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The Pathogenic A391E Mutation in FGFR3 Induces a Structural Change in the Transmembrane Domain Dimer

Krishna C. Mudumbi · Ayse Julius · Jana Herrmann · Edwin Li

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Abstract Fibroblast growth factor receptor 3 (FGFR3) is a single-pass membrane protein and a member of the receptor tyrosine kinase family of proteins that is involved in the regulation of skeletal growth and development. FGFR3 has three distinct domains: the ligand binding extracellular domain, the cytosolic kinase domain and the transmembrane domain (TMD). Previous work with the isolated FGFR3 TMD has shown that it has the ability to dimerize. Clinical and genetic studies have also correlated mutations in the TMD with a variety of skeletal and cranial dysplasias and cancer. Although the structures of the extracellular and cytosolic domains of FGFR3 have been solved, the structure of the TMD dimer is still unknown. Furthermore, very little is known regarding the effects of pathogenic mutations on the TMD dimer structure. We, therefore, carried out ToxR activity assays to determine the role of the SmXXXSm motif in the dimerization of the FGFR3 TMD. This motif has been shown to drive the association of many transmembrane proteins. Our results indicate that the interaction between wild-type FGFR3 TMDs is not mediated by two adjacent SmXXXSm motifs. In contrast, studies using the TMD carrying the pathogenic A391E mutation suggest that the motifs play a role in the dimerization of the mutant TMD. Based on these observations, here we report a new mechanistic model in which the pathogenic A391E mutation induces a structural change that leads to the formation of a more stable dimer.

K. C. Mudumbi · E. Li (✉)
Department of Biology, Saint Joseph's University, Philadelphia, PA 19107, USA
e-mail: eli@sju.edu

A. Julius · J. Herrmann
Lehrstuhl für Chemie der Biopolymere, Technische Universität München, 85354 Freising, Germany

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Introduction

Oligomerization of transmembrane helices is an important biochemical event that drives the folding of multispan membrane proteins into their three-dimensional configuration. It also drives the interaction of membrane proteins, allowing them to transduce extracellular signals and propagate them inside the cell. For example, receptor tyrosine kinases (RTKs) form dimers which are stabilized by the binding of extracellular ligands. Stabilization of the dimeric form allows the receptors to phosphorylate each other. When RTKs are activated in this way, cytosolic adaptor proteins are able to recognize and bind to the phosphorylated residues, thereby propagating cell signaling across the cell membrane (Lemmon and Schlessinger 2010). Thus, insight into the physicochemical principles of transmembrane helix dimerization is crucial for understanding and regulating the process of abnormal cell signaling.

The specificity of helix–helix interaction is achieved by the presentation of the appropriate side chains, which are recognized by the side chains of the other associating helix. The final structure achieved by this interaction can be described by a small set of parameters: the relative vertical position of the helix, the helix tilt (which is related to the crossing angle) and the rotational angle (which defines which side of the helix is facing the dimer interface). Thus, knowledge of the residues in the contact region between the two helices provides useful information regarding the structure of the dimer.

Through the study of the human erythrocyte protein glycophorin A (GpA), a high-affinity dimer, it was

proposed that a specific sequence motif in the dimer interface mediated the transmembrane helix–helix interactions in this protein. Scanning alanine mutagenesis confirmed that glycines 79 and 83 in the transmembrane domain (TMD) were crucial for its strong dimerization. These and other results gave rise to the concept of a GXXXG motif mediating GpA TMD–TMD interaction (Lemmon et al. 1994). Further examination of this sequence motif in other TMDs led to the broader SmXXXSm motif in which two small (Sm) amino acids flank three amino acids (XXX). In this motif, the small residues allow a close interaction between the two alpha helices by creating a groove in which side chains can be buried (MacKenzie et al. 1997). The SmXXXSm motif has been shown to be important in driving the dimerization of many membrane proteins including GpA (Brosig and Langosch 1998), BNIP3 (Sulistijo et al. 2003) and MCP (Melnyk et al. 2004). However, extensive studies have also demonstrated that presence of the SmXXXSm alone is not sufficient to mediate TMD–TMD interactions (Schneider and Engelman 2004; Li et al. 2012; Cymer et al. 2012).

Our studies focused on the role of SmXXXSm motifs in the dimerization of the TMD of fibroblast growth factor receptor 3 (FGFR3), a receptor tyrosine kinase and a member of the FGFR family of proteins (FGFR1-4). FGFRs play important roles in tissue repair, tumor angiogenesis, wound healing and skeletal development. Like most RTKs, FGFRs exist in a monomer–dimer equilibrium in the plasma membrane. Binding of extracellular growth factors stabilizes the dimer, leading to cross-phosphorylation of the receptors. The role of the TMD of FGFR3 has been well documented. Previous studies have shown that the isolated TMD can dimerize in micelles and liposomes (Li et al. 2005; You et al. 2005; Merzlyakov et al. 2007). Furthermore, recent biophysical studies using isolated TMDs, chimeric proteins and the full receptor have provided insights into the mechanism of pathogenesis arising from mutations in the TMD of FGFR3 (Li et al. 2006; He and Hristova 2008; He et al. 2012; Chen et al. 2011a). For instance, the A391E mutation, which has been linked to Crouzon syndrome with acanthosis nigricans, is known to increase the dimerization propensity of FGFR3 (Li et al. 2006; Chen et al. 2011a).

Although previous studies clearly show that TMDs are not merely just a link between the ligand-binding and kinase domains, very little is known regarding the structure of the FGFR3 TMD dimer. Using the software CHI, Li and Hristova (2006) produced a structural model of the wild-type FGFR3 TMD dimer in which Leu377, Val381, Phe384 and Ile387 are the critical residues in the contact region between the two helices. In this model, Ala391 faces the dimer interface and molecular dynamics simulation showed that substitution of Ala391 with Glu could lead to the formation of a hydrogen bond with the adjacent helix. Thus, this model proposes that stabilization of the TMD dimer via hydrogen bonds does not induce a structural change (Li et al. 2006).

In the work presented here, the ToxR assay was used to measure the dimerization of wild-type (WT) FGFR3 TMD and determine the role of two adjacent SmXXXSm motifs (^{374}**A**GIL**S**YGV**G**FFLFILVVAAVTL) in the dimerization process. Furthermore, the role of these motifs in the dimerization of the A391E FGFR3 TMD (^{374}**A**GIL**S**YGV**G**FFLFILVV**E**AVTL) was also examined. Our results show that, in bacterial membranes, the small A374, S378 and G382 residues that form the SmXXXSm motifs are not in the dimer interface of the WT TMD dimer. In contrast, substitution of these residues in the A391E TMD leads to changes in the affinity of the TMD to dimerize. Thus, while the motif does not appear to mediate the wild-type TMD–TMD interaction, it does in the A391E TMD. Our results are the first to provide evidence that the pathogenic A391E mutation may be inducing a structural change that leads to the formation of a more stable dimer.

Materials and Methods

Plasmids and Site-Directed Mutagenesis

ToxR plasmids carrying the different FGFR3 TMDs were created using pToxRV, in which the TMD of interest is inserted between the cytosolic ToxR domain and the periplasmic *Escherichia coli* maltose binding protein (MalE). The pToxRV_FGFR3WT construct was engineered using oligonucleotides encoding the desired sequence from A374 to L395 of WT FGFR3 TMD (^{374}AGILSYGVGFFLFILVVAAVTL) including a *Nhe*I and *Bam*HI restriction site at the 5′ and 3′ ends, respectively. Point mutations in the TMD were generated using the QuikChange® mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's protocols. Plasmids encoding GpA (WT and G83A) TMDs and ΔTMD were provided by Dr. Dieter Langosch (Technical University of Munich, Germany) and have been previously described in (Brosig and Langosch 1998; Langosch et al. 1996). All constructs were verified by DNA sequencing.

ToxR Activity Assays

Transcription activation was determined by expressing the ToxR chimeric protein in FHK12 *E. coli* cells as described (Herrmann et al. 2010; Langosch et al. 1996). FHK12 cells transformed with pToxR plasmids were grown overnight at 37 °C in LB medium supplemented with 35 μg/ml kanamycin. Expression of the chimeric protein was induced by inoculating

the overnight cultures in LB medium supplemented with 0.005 % (w/v) L-arabinose and 0.4 mM isopropyl-β-thiogalactoside (IPTG). Enzymatic activities were measured using 2-nitrophenyl-beta-D-galactopyranoside (ONPG) as the substrate at a final concentration of 0.4 % (w/v). Unless otherwise noted, relative activities were calculated with respect to the activity measured using WT FGFR3 TMD. One-sample *t* tests were carried out with the null hypothesis that the calculated relative activities are 1, to determine if there were statistical differences in TMD dimerization between WT FGFR3 TMD and all the other tested TMDs.

Western Blotting and MalE Complementation Assays

Expression levels of the chimeric ToxR proteins in FHK12 cells were evaluated by Western blotting. The MalE domain was detected using an anti–maltose binding protein rabbit antiserum (New England Biolabs, Ipswich, MA) and an anti-rabbit IgG alkaline phosphatase antibody (Sigma-Aldrich, St. Louis, MO). Proper membrane insertion of the different constructs was determined by a complementation assay using PD28 *E. coli* cells, a bacterial strain lacking MalE (Brosig and Langosch 1998).

Results

FGFR3 TMD Dimers are Weak

In the ToxR activity assay, TMD–TMD interaction directly correlates with the amount of β-galactosidase being produced in the cell. Measurement of enzymatic activity of β-galactosidase is, thus, used as a reporter of the strength of the interaction. To determine the strength of WT FGFR3 TMD dimerization, FHK12 *E. coli* cells were transformed with plasmids encoding the MalE_TMD_ToxR chimeric protein. To induce expression of the protein, transformed cells were grown with arabinose and IPTG for 6 h at 37 °C. Enzymatic activity was measured with bacterial cell lysates using ONPG as the substrate. Similar experiments were carried out using the TMD of GpA and its G83A mutant. GpA dimerizes strongly in bacterial membranes and is used frequently as a positive control in ToxR activity assays. While the GpA TMD dimerizes strongly, the TMD carrying the G83A substitution dimerizes weakly, resulting in a much lower activity (Langosch et al. 1996).

Our results show that FGFR3 TMD dimerization is very weak compared to those of WT GpA TMD and its G83A mutant (Fig. 1). Furthermore, a negative control was run in which the TMD was removed from the chimeric ToxR protein (ΔTM). This ΔTM construct, as expected, showed no activity. Western blot analysis showed that all the constructs were expressed at similar levels, and MalE complementation assays demonstrated that all constructs integrated properly in the bacterial membrane (Fig. 1).

Substitutions in A374, S378 and G382 Do Not Significantly Alter the Dimerization Propensity of WT FGFR3 TMD

The small (Sm) amino acids in the SmXXXSm motifs were replaced with other amino acids (Ala, Gly, Ser, Cys or Ile) to determine if they play an important role in TMD–TMD interaction. FHK12 cells were transformed with these plasmids, and enzymatic activities were measured as described above. The activities generated from TMDs carrying the substitutions were compared to that from WT FGFR3 TMD. Figure 2 shows that substitutions of A374, S378 and G382 to glycine, alanine, serine, cysteine or

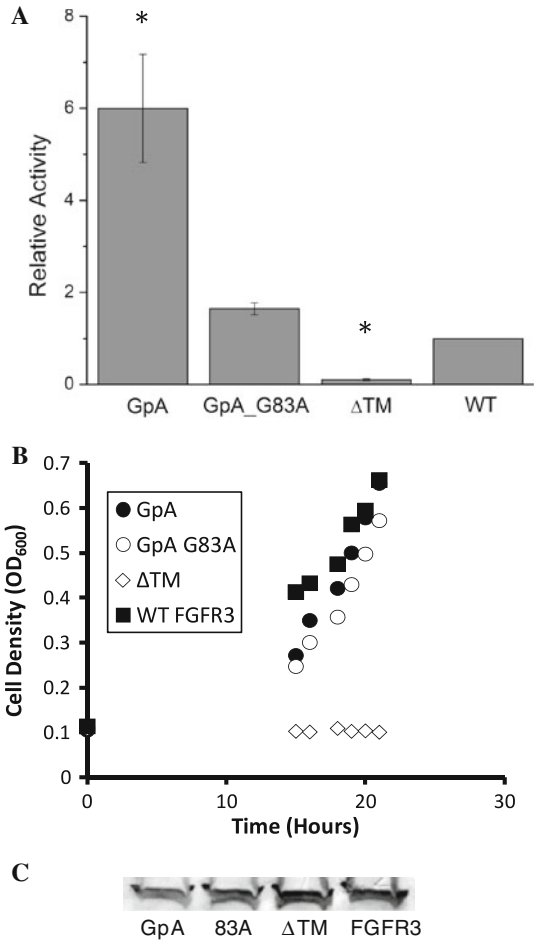

Fig. 1 Homodimerization of GpA and wild-type (WT) FGFR3 TMDs as measured by the ToxR assay. **a** β-Galactosidase activities are shown relative to the one from WT FGFR3 TMD. Data are expressed as the mean of 8–14 independent measurements. *Statistically different from WT FGFR3 TMD ($p < 0.05$). **b** Growth curves from the MalE complementation assay showing proper insertion of the TMDs in the bacterial membrane. **c** Western blot analysis of expression of the chimeric protein containing the different TMDs

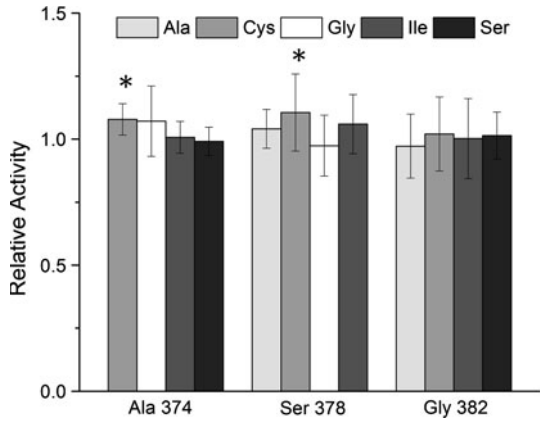

Fig. 2 Effect of single–amino acid substitutions in the SmXXXSm motifs of wild-type (WT) FGFR3 TMD. The small (Sm) residues in the SmXXXSm motifs were substituted with Gly, Ala, Ser, Cys and Ile. β-Galactosidase activities from TMD constructs are shown relative to the one from WT FGFR3 TMD. Data are expressed as the mean of 8–14 independent measurements. *Statistically different ($p < 0.05$)

isoleucine resulted in no or very little changes in dimerization. Only two substitutions, A374C and S378C, were determined to be statistically different from WT TMD. Expression levels and membrane integration were not affected by the single substitutions (data not shown).

Double Substitutions A374I/S378I and S378I/G382I Do Not Significantly Alter the Dimerization Propensity of WT FGFR3 TMD

The two small amino acids in each of the SmXXXSm motifs were replaced with isoleucine to determine if the double substitution would cause changes not observable by single substitutions. Figure 3 shows that the dimerization propensities of TMDs with the A374I/S378I and S378I/G382I substitutions were similar to that of WT. Expression levels and membrane integration were not affected by the double substitutions (data not shown).

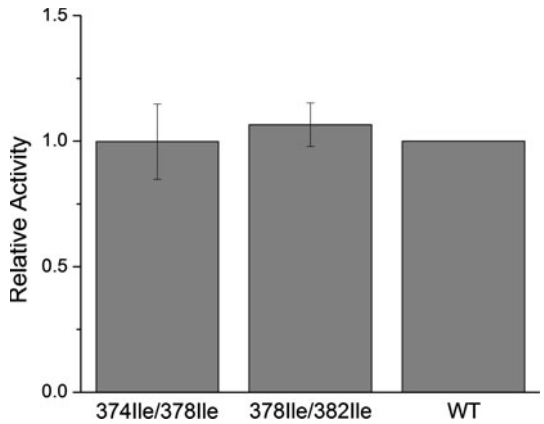

Fig. 3 Effect of double amino acid substitutions in the SmXXXSm motifs of wild-type (WT) FGFR3 TMD. The two small (Sm) residues in each of the SmXXXSm motifs were substituted with isoleucine (Ile). β-Galactosidase activities from TMD constructs are shown relative to the one from WT FGFR3 TMD. Data are expressed as the mean of 8–14 independent measurements

Substitutions in A374, S378 and G382 Significantly Alter the Dimerization Propensity of FGFR3 A391E TMD

The pathogenic A391E mutation was introduced in the ToxR plasmid containing the WT FGFR3 TMD. In ToxR assays, the homodimerization between A391E TMDs is slightly stronger than the one between WT TMDs (Fig. 4).

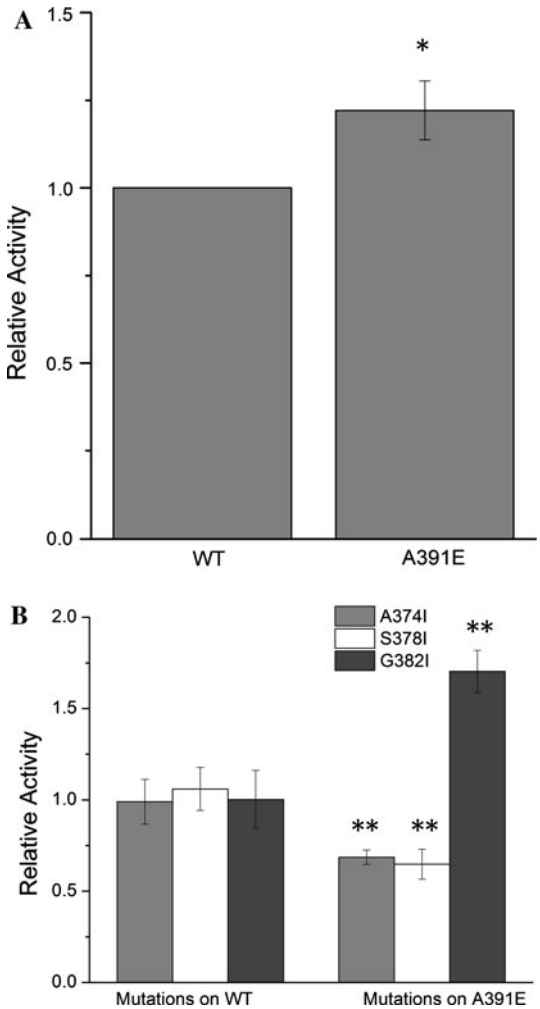

Fig. 4 Comparison of the effect of single–amino acid substitutions in the SmXXXSm motifs of wild-type (WT) and A391E FGFR3 TMDs. The small (Sm) residues in the SmXXXSm motifs were substituted with isoleucine. **a** β-Galactosidase activities from WT and A391E FGFR3 TMDs are shown relative to that of WT TMD. *Statistically different when compared to WT TMD. **b** β-Galactosidase activities from WT and A391E isoleucine mutants are shown relative to those of WT and A391E TMDs, respectively. Data are expressed as the mean of 8–14 independent measurements. **Statistically different when compared to A391E TMD ($p < 0.05$)

In the A391E TMD, substitution of the Sm residues (A374, S378 and G382) to isoleucine altered the dimerization propensity. Two substitutions (A374I and S378I) led to a decrease of dimerization of about 25 %, while one substitution (G382I) resulted in an increase of about 75 % compared to the dimerization of the A391E TMD. Similar substitutions in the WT TMD did not result in any significant change in dimerization (Fig. 4). Expression levels and membrane integration were not affected by the substitutions introduced in the A391E TMD (data not shown).

Discussion

FGFR3, like most RTKs, exists in a monomer–dimer equilibrium in cell membranes. This lateral dimerization has evolved as a means of signal transduction across the plasma membrane. The contribution of the TMD of FGFR3 in the dimerization of the full receptor is made evident in the several diseases that arise due to single amino acid substitutions in this domain (reviewed in Li and Hristova 2006). Previous studies have shown that the isolated TMD dimerizes in detergent and synthetic membranes. These studies have also demonstrated that dimerization is weak and that pathogenic mutations in the isolated TMD may or may not alter the propensity of TMDs to dimerize (Chen et al. 2011a; He and Hristova 2008; He et al. 2012; Li et al. 2005, 2006; You et al. 2006, 2007). Although many studies have focused on the strength of dimerization of WT and pathogenic mutant TMDs, very few studies have provided structural information about the TMD dimer such as tilt, rotational angle and crossing angle.

The results presented here show that the interaction between WT FGFR3 TMDs is weak (Fig. 1). As expected, it is much weaker than that of WT GpA TMD, which has a high affinity to self-associate (Langosch et al. 1996). This weak interaction was first shown using synthetic peptides corresponding to the TMD of FGFR3 and carried out in detergent and lipid vesicles (Li et al. 2005; You et al. 2005). More recently, Finger et al. (2009) used the ToxCAT assay to show that the TMD–TMD interaction of FGFR3 is approximately 10 % of WT GpA and is among the weakest from all the 58 TMDs of RTKs. Thus, our results are in agreement with previous reports regarding the strength of WT FGFR3 TMD dimerization.

Although the weak dimerization of WT FGFR3 TMD has been demonstrated previously, there are no experimental studies providing information about the dimer structure and the residues mediating the contact between the two helices. Because the SmXXXSm motif has been shown to be critical in the dimerization of several membrane proteins (Li et al. 2012), we investigated the role of two adjacent SmXXXSm motifs in the TMD of FGFR3. Our results from the ToxR activity assay show that dimerization of WT FGFR3 TMD in bacterial membranes is not dependent on the small (Sm) residues at each end of the two motifs (A374, S378 and G382). Substituting A372, S378 and G382 with either smaller or larger residues (e.g., A374G, G382I) neither diminished nor enhanced the affinity of the WT TMD to dimerize. The small, but statistically different, changes observed with the A374C and S378C substitutions indicate that the cysteine residue in one helix may be close to its counterpart in the adjacent helix but not optimally positioned (face to face) to promote a larger stabilization of the dimer through disulfide bonds.

The helix–helix interaction of WT FGFR3 TMD is relatively weak, so it is possible that small changes in dimerization arising from single mutations may not be detectable using the ToxR assay. To circumvent this possible lack of sensitivity, we introduced double substitutions on the two small amino acids on both sides of the SmXXXSm motif (A374 and S378, S378 and G382). Replacing the two Sm residues with isoleucine in the SmXXXSm motif did not result in any significant change in dimerization (Fig. 3).

The failure of single and double substitutions of the Sm residues to alter the dimerization propensity of WT FGFR3 TMD suggests that the SmXXXSm motifs ^{374}AXXX^{378}S and ^{378}SXXX^{382}G do not mediate WT TMD–TMD interactions. The absence of SmXXXSm-mediated dimerization is not surprising. Similar results have been observed with other RTKs. For example, mutations in the SmXXXSm motifs of discoidin domain receptors 1 and 2 and platelet-derived growth factor receptor beta did not change the dimerization propensity (Noordeen et al. 2006; Oates et al. 2010). Furthermore, the solved dimer structures of several TMDs with SmXXXSm motifs have shown that the motif does not participate in the dimer interface (Mineev et al. 2010; Bocharov et al. 2010; Call et al. 2010). Our results further corroborate that while SmXXXSm motifs are frequently found in TMD sequences, they are "neither necessary nor sufficient" for TM helix dimerization (Doura et al. 2004; He et al. 2011; Li et al. 2012).

Although no changes in TMD dimerization were observed with single and double substitutions in the WT FGFR3 TMD, some pathogenic mutations are known to change the dimerization propensity. For instance, the pathogenic A391E mutation, which has been associated with Crouzon syndrome with acanthosis nigricans and bladder cancer (van Rhijin et al. 2002), is known to stabilize the FGFR3 dimer. Studies using synthetic peptides, chimeric proteins and full receptors have indicated that pathogenesis arises due to FGFR3 overactivation as a result of dimer stabilization (Chen et al. 2011b; He and Hristova 2008; Li et al. 2006). Our results from the ToxR assay are in agreement with these previous studies, showing that the

A391E substitution increases the dimerization propensity of the TMD (Fig. 4).

The A391E mutation is thought to increase the dimerization propensity as a result of hydrogen bonds between the two helices. Biophysical studies have shown that the free energy of dimerization of the A391E TMD is lower than that of WT TMD. This change in free energy, of approximately −1.4 kcal/mol, is similar to the energy associated with a hydrogen bond. Furthermore, previous studies have also provided a computer model of the FGFR3 WT TMD dimer structure. In this structure, both Ala391 residues are facing the neighboring helix, but not in direct contact with each other. Thus, this model proposes that the A391E substitution does not induce a change in the crossing and rotational angles of the helices but stabilizes the same WT structure through hydrogen bonds between the side chain of Glu and the backbone atoms of the adjacent helix (Li et al. 2006). However, our results from the ToxR assay show that substitutions in A374, S378 and G382 altered the dimerization propensity of A391E TMD (Fig. 4). Dimerization of the A391E TMD was decreased by nearly 25 % in the presence of either A374I or S378I substitution. This destabilization, however, was not observed when the same mutations were introduced in the WT TMD. Similarly, the dimer stabilization induced by the G382I mutation in the A391E TMD was not observed in the WT TMD. In summary, our results indicate that although the Sm residues do not mediate the contact between WT TMDs, they do appear to be in the interface of the A391E TMD dimer. These results indicate that the dimer interface of the WT TMD dimer is different from that of the A391E TMD, most likely from a change in the crossing angle or the rotational angle, or both. Therefore, based on our assays in bacterial membranes, the pathogenic A391E mutation induces a structural change that results in the formation of a more stable dimer.

The observed destabilization of the A391E TMD dimer induced by substitutions in A374 and S378 also suggests that these two residues directly participate in the contact between the two helices. The increase in dimer stability arising from the G382I substitution in A391E TMD, however, suggests that G382 is farther away from the region of contact between the two helices but still faces each. Substitution of glycine to a much bulkier residue may not disrupt the dimer but, instead, may provide additional packing to stabilize the dimer through an increase in van der Waals interactions.

The structural determination approach described here combines mutagenesis and dimerization propensity. This approach has been successful in gaining structural information on the GpA and BNIP3 dimers (Langosch et al. 1996; Sulistijo et al. 2003). The underlying assumption in these studies is that the same TMD dimer structure exists for the original dimer and for all its respective mutants. A destabilizing or stabilizing substitution in the TMD, thus, is expected to disrupt the monomer–dimer equilibrium but not the dimer structure. Yet, we are also aware that the effects of substituting a residue can also affect both protein–lipid and protein–protein interactions and alter the dimer structure (Li et al. 2012). This is apparent in our results using the A391E FGFR3 TMD. The results presented are in agreement with others showing that the pathogenic mutation increases the propensity of the TMD to dimerize. Our experiments, however, also show that the WT TMD responds differently from the A391E TMD when the Sm residues are substituted with isoleucine. These observations indicate that the WT and A391E TMD dimers are structurally different. This difference may arise from the A391E mutation alone or from the Sm substitutions, or both. Despite these possibilities, our extensive mutagenesis in the WT TMD and the lack of changes arising from these substitutions lead us to postulate that the most likely scenario is a structural change induced by the pathogenic A391E mutation. Overall, our results provide new insights regarding the sequence dependence of FGFR3 TMD dimerization. Furthermore, they present us with a new model, based on experimental data, in which the FGFR3 overactivation induced by the pathogenic A391E mutation involves a more stable dimer that is structurally different from that of the WT TMD.

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