The EMBO Journal vol.11 no.5 pp.1885 - 1890, 1992

A confined variable region confers ligand specificity on fibroblast growth factor receptors: implications for the origin of the immunoglobulin fold

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Communicated by D.Givol

Binding of cellular growth factors to their receptors constitutes a highly specific interaction and the basis for cell and tissue-type specific growth and differentiation. A unique feature of fibroblast growth factor (FGF) receptors is the multitude of structural variants and an unprecedented degree of cross-reactivity between receptors and their various ligands. To examine receptor-ligand specificity within these families of growth factors and receptors, we used genetic engineering to substitute discrete regions between Bek/FGFR2 and the closely related keratinocyte growth factor receptor (KGFR). We demonstrate that a confined, 50 amino acid, variable region within the third immunoglobulin-like domain of Bek and KGFR exclusively determines their ligand binding specificities. Replacing the variable region of Bek/FGFR2 with the corresponding sequence of KGFR resulted in a chimeric receptor which bound KGF and had lost the capacity to bind basic FGF. We present evidence that the two variable sequences are encoded by two distinct exons that map close together in the mouse genome and follow a constant exon, suggesting that the two receptors were derived from a common gene by mutually exclusive alternative mRNA splicing. These results identify the C-terminal half of the third immunoglobulin-like domain of FGF receptors as a major determinant for ligand binding and present a novel genetic mechanism for altering receptor-ligand specificity and generating receptor diversity.

Key words: alternative splicing/chimeric receptor/immunoglobulin domains

Introduction

The fibroblast growth factor (FGF) family consists of seven closely related polypeptides involved in the control of cell growth, embryonal development, angiogenesis and malignant transformation (reviewed by Burgess and Maciag, 1989; Klagsbrun, 1989). At least five FGF receptors have recently been identified and cloned (Kornbluth *et al.*, 1988; Ruta *et al.*, 1988; Lee *et al.*, 1989; Pasquale and Singer, 1989; Dionne *et al.*, 1990; Avivi *et al.*, 1991; Keegan *et al.*, 1991; Miki *et al.*, 1990; Partanen *et al.*, 1991; Raz *et al.*, 1991; Reid *et al.*, 1990). They share a common basic structure with three extracellular immunoglobulin (Ig)-like domains, an eight amino acid 'acidic box' and a split kinase as their intracellular catalytic domain. A unique feature of this family of receptors is the multitude of structural variants of expressed forms, many of which may be regulated by alternative RNA splicing. Several members of this family of receptors were found to be expressed as either three or two Ig-like domain forms (Reid *et al.*, 1990; Johnson *et al.*, 1991), as soluble secreted forms composed of an extracellular region lacking the first Ig-like domain (Johnson *et al.*, 1991) and as receptors with shorter cytoplasmic domains (Champion-Arnaud *et al.*, 1991). Recent analysis of the human genes for Flg (FGFR1), Bek (FGFR2) and K-Sam has demonstrated a unique exon organization which may encode different variants of their Ig-like domain III (Houssaint *et al.*, 1990; Johnson *et al.*, 1991). It was shown that Ig-domain III is encoded by two separate exons, corresponding to the N-terminal and the C-terminal halves of the domain respectively. The second exon, encoding the C-terminal half of the domain, is duplicated and diversified and may thus be used alternatively in different receptors. However, the functional role of the sequences encoded by the two alternatively spliced exons has not yet been ascertained.

Another unique feature of the FGFR family is an unprecedented degree of cross-reactivity between receptors and their various ligands. For example, both Flg and Bek receptors seem to bind equally well to acidic FGF (aFGF) and basic FGF (bFGF) (Dionne *et al.*, 1990; Johnson *et al.*, 1990). The keratinocyte growth factor receptor (KGFR) differs in this respect: it binds with high affinity to keratinocyte growth factor (KGF) and aFGF but not to bFGF (Bottaro *et al.*, 1990). Sequence comparison of murine Bek (Raz *et al.*, 1991) and KGFR (Miki *et al.*, 1991) reveals a high degree of overall homology between the two receptors despite their distinct ligand specificities.

To examine receptor-ligand specificity in this family, we generated chimeric receptors by substitution of discrete regions between the two closely related receptors Bek/FGFR2 and KGFR. This resulted in a drastic change in the ligand binding profiles of the engineered receptors and identified a confined, 50 amino acid, variable region within the third Ig-like domain which exclusively determines their ligand binding specificities. Moreover, the two variable sequences are encoded by two distinct exons that mapped close together on the mouse genome, suggesting that the two receptors are derived from a common gene by alternative mRNA splicing. These results may present a novel genetic mechanism for altering receptor-ligand specificity.

Results

Expression and binding profile of chimeric receptor

To study the possible contribution of the variable half of the third Ig-domain to the binding specificity of Bek and KGFR, we generated a chimeric molecule between Bek and KGFR. Figure 1 shows the regions where KGFR and Bek differ (Miki *et al.*, 1991; Raz *et al.*, 1991) and the strategy used

for generating the KGFR/Bek chimera. While Bek possesses a typical three Ig-domain structure, the KGFR lacks both the first Ig-domain and the conserved acidic box. In addition they differ in a restricted variable sequence (v) in the C-terminal half of the third Ig-domain in which there is only 48% sequence identity between the two receptors (Figure 1A). Mouse skin RNA was used to prepare cDNA which was amplified by PCR using two oligonucleotides flanking the variable half of KGFR and overlapping the common half of Ig-domain III (Figure 1B). The shared restriction sites were used for insertion of the KGFR variable segment into the cDNA of the extracellular region of Bek. Sequence analysis of the PCR product and the chimeric construct (Figure 1C) confirmed their identity to the KGFR sequence in that region and the in-frame insertion of the KGFR variable segment, replacing the corresponding Bek sequence.

To determine the ligand-binding profiles of Bek and the chimeric receptor independently, we used the APtag expression vector (Flanagan and Leder, 1990) to express the extracellular portion of the receptor as a secreted alkaline phosphatase (AP) fused protein (Figure 1B). Both Bek−AP (BAP) and the KGFR/Bek−AP (K-BAP) constructs were co-transfected into NIH 3T3 cells with a plasmid containing a neomycin resistance gene and secretor clones were selected by assay for AP activity in the conditioned medium. Making use of the absolute requirement for heparin in FGFR binding (Yayon *et al.*, 1991), we analysed the ligand specificity of BAP and K-BAP soluble receptors by binding to heparin−Sepharose bound FGFs and measuring the associated AP activity. The soluble BAP retains its ligand binding capacity and specificity (Figure 2) like the previously described c-kit−AP (Flanagan and Leder, 1990) and the murine FGFR1−AP (Ornitz *et al.*, 1992). BAP was found to bind aFGF, bFGF and hst/Kfgf (not shown) but not KGF, in agreement with studies on cells which overexpress either Bek or Flg (Dionne *et al.*, 1990). The K-BAP chimera acquired a binding profile strikingly different from that of the parental Bek as it lost its capacity to bind bFGF and gained a full capacity to bind KGF (Figure 2). The acquired ligand binding profile of K-BAP is very similar to that reported for cellular KGF-R (Bottaro *et al.*, 1990).

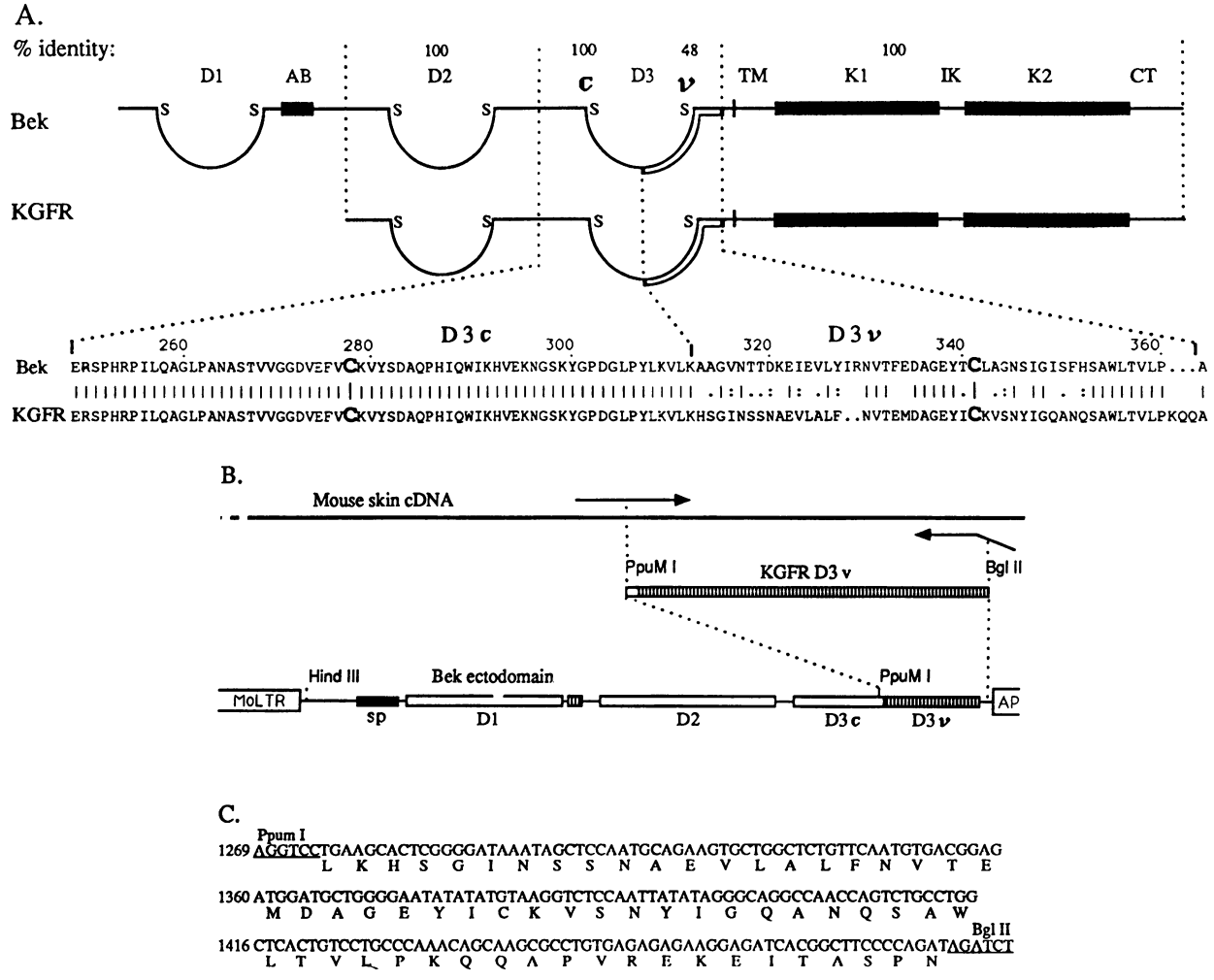

Fig. 1. Structure of Bek and KGFR and construction of their expressed ectodomains. **A.** Structure and sequence comparison of Bek and KGFR. The scheme shows the overall structure of the two receptors and the amino acid sequence identity. D1, D2 and D3, Ig-like domains; AB, acidic box, TM, transmembrane region; K1, K2, tyrosine kinase regions; IK, interkinase segment; CT, C-terminal tail. The amino acid sequence (one letter code) depicts the differences in Ig-domain III (D3) between Bek and KGFR. The N-terminal region containing constant sequence is denoted by c and the C-terminal region containing variable sequence is denoted by v. **B.** Scheme of the construction of the Bek ectodomain in the APtag vector and the exchange of the variable region of domain III with that of the KGFR. The latter was prepared by PCR on skin cDNA. **C.** The cDNA sequence of the PCR product obtained from mouse skin.

Ligand binding analysis of Bek and Bek/KGFR chimeric receptor

To analyse the ligand binding specificity of the secreted receptors further, we used competition binding analysis and affinity labelling with labelled ligand. Displacement binding analysis of BAP and K-BAP with the three ligands bFGF, aFGF and KGF was performed in the presence of heparin and using insoluble anti-AP antibody to separate receptor bound ligand. The results show that BAP binds aFGF and bFGF with dissociation constants of 7 and 9 nM respectively (Figure 3), whereas no binding of KGF to BAP could be measured. On the other hand the K-BAP receptor binds aFGF and KGF with dissociation constants of 8 and 5 nM respectively and with no detectable binding of bFGF under the same experimental conditions. Both BAP and K-BAP bind aFGF with a similar affinity resembling the ability of aFGF to bind to Bek and KGFR expressing cells (Dionne *et al.*, 1990; Miki *et al.*, 1991). The competition binding experiments confirmed the unique ligand profile of the chimeric receptor and suggest that aFGF, bFGF and KGF all interact with a common region of the receptor. Furthermore, our data indicate that bFGF and KGF bind to different sequences which are localized in this common region, whereas aFGF most probably binds to epitopes which overlap but are not identical to those involved in bFGF and KGF binding. The affinity of the different ligands to either BAP or K-BAP was 10- to 30-fold lower than that reported for the respective integral membrane receptors. As the extent of this decrease in binding affinity was similar for all ligands tested it may reflect some constraints on the receptor–AP fusion protein, similar to that observed in other soluble receptor systems (Gunther *et al.*, 1990). It is also possible that some cell associated factors other than heparan sulphate proteoglycans (Yayon *et al.*, 1991) contribute to the high affinity state of the FGFRs.

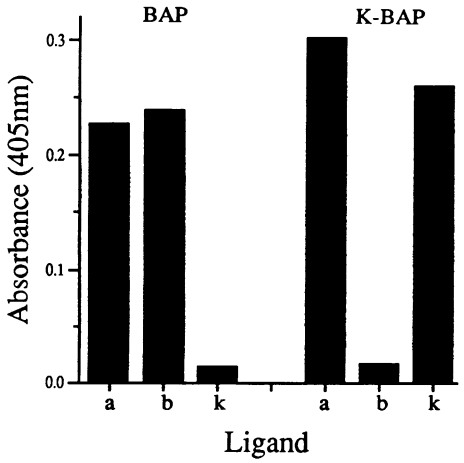

Fig. 2. Ligand binding profile of soluble receptors. The assay makes use of heparin–Sepharose-bound ligands to bind receptor–AP fusion proteins from cell medium. Conditioned medium from the indicated cell lines was incubated with 30 μl of Sepharose-bound ligand. The extent of receptor binding was determined by measuring the AP activity (absorbance at 405 nm). BAP and K-BAP correspond to the secreted receptors derived from cells transfected with the APtag expression vectors BAP or K-BAP respectively. a, b and k designate heparin–Sepharose bound aFGF, bFGF and KGF respectively.

Chemical cross-linking of the three ligands to the parental and the chimeric receptors further demonstrates the exclusive contribution of the variable segment derived from KGFR to ligand specificity (Figure 4). K-BAP could be cross-linked to either KGF or aFGF and both cross-linkings could be inhibited by an excess of either KGF or aFGF but not of bFGF. BAP on the other hand could not be cross-linked to KGF and its cross-linking to bFGF was inhibited by bFGF or aFGF but not by KGF. It is of interest to note that while Flg and Bek exist naturally in both two and three Ig-domain forms, which bind bFGF equally well (Johnson *et al.*, 1990), all forms of KGFR found to date have only two Ig-domains (Miki *et al.*, 1991). We could not, therefore, rule out the possibility that the first Ig-domain may have a specific dominant negative effect on the binding of KGF to its receptor. The fact that the chimeric K-BAP contains the N-terminal Ig-domain (domain I), while demonstrating a ligand binding profile indistinguishable from that of KGFR, rules out that possibility. It can therefore be concluded that the substituted, 50 amino acid variable region in the C-terminal half of Ig-domain III determines receptor–ligand specificity.

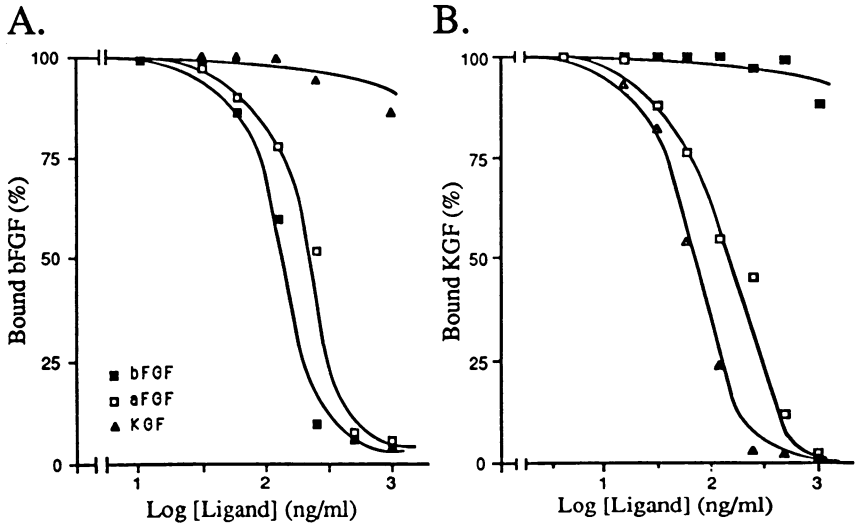

Fig. 3. Binding analysis of various FGFs to soluble receptors. The binding of the soluble receptors BAP and K-BAP was determined by displacement analysis using [^{125}I]bFGF or [^{125}I]KGF, and various FGFs. **A.** Displacement analysis of the binding of [^{125}I]bFGF to BAP. **B.** Displacement analysis of the binding of [^{125}I]KGF to K-BAP.

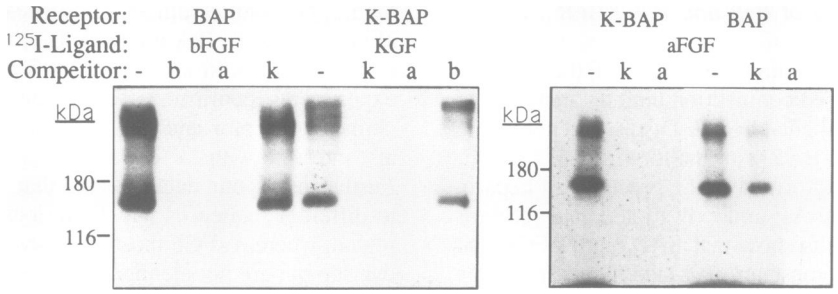

Fig. 4. Cross-linking of FGFs to secreted receptors. Conditioned medium from confluent cells secreting either BAP or K-BAP (100 μl) was reacted with the indicated labelled FGF in the presence or absence of a 100-fold excess of cold FGF. The receptor was immunoprecipitated with agarose–protein A–anti-AP followed by cross-linking with DSS and separation on an acrylamide gel. After the run the gel was dried and exposed to an X-ray film for 16 h. b, a and k: bFGF, aFGF and KGF respectively.

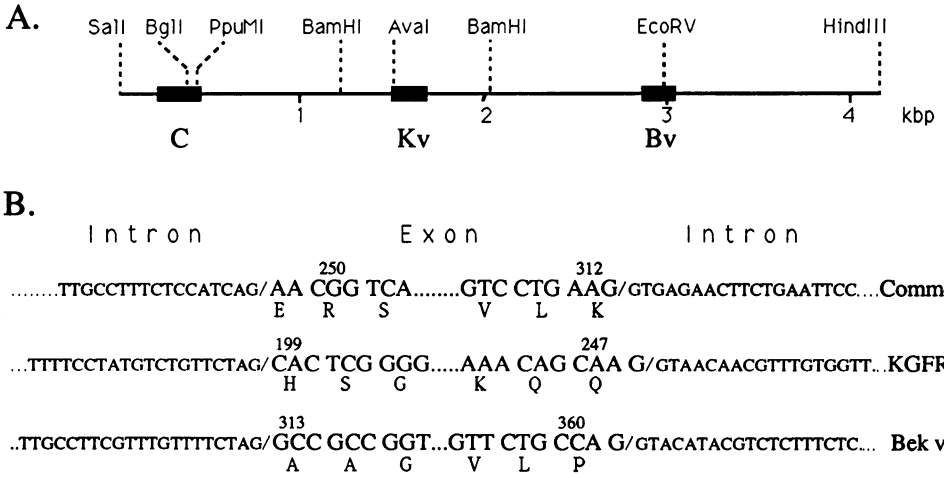

Fig. 5. Genomic structure of the murine Bek/KGFR domain III. A map of the 4.2 kb *Hin*dIII–*Sal*I fragment derived from the positively hybridizing phage isolated from a mouse DNA library in EMBL3. The library was screened with the PCR product of mouse skin cDNA (Figure 1) and with the *Ppu*MI–*Eco*RV fragment of *bek*. The exons are represented by black rectangles. C, common exon; Kv, KGFR variable exon; Bv, Bek variable exon. Unique restriction sites present in the cDNA are shown. **B.** DNA sequence of the intron–exon junctions of the three exons present in the *Hin*dIII–*Sal*I fragment. The sequence of the entire exons was determined by using internal primers derived from the cDNA sequence. The numbers of amino acids correspond to the published sequence of Bek (Raz *et al.*, 1991) and KGFR (Miki *et al.*, 1991).

Gene organization of Ig-domain III in murine Bek

The complete identity of the sequence of Bek and KGF-R except in Ig-domain I (absent in KGFR) and domain III strongly suggests that the two receptors may have derived from the same gene. By screening a mouse genomic library with probes specific for Bek and KGFR variable regions, we isolated a phage whose DNA insert contained both variable sequences. Restriction enzyme analysis and hybridization with the specific probes demonstrated that Ig-like domain III is encoded by two separate exons corresponding to the constant N-terminal and the variable C-terminal halves. The C-terminal half, however, is represented at the DNA level by two exons, separated by a 1.2 kb intron. The first exon encodes the KGFR variable region and the second encodes the Bek variable region (Figure 5). It is very likely that mutually exclusive, tissue specific splicing (Smith *et al.*, 1989) gives rise to a single third domain which is typical of either Bek or KGFR in fibroblasts and keratinocytes respectively. A similar gene structure has recently been found for human *bek* (Champion-Arnaud *et al.*, 1991), where one of the exons produces the *bek* transcript and the other contains *K-SAM*, a gene that is amplified in human stomach tumours (Hattori *et al.*, 1990). Very recently a similar arrangement of the exons encoding the third Ig-domain was also described for human *flg* and *bek* genes (Johnson *et al.*, 1991). Our results on the murine *bek* gene structure are in agreement with these studies and suggest that this arrangement of exons may be a general feature in the FGFR gene family.

Discussion

In the study reported here we have analysed the ligand binding specificity of members of the FGFR family. We chose two closely related receptors, the murine Bek/FGFR2 and KGFR because of their sequence similarity and distinct ligand specificity. KGFR lacks Ig-domain I and the acidic box and shows sequence divergence at the C-terminal half of Ig-domain III, which we termed variable sequence. By replacing the homologous variable sequence of Bek with that of KGFR we generated a chimera (K-BAP) which acquired the ligand binding specificity of KGFR. This implies that Ig-domain I has no effect on the binding of KGF and that the variable sequence in Ig-domain III confers the ability to bind KGF on the chimeric K-BAP. Additionally the chimera lost the capacity to bind bFGF but retained the ability to bind aFGF. Hence, although the variable sequence in domain III is a major element which determines the binding of KGF

and bFGF, other structural elements in Ig-domain III and possibly Ig-domain II may also contribute to the binding of ligands to FGFR.

Further insight into the structure of the ligand binding site of FGFR comes from another chimera that we have generated in which the variable segment of KGFR replaced the homologous region in Flg/FGFR1. In this case, sequence variability between KGFR and Flg is present along the entire ectodomain (Safran *et al.*, 1990; Raz *et al.*, 1991). Nevertheless, this chimera when compared with Flg showed significant changes in the binding specificity which resemble the changes observed with K-BAP. The chimera lost the capacity to bind bFGF and gained KGF binding although it bound KGF with a somewhat lower affinity than did K-BAP (data not shown). This result reinforces our conclusion that the C-terminal half of Ig-domain III is a major element in the determination of FGFR binding specificity, and extends our observations to another receptor of the same family.

Functional characterization of the variable sequence in the third Ig-domain may also provide an explanation for the unusual gene organization of this domain. It was recently shown that in the human *bek* gene the third Ig-domain is encoded by two distinct exons (Johnson *et al.*, 1991). The first exon (IIIa) encodes the N-terminal half of Ig-domain III of either Bek or KGFR. The second exon has been duplicated and diversified to give the two forms IIIb and IIIc, with KGFR and Bek each containing only one of the corresponding amino acid sequences (IIIb and IIIc respectively). Our analysis of the murine exons encoding Ig-domain III shows a very similar situation, in which exons Kv and Bv are homologous to IIIb and IIIc, and also provides the borders of all three exons (Figure 5). Our results demonstrate that the sequence of each exon indeed confers a particular ligand specificity on the expressed receptor; this represents a novel mechanism for generating two distinct receptors from the same gene using alternative exons. It is also very likely that this is accomplished by mutually exclusive alternative splicing (Smith *et al.*, 1989). The sequences of the three exons shown in Figure 5 indicate that the intron is located immediately at the end of the common sequence shared by Bek and KGFR (amino acids VLK, see Figure 1). We speculate that the presence of this intron facilitated the independent duplication and diversification of the exon encoding the C-terminal half of Ig-domain III to yield the Kv and Bv exons.

The exon–intron relationships in domain III and the contribution of domain III to the ligand specificity of FGFR may have interesting implications for the evolution of the Ig fold. It has been proposed that the Ig chains had all evolved from a primordial gene coding for an Ig-like domain that functions as an independent recognition unit (Williams and Barclay, 1988). The Ig-like domains can be divided into constant (C) and variable (V) type domains based on their Ig-fold and the number of β-strands. Comparison of domain III of FGFRs with Ig domains suggests that it may be homologous to V-type domains since it contains 63 residues between the two cysteines that may accommodate the two additional β-strands (C′ and C″) typical for V-type and absent in C-type domains. The V domain has a symmetrical β-sheet topology of eight anti-parallel strands forming a two-layered barrel in which the first four strands are related to the last ones by a dyad axis (Amzel and Poljak, 1979). The presence of such an internal homology and symmetry has led to the hypothesis that there was an ancestral gene coding for half a domain which would then have to dimerize in order to form a single functional domain (McLachlan, 1980). In the gene encoding the third Ig-like domain of Bek, the intron separates the constant and variable exons between the C″ and D β-strands, thus splitting domain III into two subdomains each having four β-strands. Although inter-domain introns in the immunoglobulin superfamily have been described before (Williams and Barclay, 1988), in the case reported here the intron is functionally involved in alternating exons which determine ligand binding specificity. Hence the arrangement of one constant and two variable exons represents an evolutionary attempt to generate diversity by alternative joining of distinct RNA segments. It is tempting to speculate that the presence of an intron in the receptor gene may have facilitated the duplication and diversification of these variable exons. While in the FGFR case receptor variability is achieved through alternative RNA splicing, in the immune system multiple exons encoding V regions undergo DNA recombination to generate the wide spectrum of recognition patterns needed in antigen–antibody interactions.

The variable portion of domain III contributes according to our analysis most of the necessary interactions for the binding of KGF. The difference in sequence between Bek and KGFR (Figure 1) can be assigned to two segments in this variable portion; one (C-terminal) corresponds to the complementarity determining region 3 (CDR3) of the Ig V region, whereas the other may partially correspond to CDR2 (Kabat *et al.*, 1991). This may imply that the binding of FGFs to their receptors is by interaction with two or more polypeptide loops protruding from the Ig-like framework of the domain; in that respect this is similar to the interactions between antigen and antibody. These structural similarities may have important implications for future modeling of FGFR–ligand interactions and for the design of receptor antagonists.

Materials and methods

Construction of Bek and chimeric KGFR/Bek in the alkaline phosphatase expression vector APtag

To prepare the extracellular portion of Bek as an AP-fused protein in the Aptag expression vector (Berger *et al.*, 1988; Flanagan and Leder, 1990) the *bek* clone in pBS (Bluescript) was subjected to PCR (Saiki *et al.*, 1988) using primer 1 (5′-GAAGCTTACCGTCCACGTGG, corresponding to the sequence beginning 50 bp upstream of the ATG and containing a *Hin*dIII site) and primer 2 (5′-TCGCGAAGATCTATCTGGGGAAGCCGT, corresponding to the sequence ending two codons 5′ to the transmembrane region and containing a *Bgl*II site). The reaction mixture (100 μl) was 67 mM Tris–HCl, pH 8.8, 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 250 μM dNTP, 0.17 mg/ml bovine serum albumin, 0.5 mM of each primer and 5 U *Taq* polymerase (Promega). After 30 cycles of 1.5 min at 94°C, 1.5 min at 65°C and 4 min at 72°C the reaction product was extracted with phenol and phenol–chloroform (1:1) and ethanol precipitated. The DNA was dissolved and after site-filling with dNTP and the Klenow enzyme was cloned into the *Eco*RV site of pBS. The *Hin*dIII–*Bgl*II fragment from this plasmid encoding the extracellular region of Bek was isolated and cloned into the *Hin*dIII and *Bgl*II digested APtag vector and was named BAP. To prepare the chimeric KGFR/Bek (K-BAP), the cDNA region corresponding to the KGFR variable region of domain III (Figure 1) was cloned from mouse skin RNA by incubating 10 μg RNA in 20 ml of 50 mM Tris–HCl pH 8.15, 6 mM $MgCl_2$, 110 mM KCl, 1 mM dithiothreitol, 20 μg/ml RNAsin, 250 μM dNTP and 1 mM primer 2 with 10 U of AMV reverse transcriptase (Genetic Resources) at 42°C for 1 h. Half of the reaction mixture (10 μl) was diluted to 100 μl with PCR buffer (see above) containing 0.5 mM primer 2 and 0.5 mM primer 3 (5′-AAGGTCCTGAAGCACTCGGGGATA) which overlaps the constant region and the KGFR variable region of domain III and contains the *Ppu*MI site. After the addition of 5 U *Taq* polymerase,

the PCR was carried out for 35 cycles as described above. The product was extracted with phenol and ethanol precipitated. The DNA was treated with Klenow enzyme and dNTP, and the PCR fragment (230 bp) was isolated by electrophoresis on an agarose gel, cloned into the *Eco*RV site of pBS and analysed by DNA sequencing. Ligation of three factors, the *Ppu*MI–*Bgl*II fragment from the latter clone, the *Hin*dIII–*Ppu*MI fragment from BAP (see Figure 1B) and the *Hin*dIII–*Bgl*II digested APtag, was employed to generate the chimeric receptor K-BAP depicted in Figure 1.

Cell lines and transfection

NIH 3T3 cells were transfected with calcium phosphate precipitate of either BAP or K-BAP together with pSV2 neo and individual clones were selected with Geneticin (800 μg/ml) and maintained in DMEM with 10% bovine calf serum. The conditioned medium was assayed for AP activity to determine whether the BAP or K-BAP fusion protein was secreted.

Ligand binding profile of secreted soluble receptors

The assay makes use of heparin–Sepharose bound ligands to bind the receptor–AP fusion proteins as determined by the AP activity on the conjugated beads. Immobilized ligand was prepared by incubating 300 μl heparin–Sepharose slurry (Pharmacia) with 1 μg of either human recombinant KGF (Amgen), human recombinant bFGF (Takeda, Japan) or human aFGF (a gift of Dr G.Neufeld). After 30 min shaking at room temperature, the beads were washed three times with 0.5 M NaCl and suspended (1:1) in 0.01 M phosphate buffer pH 7.4, 0.15 M NaCl (PBS). For receptor binding analysis, 30 μl of ligand–heparin–Sepharose beads were incubated with 10–15 μl of medium from either confluent BAP secreting cells or K-BAP secreting cells, normalized to total AP activity. After 30 min of shaking at room temperature the beads were washed three times with 0.5 M NaCl, once with 10 mM Tris–HCl pH 8.0 and incubated at 65°C for 10 min to inactivate endogenous cellular AP. The beads were assayed for AP activity by measuring absorbance at 405 nm after 15 min of incubation with 1.0 M diethanolamine (pH 9.8), 0.5 mM $MgCl_2$, 10 mM L-homoarginine, 0.5 mg/ml bovine serum albumin (Sigma) and 12 mM *p*-nitrophenylphosphate as described (Flanagan and Leder, 1990).

Binding of aFGF, bFGF and KGF to soluble FGFRs

This was analysed by displacement of labelled bFGF or KGF with unlabelled FGFs. The reaction mixture (200 μl) contained 0.4 ng of either [^{125}I]bFGF or [^{125}I]KGF, increasing amounts of unlabelled ligand, 0.2 μg heparin (Hepar, Franklin, OH), 0.2% BSA, 25 mM HEPES, pH 7.4 and 100 ml of conditioned medium from cells secreting either BAP or K-BAP. After 30 min of incubation at room temperature, 15 μl of agarose–protein A prebound to rabbit anti-human placental AP antibodies was added and the reaction was shaken for 30 min at room temperature. The beads were washed with HNTG (20 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100 and 10% glycerol) and once with 0.5 M NaCl to eliminate non-specifically bound ligands and the radioactivity associated with the beads was counted in a γ-counter.

Cross-linking of soluble secreted receptors and FGFs

Conditioned medium from confluent cells secreting either BAP or K-BAP (100 μl) was reacted with either [^{125}I]KGF, [^{125}I]aFGF or [^{125}I]bFGF (10 ng/ml) in the presence of 1 mg/ml heparin and in the absence or presence of a 100-fold excess of cold ligand followed by immunoprecipitation with agarose–protein A–anti-AP. After washing, the beads were suspended in 0.4 ml of 0.15 mM disuccinylimidyl suberate (DSS, Pierce) in PBS. After 30 min at room temperature the beads were spun and suspended in 1 ml of 150 mM glycine in 10 mM Tris–HCl pH 7.5 to terminate the cross linking reaction. Five minutes later the beads were washed with PBS, boiled for 5 min in sample buffer and the proteins analysed by PAGE on a 6% gel. After the run the gel was dried and exposed to an X-ray film for 16 h.

DNA library plasmids and growth factors

The mouse DNA library in EMBL3 phage was from Clontech. The library was screened with the PCR product of mouse skin cDNA (see Figure 1) and with the *Ppu*MI–*Eco*RV fragment of *bek*. DNA sequencing was by the chain termination method (Sanger *et al.*, 1977) using primers from internal cDNA sequences. The APtag vector was generously given by Dr P.Leder and J.Flanagan. Human recombinant KGF was from Amgen, human recombinant bFGF was from Takeda, Japan and human aFGF was from Dr G.Neufeld. Iodination of FGFs was performed on 2 μg protein by the chloramine T method and purification on heparin–Sepharose. Specific activity was between 150 000 and 250 000 c.p.m./ng.

Acknowledgements

We thank P.Leder and J.Flanagan for the APtag vector, D.Ornitz for advice, E.Arman for technical help, G.Neufeld for providing aFGF and Amgen for providing KGF. This work was supported in part by the Israel Cancer Research Fund (ICRF), the centre for molecular genetics at the Weizmann Institute and the Israel Cancer Association.

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Received on January 10, 1992; revised on February 18, 1992