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Two additional protein-tyrosine kinases expressed in human lung: Fourth member of the fibroblast growth factor receptor family and an intracellular protein-tyrosine kinase

(tyrosine kinase/growth factor/oncogenes/human genome/lung)

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ABSTRACT **The expression of protein-tyrosine kinases (PTKs; ATP:protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) was studied in normal human lung and various tumors by PCR followed by molecular cloning and sequence analysis. Six known PTKs (YES, FGR, LYN, HCK, PDGFB-R, and CSF1-R), as well as two additional members of this enzyme family, were detected in lung. One of the newly discovered sequences appears to represent a group of cytosolic PTKs. The cDNA sequence of the second unknown PTK revealed that it is a fourth member of the fibroblast growth factor receptor family. It was therefore called TKF (tyrosine kinase related to fibroblast growth factor receptor). Among a wide variety of cells and tissues tested, including human lymphocytes and macrophages, TKF was only found expressed in lung. Apart from normal lung, TKF expression could be demonstrated in some tumors of lung origin, but also in malignancies not derived from lung tissues. As fibroblast growth factors are generally involved in a variety of functions such as mitogenesis, angiogenesis, and wound healing, the specific expression of a receptor-related gene in lung only may point to yet another special function of this group of proteins.**

Since the original discovery that the transforming gene of a tumorigenic virus, the Rous sarcoma virus, is a protein kinase (1) phosphorylating tyrosine (2), a wide variety of enzymes of this class have been detected in tumor viruses as well as in normal cells. Within cells, protein-tyrosine kinases (PTKs; ATP:protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) are involved in signal transmission and aberrant forms of these genes have oncogenic potential (3).

Generally, PTKs are highly conserved in evolution. While the protooncogenes *ras* (4) and *myc* (5) have been found in bacteria, functional genes of PTKs exist in yeast (6) and *Drosophila* (7, 8). The ancient phylogenetic origin of PTKs of at least 800 million years ago (9) and the high degree of conservation of these genes suggests that tyrosine phosphorylation is a crucial requirement for the physiology of multicellular organisms and that it plays a key role in regulation of eukaryotic cell growth.

Several PTK families can be distinguished: Members of the first category of PTKs such as c-SRC (10), c-fes (11), c-abl (12), c-lck (13), c-HCK (14), and c-tkl (15) are located within the cell either associated to the cytoplasmic side of the plasma membrane or in the cytoplasm. They are all very closely related over a continuous stretch of ≈260 amino acids comprising the catalytic domain but are more or less divergent over the rest of the protein (16).

A second class of PTKs are receptor tyrosine kinases. Here, an extracellular ligand-binding domain is linked via a transmembrane region to a catalytic domain located in the cytoplasm. The signal of the extracellular ligand is transduced by triggering a phosphorylation reaction of the intracellular domain. Receptor protein kinases are divided into three distinct subgroups characterized by the following prototypes: epidermal growth factor receptor (17), the receptors for insulin (18, 19), and the platelet-derived growth factor receptor (20).

Some PTK oncogenes are implicated in human malignancies. For example, amplification of *HER2/neu* is thought to be a predictor of both overall survival and time to relapse in patients with breast cancer (21) and the rearranged *BCR–ABL* on the Philadelphia chromosome is a diagnostic karyotypic abnormality found in almost all cases of chronic myelogenous leukemia (22).

To further elucidate the physiological relevance of known PTKs and to search for additional members of this gene family as potential factors in carcinogenesis, mRNA from lung tissue was amplified by the PCR using PTK-specific primers followed by sequencing the clones. We describe here the expression of FGR, LYN, YES, HCK, PDGFB-R, and CFS1-R in normal lung. Furthermore, two additional members of the family of PTKs from normal lung tissue were detected. One of them (TKF) was characterized as a member of the fibroblast growth factor receptor (FGF-R) family, while the other appears to be a member of the intracellular PTKs.*

METHODS

Tissue Samples. Tissue samples were obtained from 32 patients undergoing surgical resection at the Nordwest Hospital in Frankfurt. Whenever possible, surrounding normal tissue was also obtained. The samples were stored at −70°C.

Cell Culture. Human lymphocytes and macrophages from peripheral blood were cultivated according to von Briesen *et al.* (23).

Nucleic Acids. High molecular weight DNA was extracted from tissues and cells according to the method of Enrietto *et al.* (24). Total RNA was isolated by the guanidinium isothiocyanate/CsCl cushion technique (25). Poly(A)$^+$ RNA was prepared by selection on an oligo(dT)-cellulose column (26). Oligonucleotide primers were synthesized by using an Applied Biosystems model 380A synthesizer with the cyanoethyl phosphoramidate chemistry.

Northern Blot Analysis. Ten micrograms of total RNA was separated on agarose/formaldehyde gels (27), transferred to nitrocellulose membranes (Amersham), and hybridized under high stringency to the probes indicated in the text (28).

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Abbreviations: PTK, protein-tyrosine kinase; FGF-R, fibroblast growth factor receptor; TKF, tyrosine kinase related to FGF-R.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. X71157).

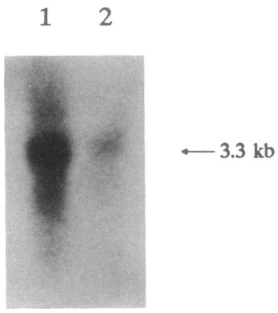

FIG. 1. Expression of the TKF gene. RNA was isolated from adult normal and malignant human lung tissues. Samples of 6 μg of total RNAs were fractionated by electrophoresis through a 2.2 M formaldehyde/1% agarose gel and transferred to a nylon filter. The blots were probed under high stringency with the 260-bp T1 fragment obtained from anchored PCR. Lanes: 1, lung carcinoma; 2, normal lung.

RNA and Anchored PCR. First-strand cDNA synthesis was done by using a PTK-specific primer [P6(2)/0.2 μM], 3 μg of total RNA, and 10 units of Moloney murine leukemia virus reverse transcriptase (50 mM Tris·HCl, pH 8.3/75 mM KCl/10 mM dithiothreitol/3 mM $MgCl_2$/500 μM dNTPs/100 μg of bovine serum albumin per ml). In RNA PCRs half of the first-strand syntheses were used as templates in 25-cycle PCRs [8.3 mM Tris·HCl, pH 8.8/41.7 mM KCl/1.25 mM $MgCl_2$/0.01% gelatin/166.7 μM dNTPs/0.2 μM each primer/5 units of *Taq* polymerase (Amersham)]. One cycle was 1 min at 96°C, 2 min at 40°C, and 3 min at 70°C.

For anchored PCR (29), 50 units of terminal transferase and 100 μM dGTP were used to add a homopolymer to 0.5–100 pg of cDNA (100 mM sodium cacodylate pH 7.1/1 mM $CoCl_2$/0.1 mM dithiothreitol/50 μg of bovine serum albumin per ml/25 mM Tris·HCl, pH 7.1). Half of a tailing reaction was used as template in a 25-cycle PCR with 0.2 μM each the anchor P12 and the specific P6(2) primer (10 mM Tris·HCl, pH 8.8/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin/0.2 mM dNTPs/5 units of *Taq* polymerase). One microliter (of 50 μl) of PCR products was used as template in a second PCR using another specific primer.

For further analysis, all PCR products were ethanol precipitated, ligated into the Bluescript KS+ vector (Stratagene), and sequenced (30).

The following primers were used: P6(2), 5′-ATCCCATAACACCACACGTC; P5(1), 5′-TTTGTCCACCGAGACCTGGC; P5, 5′-TCCACCGGGACCTGCGGGC; P6, 5′-CCAAAGGACCAGACGTCAGA; P12, 5′-GATTTCAGAGAACTAAAC$(dC)_{15}$; P5(3), 5′-TTTATCCACCGAGACCTGGC; P6(3), 5′-ATCCCATAGCACCACACGT.

Construction and Screening of a cDNA Library. A cDNA library was constructed by the method of Gubler and Hoffman (31) using a Pharmacia kit and 5 μg of poly(A)$^+$ RNA from lung tissue. Then, 1.8×10^6 recombinant λgt10 plaques were screened under high stringency (28) using a 260-base-pair (bp) TKF fragment obtained from an anchored PCR with a specific activity of 5×10^9 dpm/μg.

DNA Sequencing. PCR products and positive clones from the cDNA library were sequenced by the dideoxynucleotide chain-termination method (30) after subcloning into the Bluescript vector. Deletion fragments were created by the *Exo* III method (32).

Table 1. Expression of PTKs

Primer	PTK detected
P5/P6	YES, FGR, LYN, HCK
P5(3)/P6(3)	PDGFB-R
P5(1)/P6(2)	T1
T1/P6(2)	CSF1-R
T2/P6(2)	T2

The deduced amino acid sequences (single-letter code) for T1 and T2 are as follows: T1, ARNVLVTEDNVMKIADFGLARGVHHIDYYKKTSNGRLPVKWMAPEALFDRVYTHQS-56; T2, ARNVLVSEDNVAKVSDFGLTKEASSTQDTGKLPVKWTAPEALREKKFSTKS-51. These sequences correspond to PTK subdomains VI–IX. The sequences of the primers are given in *Methods*.

Determination of Phylogenetic Relationship. Phylogenetic relationships were determined by using the TREE program of HUSAR (Heidelberg Unix Sequence Analysis Resource, Deutsches Krebsforschungsrentrum, Heidelberg), based on the progressive alignment method of Feng and Doolittle (33) in a multiple sequence alignment. Thirty-three human PTK sequences were aligned over the entire catalytic domains (16) (amino acids 67–351 of TKF, corresponding to amino acids 265–523 of SRC). The kinase inserts of PDGFA-R, PDGFB-R, KIT, CSF1-R, and FLT were deleted prior to alignment because of their great divergence in length and sequence.

RESULTS

Identification of PTKs in Human Lung Tissue. Aligning the catalytic domains of known PTKs demonstrates that their degree of conservation is not uniform but rather alternates. Within the catalytic domain, 11 major subdomains of high conservation exist (16). Degenerate oligonucleotide primers P5 and P6 from subdomains VI and IX (see *Methods*) corresponding to the motifs His-Arg-Asp-Leu and Asp-Val-Trp-Ser-Phe-Gly were used for PCR based on cDNA from human lung tissues.

The PCR products were cloned and the complete sequences of 212 inserts were determined. A computer homology search (European Molecular Biology Laboratory, GenBank, Swiss-Prot, and National Biomedical Resource) showed that six types of clones represented known PTKs: HCK (14), FGR (34), LYN (35), YES (36), PDGFB-R (20), and CSF1-R (37). In addition, two more sequences, T1 and T2, were found (Table 1), one of which has been described independently by Partanen *et al.* (38) using the strategy of Wilks (39).

Isolation of the FGF-R-Related Gene. To clone the T1-corresponding cDNA, the sequence of T1 was elongated by 60 bp into a more specific domain by anchored PCR. This 260-bp T1-specific fragment was used to screen 1.8×10^6 recombinant clones from a cDNA library from normal lung.

Structure of the mRNA of the FGF-R-Related Gene. Northern blot analysis of mRNA from human lung using a 260-bp T1 clone as a probe showed a band of 3.3 kilobases (kb) (Fig. 1) in both normal tissue and carcinoma, which was expressed at different degrees. The complete amino acid sequence of the putative open reading frame of T1 (Fig. 2) extends from its 5′ end and ends at an in-frame stop codon at position 1212. The consensus sequence for eukaryotic translation initiation sites (40) and the ATG (methionine) codon are missing. Thus,

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  1  HPRPPATVQKLSRFPLARQFSLESGSSGKSSSSLVRGVRLSSSGPALLAGLVSLDLPLDP
 61  LWEFPRDRLVLGKPLGEGCFGQVVRAEAFGMDPARPDQASTVAVKMLKDNASDKDLADLV
121  SEMEVMKLIGRHKNIIMLLGVCTQEGPLYVIVECAAKGNLREFLRARRPPGPDLSPDGPR
181  SSEGPLSFPVLVSCAYQVARGMQYLESRKCIHRDLAARNVLVTEDNVMKIADFGLARGVH
241  HIDYYKKTSNGRLPVKWMAPEALFDRVYTHQSDVWSFGILLWEIFTLGGSPYPGIPVEEL
301  FSLLREGHRMDRPPHCPPELYGLMRECWHAAPSQRPTFKQLVEALDKVLLAVSEEYLDLR
361  LTFGPYSPSGGDASSTCSSSDSVFSHDPLPLGSSSFPFGSGSGVQT
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FIG. 2. Sequence of T1 cDNA. Putative amino acid sequence of the longest T1 clone isolated from a human lung cDNA library is shown.

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TKF    HPRPPATVQKLSRFPLARQ--FSLESGSSGKSSSSLVRGVRLSSS--GPALLAGLVSLDL  56
FLG    SQMAVAKLA.SIPLRRQVT--V.AD.SA.MN.GVL...PS.....--.TPM...VSEYE. 465
BEK    FSSQ..VHKLTK.I..R..VTV.A..S..MN.NTP...ITTRL..TADTPM...VSEYE. 468
FGFR3  KGLGSP..H.I.....K..--V....NA.MS.NTP...IA....G--EGPT..NVSE.E. 459

TKF    PLDPLWEFPRDRLVLGKPLGEGCFGQVVRAEAFGMDPARPDQASTVAVKMLKDNASDKDL 116
FLG    .E..R..L..........|.....|...L...I.L.KDK.NRVTK...|....SD.TE... 525
BEK    .E..K......K.T.....|.....|...M...V.I.KDK.KE.V....|....D.TE... 528
FGFR3  .A..K..LS.A..T.....|.....|...M...I.I.KD.AAKPV....|....D.T.... 519

TKF    ADLVSEMEVMKLIGRHKNIIMLLGVCTQEGPLYVIVECAAKGNLREFLRARRPPGPDLSP 176
FLG    S..I....M..M..K.........A...D........Y.S......Y.Q......LEYCY 585
BEK    S.......M..M..K.........A...D........Y.S......Y........MEY.Y 588
FGFR3  S.......M..M..K.....N...A...G.....L..Y.A..............L.Y.F 579

TKF    DGPRSSEGPLSFPVLVSCAYQVARGMQYLESRKCIHRDLAARNVLVTEDNVMKIADFGLA 236
FLG    NPSHNP.EQ..SKD............E..A.K...|.....|...............|.... 645
BEK    .IN.VP.EQMT.KD....T..L....E..A.Q...|.....|.......N.......|.... 648
FGFR3  .TCKPP.EQ.T.KD............E..A.Q...|.....|...............|.... 639

TKF    RGVHHIDYYKKTSNGRLPVKWMAPEALFDRVYTHQSDVWSFGILLWEIFTLGGSPYPGIP 296
FLG    .DI.........T................I....|.....|..V..............V. 705
BEK    .DINN.......T.....................|.....|..V.M.............. 708
FGFR3  .D..NL......T.....................|.....|..V.............. 699

TKF    VEELFSLLREGHRMDRPPHCPPELYGLMRECWHAAPSQRPTFKQLVEALDKVL-LAVSEE 356
FLG    .....K..K......K.SN.TN...MM..D....V............D..RIVA.TSNQ. 765
BEK    .....K..K......K.AN.TN...MM..D....V............D..RI.T.TTN.. 768
FGFR3  .....K..K......K.AN.THD..MI.................D..R..TVTSTD. 759

TKF    YLDLRLTFGPYSPSGGDASSTCSSS--DSVFSHDPLPLGSSSFPFGSGSGVQT 404
FLG    ....SMPLDQ....FP.TR.STC..GE......E...EEPCLPRHPAQLANGGLKRR 825
BEK    ....SQPLEQ....YP.TR.S...G-D.....P..M.YEPCLPQYPHINGSVKT 827
FGFR3  ....SAP.EQ...GGQ.TP.SS..G-DD...A..L..PAPP.SGGSRT 806
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FIG. 3. Comparison of catalytic domains of human FGF-Rs. The catalytic domains of TKF, FLG, BEK, and FGF-R3 as deduced from the cDNA clones are aligned. Dots replace residues similar to the putative TKF protein. Dashes represent gaps introduced to improve alignment. Motifs referred to in the text are boxed.

T1 most likely does not comprise the whole mRNA of the new gene. The 3′ noncoding region has a length of 527 bp, which encompasses the signal for poly(A) addition at position 1656 and a poly(A) sequence at its end.

A computer analytic comparison of the T1 sequence revealed that its putative translation product is related to catalytic domains of PTKs. The homology is most striking for the family of receptor tyrosine kinases and in particular to the subgroup of FGF-Rs. The relationship of T1 is closest to the products of two genes, FLG (69% homology) and BEK (68% homology), and less for those of RET, CSF1-R, FLT, KIT, and PDGF-R. Because of this relationship to PTKs of the FGF-R family, we named the gene TKF (tyrosine kinase related to the FGF-R).

A comparison of the catalytic domains of FLG (41), BEK (41), FGF-R3 (42), and TKF (Fig. 3) confirmed TKF to be a member of the PTK family. The consensus Gly-Xaa-Gly-Xaa-Xaa-Gly found in many nucleotide-binding proteins in addition to PTKs (43) is perfectly conserved in TKF at amino acid positions 76–81. A highly conserved lysine residue, which seems to be directly involved in the phosphotransfer reaction possibly mediating proton transfer (44), is located at position 105. Three invariant amino acids (Asp-Phe-Gly) implicated in ATP-binding are located between residue po-

Table 2. Expression of TKF in normal and malignant human tissues

Tissue	Expression	Number of samples studied	Tissue	Expression	Number of samples studied
Normal tissue			Tumors		
Lung	+	19	Intestine		
Intestine (colon, sigma, rectum)	−	7	Adenocarcinoma (mucigenous)	+	3
Stomach	−	1	Adenocarcinoma (not mucigenous)	+	3
Esophagus	−	2	Adenocarcinoma (not mucigenous)	−	1
Thymus	−	1	Stomach		
Spleen	−	1	Adenocarcinoma	−	1
Placenta	−	1	Esophagus		
Foreskin	−	1	Epidermoid	−	2
Normal human cells			Brain	−	3
Lymphocytes	−		Skeletal metastases		
Macrophages	−		Adenocarcinoma (breast cancer)	+	1
Tumors			Adenocarcinoma (cardia carcinoma)	+	1
Lung					
Adenocarcinoma	−	4			
Epidermoid (partially differentiated)	−	13			
Epidermoid (partially differentiated)	+	1			
Epidermoid (undifferentiated)	+	2			
Carcinoid (primary)	+	1			

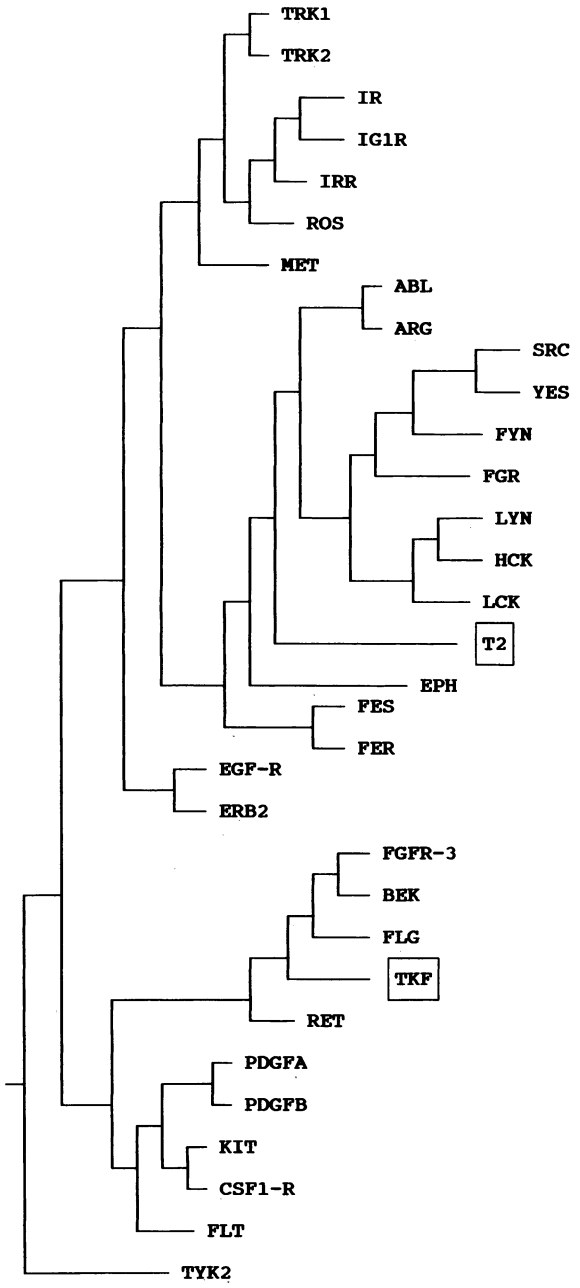

FIG. 4. Phylogenetic relationships of 33 human PTK sequences. The tree was calculated as indicated in *Methods*. It is informative only concerning the branching order. Branch lengths in this figure are not proportional to true evolutionary distances.

sitions 232 and 234. Sequences that distinguish PTKs from serine/threonine kinases, His-Arg-Asp-Leu-Ala-Ala-Arg-Asn-Val (positions 212–220) and Pro-Val-Lys-Trp-Met-Ala-Pro (positions 254–259), are conserved in comparison to other PTKs and are identical to BEK and FLG. Compared to other PTKs (16), the kinase domain has an insertion of 14 amino acids identical in length to BEK and FLG (residues 172–185 of TKF).

Expression of TKF. The expression of TKF in various cells and tissues is shown in Table 2. Very interestingly, TKF is specifically expressed in lung and not in other tissues and cells, including human lymphocytes and macrophages. In several tumors, however, including malignancies not derived from lung, TKF was found expressed as well.

Phylogeny of the PTK-Specific Catalytic Domains of TKF and T2. The sequences of the catalytic domains of 33 human PTKs (corresponding to amino acids 265–523 of $pp60^{c\text{-}SRC}$) were used to determine their phylogenetic relationships (Fig. 4). Five major branches can be distinguished: (*i*) insulin receptors, (*ii*) intracellular PTKs, (*iii*) FGF-Rs, (*iv*) platelet-derived growth factor receptors, and (*v*) epidermal growth factor receptors. Again, TKF is grouped with the family of the FGF-Rs. Within this family BEK, FLG, and FGF-R3 are more closely related to each other than to TKF. T2, in contrast, is grouping with the intracellular PTKs and appears to define a distinct group.

Table 3. Relationship of FLG and BEK sequences with other published sequences of genes from the FGF-R family

	Sequence variations
Compared to BEK (41)	
TK14 (47)	aa 308/309: 3-aa insertion
	aa 429/430 deleted
K-Sam (49)	aa 37–125 deleted
	aa 314–361, ≈50% homology
	aa 429/430 deleted
	aa 761–772 highly divergent
	C-terminal 48 aa deleted
Compared to FLG (41)	
FGF2H (45)	aa 30–119 deleted
sbFGF (48)	aa 30–119 deleted
	aa 148/149 deleted
FGF1a	aa 148/149 deleted
FGF-R (46)	aa 817 exchanged

aa, Amino acid(s).

DISCUSSION

Because of their mostly low expression rates, the role of many PTKs in the regulation of normal cells, and also in the development of malignant tumors, is not very well understood. The use of PCR technology now allows us to efficiently search for messages of even very low abundance. Among receptor PTKs, so far only the expression of ERB-B has been described in lung. We have found the expression of additional PTKs—HCK, YES, FGR, LYN, PDGFB-R, CSF1-R, and two additional genes in this tissue. Among the newly discovered genes, T2 represents an unusual group of PTKs (Fig. 4). Further studies (unpublished data) have shown that it is related to csk, recently isolated from rat brain.

The putative TKF product is significantly homologous to various human receptors of the heparin-binding growth factor family: BEK (41), FLG (41), FGF-R h2/h3 (45), FGF-r (46), TK 14 (47), FGF-Rs (48), and K-sam (49). A homology search showed that they belong to two categories (Table 3): (*i*) FGF-Rs of the BEK type: The putative proteins of BEK and TK 14 are identical (100% homology) except for the occurrence of two gaps in TK 14 due to an addition of 3 amino acids at position 309 and a deletion of 2 amino acids at position 431. K-sam, which is amplified in the stomach cancer-derived cell line KATO-III (49), is 95.9% homologous to BEK at the amino acid level with two gaps. The largest gap of 88 amino acids in K-sam occurs within the first of the three extracellular immunoglobulin-like domains of BEK. Thus, most of the structural variations of TK 14 and K-sam, which are both of tumorigenic origin, occur in the extracellular domain and might influence the ligand-mediated signal transduction by FGF. (*ii*) FGF-R of the FLG type: FLG has been isolated from a human umbilical vein endothelial cell (HUVEC) cDNA (41, 50). Comparison to a human receptor for acidic and basic FGF (46) and to a shorter form of a human FGF-R (48) shows homologies of 99.9% and 99.8%, respectively.

The alignment of FLG to diverse forms (h2/3) of a receptor for acidic and basic FGFs (45) from a HUVEC and a placenta

library shows 100% homology and one gap, which is again located in the region of the immunoglobulin-like domain. In addition, some forms of FGF-R (h4/5) cDNA (45) were observed, which encode only the extracellular domain of the FGF-R, a variant that is discussed as a secreted protein. The different receptor species differ by a few amino acids in the extracellular domain. These multiple forms of FGF-R, which are highly homologous to FLG may derive by alternative splicing, a process that has been detected in mouse cells as well (51).

In summary, the different representatives of the FGF-Rs are various modifications of two genes, *BEK* and *FLG*. Their heterogeneity is mostly due to tumorigenic abnormalities or possibly specific regulation by alternative splicing. Very recently, another member of the FGF-R family of humans has been described (42), which was called FGF-R3. The gene described here, TKF, is clearly distinct from FGF-R3 and thus represents a fourth member of this family (Fig. 3).

Generally, several different but related receptors for a certain family of growth factors exist in various species. There are two different human platelet-derived growth factor receptors (52), various receptors for insulin and insulin-like growth factor (53), and different FGF-R genes in chickens (54, 55) and mice (51, 56). The existence of seven different FGFs might indicate that the FGF-R family has a particularly high number of different members. In line with this assumption binding analyses on cells demonstrated that differences in the relative capacities of acidic and basic FGF to bind to the BEK and FLG proteins are not due to different affinities or differences in the expression of these receptors (41). Furthermore, by affinity labeling experiments using either ^{125}I-labeled acidic FGF or ^{125}I-labeled basic FGF, a variety of receptor proteins were identified (45). Changes in heparin-binding FGF gene expression and receptor phenotype have also been described during liver regeneration (57). Whether the specific expression of our TKF gene in lung only points to a very specific biochemical function remains to be determined. In any case, it does not appear to be due to invading lymphocytes and/or macrophages as both cell types have been found negative for TKF expression.

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