Constitutive Activation of Fibroblast Growth Factor Receptor-2 by a Point Mutation Associated with Crouzon Syndrome*

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The fibroblast growth factor receptors (FGFRs) are a family of ligand-activated, membrane-spanning tyrosine kinases. Mutations in several human FGFR genes have been identified as playing a role in certain disorders of bone growth and development. One of these, Crouzon syndrome, an autosomal dominant disorder causing craniosynostosis, has been associated with mutations in the human FGFR-2 gene. We report here that microinjection of Xenopus embryos with RNA encoding an FGFR-2 protein bearing a Cys332 → Tyr mutation (FGFR-2CS) found in Crouzon syndrome results in fibroblast growth factor (FGF)-independent induction of mesoderm in animal pole explants. Wild-type FGFR-2 did not induce mesoderm when injected at similar doses. The effects of the mutant receptor were blocked by co-expression of dominant negative mutants of either Raf or Ras. Analysis of the mutant receptor protein expressed in Xenopus oocytes indicates that it forms covalent homodimers, does not bind radiolabeled FGF, and has increased tyrosine phosphorylation. These results indicate that FGFR-2CS forms an intermolecular disulfide bond resulting in receptor dimerization and ligand-independent activation that may play a role in the etiology of Crouzon syndrome.

The fibroblast growth factors (FGFs) are a family of polypeptide mitogens that currently consists of nine members (1, 2). The FGFs mediate a variety of biological processes including angiogenesis, wound healing, migration, mitogenesis, neuronal survival, and mesoderm induction (2, 3). These biological effects are mediated via binding to four members of a family of high affinity membrane-spanning tyrosine kinase receptors (2, 3). The FGFs have also been shown to bind to lower affinity cell surface heparan sulfate proteoglycans (1, 2). The prototype FGF receptor (FGFR) is comprised of an extracellular domain made up of three immunoglobulin (Ig)-like domains designated IgI–IgII, a hydrophobic membrane-spanning region, and a cytoplasmic tyrosine kinase domain (2, 3). The amino acid sequences of individual members of the FGFR family are highly conserved among vertebrate species (2). The IgII domain of FGFR-3 is encoded by three exons and is generated by alternative splicing of IgIIa with one of two alternative exons designated IgIIb and IgIIc (4, 5). This alternative splicing generates receptor isoforms with varying ligand binding specificities (6–8). Like the FGFs themselves, the FGFRs have unique but overlapping spatiotemporal patterns of expression during vertebrate development (9–11). The unique patterns of expression of both FGFs and their receptors during vertebrate development suggest that each may have a specialized function. Recent experimental evidence indicates that when FGF function is disrupted by genetic manipulation, major defects in embryonic development occur (12–16).

Within the last year, several mutations have been identified in FGFR genes that appear to be the cause of several human disorders of bone growth and development (2). One of these, Crouzon syndrome, is characterized by craniosynostosis, an abnormality of skull development in which the sutures of the growing bones fuse prematurely (17). A variety of mutations in exons IgIIa and IgIIc of FGFR-2 have been identified in Crouzon syndrome (17–19). These mutations may either directly (Cys342 → Tyr/Cys342 → Arg/Cys342 → Ser/Cys342 → Phe; Cys378 → Phe; Tyr328 → Cys; Ser347 → Cys; Ser354 → Cys) or indirectly (Ser267 → Pro; Glu289 → Pro; Tyr340 → His) result in the creation of a free cysteine residue that could result in covalent dimerization resulting in ligand-independent activation of the mutant receptor (2). Here we report that mutation of Cys342 → Tyr of Xenopus FGFR-2, analogous to the Cys342 → Tyr mutation most commonly found in Crouzon syndrome, promotes activation of the mutant receptor in the absence of ligand.

EXPERIMENTAL PROCEDURES

Materials—C4-Raf cDNA and N17-Ras cDNA were gifts of Dr. U. Rapp (National Cancer Institute) and Dr. T. Sargent (NIH), respectively. Affinity-purified rabbit antibodies to FGFR were prepared as described previously (20). A murine monoclonal antibody to phosphorylase, PY20, was obtained from Transduction Laboratories. Recombinant FGFR-1 was a gift from Dr. W. Burgess (Holland Laboratory).

In Vitro Mutagenesis—A BamHI fragment encoding the entire open reading frame of Xenopus FGFR-2 (10) was subcloned into pTZ19U (Bio-Rad). Mutagenesis of Cys310 → Tyr in Xenopus FGFR-2 was performed by the method of Kunkel et al. (21) using the mutagenic primer 5’-TCCAGCTATATAATTTATATCCATC-3’ to yield FGFR-2CS. The presence of the Cys310 → Tyr mutation and the absence of other mutations were confirmed by sequence analysis.

Plasmid Construction—All constructs for in vitro transcription were cloned into the BglII site of the SP64T or SP64T3 vectors (gifts of Dr. D. Metton, Harvard University). Synthesis of capped mRNA for microinjection was performed with SP6 RNA polymerase using a Message Machine kit (Ambion).

Embryo Injections—Eggs were collected from Xenopus laevis females and fertilized in vitro as described previously (22). Embryos were dejellied 30–60 min after fertilization with 2% cysteine, pH 8.0, and maintained at 17°C. At the two-cell stage, embryos were transferred to 1× MMF (5 mM HEPES, pH 7.8, 100 mM NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, and 0.1 mM EDTA) containing 50 μg/ml gentamicin and 5% BSA, bovine serum albumin.

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1 The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; BSA, bovine serum albumin.

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Ficoll. Each blastomere of two-cell embryos was injected in the animal pole with 5–10 nl of the indicated amount of RNA.

Animal Cap Assays and RNA Gel Blot Analysis—Animal pole ectoderm (animal caps) was dissected from stage 8–9 embryos (23) and incubated in 0.5 × MMR containing 1 mg/ml bovine serum albumin (BSA) and 50 μg/ml gentamicin in the presence or absence of 200 ng/ml recombinant FGF-1 at 22 °C. Animal caps were collected at stage 10.5 or 18 for Xbra mRNA analysis and stage 22–24 for muscle α-actin mRNA analysis unless otherwise noted. Total RNA was isolated and analyzed by RNA gel blot as previously described (24–26). To control for RNA loading, blots were hybridized to a Xenopus 18 S rRNA oligonucleotide probe (26).

Oocyte Injections and Immunoblot Analysis—Oocytes were collected and staged according to established procedures (27). Oocytes were defolliculated by mild collagenase treatment and maintained in 1 × MBS (27) containing 1 mg/ml BSA and 50 μg/ml gentamicin at 18 °C. Oocytes were injected with 10–20 nl of RNA at the indicated concentrations and cultured as described above for 1–2 days before immunoblot or cross-linking analysis. Oocytes were extracted in cold lysis buffer (20 mm HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1.0 mM EGTA, and 0.1 mM NaVO₄) extracted with 1 volume of MBS containing 1 mg/ml bovine serum albumin (Upjohn) (binding buffer) in the presence of 20 ng/ml 125I-FGF-1 (8–10 × 10⁴ cpm/ng) prepared as described previously (29). Oocytes were incubated at 4°C for 1 h followed by extensive washing with binding buffer. Ligand-receptor complexes were cross-linked with disuccinimidyl suberate (0.3 mM; Pierce) for 15 min at 4°C as described (29). Oocyte lysates were prepared as described above, and equal amounts of protein were immunoprecipitated with an FGFR antibody (20). Immune complexes were immobilized onto Protein G-Sepharose beads (Pharmacia Biotech Inc.), washed extensively with lysis buffer and kinase reactions performed as described (20). Samples were separated on 6% SDS-polyacrylamide gels, and tyrosine-phosphorylated proteins were visualized by autoradiography (20).

FGF Receptor Cross-linking—Oocytes were injected with 5 ng of either FGFR-2 or FGFR-2CS RNA and cultured for 2 days prior to cross-linking analysis. Thirty oocytes expressing either FGFR-2 or FGFR-2CS were incubated in 0.5 ml of 1 × MBS containing 1 mg/ml BSA and 10 units/ml heparin (Upjohn) (binding buffer) in the presence of 20 ng/ml 125I-FGF-1 (8–10 × 10⁴ cpm/ng) prepared as described previously (29). Oocytes were incubated at 4°C for 1 h followed by extensive washing with binding buffer. Ligand-receptor complexes were cross-linked with disuccinimidyl suberate (0.3 mM; Pierce) for 15 min at 4°C as described (29). Oocyte lysates were prepared as described above, and ligand-receptor complexes were partially purified by wheat germ agglutinin-agarose adsorption (30), followed by analysis on 7.5% SDS-polyacrylamide gels. Cross-linked complexes were visualized by autoradiography.

RESULTS AND DISCUSSION

Several mutations have been identified in the IgII domain of human FGFR-2 in individuals with Crouzon syndrome (17, 18, 31). The most frequently identified mutation is Cys³⁴² → Tyr, resulting in the generation of a free cysteine residue that may then be available to form intermolecular disulfide bonds. To test this possibility we created a mutation in a Xenopus FGFR-2 cDNA (Cys³⁴² → Tyr) analogous to the human Cys³⁴² → Tyr mutation. Fifty picograms of RNA transcripts from wild-type FGFR-2 or FGFR-2CS plasmids were injected into both blastomeres of two-cell stage Xenopus embryos. At the blastula stage, animal pole ectoderm (animal caps) was dissected, cultured, and assayed for elongation (32). By late neurula stages animal caps from embryos injected with FGFR-2CS RNA (Fig. 1d) elongated in a manner similar to control caps treated with FGF-1 (Fig. 1b). Animal caps injected with similar amounts of wild-type FGFR-2 RNA (Fig. 1c) remained spherical and resembled un.injected control animal caps (Fig. 1a). Injection of greater than 200 pg of FGFR-2CS RNA results in cleavage arrest in blastula stage embryos.² FGF-mediated mesoderm induction and animal cap elongation have been shown previously to be blocked by the expression of a dominant negative Raf (C4-Raf) or dominant negative Ras (N17-Ras) (33, 34). Consistent with this observation, co-expression of either a dominant negative Raf (Fig. 1e) or dominant negative Ras (Fig. 1f) with FGFR-2CS inhibited FGF-independent animal cap elongation.

We also assessed mesoderm induction by assaying for the expression of mesoderm-specific molecular markers. Xbra, the Xenopus homolog of brachyury, is expressed broadly in the presumptive mesoderm of gastrulating embryos and has been shown to be a useful molecular marker for mesoderm induction by growth factors such as FGF (35). Wild-type FGFR-2 or FGFR-2CS RNAs were injected into the animal pole of both blastomeres of two-cell stage embryos. Animal caps were dissected from blastula stage embryos (stages 8–9) and cultured until sibling controls reached the indicated stages (stage 10.5 or 18). RNA was isolated and analyzed by RNA gel blot hybridization. Fig. 2A shows that Xbra mRNA is expressed in animal caps in a dose-dependent manner following embryo injection with FGFR-2CS RNA. Injection of as little as 20 pg of FGFR-2CS RNA was sufficient to induce expression of Xbra mRNA, and the level detected was similar to that induced by FGF. No Xbra mRNA expression was detected in caps injected with a similar amount of wild-type FGFR-2 RNA or in un injected control animal caps. Xbra transcripts were not detected in animal caps co-expressing FGFR-2CS and either a dominant negative Ras or a dominant negative Raf.

Muscle-specific α-actin mRNA, a late marker for mesoderm

² K. M. Neilson and R. E. Friesel, unpublished observation.
Fig. 2. Induction of molecular markers of mesoderm formation by FGFR-2CS. Embryos at the two-cell stage were injected with either FGFR-2 or FGFR-2CS RNA in the indicated amounts. For experiments involving dominant negative Raf and Ras mutants, 50 pg of FGFR-2CS RNA was co-injected with 100 pg of C4-Raf RNA or N17-Ras RNA. A, animal caps were dissected at stage 8–9 and harvested at either stage 10.5 or 18 as indicated. RNA was isolated and analyzed by RNA gel blot hybridization. The blot was rehybridized with an 18S rRNA oligonucleotide probe to serve as an RNA loading control. B, embryos were injected and animal caps dissected as described in A. Animal caps were harvested when sibling control embryos reached stage 22–24, and RNA was isolated and analyzed by RNA gel blot hybridization for muscle α-actin mRNA expression (arrow). Cytoskeletal actin transcripts (upper two bands) serve as an internal control for RNA loading.

A noteworthy feature of most Crouzon syndrome mutations identified thus far is the creation or loss of cysteine residues in the IgIII domain of FGFR-2 (2). Both Cys278 and Cys342 are predicted to form a disulfide bond essential to formation of the Ig domain. These two cysteine residues are conserved throughout the FGFR family. Mutation of either of these two residues could result in destabilization of the structure of the Ig domain and the creation of a free cysteine residue. The creation of a free cysteine residue either directly or indirectly predicts a possible mechanism for the FGFR-independent mesoderm-inducing effects of FGFR-2CS: FGFR dimerization and ligand-independent activation by formation of an intermolecular disulfide bond. To examine this possibility, we microinjected wild-type FGFR-2 or FGFR-2CS RNA into Xenopus oocytes. Lysates from uninjected or injected oocytes were analyzed by reducing or nonreducing SDS-polyacrylamide gel electrophoresis and immunoblotting with an FGFR antibody (30). Under reducing conditions, both FGFR-2 and FGFR-2CS migrated as monomeric forms of 110 and 125 kDa, whereas under nonreducing conditions FGFR-2CS displayed an additional species at ~260–280 kDa (Fig. 3A), consistent with the size of a disulfide-linked homodimer. These same protein samples were then subjected to immunoblot analysis with a monoclonal antibody to phosphotyrosine (pTyr, PY20). C, lysates from injected oocytes were subjected to immunoprecipitation with an anti-FGFR antibody, and in vitro kinase assays were performed as described under "Materials and Methods." Electrophoresis conditions are indicated beneath each panel. The molecular mass markers (in kilodaltons) are shown to the right.
These data establish that creation of a free, exposed cysteine residue in the IgIIIdomain of FGFR-2 results in the formation of an intermolecular disulfide bond and ligand-independent activation of this receptor. These results indicate a potential mechanism for the dominant phenotypic effects observed in Crouzon syndrome and other craniosynostoses involving mutations in FGFR genes (2). It is likely that most of these FGFR mutations are activating ones, and the heterogeneity of clinical features observed in these syndromes may reflect the degree to which individual mutations activate the receptor. Alternatively, the variation in phenotype among individuals with identical point mutations may indicate that other genes may be involved that modify the effects of mutated FGFRs. A systematic analysis of each FGFR mutation, as described here, may shed light on subtle functional differences between different mutations, which may account for phenotypic variability.

The Xenopus system has proven to be a very useful model for the functional analysis of mutant signal transduction molecules in vivo (41). In particular, much has been learned about FGFR receptor structure and function employing this system (12, 30, 42). Although the induction of mesoderm in Xenopus animal cap by a constitutively activated FGFR-2 does not explain all of the events that lead to craniosynostosis, it does provide an excellent assay system to determine the functional consequences of the mutations associated with these syndromes. Particularly advantageous is the ability to demonstrate a dose-response relationship between the amount of RNA injected and the observed biological effects (see Fig. 2). This type of analysis may be particularly important since all of the FGFR mutations identified thus far are heterozygous, indicating that homozygous mutants may be lethal, and thus level of expression may be linked to severity of disease. While the full mechanistic details on the etiology of Crouzon syndrome remain to be established, our results demonstrate that a mutation associated with this syndrome results in constitutive activation of FGFR-2 with potentially adverse biological consequences for vertebral development.

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