 30 μCi/ml ^{35}S-methionine (New England Nuclear, Boston, MA) for 5 hours. Cell lysate and conditioned media were collected and analyzed by SDS-PAGE.

Other cell types: NBT-II cells, a rat bladder carcinoma cell line (ATCC), were grown in DMEM medium (Gibco, Grand Island, NY) + 10% FBS. KATO-III cells, a human stomach carcinoma cell line (ATCC), were grown in RPMI 1640 (Gibco, Grand Island, NY) + 10% FBS.

DNA Synthesis Assays

For measurement of ^{3}H-thymidine incorporation into DNA following sodium chlorate treatment, dermal fibroblasts cells were processed as described in the previous chapter (Materials and Methods). Control cultures and sodium chlorate treated cultures were harvested separately and analyzed for relative incorporation of ^{3}H-thymidine into TCA-insoluble material.

Preparation of Cell Lysates and Western Blot Analysis

Dermal fibroblasts were lysed in ice-cold M-RIPA buffer (50 mM TRIS-HCl pH 7.4; 1% NP-40; 0.1% deoxycholic acid; 150 mM NaCl; 10 mM sodium pyrophosphate; 10 mM NaF; 4 mM disodium EDTA; 0.02 mM leupeptin; 2 mM sodium vanadate; 1 mM phenylmethylsulfonyl flouride (PMSF)) and scrapped from the culture dish. Nuclei were removed by centrifugation and the lysates were incubated with anti-FGFR antibody that had been preadsorbed to protein A-Sepharose (Sigma Chemical Co., St. Louis, MO). Incubation was performed overnight at 4 ^{o}C on a rocker table. For each sample point, conditioned media and cell lysates were collected from approximately 5-6 x 10^{6} cells. Conditioned media were collected, and immunoprecipitated in the presence of protease inhibitors as described above for cell lysates. Following overnight incubation, the antibody/Protein A-Sepharose complex was washed twice with ice-cold M-RIPA buffer, centrifuged, and bound material was eluted by SDS-PAGE sample buffer. The samples were electrophoresed through 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The blots were preincubated with TTBS (10 mM TRIS-Hcl pH 7.4; 150 mM NaCl; 0.05% Tween-20) supplemented with 5% (w/v) dry non-fat milk (blocking solution). Primary antibody incubation was performed overnight at 4 ^{o}C on a rocker table in blocking solution described above. Primary antibodies included: (1) anti-FGFR-1 mouse monoclonal antibody (UBI) at 5 µg/ml; (2) anti-phosphotyrosine mouse monoclonal antibody (Sigma cat.#P-3300) at 1:10,000 dilution; (3) anti-FGFR-1/FGFR-3

rabbit polyclonal antibody SB102 [96] (a gift from M. Haymen, PhD. Stony Brook, NY) at a dilution of 1:1000. This antibody is directed against the tyrosine kinase domain of FGFR-3, but cross reacts with FGFR-1. Following primary antibody incubation, the blots were washed in several changes of TTBS at room temperature and incubated in the appropriate secondary antibody conjugated to horseradish peroxidase (Biorad goat-anti-mouse HRP; Biorad goat-anti-rabbit HRP) and washed thoroughly with TTBS. Blots were analyzed using the chemiluminescence technique available from New England Nuclear (Wilmington, DE). Specificity of the antibodies was determined by simultaneous loading of a human FGFR-1 positive control on all blots. The human FGFR-1 preparation used is available from UBI (Cleveland, OH) and is a baculovirus product encoding the two-immunoglobulin domain form of human FGFR-1.

Oligonucleotide Probes

Human cDNA probes corresponding to the extracellular domains of FGFR-1 IIIc (pCD115) and FGFR-2 IIIc (pcD116) [71], were prepared by the random primer extension labelling system available from NEN (Boston, MA). A cRNA probe corresponding to the 117 bp region encoded by the ligand specific exon of FGFR-2 IIIb, was synthesized from a template cloned from human keratinocytes (see below). A human cDNA FGFR-3 probe corresponding to the region encoding the signal sequence and a portion of the first immunoglobulin domain was prepared by collecting a 362 bp Pvu II fragment from digesting the construct He8/pGEM3Zf+ [82]. A human cDNA FGFR-4 probe corresponding to the region encoded by the signal sequence and the entire first immunoglobulin domain was prepared by collecting a 192 bp Hinf I fragment from a digest of the construct He6/pGEM3Zf+ [82].

Positive controls for probe hybridization of northern blots were generated from two cell types. Positive controls for FGFR-3 and FGFR-4 were obtained from KATO-III

cells, a human stomach carcinoma cell line (ATCC). The cells were grown and polyA+ mRNA collected as described in the previous chapter (Materials and Methods). NBT-II cells, a rat bladder carcinoma cell line (ATCC), were grown and poly A+ mRNA isolated as a positive control for the FGFR-2 exon IIIb (KGF receptor). NBT-II cells did not express detectable levels of FGFR-2 exon IIIc.

Northern Blots

PolyA+ mRNA and northern blot analysis was conducted as previously described (see previous chapter; Materials and Methods). Growth factor receptor northern blots, excluding blots hybridized with the FGFR-2 IIIb probe, were hybridized with cDNA probes for 18 hours at 42-45 ^{o}C using 1-5 x 10^{6} cpm/ml cDNA probe in hybridization solution. Northern blots hybridized with a cRNA probe specific to FGFR-2 IIIb (KGF receptor) were incubated for 18 hours at 60-65 ^{o}C using 1-5 x 10^{6} cpm/ml cRNA probe in hybridization solution.

Polymerase Chain Reaction

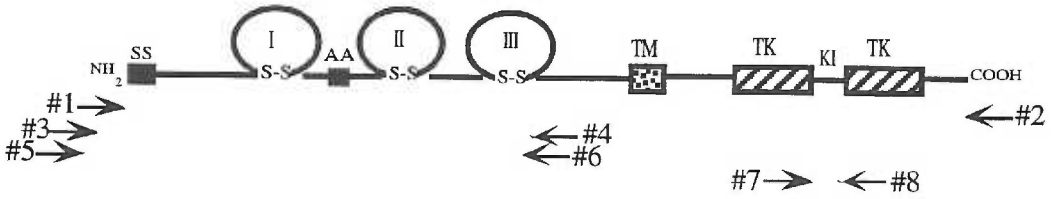
Single stranded cDNA was prepared by simultaneous priming of poly A+ mRNA with oligo-dT and random hexamers according to the method of Maniatis [156]. Polymerase chain reaction (PCR) was performed with 10-20 pmol of each oligonucleotide primer in 50 mM KCl, 1.5-3.5 mM $MgCl_2$, 10 mM TRIS-HCl pH 8.3, 0.01% gelatin, 0.2 mM each dATP, dGTP, dCTP, and dTTP, 1.25 U Taq DNA polymerase (Bethesda Research Laboratories; Bethesda, MD) in a final volume of 25 µl, according to the method of Maniatis [156]. Reactions for specific primers were performed for 35 cycles with cycling times of 93 ^{o}C for 1 min., 60 ^{o}C for 1 min., and 72 ^{o}C for 2.5 min. Reactions for degenerate primers were performed for 40 cycles with cycling times of 95 ^{o}C for 1 min., 55 ^{o}C for 1 min., and 72 ^{o}C for 1 min.

PCR primers have the following sequences (see Figure 21):

Table 2:

PRIMER NAME	FGFR SPECIFICITY
1.) 5'R1H33.1.93-TATTAAGCTTCCATGGAGATGTGGAGCCTTG	FGFR-1
2.) 3'R1Xba13.1.93-GTCATCTAGAAGGGTTACAGCTGACGGT	FGFR-1
3.) P1a-CGAGCTCACTGTGGAGTATCCATG	FGFR-1
4.) P1b-GTTACCCGCCAAGCACGTATAC	FGFR-1
5.) 5'Bek(-117)-CGCCTTCGGTTCCTGAG	FGFR-2
6.) 3'KGFR-AGACTGGTTGGCCTG	FGFR-2IIIb
7.) 5'MEM(tk)-GCGGGATCCGTTTTTTTGTAGTAATC	all FGFRs-degenerate
8.) 3'DYY(tk)-GCGCTGCAGATGGAAATGATGAAAAT	all FGFRs-degenerate

Figure 21: Schematic representation of the protein structure of an FGFR showing the



approximate locations where PCR primers utilized for cloning purposes hybridize to a particular FGFR gene. Small arrows below the diagram represent specific primers listed in Table 2. Primers with their arrowheads pointing to the right are 5' primers and primers with their arrowheads pointing to the left are 3' primers. SS: signal sequence; AA: acidic box; S-S: disulfide bonds forming Ig loops; TM: transmembrane domain; TK: tyrosine kinase domain; KI: kinase insert domain.

Cloning

PCR products were cloned directly into vectors containing 3' dT-overhangs at the insertion sites. This cloning system takes advantage of the template independent activity

of thermostable polymerases used in PCR that add single deoxyadenosines to the 3'-end of all duplex molecules. These A-overhangs were used to insert the PCR products into the vectors described above.

The FGFR-2 IIIb construct (corresponding to the extracellular domain of FGFR-2 IIIb (KGF receptor)) was generated by cloning a 920bp PCR product obtained from normal human keratinocyte cDNA utilizing the primers detailed above, 5'Bek(-117) and 3'KGFR, into pCR1000 vector (Invitrogen: Sand Diego, CA) (pCR1000KGFR(mixC) see Figure 22). The PCR product was purified by ion-exchange using chromatographic beads (Qiagen; Chatsworth, CA) according to the manufacturer's method and cloned directly into pCR1000 vector. This construct was later used for cRNA probe generation and northern blot analysis. The FGFR-2 IIIb construct was linearized with Ava I and T7 RNA polymerase was used to generate antisense RNA.

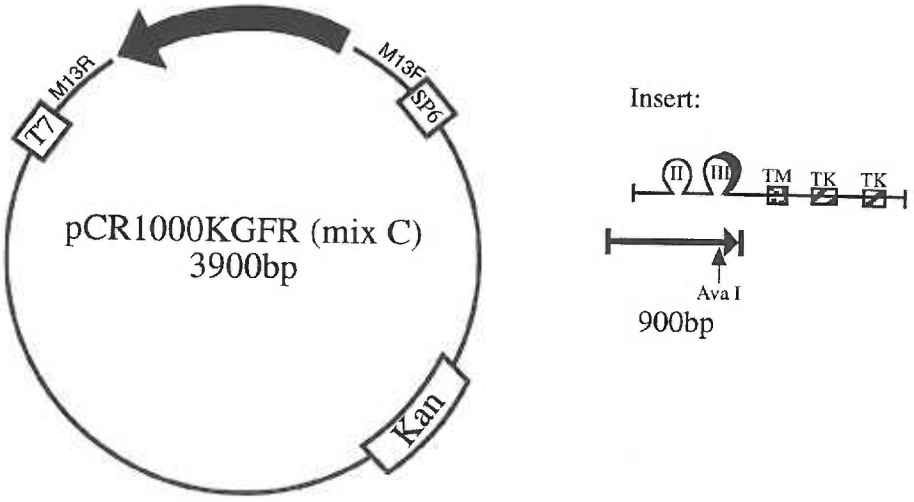


Figure 22: Diagram of plasmid construct. The insert encodes a 900 bp fragment representing FGFR-2 IIIb. The smaller diagram at the right shows a schematic of an FGFR protein indicating the portion of the molecule that is contained in the plasmid. The Ava I site indicates the cut site used to generate a FGFR-2 IIIb specific cRNA probe (see text). T7 and SP6: RNA polymerase promoters. M13R and M13F: sequencing

primer sites. Kan: kanamycin resistance gene. Bold arrow indicates the inserted FGFR-2 IIIb sequence with the 3' end located at the arrow head.

A full-length FGFR-1 construct (corresponding to -19 bp 5' to ATG start site and +47 bp 3' TGA stop) was generated by cloning a 2300 bp PCR product obtained from dermal fibroblast cDNA utilizing the primers, 5'R1H33.1.93 and 3'R1Xba13.1.93, into pCRII (Invitrogen). The PCR product was purified by ion-exchange as above, prior to cloning. The full-length insert was removed from the plasmid with Hind III/Xba I and subcloned into the vector pRC/CMV (Invitrogen) using the same restriction enzymes (pRC/CMV2300c1.1 see Figure 23). This construct was used to generate full-length sense RNA for controls in RNase protection assays.

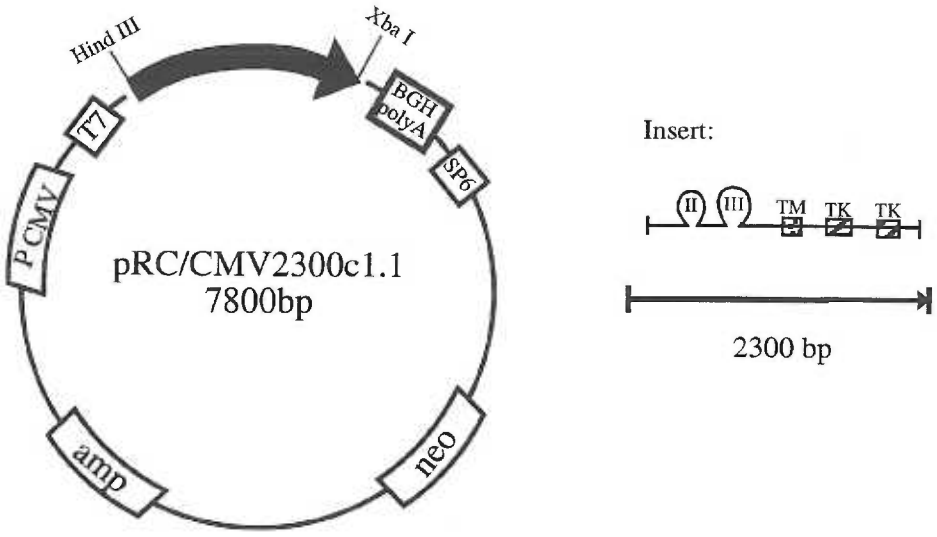


Figure 23: Diagram of plasmid construct. The insert encodes a full-length 2300 bp FGFR-1 IIIc open reading frame. The smaller diagram at the right is a schematic of an FGFR protein indicating the portion of the molecule that is contained in the plasmid. Hind III and Xba I indicate the cloning sites used to insert this sequence. T7 and SP6: RNA polymerase promoters. P CMV: CMV promoter (a mammalian promoter). BGH poly A: sequence from the bovine growth hormone gene encoding polyadenylation sites. Amp and neo: 0 and neomycin resistance genes. Bold arrow indicates the inserted

FGFR-1 IIIc sequence with the 3' end located at the arrow head.

Another FGFR-1 construct (different than that stated above) (corresponding to -43 bp 5' to ATG start site and +47 bp 3' TGA stop) was generated by cloning a 2000 bp PCR product obtained from dermal fibroblast cDNA utilizing the primers, P1a and 3'R1Xba13.1.93, into pTZBlue (Novagen; Madison, WI). The insert was removed from the above vector with Xba I/BamHI, Klenow filled and blunt-end ligated into Not I linearized pRC/CMV (pRC/CMV2000c2.2 see Figure 24). This construct was used to generate sense RNA for controls in RNase protection assays.

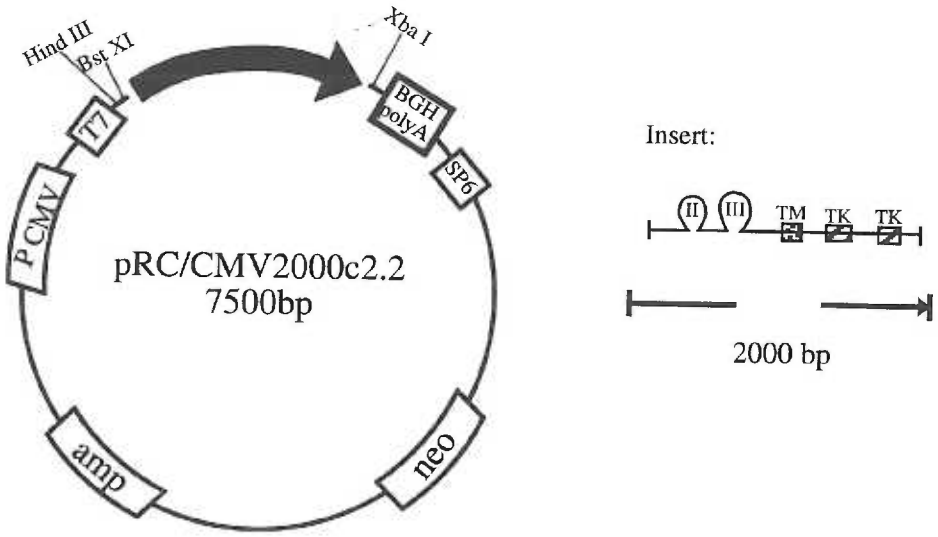


Figure 24: Diagram of plasmid construct. The insert encodes a complete 2000 bp FGFR-1 IIIc open reading frame with two deletions indicated by the interrupted line at the right. The smaller diagram at the right is a schematic of an FGFR protein indicating the portion of the molecule that is contained in the plasmid. Hind III, Bst XI, and Xba I indicate the cloning sites present in the polycloning region. T7 and SP6: RNA polymerase promoters. P CMV: CMV promoter (a mammalian promoter). BGH poly A: sequence from the bovine growth hormone gene encoding polyadenylation sites. Amp and neo: ampicillin and neomycin resistance genes. Bold arrow indicates the inserted FGFR-1 IIIc sequence with the 3' end located at the arrow head.

A FGFR-1 IIIc construct encoding only the extracellular region of the molecule was generated by cloning a 870 bp PCR product obtained from dermal fibroblast cDNA utilizing primers P1a and P1b into the vector pCRII vector (pCRII877c2; see Figure 25). This construct was linearized with Pvu II at a site located in the second immunoglobulin domain, and used to generate antisense riboprobe for RNase protection.

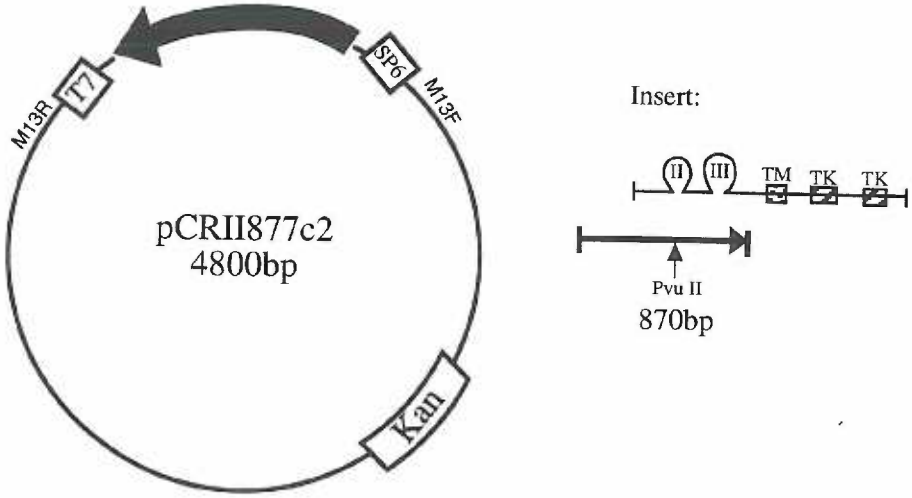


Figure 25: Diagram of plasmid construct. The insert encodes a 870 bp fragment representing the extracellular portion of FGFR-1 IIIc. The smaller diagram at the right is a schematic of an FGFR protein indicating the portion of the molecule that is contained in the plasmid. The Pvu II site indicates the cut site used to generate a specific cRNA probe (see text). T7 and SP6: RNA polymerase promoters. M13R and M13F: sequencing primer sites. Kan: kanamycin resistance gene. Bold arrow indicates the inserted FGFR-1 IIIc sequence with the 3' end located at the arrow head.

RNase Protection

RNase protection assays were conducted according to the method of Maniatis [156]. RNA samples were incubated in 75% formamide + 5 x 10^5 cpm riboprobe overnight at 53 ^{o}C. Samples were digested in RNase A and RNase T_1. Controls included; (1) undigested probe, (2) digested probe, (3) probe combined with yeast

tRNA, (4) probe combined with sense RNA generated from pRC/CMV2300, (5) probe combined with sense RNA generated from pRC/CMV2000. Size standards were generated by end-labeling a 1 kb DNA ladder (BRL) with T4-polynucleotide kinase [156]. Samples were analyzed on 5% polyacrylamide gels containing 8 M urea.

A RNase protection probe was generated by linearizing pCRII877c2 with Pvu II and using T7 RNA polymerase . This reaction created a 370 bp riboprobe encoding a portion of immunoglobulin domain two and the entire third immunoglobulin domain of FGFR-1 IIIc (see Figure 25).

DNA Sequencing

cDNA inserts were mapped with several restriction enzymes, and were sequenced by the chain-termination method according to the manufacturer's protocol using a Sequenase Kit (USB; Cleveland, OH) [157]. Universal primers (M13) were used to generate the initial sequence and then specific primers were synthesized on the basis of this information to complete the sequencing of the clones.

In Vitro Autophosphorylation Assays

Dermal fibroblasts were grown as described in the previous chapter (Materials and Methods) serum-free medium. The cells were solubilized in M-TG buffer (1% triton X-100; 10% glycerol; 1% aprotinin; 20 mM Hepes pH 8.0; 1 mM PMSF; 25 μM sodium vanadate; 150 mM NaCl) on ice and centrifuged at 15000 x g for 10 min. at 4 C. Proteins were immunoprecipitated with anti-FGFR-1 mouse mAb (UBI) prebound to protein A sepharose. The samples were washed three times with M-TG buffer and incubated with 10 μCi $\{\gamma\text{-}^{32}p\}$ATP in kinase buffer (0.1M DTT; 1.0 M Hepes pH 8.0; 0.2 M $MnCl_2$; 0.5 M sodium vanadate; 10% NP40; 0.2 mM ATP) for 10 min. on ice. Reactions were stopped by addition of Laemmli sample buffer [158] and proteins were

separated on 7.5% SDS-PAGE. The gel was dried and exposed to Kodak X-Omat film.

In Vitro Translation

Plasmid constructs cloned into pRC/CMV vectors representing complete open reading frames for human FGFR-1, pRC/CMV2300c1.1 and pRC/CMV2000c2.2, were used in an *in vitro* translation assay. A Progmega kit, TnT Coupled Reticulocyte Lysate System (Promega; Madison, WI) was used according to the manufacturer's instructions. This kit couples transcription and translation in the same reaction tube. 1.0 µg of circular plasmid was combined with T7 RNA polymerase, 40 µCi ^{35}S-methionine (1000 Ci/mmol), and other kit reagents and incubated at 30 ^{o}C for 60 min. The results of translation were analyzed on 7.5% SDS-PAGE. The gels were treated with a flourographic reagent at exposed to film at -80 ^{o}C for 7-24 hours.

Chapter 4: Summary and Future Directions

The original hypothesis stated that FGFs and their receptors are involved in the growth regulation of human skin-derived cells, specifically dermal fibroblasts. The results described in my thesis support this hypothesis in two ways.

First, dermal fibroblasts grown in serum-free medium wre shwon to produce and respond to aFGF and bFGF. The production of aFGF and bFGF was shown to be enhanced in these cells when treated with serum. This suggests that dermal fibroblasts can possibly control their own growth through an autocrine action by proliferating in response to "self-manufactured" FGF. Moreover, dermal fibroblast-derived aFGF and bFGF may regulate the growth of neighboring cells.

Second, dermal fibroblasts wre shown to express a single high-affinity FGFR gene, FGFR-1. These cells produce both a membrane-bound and a secreted form of FGFR-1. The secreted form of FGFR-1 appears to be down-regulated by serum-treatment of the cells. Both receptors are phosphorylated on tyrosine residues, suggesting that the receptors are involved in some sort of cellular signaling cascade. In addition, dermal fibroblasts require sulfation of cell surface molecules in order to proliferate in response to aFGF and bFGF.

The results detailed in this thesis generate many new questions. Perhaps the most interesting is whether a membrane-bound FGFR-1/secreted FGFR-1 dimer can form? If it can, one must ask what effect these dimers have on mitogenic signal transduction in dermal fibroblasts? These questions may be answered by undertaking the series of experiments described below. First, it must be established whether or not the secreted FGFR-1 identified in this study is capable of binding ligand. Next, it must be determined

whether or not the secreted FGFR-1 could transduce a FGF-induced signal in the absence of other FGFRs. This could be performed by utilizing cells that do not contain endogenous FGFRs that are stably transfected with cDNA encoding the secreted FGFR-1. One prediction is that this stable transfectant would not transduce an FGF-induced signal by itself. By submitting cells that are stably cotransfected with both a cDNA encoding the secreted FGFR-1 form and a cDNA encoding the membrane-bound FGFR-1 form to mitogenic assays one could address these questions directly. It may be that the role of the secreted FGFR-1 in the cotransfected cell is a dominant-negative one. Alternatively, the cotransfected cells may exhibit an enhanced response to FGF by effecting ligand localization to the membrane-bound FGFR-1.

A relationship between the results detailed in chapters 2 and 3 of this thesis can be made. Dermal fibroblasts produce multiple molecular weight forms of aFGF and bFGF grown in serum-free medium. Under these same conditions, dermal fibroblasts express both a membrane-bound and a secreted form of FGFR-1. Upon serum stimulation, dermal fibroblasts increase their production of aFGF and bFGF, whereas the expression of the secreted form of FGFR-1 is decreased. If the secreted form of FGFR-1 expressed by dermal fibroblasts exhibits a dominant-negative effect by dimerizing with the membrane-bound form of FGFR-1, then the decreased expression resulting from serum-treatment of the cells may result in the release of this negative constraint. This, in turn, allows the cells to respond aFGF and bFGF.

Dermal fibroblasts grown in serum-free medium proliferate in response to aFGF and bFGF, presumably when the expression of the secreted form of FGFR-1 is at its greatest. This may be an artifact of the non-physiologic doses of aFGF and bFGF used in these mitogenic assays. The mechanism(s) by which aFGF and bFGF is externalized from dermal fibroblasts in order to interact with either the secreted form of FGFR-1 or

the membrane-bound form of FGFR-1 was not directly addressed in this study.

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