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Apert syndrome mutations in fibroblast growth factor receptor 2 exhibit increased affinity for FGF ligand

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Dominantly acting mutations of the fibroblast growth factor (FGF) receptor 2 (*FGFR2*) gene have been implicated in various craniosynostosis syndromes. Apert syndrome, characterized in addition by syndactyly of the limbs, involves specific mutations at two adjacent residues, Ser252Trp and Pro253Arg, predicted to lie in the linker region between IgII and IgIII of the FGFR2 ligand-binding domain. We have analysed the interaction of FGF ligands with wild-type and Apert-type mutant FGFR2 ectodomains in solution. Wild-type and Apert-type receptors form a complex with FGF ligands with a stoichiometry of 2:2 (ligand:receptor). The kinetics and specificity of ligand binding to wild-type and Apert mutant receptors have been analysed using surface plasmon resonance techniques. This reveals that Apert mutations, compared with wild-type, exhibit a selective decrease in the dissociation kinetics of FGF2, but not of other FGF ligands examined. In contrast, the substitution Ser252Leu in FGFR2, previously observed in several asymptomatic individuals, exhibited wild-type kinetics. These findings indicate that Apert syndrome arises as a result of increased affinity of mutant receptors for specific FGF ligands which leads to activation of signalling under conditions where availability of ligand is limiting.

INTRODUCTION

The fibroblast growth factors (FGFs) are a family of at least 10 evolutionary conserved polypeptide ligands with wide-ranging actions during embryonic, fetal and adult life (reviewed in ref. 1). The biological effects of FGFs are mediated through a family of four FGF receptors, FGFR1–4, which are members of the intrinsic tyrosine kinase class of transmembrane growth factor receptors (reviewed in refs 1–3).

Specific mutations in the genes encoding FGFR1, 2 and 3 have, in humans, been associated with Crouzon, Pfeiffer, Jackson–Weiss, Apert, Beare–Stevenson and Muenke syndromes (4–11) whose defining feature is craniosynostosis: the premature fusion of skull sutures. Apert syndrome is, in addition, characterized by bony fusions of the digits of the hands and feet, whereas the limbs appear clinically normal in Crouzon syndrome. All mutations identified to date in these syndromes are dominantly inherited but are clustered in different regions of the *FGFR* genes (reviewed in ref. 12). This suggests that the phenotypic consequences of these *FGFR* mutations involve gain-of-function mechanisms with specific effects on skeletogenesis. This is supported by the demonstration that activation of FGFR signalling by application of ectopic FGF to the developing suture results in the induction of osteogenic differentiation (13).

Apert syndrome exhibits a striking clustering of mutations in two specific and adjacent amino acids, Ser252 and Pro253 of FGFR2 (5). The target Ser/Pro motif embedded in the predicted linker region is conserved within most vertebrate (but not invertebrate) FGFRs (1). These residues are, from modelling studies (14,15), predicted to lie in a region which connects two domains of the extracellular region of FGFR2: IgII and IgIII (Fig. 1). These domains together form the recognition site for FGF ligands (16–19). A remarkable feature of Apert mutations is their specificity: in our series, 190 of 192 unrelated patients exhibit either Ser252Trp or Pro253Arg substitutions (5,20–22; A.O.M. Wilkie, unpublished data). Equivalent Pro/Arg substitutions have been described in *FGFR1* (Pro252Arg) and *FGFR3* (Pro250Arg) associated with Pfeiffer (7) and Muenke (23) syndromes, respectively. We have described three additional rare mutations of the FGFR2 linker region in association with a variety of phenotypes: Ser252Phe in Apert syndrome, Ser252Leu with a normal or mild Crouzon phenotype and the double mutation Ser252Phe/Pro253Ser with atypical Pfeiffer syndrome (22). The existence of these two latter mutations suggests that the phenotypic consequences of mutation in the linker region depend upon the exact nature of the amino acid substitution involved.

The conservation and physical clustering of *FGFR2* mutations in Apert syndrome may be contrasted with those observed in the Crouzon/Pfeiffer/Jackson–Weiss group where the mutations are both more dispersed in the extracellular domain of FGFR2 and more heterogeneous in nature. It has been noted, however, that the latter mutations frequently correspond to either loss or gain of cysteine residues or involve substitutions predicted to result in disruption of disulfide bonds (12,14). Indeed, Crouzon mutations have been shown to elicit receptor activation, in the absence of

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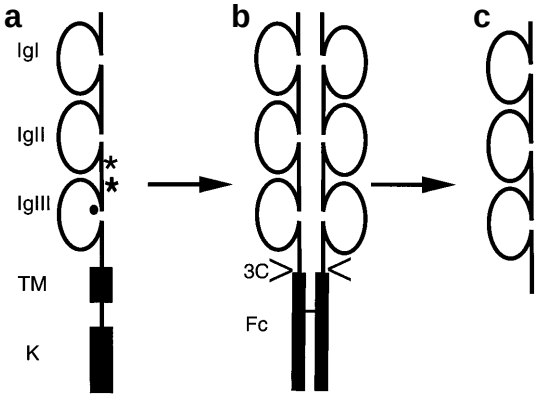

Figure 1. Schematic diagram of the FGFR2 constructs employed in this study. (**a**) Full-length FGFR2. IgI–III represent the three Ig-like domains of the extracellular region of the receptor. TM, transmembrane domain; K, kinase domain. The location of the mutations employed in this study are indicated: *, Ser252 and Pro 253; •, Ser267. (**b**) The FGFR2 ectodomain is fused to human Fc to produce the dimeric form of soluble receptor. The cleavage sites for rhinovirus 3C protease at the junction between FGFR2 and Fc are indicated by >. (**c**) Cleavage of the dimeric soluble form of FGFR2 by rhinovirus 3C releases the soluble monomeric form of FGFR2.

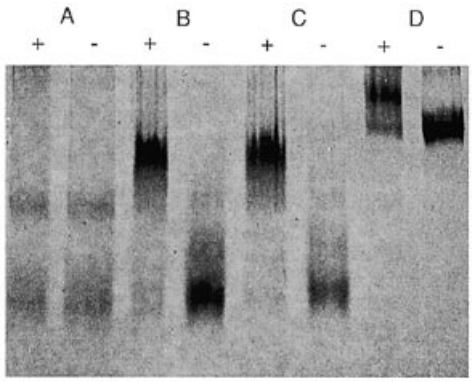

Figure 2. Native PAGE of soluble FGFR2 ectodomains. Lanes marked + and – represent the presence and absence of a 2-fold molar excess of FGF2 ligand, respectively. (**A**) Crouzon-type mutant Ser267Pro, monomeric form. (**B**) Apert-type mutant Ser252Trp monomeric form. (**C**) Wild-type receptor. monomeric form. (**D**) Wild-type receptor dimeric form.

ligand, via the formation of disulfide-bonded receptor dimers (24–26). This suggests that the mechanisms of the gain-of-function phenotype observed in different craniosynostosis-associated mutations of *FGFR2* may be quite distinct despite the overall similarity in phenotype. It is important, therefore, to define alterations in receptor function produced by Apert mutations.

An unexpected feature of craniosynostosis-associated mutations of *FGFR2* is their tissue specificity. Simple constitutive activation of FGFR signalling could not, of itself, account for the observed phenotypes since it has been demonstrated in a variety of animal models that activation of FGFR signalling by ectopic expression of secreted ligand results in severe embryonic phenotypes (27–30). This could be explained if the syndrome manifestations required additional features of FGFR function specific to the developing sutures and digits. We have suggested previously (14) that Apert mutations in the linker region of FGFR2 may result in altered specificity or affinity for FGF ligands. The requirement for specific ligands to elicit altered FGFR signalling could, in principle, account for the tissue specificity of craniosynostosis-associated mutations.

Here we test this hypothesis by examining the interaction between different FGF ligands and mutant forms of FGFR2 using biophysical techniques. We report that Apert-type mutant receptors, but not the phenotypically mild mutation Ser252Leu, exhibit a decrease in the kinetics of ligand–receptor dissociation which leads to enhanced receptor occupancy at low concentrations of ligand. This phenomenon is specific since it is most obviously manifest in the presence of FGF2 but not other FGF family ligands examined. Therefore, although there are shared phenotypic manifestations, the pathological mechanisms of Apert and Crouzon syndromes are distinct. Based on these findings, we propose that Apert syndrome is caused by activation of FGFR2 signalling in the presence of limiting amounts of FGF ligand.

RESULTS

Expression of recombinant FGFR2 ectodomains

All *FGFR2* receptor mutations studied here were expressed in the form of a soluble ectodomain comprising Ig domains I–III (Fig. 1b). The FGFR2 isoform employed in these experiments was the IIIc splice variant (31). FGFR2 soluble ectodomains were expressed in a modified form of the pIG vector (32) which yields a secreted fusion protein between the receptor ectodomain and the Fc portion of human Ig. The inclusion of a cleavage site for the rhinovirus 3C protease at the junction between the receptor ectodomain and the Fc region allows the receptor to be produced in two forms: dimeric corresponding to the Fc fusion and monomeric following cleavage with the 3C protease (Fig. 1c). Polyacrylamide gel electrophoresis (PAGE) under reducing conditions showed that all receptor forms were expressed as a single species of the predicted mass (data not shown).

Native PAGE and determination of stoichiometry

Monomeric and dimeric forms of wild-type FGFR2 were subjected to non-denaturing (native) PAGE in the presence and absence of a 2-fold molar excess of FGF2 to establish the form and stoichiometry of the ligand–receptor complex (Fig. 2). Monomeric wild-type FGFR2 and dimeric wild-type FGFR2 Fc both migrated as single species and exhibited significant differences in mobility reflecting their relative molecular mass. Both monomeric and dimeric wild-type FGFR2 were able to bind FGF2 as shown by migration retardation in the presence of ligand (Fig. 2). The receptor–ligand complexes also migrated as a single species indicating the formation of a stable and uniform complex.

The stoichiometry of the receptor–ligand complexes formed in solution was determined by native PAGE followed by transfer of the retarded complex to PVDF membrane and five cycles of amino acid sequencing. This revealed a stoichiometry of 1:1 (ligand:receptor). Since the mobility of the monomeric complex with ligand corresponds to that of a receptor dimer, it follows that the monomeric receptor–ligand complex contains two molecules of ligand and two molecules of receptor.

The same approach was employed to analyse the interaction between FGF2 and mutant forms of FGFR2. Apert mutations Ser252Trp and Pro253Arg exhibited essentially identical behaviour to the wild-type counterpart in this assay (Fig. 2). Both native mutant receptor and receptor–ligand complexes migrated as single species. A Crouzon mutation Ser267Pro (33) exhibited a different behaviour in native PAGE. It migrated as two forms: one corresponding in mobility to native wild-type receptor and a second retarded form whose migration corresponded to that of a receptor dimer. This shows that Crouzon-type mutant receptors undergo spontaneous dimerization in solution in the absence of ligand. Unlike the Apert mutations examined, the Crouzon mutant did not exhibit retarded migration in the presence of FGF2 (Fig. 2), indicating that, under the conditions of this assay, the mutant receptor was unable to form a stable complex with FGF2. These findings are in accord with previous studies of Crouzon mutant receptors *in vitro* (24–26).

The findings show that Apert-type mutant receptors are identical to their wild-type counterparts in their ability to form a complex with FGF ligand and, unlike the Crouzon-type mutation examined, do not undergo spontaneous dimerization in the absence of ligand. This suggests that the defect induced by Apert mutations may involve alterations in the kinetics and/or affinity of the ligand–receptor interaction.

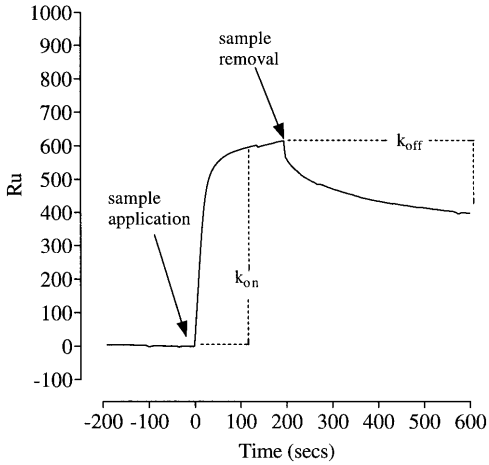

Figure 3. Schematic diagram of surface plasmon resonance sensogram data used in this study. FGFR2 is immobilized on a sensor chip and subjected to a buffer flow rate of 100 µl/min (–200–0 s). At time 0 s, the FGF sample is applied to the chip. The formation of a complex between ligand and receptor results in an increase in Ru. The rate of complex formation k_{on} is calculated from the rate of increase in Ru in the presence of sample (0–195 s). After 195 s, sample injection is stopped and the complex formed begins to dissociate. The rate of dissociation k_{off} is calculated from the rate of decrease in Ru from 200 to 600 s.

Surface plasmon resonance studies: kinetic analysis

The interaction between mutant forms of FGFR2 and FGF ligands was analysed by surface plasmon resonance (SPR) techniques (34,35) in order to define the kinetics and specificity of receptor–ligand interactions in more detail. In this technique, one partner in a receptor–ligand interaction is covalently immobilized to a sensor chip and the other interactant is passed over the chip in solution. As a complex between the two partners is formed, an increase in signal (expressed as resonance units, Ru) occurs which is proportional to the mass of protein bound to the chip (Fig. 3). The rate of increase in signal observed can be used to calculate the association rate (k_{on}) of the interaction. When the interacting partner is removed from the sample buffer, the complex formed on the chip undergoes dissociation and the rate of dissociation (k_{off}) is calculated from the decrease in observed signal. A specific feature of the instrument employed in this study is that the sensor chip is divided into four separate channels which permits the simultaneous comparison of up to three interaction partners with a single ligand. The major attraction of SPR for these experiments is, therefore, the ability to define, with precision, the kinetic properties of the FGF–FGFR interaction by measurement of the rate of ligand association and dissociation and to compare, in parallel, the binding of a ligand to both mutant and wild-type receptors.

Kinetic studies of receptor–ligand interactions were performed using sample injections of 326 µl at a flow rate of 100 µl/min and immobilized dimeric FGFR2 concentrations between 3000 and 7000 Ru. All data were collected by passing ligand simultaneously over all four channels of sensor chips in which one channel had been conjugated with bovine serum albumin (BSA), one channel with control wild-type FGFR2 and the remaining two channels with mutant receptors. Data from the BSA channel were subtracted from the sample channels before data analysis to remove changes in signal arising from refractive index effects and non-specific interaction with the sensor chip. In each experiment, the behaviour of mutant receptors is directly compared with wild-type as they are exposed simultaneously to the same ligand. The sensor chip was regenerated by application of 10 mM HCl after each sample application cycle.

Application of a range of concentrations of FGF2 (20–50 nM) to immobilized wild-type FGFR2 and Apert-type mutants Ser252Trp and Pro253Arg (Fig. 4a) yielded a rapid increase in signal resulting from specific binding of ligand, followed by a decrease in signal upon removal of the ligand from the sample buffer resulting from dissociation of the receptor–ligand complex. A parallel comparison of FGF2 binding to wild-type and the Apert-type mutant receptors Ser252Trp and Pro253Arg revealed a significant difference in the kinetics of ligand dissociation (Fig. 4b). The overall rate of dissociation of FGF2 from both mutant receptors was significantly slower than that from wild-type receptor (Fig. 4b). In addition, the overall rate of dissociation of FGF2 from Ser252Trp was slower than that from Pro253Arg (Fig. 4b). Calculation of the apparent association rate (k_{on}) of FGF2 with receptors did not reveal any significant differences between wild-type and mutant receptor, being in all cases in the region of 10^6/M/s (Table 1). Calculation of the apparent affinity of the interactions (expressed as the ratio k_{off}/k_{on}) revealed that Ser252Trp exhibited a 6-fold higher affinity and Pro253Arg a 2-fold higher affinity for FGF2 compared with wild-type (Table 1). These findings reveal that FGF2 dissociates significantly more slowly from the Apert-type mutant receptors Ser252Trp and Pro253Arg than from the wild-type counterpart.

These studies were extended in two ways. Having established that Apert-type mutant receptors exhibit different kinetic properties from wild-type in their interaction with FGF2, we examined the ligand specificity of this phenomenon. Wild-type and Apert-type mutant receptors were tested for their ability to bind a range of different FGF ligands (Fig. 5a–c). In all cases, there was no significant difference between wild-type and mutant receptors in the value of k_{on} (Table 1). This experiment revealed that there

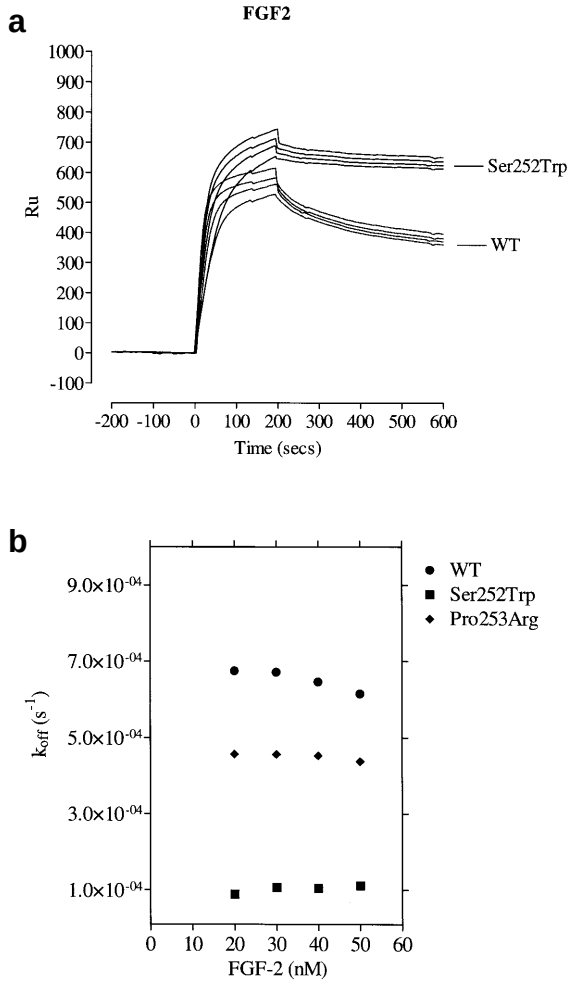

Figure 4. (**a**) Sensogram data of varying concentrations of FGF2 (20–50 nM) binding to immobilized wild-type FGFR2 and Ser252Trp Apert-type mutant receptor in parallel channels. The sensogram of FGF2 binding to Pro253Arg in the same experiment has been omitted for the sake of clarity. (**b**) Plot of calculated k_{off} versus FGF2 ligand concentration using the data from (a). The standard error of the mean (SEM) values were <5% of the total.

was a much milder effect on k_{off} for the interaction with FGF1 (Fig. 5a and b, Table 1) and no significant difference between Apert-type and wild-type receptors in the apparent value of k_{off} in the interaction with FGF4 (Fig. 5c and d, Table 1) and FGF6 (not shown).

These findings indicate that kinetic differences between wild-type and mutant IIIc receptors are most obviously manifest in the kinetics of interaction with FGF2, and that with respect to other FGF ligands Apert-type mutants more closely resemble their wild-type counterparts.

Several asymptomatic individuals with the substitution Ser252Leu recently have been identified (22). A study of this mutant thus would define whether kinetic differences in FGF2 dissociation are correlated with phenotypic features. The Ser252Leu receptor mutant was compared with wild-type and Apert-type mutant receptor (Fig. 6). This revealed that the FGFR2 mutant Ser252Leu exhibited kinetic behaviour which was identical to wild-type rather than Apert-type receptor. There is, therefore, a direct relationship between kinetic features of ligand dissociation and the phenotype of human patients. In particular, the severity of craniofacial phenotype for the series of FGFR2 mutations Ser252Trp, Pro253Arg and Ser252Leu (22,36) correlates with effects on FGF2 dissociation kinetics.

Table 1. Summary of kinetic data shown in Figures 4 and 5

		Wild-type	Ser252Trp	Pro253Arg
FGF1	k_{on} (/M/s)	8.02×10^5	8.83×10^5	7.22×10^5
	k_{off} (/s)	6.35×10^{-4}	3.86×10^{-4}	5.06×10^{-4}
	k_{off}/k_{on} (M)	7.91×10^{-10}	4.4×10^{-10}	7.0×10^{-10}
FGF2	k_{on} (/M/s)	1.33×10^6	1.34×10^6	1.18×10^6
	k_{off} (/s)	6.52×10^{-4}	1.02×10^{-4}	4.51×10^{-6}
	k_{off}/k_{on} (M)	4.9×10^{-10}	0.76×10^{-10}	3.81×10^{-6}
FGF4	k_{on} (/M/s)	1.48×10^6	1.1×10^6	1.09×10^6
	k_{off} (/s)	6.1×10^{-4}	5.03×10^{-4}	5.13×10^{-4}
	k_{off}/k_{on} (M)	4.12×10^{-10}	4.55×10^{-10}	4.7×10^{-10}

k_{on} was derived as described in Materials and Methods. The standard error was in all cases <15% of the calculated value. k_{off} is expressed as the mean value of data points in Figures 4b, 5b and 5d. The apparent affinity K_d is expressed as the ratio k_{off}/k_{on}.

DISCUSSION

The principal finding reported here is that FGFR2 receptors harbouring mutations of the Apert type (Ser252Trp, Pro253Arg) are fully functional in terms of ligand binding and complex formation but exhibit a selective decrease in the kinetics of ligand dissociation in the presence of FGF2. We cannot exclude the possibility that these mutations also affect the interaction with other receptor co-factors or ligands. However, it is significant that this effect was not seen with the phenotypically mild mutation Ser252Leu, which can also be generated by point mutation at amino acid 252. In addition, the dissociation rate effect was more pronounced with the mutant Ser252Trp than Pro253Arg. We conclude, therefore, that Apert-type mutations result in a selective alteration in the interaction with FGF ligands. Similar conclusions have been reached from studies of FGFR1 kinase activity in cells transfected with normal and Apert-type mutant receptors (L. Yu, W. Yuan, M.C. Naski, A. Chellaiah and D.M. Ornitz, personal communication). This provides insight into structural aspects of FGF–receptor interactions and provides an attractive biological explanation for the craniosynostosis phenotype of Apert syndrome. The syndactyly phenotype may have a distinct basis since it shows a reverse relationship with mutation type, being more severe in the Pro253Arg mutation (20). One possibility, not examined here, is that this is mediated by an equivalent mechanism via ligand interaction with the alternatively spliced IIIb splice variant of FGFR2.

Structural aspects of FGF–receptor interactions

The data presented here show that, under our experimental conditions, FGF2 interacts with monomeric receptor ectodomains to form a complex of two molecules of ligand and two molecules of receptor in accord with the previous findings (37). Molecular modelling of FGFR ectodomains (14,15) combined with mutagenesis studies (16–19) have shown that FGF2 binds two FGFR ectodomains through interaction with two distinct

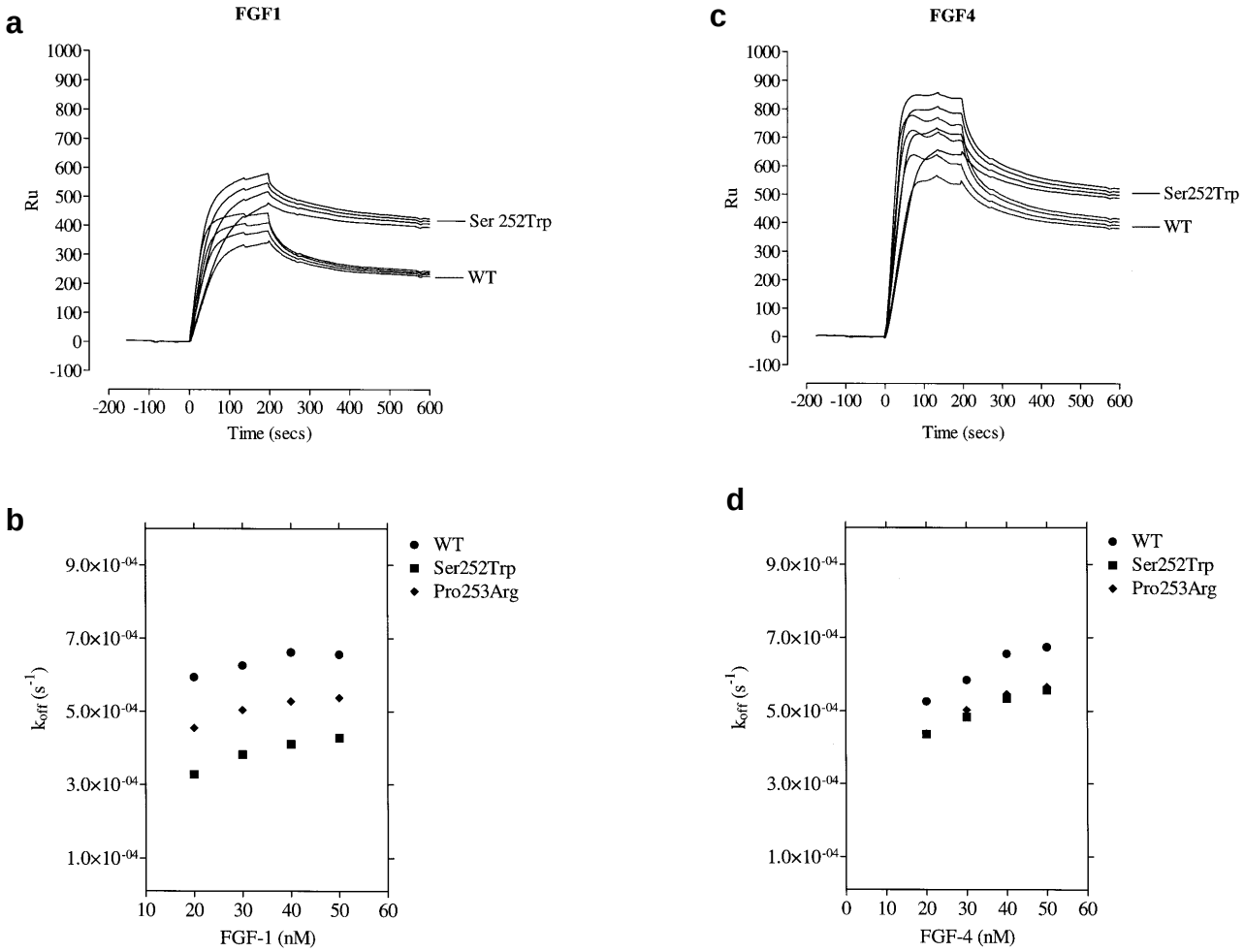

Figure 5. (**a**) Sensogram data of varying concentrations of FGF1 (20–50 nM) binding to immobilized wild-type FGFR2 and Ser252Trp Apert-type mutant receptor in parallel channels. The sensogram of FGF1 binding to Pro253Arg in the same experiment has been omitted for the sake of clarity. (**b**) Plot of calculated k_{off} versus FGF1 ligand concentration using the data from (a). The standard error of the mean (SEM) values were <5% of the total. (**c**) Sensogram data of varying concentrations of FGF4 (20–50 nM) binding to immobilized wild-type FGFR2 and Ser252Trp Apert-type mutant receptor in parallel channels. The sensogram of FGF4 binding to Pro253Arg in the same experiment has been omitted for the sake of clarity. (**d**) Plot of calculated k_{off} versus FGF4 ligand concentration using the data from (c). The SEM values were <5% of the total.

sites, located in the IgII and IgIII domains, respectively. These domains are separated by the linker region in which the Apert mutations are predicted to lie.

The nature of the linker domain between IgII and IgIII recently has been characterized by the X-ray structure of interleukin-1 (IL-1) and IL-1 receptor antagonist complexed to the IL-1 receptor (IL-1R; 38,39). This has revealed that IL-1, which is related to FGF in three-dimensional structure, binds to the IL-1R (which, like FGFR2, has three Ig-like domains in its extracellular region) via two ligand epitopes interacting with complementary binding sites in two adjacent Ig domains of the IL-1R. In addition, the IL-1R has an extended flexible linker region between IgII and IgIII analogous to the predicted linker region of FGFR2 connecting IgII and IgIII (14,15). The two key features of the IL-1R linker region are that it does not participate directly in interaction with ligand but does prevent intermolecular interactions between the second and third Ig domains which are thus free to undergo inter-domain changes in conformation.

Taken together, this suggests that the interaction of FGF2 with FGFR2 involves some form of conformational change in receptor, or receptor–ligand complex arising from the simultaneous interaction of FGF2 with IgII and IgIII. This conformational change may be accentuated by specific mutations in the linker region resulting in a decreased rate of ligand dissociation. The finding that the Ser252Leu mutant did not affect this process indicates that the identity of specific residues in the linker domain is significant. The Ser252–Pro253 linker motif is highly conserved in vertebrate FGFRs but is conspicuously absent in invertebrate FGFRs (1). It is notable that all known Apert-type linker substitutions identified to date involve the introduction of residues with bulky side chains such as Trp, Arg or Phe which may act to increase the rigidity of the linker region. This model suggests the possible existence of ‘super-Apert’ mutations which would show enhanced effects on ligand dissociation and might thus be predicted to exhibit more severe phenotypes than those

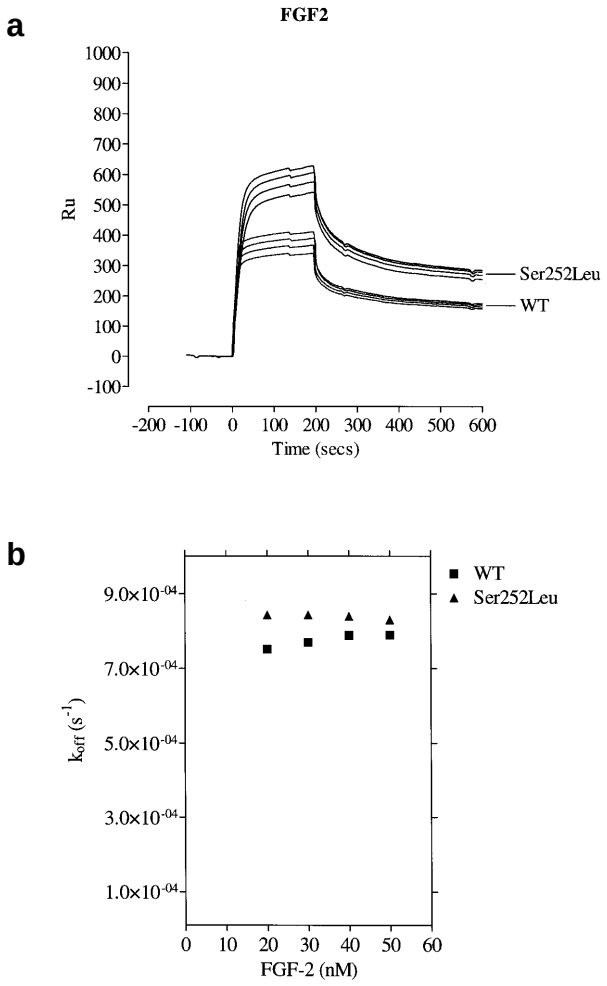

Figure 6. (**a**) Sensogram data of varying concentrations of FGF2 (20–50 nM) binding to immobilized wild-type FGFR2 and Ser252Leu asymptomatic mutant receptor in parallel channels. (**b**) Plot of calculated k_{off} verasus. FGF2 ligand concentration using the data from (a). The standard error of the mean (SEM) values were <5% of the total.

hitherto defined. This may be explored by a more systematic survey of linker domain substitutions.

The biological action of Apert mutations

The data presented here suggest that Apert mutations may lead to receptor activation as a consequence of interaction of the mutant receptor with FGF2. One attraction of this model is that it provides an explanation for the tissue specificity of Apert syndrome. It predicts that a phenotype is only manifest in situations where FGFR2 signalling occurs under conditions where receptor occupancy is controlled by ligand availability, since mutant receptors will exhibit higher receptor occupancy at subsaturating concentrations of ligand or where the biological response is influenced by the duration of receptor signalling.

A recent investigation of FGFR2 and FGF2 expression in the developing mouse skull (13) indicates that this condition is met in the case of the coronal suture. This study shows that FGFR2 is expressed in a narrow gap of the coronal suture between, and distinct from, domains undergoing osteogenic differentiation. This cell type thus represents the primary target for the effect of Apert mutations in the skull. FGF2 is highly expressed in the adjacent osteoid plates but is present at lower levels in the domain of FGFR2 expression of the coronal suture. Pre-osteogenic cells expressing FGFR2 are, therefore, normally exposed to low levels of FGF2 stimulation. This is strengthened further by the finding that enhancement of FGFR2 signalling by exposure to ectopic FGF2 leads to down-regulation of FGFR2 and enhanced osteogenic differentiation (13). This is consistent with the rate of osteogenic differentiation in the coronal suture being under the control of FGF2 availability. It is, however, presently unknown whether other FGF ligands exist which exhibit similar preferential affinity for Apert-type mutant receptors and are expressed in the suture. In this respect, the phenotype of FGF2 null mutant mice (40,41) does not reveal any overt effects on skeletogenesis.

FGF2 availability may also be subject to additional levels of control. A striking feature of FGF2 (and FGF1) is the absence of a functional secretory signal sequence. It has generally been concluded that, in contrast to other FGF ligands, FGF2 bioavailability is restricted by intracellular localization. However, the existence of specific mechanisms for export of non-secreted FGF ligands has been inferred previously from the study of pancreatic tumour progression (42), and several potential mechanisms have been suggested (43–46). Suture fusion may represent a physiological system in which FGF2 selectively is made available to target cell receptors by export and this could also contribute to the tissue specificity of the FGFR mutations.

General implications

The results presented here indicate that Apert syndrome is the consequence of a novel type of gain-of-function mutation which results in enhanced receptor occupancy by ligand and/or prolongation of the duration of receptor signalling. There are a number of biological systems in which quantitative changes in receptor occupancy and signalling have been shown to have profound biological consequences. For example, mutant forms of insulin which exhibit decreased rates of dissociation from the insulin receptor display a disproportionately high mitogenic potency as a consequence of sustained activation of receptor phosphorylation (47; see ref. 48 for a theoretical treatment). It was also demonstrated recently, using genetic approaches in the mouse, that oligodendrocyte precursor population size is strictly dependent upon platelet-derived growth factor A (PDGF-A) gene dosage and consequent ligand concentration (49). It will thus be important to determine the extent to which this class of mechanism operates to elicit pathological effects in other receptor signalling pathways.

MATERIALS AND METHODS

Site-directed mutagenesis of FGFR2

A cDNA encoding the human FGFR2 IIIc isoform (hBEK, clone TK 14; 31) was obtained from R. Breathnach (INSERM U211, Nantes, France) and used as a template for subsequent PCR amplification. An amplified *Eco*RI–*Bam*HI fragment encoding the three extracellular Ig domains of human FGFR2 (amino acids 1–362 of hBEK) was subcloned into the vector pIG (50). Cloning sites used in the vector were an *Eco*RI site 5′ of the first ATG codon and a *Bam*HI site 3′ of codon 362 upstream of the human IgG1-Fc gene. Mutations were introduced into the receptor by

splice overlap PCR techniques. The sequence of oligonucleotides employed is available on request. All PCR cloned inserts were sequenced by the chain termination method.

Production of soluble ectodomains of FGFR2

The pIG–FGFR2 constructs were transiently transfected into human epithelial kidney 293/tsA1609neo cells (51) by calcium phosphate precipitation as described previously for pIG–IL-11R constructs (50). Dimeric FGFR2 ectodomain–Fc fusion proteins were recovered from culture supernatants by binding to protein A–Sepharose (Pharmacia) and elution with 0.1 M citric acid, pH 3, after extensive washing with phosphate-buffered saline (PBS). Monomeric forms of FGFR2 ectodomain were obtained by protease 3C cleavage *in situ* on the protein A column at room temperature overnight in 50 mM Tris, pH 8, 150 mM NaCl, 1 mM dithiothreitol using ~2.5 µg enzyme/mg receptor. Cleaved FGFR2 was collected in the same buffer. After elution from the columns, fraction samples were analysed by SDS–PAGE using a Phastgel system (Pharmacia) and extensively dialysed against PBS. Estimation of protein concentration in the preparations of receptor was carried out using a Coomassie assay kit (Pierce).

Production of FGF ligands

FGFs 2 and 6 were obtained by expression of cDNAs from prokaryotic expression vectors: human FGF2 was expressed in pFC80, a gift from Dr A. Isacchi (Pharmacia, Milan), and human FGF6 from pMB40 (52), a gift of Professor D. Birnbaum.

Expressed proteins were purified by affinity chromatography on HiTrap Heparin columns (Pharmacia). Purified recombinant human FGF1 was purchased from R&D Systems (Abingdon, UK). Purified recombinant human FGF4 was a gift of Dr David Rogers (Genetics Institute). Protein concentration was determined in all cases by amino acid composition (Alta Bioscience, Birmingham, UK). All FGF ligands were buffer exchanged into HBS (10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA, 0.05% surfactant P20) by gel filtration (Pharmacia Hitrap) before use. Gel filtration studies revealed that ligands employed in these experiments were monomeric.

Non-denaturing (native) PAGE

Soluble ligand–receptor complexes were produced by mixing 3–6 µg of receptor with a 2-fold molar excess of FGF2 and agitating at 4°C for 2 h in a volume of 20 µl followed by electrophoretic separation in a non-denaturing 7.5% polyacrylamide gel consisting of 24 mM Tris, pH 8.6, 149 mM glycine, 10% glycerol, 0.1% ammonium persulfate and 0.005% TEMED. Electrophoresis was performed in a Bio-Rad Mini Protean II system. Each gel was run at 15 mA in 24 mM Tris, pH 8.3, 149 mM glycine. After electrophoresis, proteins were either visualized by staining with Coomassie blue or transferred onto PVDF membrane (Immobilon) for protein sequencing. Blotting was carried out in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] pH 11 buffer containing 10% methanol at 30 V at 4°C for 17 h. The membrane was then stained with Coomassie blue according to the manufacturer's recommendations, air dried and the appropriate bands excised with a scalpel blade and subjected to N-terminal sequencing (Alta Bioscience, Birmingham, UK). The molar ratio of ligand and receptor present in the blotted species was determined from the relative recovery of serine derived from FGFR2 in cycle 3 and FGF2 in cycle 4.

Surface plasmon resonance (SPR)

A BIAcore 2000 instrument (Pharmacia Biosensor) was employed to analyse the interaction between soluble FGFR2 ectodomains and FGF ligands. Purified wild-type and mutant FGFR2 ectodomains were immobilized by amine coupling (35) in adjacent flow cells of CM5 research-grade sensor chips using 10 mM sodium acetate buffer at pH 4 for monomeric FGFR2 and pH 4.5 for dimeric receptor. All experiments were performed at 24°C using a HBS buffer (Pharmacia Biosensor comprising 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20). In every experiment, there were two control channels, coupled with wild-type FGFR2 and BSA, respectively, and two experimental channels coupled with mutant receptors. Control experiments revealed that relative binding kinetics of wild-type and mutant receptors were unaffected by the identity of the channel employed. For each sensor chip, a similar number of response units (Ru) were immobilized in each of the four flow cells; this varied between 3000 and 7000 Ru which corresponds to ~3–7 $\times 10^{-14}$ mol/mm^2 for dimeric FGFR2. It is not possible precisely to equalize the extent of receptor coupling to individual channels of a chip so the maximum response levels vary from channel to channel, reflecting the amount of immobilized receptor. Estimates of the maximum response level derived from steady-state binding indicated that the majority (>90%) of immobilized receptor was able to bind ligand.

Kinetic studies were performed at a flow rate of 100 µl/min using injection volumes of 326 µl (195 s) and ligand concentrations of 20–50 nM. Under these conditions, the apparent ligand dissociation rate was observed to be independent of flow rate, indicating minimal ligand rebinding (data not shown). Dissociation was allowed to proceed for 400 s before chip regeneration by injection of 330 µl of 10 mM HCl.

Data analysis

Kinetic data were evaluated using the BiaEvaluation software package (Pharmacia Biosensor) according to the manufacturer's recommendations. Data were collected from four serial injections of 20–50 nM ligand (in 10 nM increments), normalized by subtraction of data derived from the parallel BSA channel and overlaid. The apparent association rate k_{on} was determined from linear curve fitting to a plot of ligand concentration against slope of a plot of dR/dT versus C (type 3 association model). The apparent dissociation constant k_{off} was determined by non-linear curve fitting of the data to a homogeneous single site model. Binding data were exported to the data analysis package Prism (Graphad software) for graphical analysis and manual inspection.

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REFERENCES

1. Coulier, F., Pontarotti, P., Roubin, R., Hartung, H., Goldfarb, M. and Birnbaum, D. (1997) Of worms and men: an evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor families. *J. Mol. Evol*., **44**, 43–56.
2. Givol, D. and Yayon, A. (1992) Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. *FASEB J*., **6**, 3362–3369.
3. Johnson, D.E. and Williams, L.T. (1993) Structural and functional diversity in the FGF receptor multigene family. *Adv. Cancer Res*., **60**, 1–41.
4. Reardon, W., Winter, R.M., Rutland, P., Pulleyn, L.J., Jones, B.M. and Malcolm, S. (1994) Mutations in the fibroblast growth factor receptor 2 gene cause Crouzon syndrome. *Nature Genet*., **8**, 98–103.
5. Wilkie, A.O.M., Slaney, S.F., Oldridge, M., Poole, M.D., Ashworth, G.J., Hockley, A.D., Hayward, R.D., David, D.J., Pulleyn, L.J. and Rutland, P. (1995) Apert syndrome results from localized mutations of FGFR2 and is allelic with Crouzon syndrome. *Nature Genet*., **9**, 165–172.
6. Rutland, P., Pulleyn, L.J., Reardon, W., Baraitser, M., Hayward, R., Jones, B., Malcolm, S., Winter, R.M., Oldridge, M., Slaney, S.F., Poole, M.D. and Wilkie, A.O.M. (1995) Identical mutations in the FGFR2 gene cause both Pfeiffer and Crouzon syndrome phenotypes. *Nature Genet*., **9**, 173–176.
7. Muenke, M., Schell, U., Hehr, A., Robin, N.H., Losken, H.W., Schinzel, A., Pulleyn, L.J., Rutland, P., Reardon, W., Malcolm, S. and Winter, R.M. (1994) Mutations in the fibroblast growth factor receptor-1 gene in Pfeiffer syndrome. *Nature Genet*., **8**, 269–274.
8. Jabs, E.W., Li, X., Scott, A.F., Meyers, G., Chen, W., Eccles, M., Mao, J.I., Charnas, L.R., Jackson, C.E. and Jaye, M. (1994) Jackson–Weiss and Crouzon syndromes are allelic with mutations in fibroblast growth factor receptor 2. *Nature Genet*., **8**, 275–279.
9. Bellus, G.A., Gaudenz, K., Zackai, E.H., Clarke, L.A., Szabo, J., Francomano, C.A. and Muenke, M. (1996) Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes. *Nature Genet*., **14**, 174–176.
10. Meyers, G.A., Day, D., Goldberg, R., Daentl, D.L., Przylepa, K.A., Abrams, L.J., Graham, J.M. Jr, Feingold, M., Moeschler, J.B., Rawnsley, E., Scott, A.F. and Jabs, E.W. (1996) FGFR2 exon IIIa and IIIc mutations in Crouzon, Jackson–Weiss, and Pfeiffer syndromes: evidence for missense changes, insertions, and a deletion due to alternative RNA splicing. *Am. J .Hum. Genet*., **58**, 491–498.
11. Przylepa, K.A., Paznekas, W., Zhang, M., Golabi, M., Bias, W., Bamshad, M.J., Carey, J.C., Hall, B.D., Stevenson, R., Orlow, S., Cohen, M.M. Jr and Jabs, E.W. (1996) Fibroblast growth factor receptor 2 mutations in Beare–Stevenson cutis gyrata syndrome. *Nature Genet*., **13**, 492–494.
12. Wilkie, A.O.M. (1997) Craniosynostosis: genes and mechanisms. *Hum. Mol. Genet*., **6**, 1647–1656.
13. Iseki, S., Wilkie, A.O.M., Heath, J.K., Ishimaru, T., Eto, K. and Morriss-Kay, G.M. (1997) FGFR2 and osteopontin domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGF2. *Development*, **124**, 3375–3384.
14. Wilkie, A.O.M., Morriss-Kay, G.M., Jones, E.Y. and Heath, J.K. (1995) Functions of fibroblast growth factors and their receptors. *Curr. Biol*., **5**, 500–507.
15. Bateman, A. and Chothia, C. (1995) Outline structures for the extracellular domains of the fibroblast growth factor receptors. *Nature Struct. Biol*., **2**, 1068–1074
16. Cheon, H.G., LaRochelle, W.J., Bottaro, D.P., Burgess, W.H. and Aaronson, S.A. (1994) High-affinity binding sites for related fibroblast growth factor ligands reside within different receptor immunoglobulin-like domains. *Proc. Natl Acad. Sci. USA*, **91**, 989–993.
17. Gray, T.E., Eisenstein, M., Shimon, T., Givol, D. and Yayon, A. (1995) Molecular modeling based mutagenesis defines ligand binding and specificity determining regions of fibroblast growth factor receptors. *Biochemistry*, **34**, 10325–10333.
18. Wang, F., Kan, M., Xu, J., Yan, G. and McKeehan, W.L. (1995) Ligand-specific structural domains in the fibroblast growth factor receptor. *J. Biol. Chem*., **270**, 10222–10230.
19. Zimmer, Y., Givol, D. and Yayon, A. (1993) Multiple structural elements determine ligand binding of fibroblast growth factor receptors. *J. Biol. Chem*., **268**, 7899–7903.
20. Slaney, S.F., Oldridge, M., Hurst, J.A., Moriss-Kay, G.M., Hall, C.M., Poole, M.D. and Wilkie, A.O.M. (1996) Differential effects of FGFR2 mutations on syndactyly and cleft palate in Apert Syndrome. *Am. J. Hum. Genet*., **58**, 923–932.
21. Moloney, D.M., Slaney, S.F., Oldridge, M., Wall, S.A., Sahlin, P., Stenman, G. and Wilkie, A.O.M. (1996) Exclusive paternal origin of new mutations in Apert syndrome. *Nature Genet*., **13**, 48–53.
22. Oldridge, M., Lunt, P., Zackai, E.H., McDonald-McGinn, D.M., Muenke, M., Moloney, D.M., Twigg, S.R.F., Heath, J.K., Howard, T.D., Hoganson, G., Gagnon, D.M., Jabs, E.W. and Wilkie, A.O.M. (1997) Genotype–phenotype correlation for nucleotide substitutions in the IgII–IgIII linker of FGFR2. *Hum. Mol. Genet*., **6**, 137–143.
23. Bellus, G.A., Gaudenz, K., Zackai, E.H., Clarke, L.A., Szabo, J., Francomano, C.A. and Muenke, M. (1996) Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes. *Nature Genet*., **14**, 174–176.
24. Galvin, B.D., Hart, K.C., Meyer, A.N. and Webster, M.K. (1996) Constitutive receptor activation by Crouzon syndrome mutations in fibroblast growth factor receptor (FGFR)2 and FGFR2/Neu chimeras. *Proc. Natl Acad. Sci. USA*, **93**, 7894–7899.
25. Neilson, K.M. and Friesel, R. (1996) Ligand-independent activation of fibroblast growth factor receptors by point mutations in the extracellular, transmembrane, and kinase domains. *J. Biol. Chem*., **271**, 25049–25057.
26. Robertson, S.C., Meyer, A.N., Hart, K.C., Galvin, B.D., Webster, M.K. and Donoghue, D.J. (1998) Activating mutations in the extracellular domain of the fibroblast growth factor receptor 2 function by disruption of the disulfide bond in the third immunoglobulin-like domain. *Proc. Natl Acad. Sci. USA*, **95**, 4567–4572.
27. Cohn, M.J., Izpisua-Belmonte, J.C., Abud, H., Heath, J.K. and Tickle, C. (1995) Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell*, **80**, 739–746.
28. Abud, H., Skinner, J.A., McDonald, F.J., Bedford, M.T., Lonai, P. and Heath J.K. (1996) Ectopic expression of FGF-4 in chimeric mouse embryos induces the expression of early markers of limb development in the lateral ridge. *Dev. Genet*., **181**, 51–65.
29. Crossley, P.H., Martinez, S. and Martin, G.R. (1996) Midbrain development induced by FGF8 in the chick embryo. *Nature*, **380**, 66–68.
30. Storey, K.G., Goriely, A., Sargent, C., Burns, H.D., Abud, H. and Heath, J.K. (1998) Early posterior neural tissue is induced by FGF in the chick embryo. *Development*, **125**, 473–484.
31. Champion-Arnaud, P., Ronsin, C., Gilbert, E., Gesnel, M.C., Houssaint, E. and Breath, R. (1991) Multiple mRNAs code for proteins related to the BEK fibroblast factor receptor. *Oncogene*, **6**, 979–987.
32. Karow, J., Hudson, K.R., Hall, M.A., Vernallis, A.B., Taylor, J.A., Gossler, A. and Heath, J.K. (1996) Mediation of interleukin-11-dependent biological responses by a soluble form of the interleukin-11 receptor. *Biochem. J*., **318**, 489–495.
33. Oldridge, M., Wilkie, A.O.M., Slaney S.F., Poole, M.D., Pulleyn, L.J., Rutland, P., Hockley, A.D., Wake, M.J.C., Goldin, J.H., Winter, R.M., Reardon, W. and Malcolm, S. (1995) Mutations in the third immunoglobulin domain of the fibroblast growth factor-2 gene in Crouzon syndrome. *Hum. Mol. Genet*., **4**, 1077–1082.
34. Fagerstam, L.G., Frostell, A., Karlsson, R., Kullman, M., Larsson, A., Malmqvist, M. and Butt, H. (1990) Detection of antigen–antibody interactions by surface plasmon resonance. Application to epitope mapping. *J. Mol. Recogn*., **3**, 208–214.
35. Karlsson, R., Michaelsson, A. and Mattsson, L. (1991) Kinetic analysis of monoclonal antibody–antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods*, **145**, 229–240.
36. Slaney, S.F., Oldridge, M., Hurst, J.A., Moriss-Kay, G.M., Hall, C.M., Poole, M.D. and Wilkie, A.O. (1996) Differential effects of FGFR2 mutations on syndactyly and cleft palate in Apert syndrome. *Am. J. Hum. Genet*., **58**, 923–932.
37. Spivak-Kroizman, T., Lemmon, M.A., Dikic, I., Ladbury, J.E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J. and Lax, I. (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation and cell proliferation. *Cell*, **79**, 1015–1024.
38. Vigers, G., Anderson, L.J., Caffes,P. and Brandhuber, B.J. (1997) Crystal structure of the type-1 interleukin-1 receptor complexed with interleukin-1 beta. *Nature*, **386**, 190–193.
39. Schreuder, H., Tardif, C., Trump-Kallmeyer, S., Soffientini, A., Sarubbi, E., Akeson A., Bowlin, T., Yanofsky S. and Barrett, R.W. (1997) A new cytokine receptor binding mode revealed by the crystal strucutre of the IL-1 receptor with an antagonist. *Nature*, **386**, 194–199.
40. Ortega,S., Ittmann, M., Tsang, S.H., Ehrich, M. and Basilico, C. (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. *Proc. Natl Acad. Sci. USA*, **95**, 5672–5677.

41. Zhou, M., Sutliff, R.L., Paul, R.J., Lorenz, J.N., Hoying, J.B., Haudesnschild, C.C., Yin, M.Y., Coffin, J.D., Kong, L., Kranias, E.G., Luo, W.S., Boivin, G.P., Duffy, J., Pawlowski, S.A. and Doetschman, T. (1998) Fibroblast growth factor 2 control of vascular tone. *Nature Med*., **4**, 201–220.
42. Kandel, J., Bossy-Wetzel, E., Radvanyi, F., Klagsbrun, M., Folkman, J. and Hanahan, D. (1991) Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell*, **66**, 1095–1104.
43. Tarantini, F., Gamble, S., Jackson, A. and Maciag, T. (1995) The cysteine residue responsible for the release of fibroblast growth factor-1 residues in a domain independent of the domain for phosphatidylserine binding. *J. Biol. Chem*., **270**, 29039–29042.
44. Shin, J.T., Opalenik, S.R., Wehby, J.N., Mahesh, V.K., Jackson, A., Tarantini, F., Maciag, T. and Thompson, J.A. (1996) Serum-starvation induces the extracellular appearance of FGF-1. *Biochim. Biophys. Acta*, **1312**, 27–38.
45. Shi, J., Friedman, S. and Maciag, T. (1997) A carboxyl-terminal domain in fibroblast growth factor (FGF)-2 inhibits FGF-1 release in response to heat shock *in vitro*. *J. Biol. Chem*., **272**, 1142–1147.
46. Cheng, G.C., Briggs, W.H., Gerson, D.S., Libby, P., Grodzinsky, A.J., Gray, M.L. and Lee, R.T. (1997) Mechanical strain tightly controls fibroblast growth factor-2 release from cultured human vascular smooth muscle cells. *Circ. Res*., **80**, 28–36.
47. Hansen, B.F., Danielsen, G.M., Drejer, K., Sorensen, A.R., Wiberg, F.C., Klein, H.H. and Lundemose, A.G. (1996) Sustained signalling from the insulin receptor after stimualtion with insulin analogues exhibiting increased mitogenic potency. *Biochem. J*., **315**, 271–279.
48. Shymko, R.M., De Meyts, P. and Thomas, R. (1997) Logical analysis of timing dependent receptor signalling specificity: applications to the insulin receptor metabolic and mitogenic signalling pathways. *Biochem J*., **326**, 463–469.
49. Calver, A.R., Hall, A.C., Yu, W.-P., Walsh, F., Heath, J.K., Betsholtz, C. and Richardson, W.D.R. (1998) Oligodendrocyte population dynamics and the role of PDGF *in vivo*. *Neuron*, **20**, 869–882.
50. Karow, J.H.K., Hall, M.A., Vernallis, A.B., Taylor, J.A., Gossler, A. and Heath, J.K. (1996) Mediation of interleukin-11-dependent biological responses by a soluble form of the interleukin-11 receptor. *Biochem. J*., **318**, 489–495.
51. DuBridge, R.B., Tang, P., Hsia, H.C., Leong, P.M., Miller, J.H. and Calos, M.P. (1987) Analysis of mutation in human cells by using an Epstein–Barr virus shuttle system. *Mol. Cell. Biol*., **7**, 379–387.
52. Pizette, S., Coulier, F., Birnbaum, D. and DeLapeyriere, O. (1996) FGF6 modulates the expression of fibroblast growth factor receptors and myogenic genes in muscle cells. *Exp. Cell Res*., **224**, 143–151.