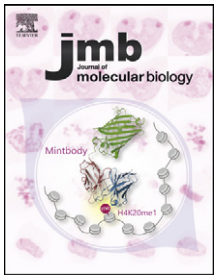

Pathogenic Cysteine Removal Mutations in FGFR Extracellular Domains Stabilize Receptor Dimers and Perturb the TM Dimer Structure

Sarvenaz Sarabipour and Kalina Hristova

Department of Materials Science and Engineering, *Johns Hopkins University, Baltimore, MD 21212, USA*

Correspondence to Kalina Hristova: *kh@jhu.edu*
http://dx.doi.org/10.1016/j.jmb.2016.08.026
Edited by Todd Yeates

Abstract

Missense mutations that introduce or remove cysteine residues in receptor tyrosine kinases are believed to cause pathologies by stabilizing the active receptor tyrosine kinase dimers. However, the magnitude of this stabilizing effect has not been measured for full-length receptors. Here, we characterize the dimer stabilities of three full-length fibroblast growth factor receptor (FGFR) mutants harboring pathogenic cysteine substitutions: the C178S FGFR1 mutant, the C342R FGFR2 mutant, and the C228R FGFR3 mutant. We find that the three mutations stabilize the FGFR dimers. We further see that the mutations alter the configuration of the FGFR transmembrane dimers. Thus, both aberrant dimerization and perturbed dimer structure likely contribute to the pathological phenotypes arising due to these mutations.

Fibroblast growth factor (FGF) receptors (FGFR1–4) comprise a family of transmembrane (TM) tyrosine kinases that bind 18 fgf ligands with high affinity in the presence of heparan sulfate [1]. They are composed of extracellular (EC) portions built of three Ig-like domains (D1, D2, and D3), single TM domains, and intracellular kinase domains [2,3]. These receptors play key roles in the regulation of cell differentiation, migration, proliferation, and apoptosis [1]. They are required for embryonic development, lung morphogenesis, osteogenesis, and limb bud development [4,5].

The FGFRs belong to the large family of receptor tyrosine kinases (RTKs) [6]. Like all RTKs, they are activated upon lateral dimerization in the cellular membrane, which brings the two kinases in close proximity [1,6,7]. In the dimer, the two kinases activate each other by cross-phosphorylating the tyrosines in the kinase activation loop. For many years, dimerization was believed to occur only in response to ligand binding [1]. However, recent work has demonstrated that dimerization can also occur in the absence of ligand because the FGFRs have intrinsic sequence-specific propensities for lateral interactions [8,9].

The FGFRs are known to harbor many pathogenic mutations [10,11]. As germ-line mutations, they cause developmental abnormalities of the skeletal system [1,4,10,12–15]. As somatic mutations, they have been linked to various cancers [16,17]. Some of these mutations are believed to cause pathologies by stabilizing the active FGFR dimers [18]. In particular, mutations that introduce or remove cysteine residues are believed to belong to this category [19]. Indeed, the unpaired cysteines can form disulfide bonds that bridge two receptors, leading to constitutive dimerization.

Here, we study the effect of three cysteine mutations in FGFR1, FGFR2, and FGFR3 on the stability of full-length FGF dimers. The first mutation that we investigate is the C178S mutation in the first Ig-like domain (D1) of FGFR1, which is associated with Kallman syndrome [20]. The phenotype includes severe ear anomalies (hypoplasia of the external ear), failure to start puberty, infertility, and complete lack of sense of smell. It occurs in 1 in 10,000 men and 1 in 50,000 women [21]. The second mutation that we study is the C342R mutation in the third Ig-like domain (D3) of FGFR2. This mutation is found in individuals with Crouzon syndrome, Jackson–Weiss syndrome, Pfeiffer syndrome, and Antley–Bixler-like syndrome [11,14,22–31]. Crouzon syndrome is characterized by the premature fusion of skull sutures (craniosynostosis) and has an incident rate of 1 in 25000 individuals [32]. The Jackson–Weiss syndrome phenotype is

characterized by craniosynostosis and foot abnormalities. The features of Pfeiffer syndrome are short fingers and soft-tissue syndactyly, while the Antley–Bixler-like syndrome phenotype includes craniofacial and limb abnormalities [13,14,24,33]. The third mutation that we investigate is C228R, which is located in the second Ig-like domain (D2) of FGFR3 and linked to colorectal cancer carcinoma [33], the most common type of intestinal cancer with 140,000 new cases each year in the United States alone.

These three pathogenic mutations substitute a cysteine in an Ig domain in the EC portions of the receptors. The Ig domains in the wild-type FGF receptors (D1, D2, and D3) are each stabilized by two intramolecular disulfide bonds. Thus, the loss of a cysteine in an Ig domain creates an unpaired cysteine. Specifically, the introduction of the studied mutations leaves C230 in FGFR1, C278 in FGFR2, and C176 in FGFR3 unpaired. These unpaired cysteines can then engage in intermolecular disulfide bond formation, which can lead to dimer stabilization.

Here, we investigate if the stabilities of the full-length FGFR dimers carrying the mutations are increased. In particular, we measure the stabilities of the mutant FGFR dimers using a quantitative FRET method termed quantitative imaging FRET (QI-FRET) [34,35], and we compare them to previously published stabilities of the wild-type FGFR dimers [8]. The QI-FRET method yields dimer stabilities and structural insights about the dimers [35–37]. This method allows us to acquire data over a broad receptor concentration range and collect binding curves. The technique also allows us to monitor structural perturbations on the cytoplasmic side of the membrane as a result of pathogenic mutations in the EC domain of the receptors [8]. The technique has been described previously as a detailed step-by-step protocol [34]. Non-interacting monomeric RTK variants, namely truncated ErbB receptors lacking the intracellular domains, have been identified and serve as negative controls in the QI-FRET experiments [38]. Furthermore, sequence-specific interactions within FGFR homodimers have been demonstrated through extensive mutagenesis and deletions [8,36,39].

The three mutations, C178S in FGFR1, C342R in FGFR2, and C228R in FGFR3, were introduced in the full-length and truncated FGFR-YFP (FGFR-yellow fluorescent protein) and FGFR-mCherry plasmids, which have been used in previous work [8]. YFP and mCherry comprise a FRET pair that allows FRET detection of FGFR dimerization. The experiments were performed in plasma membrane vesicles derived from CHO cells, which bud off from cells when the cells are incubated with an osmotic buffer [40]. These vesicles are a simplified yet highly relevant model of the plasma membrane [41].

CHO cells were co-transfected with $FGFR1_{C178S}$-YFP and $FGFR1_{C178S}$-mCherry, $FGFR2_{C342R}$-YFP and $FGFR2_{C342R}$-mCherry, or $FGFR3_{C228R}$-YFP and $FGFR3_{C228R}$–mCherry. Twenty four hours after transfection, the cells were treated with chloride salt buffer overnight at 37 °C to induce the shedding of plasma membrane vesicles bearing the receptors [40]. Vesicles were collected in four chambered slides and imaged in a confocal laser scanning microscope [34]. Each vesicle was imaged in three scans: a donor scan, a FRET scan, and an acceptor scan, as previously described [34]. The intensity of each vesicle image was quantified using a Matlab program, which yields the donor concentration, the acceptor concentration, and the FRET efficiency for each vesicle [34] (see Supplementary Data). Figure 1, top row, shows the FRET efficiency as a function of total receptor (donor label + acceptor label) concentration. Figure 1, middle row, shows the donor *versus* acceptor concentration in each vesicle. For each construct, more than 300 individual vesicles were imaged in at least three independent experiments. As discussed in detail previously, such a large number of data points is required for robust analysis of the FRET data [35]. Because the technique uses a standard confocal microscope, there is white noise associated with image acquisition (discussed in Ref. [34]). Since the underlying factor is white noise, the uncertainties can be reduced by acquiring a large number of data points [34].

A monomer–dimer equilibrium model with two optimizable parameters, the dimerization constant K and the intrinsic FRET (I-FRET) efficiency, was fitted to the data in Fig. 1 [see equation (8)], yielding the optimal K and I-FRET values for the receptors. The dimerization constant reports on the propensities of the receptors to form dimers, while the I-FRET depends on the separation between the fluorescent proteins and thus reports on the dimer structure [8,37].

Next, each data set in Fig. 1 was divided by the optimal I-FRET to obtain the dimeric fraction in each vesicle as a function of the total receptor concentration in the vesicle. Then, the dimeric fractions for similar receptor concentrations were averaged within the bins of width 500 receptors/μm^2 in Fig. 1, bottom row. The theoretical dimeric fraction is plotted with a solid line for each receptor and for the optimal value of K determined in the fit. The optimal values for the dissociation constant $K_{diss} = 1/K$, the dimerization free energy and the I-FRET are shown in Table 1.

We see that the three mutations stabilize the FGFR dimers. The C178S FGFR1 mutation increases the dimerization propensity of FGFR1 by −1.2 kcal/mol (Table 1). The FRET data for the other two mutants do not depend on the concentration, indicative of constitutive association.

FGFRs are known to form dimers, and we thus expect that $FGFR2_{C342R}$ and $FGFR3_{C228R}$ form constitutive dimers. In Fig. S5 in the Supplementary Data, the FRET efficiency for $FGFR2_{C342R}$ and $FGFR3_{C228R}$ is plotted as a function of the acceptor