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Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction

Satu Vainikka, Juha Partanen, Paola Bellosta[1], Francois Coulier[2], Claudio Basilico[1], Michael Jaye[3] and Kari Alitalo[4]

Cancer Biology Laboratory, Departments of Pathology and Virology and Transplantation Laboratory, University of Helsinki, Haartmanink. 3, 00290 Helsinki 29, Finland, [1]New York University Medical Center, 550 First Avenue, New York, NY 10016, USA, [2]INSERM, Unite 119, 27, Boulevard Lei Roure, 13009 Marseille, France and [3]Rhone-Poulenc Central Research, 500 Arcola Road, Collegeville, PA 19426-0107, USA

[4]Corresponding author

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Fibroblast growth factor (FGF) receptor (FGFR) gene family consists of at least four receptor tyrosine kinases that transduce signals important in a variety of developmental and physiological processes related to cell growth and differentiation. Here we have characterized the binding of different FGFs to FGFR-4. Our results establish an FGF binding profile for FGFR-4 with aFGF having the highest affinity, followed by K-FGF/*hst*-1 and bFGF. In addition, FGF-6 was found to bind to FGFR-4 in ligand competition experiments. Interestingly, the FGFR-4 gene was found to encode only the prototype receptor in a region where both FGFR-1 and FGFR-2 show alternative splicing leading to differences in their ligand binding specificities and to secreted forms of these receptors. Ligands binding to FGFR-4 induced receptor autophosphorylation and phosphorylation of a set of cellular polypeptides, which differed from those phosphorylated in FGFR-1-expressing cells. Specifically, the FGFR-1-expressing cells showed a considerably more extensive tyrosine phosphorylation of PLC-γ than the FGFR-4-expressing cells. Structural and functional specificity within the FGFR family exemplified by FGFR-4 may help to explain how FGFs perform their diverse functions.

Key words: alternative splicing/fibroblast growth factor/FGF receptor/signal transduction

Introduction

FGFs are a family of seven heparin-binding polypeptides that have mitogenic activity towards a wide variety of cells of mesenchymal, neuronal and epithelial origin (Burgess and Maciag, 1989). In addition to the first and best characterized members of the FGF gene family, aFGF (Jaye *et al.*, 1986) and bFGF (Abraham *et al.*, 1986), at present five other FGFs are known. These include *int*-2 (Dickson and Peters, 1987), K-FGF/*hst*-1 (Delli Bovi *et al.*, 1987; Yoshida *et al.*, 1987), FGF-5 (Zhan *et al.*, 1988), FGF-6 (Marics *et al.*, 1989) and KGF (Finch *et al.*, 1989). Besides their mitogenic activities, aFGF and bFGF have also been shown to have roles in cell differentiation and angiogenesis (Folkman and Klagsbrun, 1987; Burgess and Maciag, 1989). Some of these FGFs are also of importance in carcinogenesis, for example *int*-2 and K-FGF/*hst*-1 were cloned as oncogenes (Sakamoto *et al.*, 1986; Dickson and Peters, 1987; Delli Bovi *et al.*, 1987).

FGFs act by binding and activating cell surface receptor tyrosine kinases. The signal-transducing FGF receptors are encoded by a family of related genes and thus far four members of this family have been cloned. These include FGFR-1 (*flg*) (Ruta *et al.*, 1988), FGFR-2 (*bek*) (Dionne *et al.*, 1990; Houssaint *et al.*, 1990), FGFR-3 (Keegan *et al.*, 1991) and FGFR-4 (Partanen *et al.*, 1991). Related proteins that bind FGFs have also been purified from chicken and mouse and described in *Xenopus* and *Drosophila* (Lee *et al.*, 1989; Mansukhani *et al.*, 1990; Musci *et al.*, 1990; Reid *et al.*, 1990; Glazer and Shilo, 1991). Each of these receptors consists of an extracellular ligand binding region with two or three immunoglobulin (Ig) like loops and an intracellular tyrosine kinase domain split by a short kinase insert. The binding of FGF to the extracellular domain of the FGF receptor leads to the activation of its intrinsic tyrosine kinase activity concomitant with tyrosine autophosphorylation and tyrosine phosphorylation of target substrates (Ullrich and Schlessinger, 1990). In recent studies phospholipase C-γ (PLC-γ) has been shown to be a major substrate of FGFR-1 (Burgess *et al.*, 1990). However, little is known about the possible specificity of signal transduction by the different FGFRs.

It has been shown that FGFR-1 binds aFGF and bFGF equally well, but binds K-FGF with ~10 times lower affinity (Dionne *et al.*, 1990; Johnson *et al.*, 1990; Wenström *et al.*, 1991; Manshukani *et al.*, 1992). FGFR-2 binds aFGF, bFGF and K-FGF equally well, but does not bind FGF-5 or KGF (Dionne *et al.*, 1990; Manshukani *et al.*, 1992). FGFR-3 is also activated by aFGF and bFGF (Keegan *et al.*, 1991). The complexity of different FGFRs is further increased by differential RNA splicing, which may produce receptors with different ligand binding specificities (Johnson *et al.*, 1990; Miki *et al.*, 1991). Alternative exon usage in the second half of the third Ig loop results in a FGFR-2 protein, which behaves as a receptor for KGF (Miki *et al.*, 1992). This form binds aFGF and KGF with high affinity, but shows only low affinity for bFGF. A similar variant of FGFR-1 has also been described (Johnson *et al.*, 1991; Werner *et al.*, 1992).

The distinct but overlapping expression patterns of FGFR in cells and tissues (Partanen *et al.*, 1991; Stark *et al.*, 1991; Korhonen *et al.*, 1992a,b; Orr-Urtreger *et al.*, 1992) suggest that they are capable of transducing specific ligand-induced signals. In this report, we analyse the FGFR-4 ligand binding specificity, tyrosine kinase activation and substrate phosphorylation and compare these properties with those of FGFR-1 in stably transfected cells.

Results

FGFR-4 expression in NIH3T3 and CHO cells

For the analysis of FGF binding to FGFR-4, NIH3T3 cells were transfected with the LTR2HX expession vector containing FGFR-4 under the control of MoMuLV LTR promoter, together with a plasmid encoding neomycin phosphotransferase. Neomycin-resistant clones were selected and screened by Western blotting using rabbit antiserum against FGFR-4. Ten highly expressing clones were found and two of those clones showing abundant tyrosine phosphorylation of cellular polypeptides after aFGF stimulation were selected for further analysis. As controls, we used FGFR-1 transfected and neomycin-resistant NIH3T3 cells (Dionne *et al.*, 1990). Dihydrofolate reductase (DHFR)-deficient CHO cells were transfected with the FGFR-4 expression vector pSV2SE together with a plasmid encoding DHFR and two FGFR-4-expressing clones were selected for binding analyses.

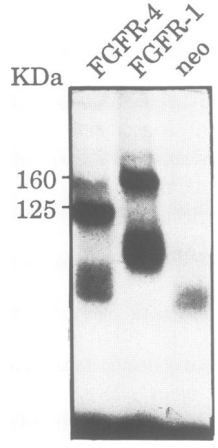

Fig. 1. Crosslinking of [^{125}I]aFGF to FGFR-1 and FGFR-4 transfected NIH3T3 cells. After the crosslinking reaction the cell lysates were run in a 7.5% SDS–polyacrylamide gel and labelled bands were detected by autoradiography. The mobilities of the affinity-labelled FGFR-4 and FGFR-1 polypeptides are shown in kDa. The structures of the lower molecular weight receptors are unknown, although they have been observed in several previous studies [see Jaye *et al.* (1992) for a review].

Comparison of aFGF, bFGF and K-FGF binding to FGFR-4

When radiolabelled aFGF was cross-linked to FGFR-4 and FGFR-1 expressing NIH3T3 cells and analysed by gel electrophoresis, major polypeptides of 125 and 160 kDa were detected, respectively (Figure 1), agreeing with our earlier determinations using transient expression in Cos cells (Partanen *et al.*, 1991). The genesis of the lower molecular weight bands is unknown, but they were also abolished in the presence of a 50-fold excess of non-radioactive aFGF (data not shown; see Partanen *et al.*, 1991). As is evident from the long autoradiographic exposure shown in Figure 1, the corresponding neomycin phosphotransferase-transfected cells (neo) express very little FGF receptors.

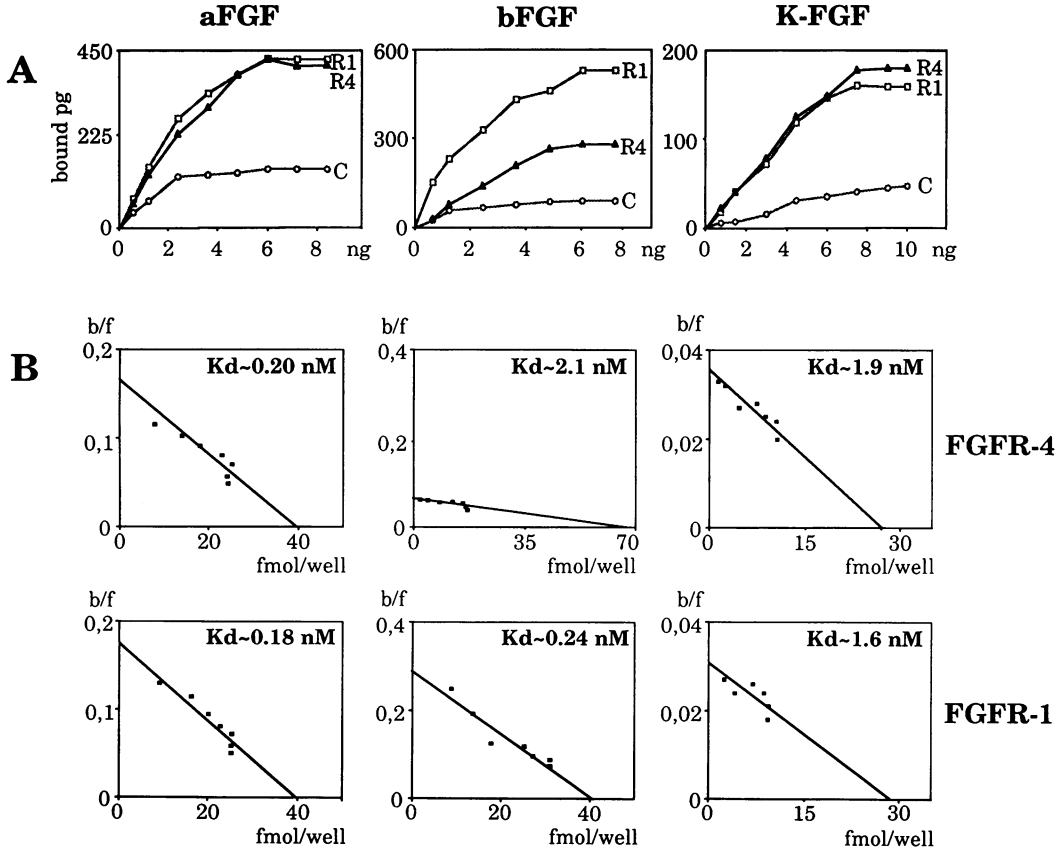

Fig. 2. Binding of aFGF, bFGF and K-FGF to FGFR-1 and FGFR-4-expressing cells. **A.** The FGFR-1, FGFR-4 and neomycin phosphotransferase transfected cells were incubated for 2 h at 4°C with different concentrations of the labelled ligands. After three washes, the cell-associated radioactivity was measured. Non-specific binding was estimated by parallel determinations with a 100-fold excess of unlabelled ligand. Shown are mean values of three experiments. The data were also subjected to Scatchard analyses (**B**).

Figure 2A shows that aFGF binds to FGFR-4 and FGFR-1-expressing NIH3T3 cells in a similar manner, with saturation of the binding sites occurring at ~6 ng/10^5 cells used in the assay. The Scatchard plots corresponding to these analyses are shown in Figure 2B. On basis of these results, both the FGFR-4 and FGFR-1 transfected cells contain ~1.8 × 10^5 binding sites for aFGF. The affinity of aFGF for both FGFR-4 and FGFR-1 estimated from the Scatchard plots is ~0.2 nM. bFGF had a similar affinity for FGFR-1, but a 10-fold lower affinity for FGFR-4 (2.1 nM). K-FGF bound to both receptors with a low affinity, ~1.9 and 1.6 nM, respectively. Similar results were obtained from experiments using FGFR-4 and FGFR-1 transfected CHO cells, which lack endogenous FGF receptors (data not shown).

Competition for FGF binding sites

One difficulty in performing comparative binding experiments is the accurate estimation of the specific activities of the different radiolabelled ligands. Reliable comparisons of binding affinities therefore require both binding and competition analyses. For the latter, the binding of radiolabelled aFGF was competed with increasing concentrations of different non-radioactive FGFs. Figure 3A shows the relative amount of radioactive ligand bound at each concentration of the non-radioactive FGF in transfected NIH3T3 cells. Comparison of the panels shows that bFGF competes much less efficiently for FGFR-4 than for FGFR-1. In contrast, non-radioactive K-FGF displaces radiolabelled aFGF nearly equally well from both FGFR-4 and FGFR-1. Also, FGF-6 is capable of aFGF displacement from both receptors, to the same degree as K-FGF. However, only a background amount of aFGF radioactivity (maximally ~10%) is displaced from either receptor by FGF5 or KGF. Figure 3B shows the results of a similar competition analysis using radioactive K-FGF. The results show that aFGF is a better competitor for both receptors than K-FGF, whereas bFGF is a better competitor than K-FGF for FGFR-1, but equally good with K-FGF for FGFR-4. These results thus agree with the competition analysis using labelled aFGF. FGF-6 and K-FGF were about equally efficient in displacing radiolabelled K-FGF from both receptors. Results of analyses using transfected CHO cells, radiolabelled aFGF and unlabelled aFGF, bFGF and K-FGF as competitors were consistent with these data except that bFGF had a slightly higher affinity for FGFR-4 than in the NIH3T3 cells (data not shown).

FGFR-4 lacks alternative exons encoding the third Ig loop

We were interested in exploring possible alternatively spliced forms of the FGFR-4 in the region encoding the third Ig loop, because such forms of FGFR-1 and FGFR-2 have been shown to differ in their ligand binding properties. Genomic clones of FGFR-4 in bacteriophage λ were isolated and analysed. Figure 4A shows a comparison of the genomic structures of FGFR-4, FGFR-1 and FGFR-2 based on the DNA sequence of a 1320 bp FGFR-4 DNA fragment encoding the region from the third Ig loop to the tyrosine kinase domain. From this analysis it is evident that FGFR-4 has only one exon for the carboxyl-terminal half of the third Ig-like loop, unlike FGFR-1 and FGFR-2, which have

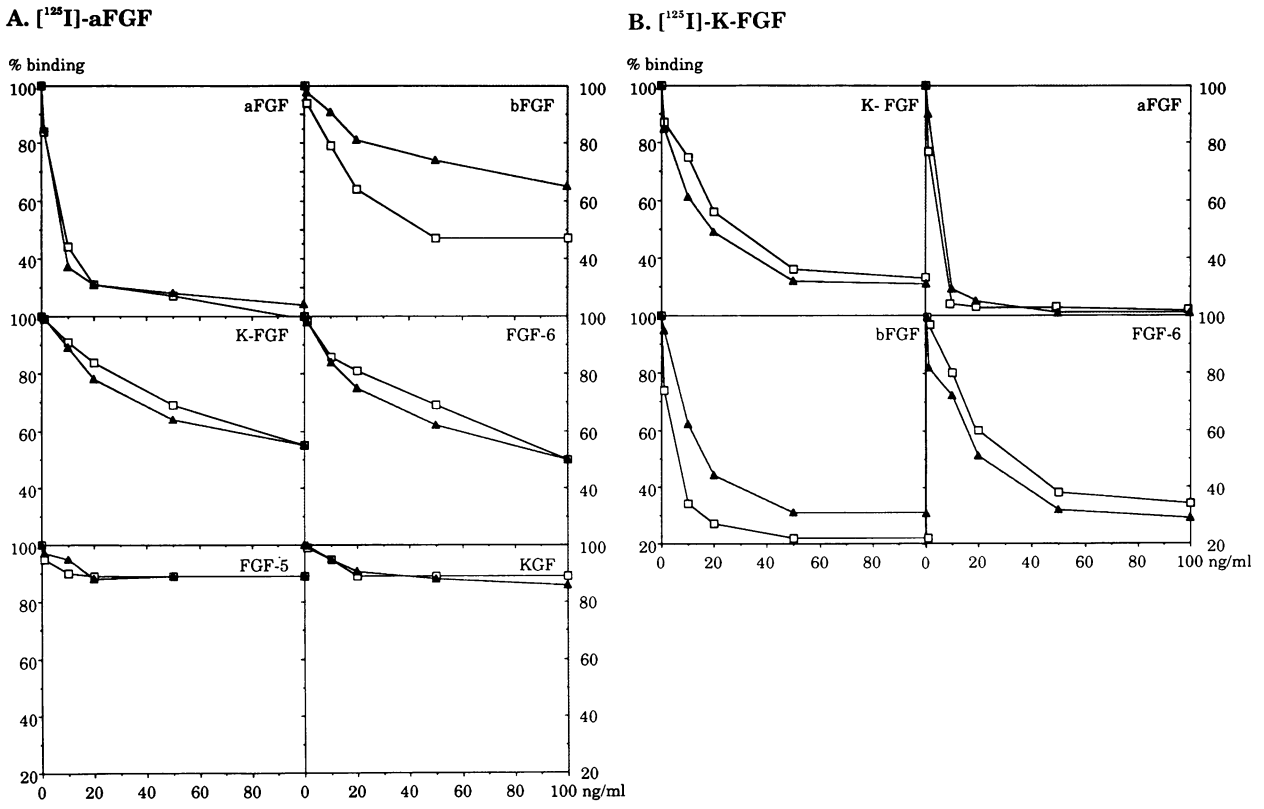

Fig. 3. Competition of [^{125}I]aFGF (**A**) and [^{125}I]K-FGF (**B**) binding to FGFR-1- and FGFR-4-expressing cells with the indicated ligands. About 6 ng/ml of labelled aFGF was added to the cell cultures in the presence of increasing concentrations of unlabeled FGFs and the percentage of remaining bound radioactivity was determined. Closed triangles, FGFR-4; open squares, FGFR-1.

alternative exons IIIb and IIIc. The positions of the introns are conserved, but the introns of the FGFR-4 gene are much shorter than the corresponding introns of FGFR-1 or FGFR-2. Figure 4B shows a comparison of the amino acid sequences of the different exons encoding the third Ig loops. It also shows that exons IIIa are very similar in sequence between the different receptors, but the sequence of the unique exon of FGFR-4 has similarities to both exon IIIb and to exon IIIc, although it shares a greater number of identical residues with exon IIIc of FGFR-1 and FGFR-2. We therefore call it exon IIIc′ of the FGFR-4.

Ligand-induced tyrosine kinase activity and receptor autophosphorylation

In order to see whether FGFs induce FGFR-4 tyrosine kinase activity, receptor-expressing NIH3T3 cells were first stimulated for 5−30 min with 20 ng/ml aFGF, lysed and cell lysates were subjected to Western blotting with a polyclonal phosphotyrosine-specific antiserum. Figure 5A shows that tyrosyl phosphorylation of several cellular polypeptides is intense after a 5 min stimulation of the FGFR-4-expressing cells and declines slowly thereafter, whereas a similar treatment of the neomycin-resistant control cells shows barely detectable amounts of phosphotyrosine. The major aFGF-induced tyrosyl phosphorylated polypeptides have apparent molecular weights of 110 and 165 kDa, intermediate ones migrate at 75 and 62 kDa and weak bands at 145, 85 and 49 kDa mobilities. A more detailed analysis of the 110 kDa band revealed a polypeptide doublet, whose more slowly migrating component represents autophosphorylation of FGFR-4, deciding from a secondary probing with FGFR-4-specific antiserum (data not shown). Also, immunoprecipitation of FGFR-4 followed by anti-phosphotyrosine immunoblotting gave a similarly migrating band (Figure 5B).

Tyrosine phosphorylation of cellular proteins upon FGFR-4 stimulation

Further stimulations were done for 5 min with different concentrations of aFGF. Figure 6 shows a typical result of such a Western blotting experiment, probed with anti-phosphotyrosine specific monoclonal antibodies. Figure 6 shows that stimulation with as little as 0.1 ng/ml aFGF induces tyrosine phosphorylation of several cellular polypeptides in FGFR-4-expressing cells, and this response is not increased with higher concentrations of the growth factor.

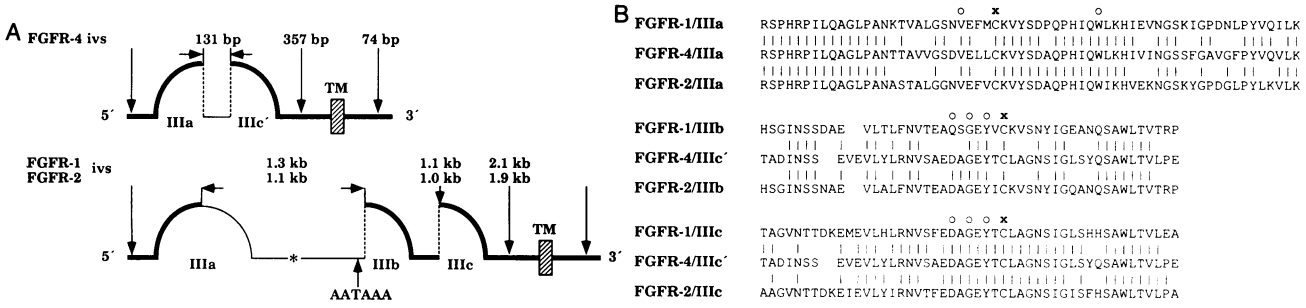

Fig. 4. Comparison of the genomic structures (**A**) and deduced amino acid sequences (**B**) of FGFR-4, FGFR-1 and FGFR-2 in the region encoding the III Ig loop. The schematic drawing in (A) is based on analysis of the FGFR-4 genomic sequence (submitted to the EMBL database; accession number X57205) and comparison with data published by Johnson *et al.* (1991). The sites of the introns (ivs) have been marked with arrows along the cDNA (thick line) and their sizes are given (Johnson *et al.*, 1991). Thus, the III Ig loop of FGFR-4 is encoded in exons IIIa and IIIc′, separated by a 131 bp ivs (thin line). In FGFR-1 and FGFR-2, the III Ig loop is encoded by exon IIIa and either exon IIIb or IIIc, depending on the splicing of the transcripts. Asterisk, stop codon; AATAAA, polyadenylation site; TM, transmembrane region. In (B) amino acid sequence identities have been compared between the different III Ig loop exons shown in (A). A few gaps have been introduced for better alignment. Cysteines (x) and other consensus residues (○) of the predicted immunoglobulin-like domain are indicated (Partanen *et al.*, 1991).

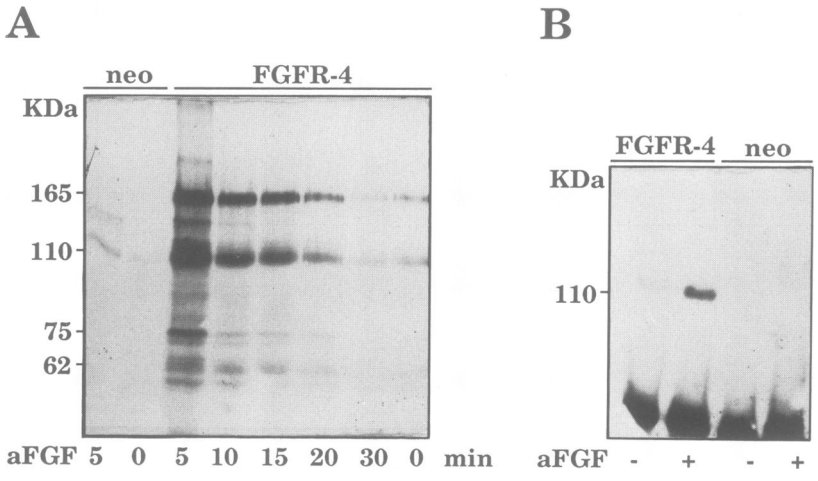

Fig. 5. Tyrosine phosphorylation of polypeptides in aFGF stimulated FGFR-4-expressing cells. Serum starved cells were stimulated with aFGF for the indicated times. Cell lysates were subjected to Western blotting using rabbit anti-phosphotyrosine antiserum (**A**). (**B**) Stimulated cells were lysed and immunoprecipitated with FGFR-4-specific antiserum followed by Western blotting using anti-phosphotyrosine specific monoclonal antibodies.

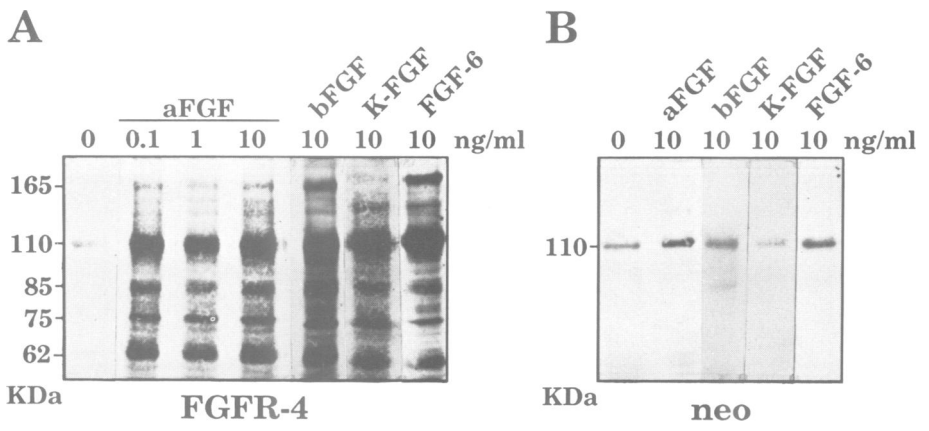

Fig. 6. Tyrosine phosphorylation induced by different FGFs. FGFR-4-expressing cells (**A**) and neomycin-resistant control cells (**B**) were stimulated with the ligands at the concentrations indicated and analysed by Western blotting using anti-phosphotyrosine monoclonal antibodies. The molecular weights of the major substrates are given.

Major tyrosine-phosphorylated polypeptides have molecular weights of 110 (a doublet band), 62, 85, 75 and 165 kDa. The different intensities of tyrosine-phosphorylated bands in Figures 5 and 6 are due to a small difference in the sodium orthovanadate treatment of the cells and to the use of different anti-phosphotyrosine antibodies for detection (data not shown). Tyrosine phosphorylation was also seen in FGFR-4-expressing cells after stimulation with bFGF, K-FGF and FGF-6 (Figure 6A), but not with FGF-5 or KGF (data not shown). Titration of the FGF concentrations needed for the induction of cellular phosphotyrosyl polypeptides gave values consistent with the ligand binding determinations (data not shown). However, the neomycin-resistant control cells did not show a substantial increase of tyrosine phosphorylation after addition of any of these ligands (Figure 6B). In FGFR-1-expressing cells 165 kDa and 110 kDa tyrosyl-phosphorylated polypeptides were prominent and autophosphorylated FGFR-1 migrated with a molecular weight of 145 kDa. Polypeptides comigrating with the 85, 75 and 62 kDa bands seen in FGFR-4-expressing cells were phosphorylated only weakly (data not shown).

Because of the differences in tyrosine-phosphorylated polypeptides of FGFR-4- and FGFR-1-expressing cells that had been stimulated with aFGF, we compared anti-phosphotyrosine immunoprecipitates of aFGF treated cells. Figure 7 shows that polypeptides of 110 (a doublet band including FGFR-4), 85, 75 and 62 kDa were tyrosine-phosphorylated in FGFR-4-expressing cells. In comparison, polypeptides of 150, 145 (FGFR-1), 92, 66, 49 and 42 kDa were tyrosine-phosphorylated in FGFR-1-expressing cells.

Differential tyrosine phosphorylation of PLC-γ in FGFR-4- and FGFR-1-expressing cells

One of the polypeptide bands differentially phosphorylated in FGFR-4- and FGFR-1-expressing cells had a molecular weight of ~150 kDa. This is similar to the size of PLC-γ, which has been reported to be a major substrate for FGFR-1 (Burgess *et al.*, 1990). We therefore examined tyrosine phosphorylation of PLC-γ in FGFR-4-expressing cells. As can be seen from the anti-phosphotyrosine immunoblotting analysis of PLC-γ immunoprecipitates shown in Figure 8A, ligand-induced tyrosine phosphorylation of PLC-γ in FGFR-1-expressing cells is considerably more extensive than in FGFR-4-expressing cells or neo control cells. The amount

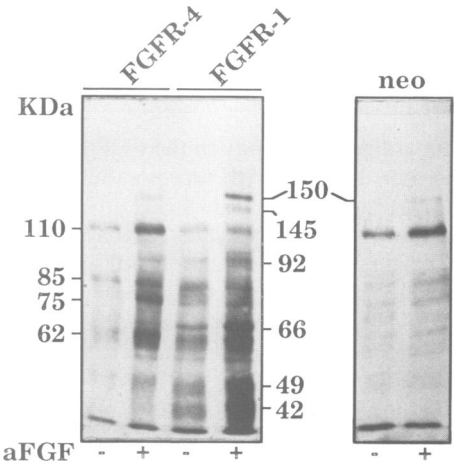

Fig. 7. Immunoprecipitation of tyrosine-phosphorylated polypeptides from FGFR-4- and FGFR-1-expressing cells. The cells were stimulated for 5 min with aFGF. Cell lysates were immunoprecipitated using anti-phosphotyrosine conjugated to agarose (UBI) and analysed by Western blotting with anti-phosphotyrosine antibodies. The molecular weights of major aFGF-induced phosphotyrosyl polypeptides are given.

of PLC-γ immunoprecipitated with anti-phosphotyrosine antibodies is also greater in FGFR-1-expressing cells (Figure 8B). Western blotting with specific antibodies showed that this difference was not due to different amounts of PLC-γ protein in the cell clones (Figure 8C). In agreement with the data obtained from transfected NIH3T3 cells, anti-phosphotyrosine immunoprecipitates of Cos cells transiently expressing FGFR-1 and PLC-γ contained tyrosyl phosphorylated PLC-γ bands, but such bands were not detected in similar immunoprecipitates from cells expressing FGFR-4 and PLC-γ (data not shown). Taken together, these results suggest that FGFR-4 binds and phosphorylates PLC-γ much less efficiently than FGFR-1.

Discussion

FGFR-4 is yet another member of the FGF receptor family, which currently includes four members having 55–70% amino acid sequence identity with each other. A major

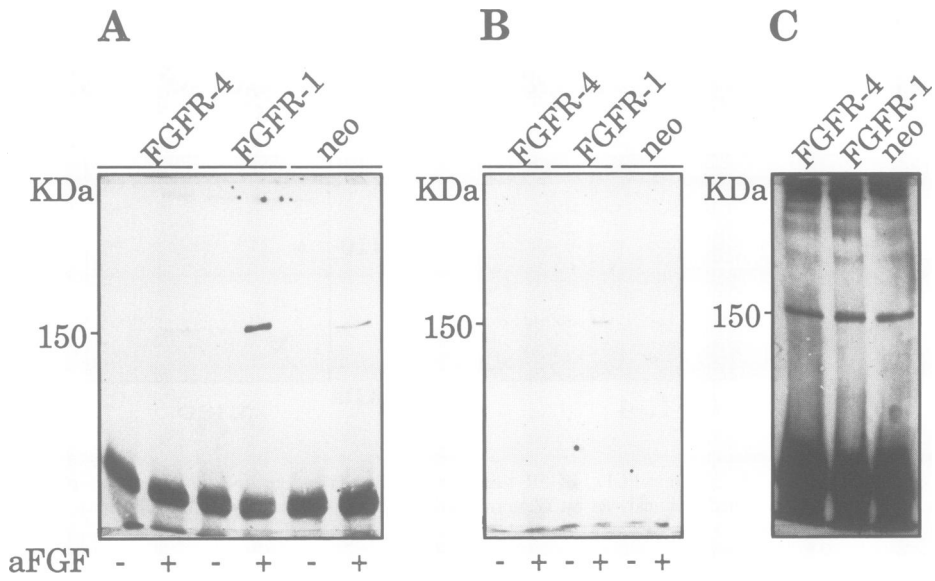

Fig. 8. Tyrosine phosphorylation of PLC-γ after aFGF stimulation. The cell lysates were immunoprecipitated with anti-PLC-γ (**A**) or monoclonal anti-phosphotyrosine antibodies (**B**) and analysed by Western blotting with the anti-phosphotyrosine or anti-PLC-γ antibodies, respectively. The same cells were also immunoprecipitated with anti-PLC-γ and analysed by Western blotting using the same antiserum (**C**).

question regarding the biology of the FGF receptor system is how the four different FGF receptors transduce diverse signals for regulation of cell growth and differentiation from seven different FGFs. In this study we have examined the FGFR-4 ligand binding specificity, ligand-induced tyrosine kinase activation and subsequent phosphorylation of cellular polypeptides. Our experiments reveal an unexpected divergence of FGFR-4 structure and function from other receptors of this family.

According to our results, aFGF binds FGFR-1 and FGFR-4 with similar affinities, as does K-FGF, although with ~10 times lower affinity. In contrast, bFGF binds FGFR-4 in transfected NIH3T3 cells with ~10-fold lower affinity than aFGF. Differences in these ligand binding affinities of FGFR-1 and FGFR-4 could also be confirmed in CHO cells, which lack detectable endogenous FGF binding. Structural determinants of FGFRs responsible for the differences in FGF binding properties seem to reside in the carboxyl-terminal part of the third Ig loop: the FGFR-1 and FGFR-2 forms containing exon IIIb bind aFGF and KGF, but bFGF only weakly, while FGFR-1 and FGFR-2 forms containing exon IIIc bind aFGF and bFGF with similar affinities, but do not bind KGF (Dionne *et al.*, 1990; Keegan *et al.*, 1991; Miki *et al.*, 1992; Yayon *et al.*, 1992). Thus it is interesting to find that FGFR-4 lacks alternative exons encoding the second half of the third Ig-like loop. In addition, FGFR-1 and possibly FGFR-2 encode secreted forms of these receptors which can be translated from mRNAs polyadenylated in the retained intron between the IIIa and IIIb exons (Johnson *et al.*, 1991; Werner *et al.*, 1992). Such secreted forms of FGFR-4 cannot exist, because there are no stop codons or polyadenylation sites in the corresponding intron of FGFR-4.

One can speculate that the exons IIIb and IIIc of FGFR-1 and FGFR-2 were generated by the mechanism of exon duplication and that FGFR-4 did not participate in these events. However, another possibility is that FGFR-4 evolved from a gene that originally contained two alternative exons in this region, but a deletion of DNA containing a 3′ portion of IIIb and a 5′ portion of IIIc along with the intervening sequence may have occurred by for example homologous recombination thus creating exon IIIc′ of FGFR-4. One cannot distinguish between these alternatives on the basis of analysis of sequence identities between the different exons. However, similarities between the third Ig loop exons of FGFR-4 and KGFR, and their differences from FGFR-1, provide a framework for targeted mutagenesis studies on the specificity of bFGF and KGF binding.

Consistent with the binding data, FGFR-4 was tyrosine-phosphorylated upon FGF stimulation and extensive tyrosine phosphorylation of cellular proteins was seen in FGFR-4-expressing cells upon stimulation with aFGF, bFGF,K-FGF or FGF-6. Major tyrosine-phosphorylated, as yet uncharacterized, bands of 85, 75 and 62 kDa were identified in aFGF-stimulated FGFR-4-expressing cells. For comparison, tyrosine phoshorylation of 150, 92, 66, 49 and 43 kDa bands was seen in FGFR-1 transfectants. Some of these bands most probably represent substrates for these receptors.

Specifically, our results showed that FGFR-4 activation in transfected NIH3T3 cells does not lead to a significant increase in the tyrosine phosphorylation of PLC-γ, which is the major substrate for FGFR-1 (Burgess *et al.*, 1990). These results were not due to differences in the PLC-γ content of the FGFR-4- and FGFR-1-expressing cells as shown by immunoprecipitation and Western blotting with specific antibodies. Similar and consistent results were obtained when anti-phosphotyrosine immunoprecipitates were analysed in Western blotting with anti-PLCγ antibodies. Also, our Scatchard analyses showed that the two cell lines contained similar numbers of FGFR-1 and FGFR-4 receptors and the amount of tyrosine phosphorylation of the two receptors did not explain the difference in PLC-γ phosphorylation. This suggests that there is a difference between these two receptors in signal transduction via PLC-γ.

Autophosphorylated Tyr766 is a binding site for PLC-γ in FGFR-1 (Mohammadi *et al.*, 1991). Since the analogous residue is conserved in all FGFRs (Tyr754 in FGFR-4), it is interesting that FGFR-4 differs from FGFR-1 in phos-

phorylation of PLC-γ. It has been demonstrated that association of several tyrosine phosphorylated receptors with their substrates occurs between the phosphorylation sites and domains (~100 amino acid residues), termed SH2 (*src* homology 2) domains, which are common to many of the known substrates (Moran *et al.*, 1990; Cantley *et al.*, 1991). It has also been shown that different SH2 domains and receptor autophosphorylation sites interact with different affinities with each other (Y.Schlessinger, T.Pawson, personal communications). The binding between FGFR-1 and PLC-γ SH2 region was retained in a 28 amino acid peptide containing the Tyr766 autophosphorylation site, derived from the carboxyl-terminal tail of FGFR-1 (Mohammadi *et al.*, 1991). FGFR-4 differs from FGFR-1 and FGFR-2 in 17 and 15 amino acid residues of this peptide respectively, whereas FGFR-1 and FGFR-2 differ from each other in only seven residues of this peptide. It will therefore be interesting to find out which parts of the flanking amino acid sequences around this critical tyrosyl residue are needed for creating a binding site for PLC-γ and whether other possible signalling molecules bind this site in the FGFR-4.

Our studies together with already published data on the structure and function of FGFRs suggest that this subfamily of receptor tyrosine kinases is yet the most versatile of the characterized ones. FGFR diversity thus includes four distinct genes encoding receptors with distinct but overlapping ligand binding specificities. Alternative splicing of FGFR-1 alone is predicted to generate several variants of this receptor including secreted forms and forms differing significantly in their affinities for the different FGFs and in the activity regulation of their tyrosine kinases (for reviews see Jaye *et al.*, 1992; Partanen *et al.*, 1992). At least some of these four receptors may undergo activation by heterodimerization, creating further complexity in their function (Bellot *et al.*, 1991). Furthermore, binding to a low affinity heparan sulfate proteoglycan receptor is a necessary requirement for FGF binding and activation of the high affinity receptor (Yayon *et al.*, 1991) and the possibility of intracellular FGFRs and ligands has been suggested (Hou *et al.*, 1991). Data presented here add to our understanding of this diversity by describing a distinct ligand binding specificity for FGFR-4, a novel type of genomic strategy for FGFR-4 expression and, for the first time, differences in signal transduction between different FGFRs. Understanding of how this complexity is put to work for the diverse and important functions of FGFs in development and in the physiology of complex organisms is a major challenge for future work.

Materials and methods

Antisera

Peptides corresponding to the carboxyl-terminal 16 amino acids of FGFR-4 were synthesized and coupled to keyhole limpet hemocyanin (KLH) using glutaraldehyde as a cross-linking reagent. Rabbits were immunized with the KLH peptide emulsified in Freund's adjuvant to generate polyclonal anti-FGFR-4 antisera. Rabbit antiserum against phosphotyrosine was obtained from Upstate Biotechnology Inc. and the PY20 mouse monoclonal anti-phosphotyrosine antibodies from Zymed. Production of antisera against PLC-γ, was described in Margolis *et al.* (1989).

Expression of FGFR-4 and FGFR-1

The full length FGFR-4 coding sequence was inserted into the *Hin*dIII–*Xho*I site of the LTRpoly mammalian expression vector (Mäkelä *et al.*, 1992). NIH3T3 cells (60% confluent) were cotransfected with 5 mg of this pLTR2HX construct and 0.25 μg of the pSVneo vector containing the neomycin phosphotransferase gene, using the Transfectace reagents (Gibco). One day after the transfection the cells were transferred into the selection media (containing 0.5 mg/ml geneticin). Colonies of geneticin-resistant cells were isolated and analyzed for expression of the FGFR-4 protein. Cells were lysed in boiling lysis buffer containing 3.3% SDS and 0.125 M Tris pH 6.8. Protein concentrations of samples were measured by the BCA method (Pierce) as described by the manufacturer. About 50 mg protein of each lysate was analysed for the presence of FGFR-4 by 6% SDS gel electrophoresis and Western immunoblotting using antisera against the carboxyl-terminus of FGFR-4 and the ECL method (Amersham). Two of the clones, Nr4-3 and Nr4-6 were selected for further analysis. CHO DG-44 cells ($DHFR^-$) were transfected by calcium phosphate precipitation with 5 μg of the pSV2SE plasmid (Partanen *et al.*, 1991) containing the full length FGFR-4 cDNA together with the vector encoding DHFR as a selectable marker. Positive clones were selected in medium lacking hypoxanthine and thymidine, and screened for the expression of FGFR-4 using [^{125}I]aFGF. CHO cells expressing FGFR-1 were obtained as previously described by Mansukhani *et al.* (1990). Cos cells were transfected by DEAE-dextran method (McCutchan, 1968, # 1034) with 2.5 μg of PLC-γ expression vector together with either 2.5 μg of FGF-4 expression vector SV2SE or 2.5 μg of FGFR-1 expression vector SV7d (Wennström *et al.*, 1991).

FGF receptor cross-linking

aFGF (a kind gift of Dr Ralf Pettersson, Ludwig Institute for Cancer Research, Stockholm) was iodinated by the chloramine T method (Hunter and Greenwood, 1962) and the labelled aFGF was purified by a heparin–sepharose column. Confluent monolayers of transfected NIH3T3 cells in 90 mm culture dishes were washed twice with binding buffer (DMEM containing 0.1% gelatin and 50 mM HEPES, pH 7.5). About 25 ng of [^{125}I]aFGF was added to the binding buffer, which was then incubated on ice for 90 min. The cells were washed once with binding buffer, twice with PBS and incubated at 4°C in PBS containing 0.3 mM disuccinimidyl suberate for 20 min. The cells were then washed once with 10 mM HEPES, pH 7.5, 200 mM glycine and 2 mM EDTA, and once with PBS, lysed in 20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1.0 mM EDTA and 1 mg/ml aprotinin, and centrifuged for 10 min at 10 000 *g*. Aliquots of the supernatants were boiled in an equal volume of 2× electrophoresis sample buffer and analysed by SDS–PAGE.

Receptor binding and competition assays

aFGF, bFGF (kindly provided by Laura Bergonzoni, Farmitalia Carlo-Erba, Milan) and K-FGF were iodinated by the chloramine T method and the labelled FGFs were purified in a heparin–sepharose column. Sepcific activities obtained were 1.5×10^4 c.p.m./ng for aFGF, 2×10^4 c.p.m./ng for bFGF and 1.75×10^4 c.p.m./ng for K-FGF. Transfected NIH3T3 cells on gelatinized 24-well plates (10^5 cells/well) were placed on ice and washed twice with cold binding buffer containing heparin (DMEM, 1 mg/ml BSA, 5 U/ml heparin and 50 mM HEPES, pH 7.4) and incubated for 2 h at 4°C with increasing amounts of iodinated FGFs in binding buffer without heparin. When radiolabelled aFGF or K-FGF was used, the cells were washed three times with binding buffer, lysed in 0.3 M NaOH and the solubilized radioactivity was determined in gamma counter. After the binding of radiolabelled bFGF, the cells were washed three times with PBS and twice with 2 M NaCl and 20 mM HEPES pH 7.5. The receptor bound ligand was released by two washes with 2 M NaCl and 20 mM sodium acetate pH 4.0 (Moscatelli, 1987), and measured by gamma counting. The same determinations were done in the presence of a 100-fold excess of unlabelled ligand to estimate the non-specific binding. CHO cells expressing the FGFR-4 and FGFR-1 were incubated with radiolabelled ligand as described. After 2 hours the cells were washed with ice cold Tris and [^{125}I]FGF bound to high affinity receptors was removed by extraction with 2 M NaCl buffered at pH 4.0. In competition assays transfected NIH3T3 or CHO cells on gelatinized 24-well plates (10^5 cells/well) were placed on ice and increasing concentrations of different FGFs were incubated with ~6 ng/ml of indicated radiolabelled ligand. Binding assays were performed and bound radioactivity was measured as described above.

Isolation and analysis of genomic clones of FGFR-4

Human genomic library in λEMBL-3 SP6/T7 (Clontech) was screened with a probe corresponding to nucleotides 198–529 of the human FGFR4 cDNA (EMBL library accession no X57205). Several overlapping clones were isolated and the insert of one of these clones (G2-9) was subcloned as *Sac*I fragments into pGEM3Zf(+). A 5.0 kb subclone hybridizing to a PCR probe derived from the amino-terminal region of the third Ig loop, as well as to a probe derived from the transmembrane region, was analysed further. The appropriate segment containing the third Ig loop was sequenced from both strands using specific primers.

Analysis of tyrosine phosphorylation in FGFR-expressing cells

Cells were serum starved overnight in medium containing 0.5% FCS, pre-incubated with 0.1 mM (experiment of Figure 5) or 0.3 mM (all other experiments) Na_3VO_4 for 15 min and stimulated for the indicated times with 50 ng/ml aFGF. Tyrosyl phosphoproteins in cell lysates were analysed by 6% SDS–PAGE and Western blotting, using anti-phosphotyrosine antibodies followed by ECL detection.

Immunoprecipitation and immunoblotting

Cell monolayers were serum starved overnight in medium containing 0.5% FCS, pre-incubated with 0.3 mM Na_3VO_4 and stimulated for 5 min with 50 ng/ml aFGF at 37°C, washed once with cold PBS and scraped into immunoprecipitation buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P40, 0.1% SDS and 0.1 U/ml aprotinin). The lysates were sonicated and centrifuged for 10 min at 10 000 *g*. The supernatant was incubated for 2 h on ice with specific antisera against FGFR-4, PLC-γ or phosphotyrosine. Protein A–Sepharose (Pharmacia) was added and incubation was continued for 45 min with rotation. The precipitates were washed four times with immunoprecipitation buffer, once with PBS and once with water before analysis by 6% SDS–PAGE. Phosphorylation in FGFR-4, PLC-γ and phosphotyrosine immunoprecipitates was detected by Western blotting using the anti-phosphotyrosine antibodies and the amounts of PLC-γ using the specific antibodies.

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