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Analysis of the activating mutations within the activation loop of leukemia targets Flt-3 and c-Kit based on protein homology modeling

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Abstract

Molecular modeling provides a mechanistic hypothesis at the molecular level for the constitutive activation recently observed and reported for tyrosine protein kinases Flt-3 and c-Kit. Three-dimensional homology models for the active and inactive forms of these two kinases were made. Comparison of these models at the molecular level reveals that mutations of specific residues located in the activation loop (D835X and 836-deletion in Flt-3; D816V in c-Kit) as well as a 6-base pair (6-bp) insertion at residue 840 in Flt-3 operate in a similar way. Each mutation tends to weaken the forces that maintain the activation-loop folded inwards. None of the mutations are found to particularly stabilize the active state directly. The reason why the equilibrium is shifted towards the gate-open conformation of the protein is because, at least in these models, the mutations are found to critically destabilize the inactive conformational state of the kinase.

Keywords: Homology modeling; Protein tyrosine kinases; Activation loop; Mutations; Oncology

1. Introduction

Type III receptor tyrosine kinases (RTKs) Flt-3 and c-Kit are two hematopoietic progenitor cell receptors that promote stem cell proliferation, development and survival [1,2]. They also have a role in leukemogenesis, being both highly expressed and frequently mutated in adult and pediatric acute myelogenous leukemia (AML) [3]. Mutations in these proteins result in a constitutive activation of their signaling cascades leading to ligand-independent growth and contributing to malignant transformation.

The most frequent activating mutation identified in Flt-3 corresponds to an in-frame internal tandem duplication (ITD) within the juxtamembrane domain [4]. This mutation has been seen in approximately 25% of AML patients [5]. Additionally, mutations in the activation loop of Flt-3 have been detected in 7% of AML patients, and comprise either the deletion of I836 or point mutation of D835 to a number of residues with varying incidence (Table 1) [6,7]. Similar point mutations in codon 816 of the c-Kit gene, which corresponds to codon 835 of Flt-3, have been found in human mast cell leukemia and AML cases as well [8]. More recently, a 6-base pair (6-bp) insertion in the activation loop of Flt-3 between codons 840 and 841 has been reported in patients with AML [9]. The Flt-3-840GS mutant is shown to be hyperphosphorylated on tyrosine and to confer growth factor-independent growth to Ba/F3 cells. These latest results indicate that in addition to the first described mutations in the catalytic domain and in the juxtamembrane, further activating length mutations exist in the Flt-3 gene [9].

The presence of any of these mutations in AML patients correlates with an increased risk for relapse and is an indicator of poor prognosis in response to currently available therapy [10]. The transforming potential of the affected proteins has been shown to be dependent on their kinase activity, a finding that has led several pharmaceutical companies to investigate the development of small molecule kinase inhibitors for treating Flt-3- and c-Kit-driven leukemias [11]. In the present study, we analyze the role of the activating mutations located in the kinase domain and propose a mechanistic hypothesis at the molecular level to explain the constitutive activation observed for the mutants of these two protein kinases.

Kinases Flt-3 and c-Kit share strong sequence similarity. Both are members of the PDGFR (platelet-derived growth factor receptor) family, which also includes PDGFR-α,

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Table 1
Activating mutations experimentally found in the second tyrosine kinase domain of Flt-3 (and c-Kit)[6–10]

Point mutation[a]	*N* (frequency)	Residue change	Refs.
835 GAT → TAT	34	Asp → Tyr	[6,7]
835 GAT → TAT	18	Asp → His	[6,7]
835 GAT → TAT	8	Asp → Glu	[6,7]
835 GAT → TAT	5	Asp → Val	[6,7]
835 GAT → TAT	4	Asp → Gly	[6,7]
835 GAT → TAT	2	Asp → Asn[b]	[6,7]
Deletion			
836 ATC → Del	13	Ile → Del	[6]
Insertion			
840 TCC → Add	NA	Ser → SerGlySer	[9]

Present modeling study focuses on the role of these three groups of mutations.
[a] Analogous point mutations in c-Kit: D816V, D816Y, D816F [8].
[b] Combined with other mutations [10].

PDGFR-β, c-FMS, and macrophage-colony stimulating factor 1 receptor. PDGFRs are contained within the RTK type III subfamily that is distinguished by five immunoglobin-like repeats in the extracellular domain and a split tyrosine kinase in the intracellular domain. Relatively little is known about the biological consequence(s) of activation of diverse signaling pathways by RTKs in hematopoietic cells [12]. Even less is known about the mechanisms that take place at the molecular level in each of these proteins individually.

Central to the activation of most RTKs is the phosphorylation of one or more tyrosines within the so-called activation loop (A-loop). Typically 20–30 residues in length, the A-loop is centrally located in the catalytic domain. Functionally, activation loops serve as gates across the active site. RTKs have been described as molecular switches that can adopt at least two extreme conformations: an “on” state that is maximally active (also known as “active”, “open” or “gate-open” conformation), and an “off” state that has minimal activity (also referred to as “inactive”, “closed” or “gate-closed” conformation) [13,14]. All RTKs catalyze the same reaction, the transfer of the γ-phosphate of ATP to the hydroxyl group of tyrosine. RTKs are usually kept off. Upon activation, the kinases adopt catalytically active “on” conformations that are structurally very similar. In these on states, the A-loop is outwards or in an open conformation. The inactive states of RTKs are less subject to the chemical constraints that the active states must satisfy, and so different classes of kinases have evolved distinct off states in which the adoption of the catalytically active conformation is impeded in different ways. There is some evidence (from the crystallographic temperature factors of a few RTKs) that the A-loop of the unphosphorylated (inactive) kinase is very mobile [15]; therefore, the A-loop is likely to exist in multiple conformations suggesting an even broader range of regulatory properties for the A-loop than expected from a simple off-versus-on switch for kinase activation [16].

To investigate the role of point mutations located in the A-loop of Flt-3 (comprising residues 829–851) and c-Kit (residues 810–832), it would be helpful to have crystallographic or other structural information available. The structure of unresolved proteins (such as Flt-3 and, until recently, c-Kit [17][1]) can be obtained through homology modeling, a computational method widely used for building protein models based on the known three-dimensional structure of similar proteins [18]. The in-silico approach has proven to be an efficient means of providing structural data for a variety of proteins [19]. In a recent study, computer models for two RTKs (PDGFR and a Flt-3 mutant) were applied to design structure-based approaches for more selective PDGFR-β receptor inhibitors [20].

The accuracy of the protein models constructed by this computational method depends on the degree of amino acid sequence homology between the target and template proteins [21]. In general, homology modeling with a sequence identity of less than 30% is quite challenging, while that with identity greater than 30% is expected to yield a model accurate enough to be used for subsequent modeling work. To analyze protein–ligand interaction and design novel compounds in a structure-based manner, a sequence identity of more than 50% is usually required. Tyrosine kinases that share a sequence identity with Flt-3 and c-Kit in the 30–50% range, and for which crystallographic data exists, include VEGFR-2 [22], FGFR-1 [23], c-Abl kinase [24], and the insulin receptor kinase [25].

In this paper, we explore and discuss possible mechanisms by which the individual Flt-3 and c-Kit mutations in the catalytic domain confer constitutive activation to the receptor tyrosine kinases. The mechanism of kinase activation by ITD is not covered in the present study. The current results, based on protein homology models of the kinase domain of the studied proteins, lead to a better understanding of how the molecular interactions that maintain the equilibrium between different conformational states in the split kinase domain of wt-Flt-3 and wt-c-Kit are disrupted in the mutants, and provide new insight into existing mechanistic assumptions.

2. Molecular modeling

2.1. Targets

The sequences of Flt-3 and c-Kit, with 993 and 976 amino acids, respectively, were retrieved from the Swiss-Protein Database [26]. The accession numbers for Flt-3 and c-Kit are, respectively, P36888 and P10721 (Swiss-Prot [26]) or NP_004110 and NP_000213 (RefSeq [27]).

[1] The structure of c-Kit was solved by Syrrx while our work was in progress. The X-ray coordinates were deposited in the Brookhaven PDB in June 2003 and released in August 2003 (code 1PKG), when the present modeling work was already completed.

2.2. Template

The insulin receptor kinase, IRK, was used as a template for the construction of the three-dimensional homology models of the target proteins. From protein–protein BLAST searches [28], IRK was found to be the most similar protein kinase to Flt-3 and c-Kit (32% sequence identity) for which both forms, active and inactive, have been solved. The public crystal structures for the two forms of IRK were obtained from the Brookhaven Protein Data Bank (reference codes: 1IR3, active, and 1IRK, inactive) [26] and used to generate respectively the gate-open and gate-closed conformations of each target kinase.

2.3. Homology building

The construction of the initial models was carried out using Chemical Computing Group's Molecular Operating Environment software, MOE 2000.02 [29], running on a Silicon Graphics workstation. Intermediate models were generated and then minimized to RMSD of 0.005. For each homology model, the best intermediate was chosen as the final model.

2.4. Refinement of the models

Major adjustments were needed to remove inserts from the target protein kinases, Flt-3 and c-Kit. The target proteins belong to a group of receptors (mainly comprised by members of the PDGFR family) that contain a large insertion of ca. 100 residues in the tyrosine kinase domain. The template protein (and related family members) does not contain such an insert. It has been reported that a majority of the kinase insert domain in VEGFR2 (a class V kinase related to the PDGFR family) is not necessary for kinase activity [30]. The major part of the approximately 70-residue kinase domain inserts of Flt-3 (residues 707–782) and c-Kit (residues 694–762) was removed from the homology models. Scission of the kinase insert domain was done with minimal alteration of the surrounding protein walls. Structural rearrangements as well as subsequent minor manipulations on the side chains and loops of the final models were performed using Merck's in-house molecular modeling package, MIX[2].

2.5. Assessment of the overall quality of the models

For the two models of Flt-3 (active and inactive conformations) and the two models of c-Kit (active and inactive), evaluation of their overall quality was performed using available protein analysis programs [31]. Upon completion of the present modeling work, the crystallographic coordinates of the active conformation of c-Kit were made publicly available [17].[1] Our 3D-homology model for the active conformational state of c-Kit was then compared to the experimentally released 2.9 Å-resolution structure. The homology model was found to be in very good agreement with the structure provided by X-ray crystallography (RMS < 3.0 Å), especially around the region of interest, i.e. conserved nucleotide binding pocket and A-loop in the C-lobe, as well as Gly-rich loop in the N-lobe (RMS < 2.5 Å). Other parts of the C-lobe in our model (not directly involved with the key kinase regions studied herein) showed slightly larger deviations from the X-ray structure, in part due to the lack of the kinase insertion domain.

2.6. Molecular Dynamics (MD) simulations

The homology model of Flt-3 in the inactive conformation was used as the starting structure for MD simulation of the wild-type Flt-3 and for construction of the starting structures for the mutants. All D835X mutants were generated replacing the original Asp side chain with the corresponding side chain in a similar orientation. All simulations were performed using the QUANTA2000 molecular modeling package (http://www.accelrys.com). Each mutant was first energy-minimized using 100 cycles of steepest descent and 1000 cycles of adopted-basis Newton Raphson methods. The systems were heated to 310 K (physiological temperature) with a 5 K rise every 50 steps per 3100 steps. After heating, the systems were allowed to equilibrate for 50 ps. This was followed by a 1.0 ns MD simulation for each mutant (as well as the wild-type protein).

3. Results and discussion

All results presented herein are based on the four 3D homology models shown in Fig. 1. These models depict the catalytic core of approximately 300 amino acids characteristic of all protein kinases. The catalytic core comprises a bilobal scaffold that has an N-terminal small lobe (above) composed almost entirely of β-sheet, and a C-terminal large lobe (below) in which α-helices dominate. Within this core, the catalytic site is at the interface of the two lobes. All mutations discussed below are located in the A-loop, in the upper region of the C-lobe of the split kinase domain (Fig. 1). Activation by ITD goes beyond the scope of the present investigation.

3.1. Point mutations

The A-loop represents a hot-spot region for activating mutations in class III RTKs. Point mutations involving an Asp residue have been described for Flt-3 (D835) [6,7] and c-Kit (D816) [8]. Such an Asp codon is highly conserved in RTKs. Since substitutions to Y or V in murine c-FMS [32], murine c-Kit [33], and murine Flt-3 [34] also result in constitutive activation of the receptors, the Asp within the

[2] Code developed by the Molecular Systems group at Merck Research Laboratories.

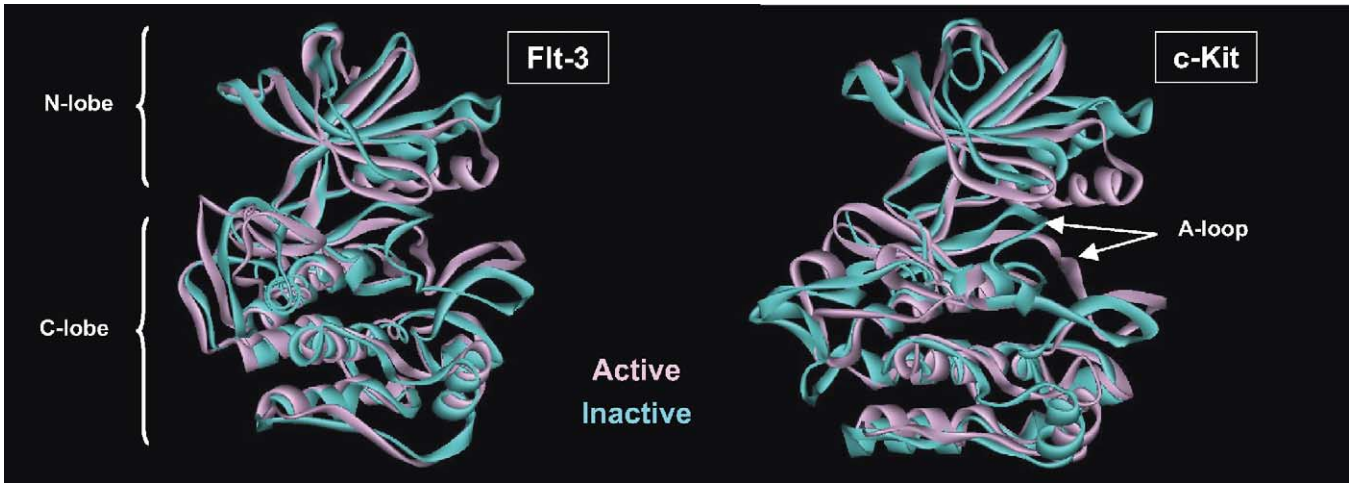

Fig. 1. 3D-homology models for the active and inactive conformational states (pink and light blue, respectively) of proteins Flt-3 (left) and c-Kit (right).

A-loop has been suggested to have some kind of regulatory role in RTKs. The absence of structural data for the catalytic domain of these proteins, however, makes this assumption difficult to prove. To the best of our knowledge, no detailed structure-function analyses for the role of this conserved Asp in Flt-3 have been published yet.

Inspection of the Flt-3 homology models in Fig. 1 reveals an important structural feature that confers upon Asp835 a key role. The side chain of D835 in Flt-3 is found to make H-bond interactions with the NH backbone of the two preceding amino acids, R834 and A833 (Fig. 2), adopting a conformation similar to the well established Asx turn [35].

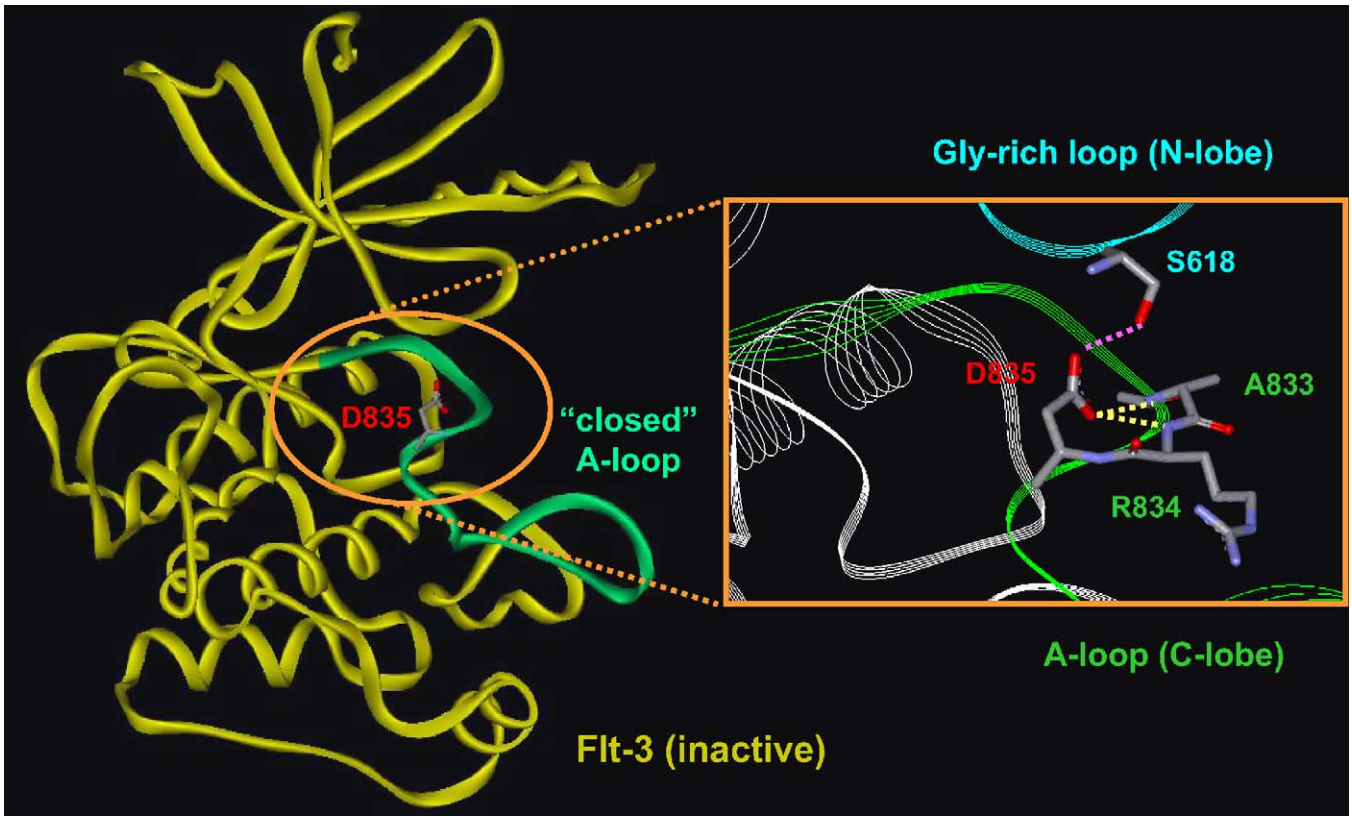

Fig. 2. Ribbon diagram of the inactive conformational state of Flt-3 (left). Activation loop highlighted in green. Zoomed area (right) shows H-bond interactions (dotted lines) identified in the vicinity of residue D835 based on the homology model. Only key neighboring residues in 3D are highlighted: S618; from the Gly-rich loop (blue ribbon) and A833, R834; from the A-loop (green ribbon). Carbon atoms are colored gray; oxygen atoms red; and nitrogen atoms blue.

Such polar interactions help maintain the loop in that particular folded conformation, anchoring the wt-Flt-3 protein in its inactive conformational state.

As seen from Fig. 2, there is also room for D835 to make an additional H-bond interaction with the OH group of S618, a polar residue in the N-lobe. S618 is located in the highly conserved Gly-rich loop of the kinase. Residues in this loop have been reported to be exquisitely sensitive to what occupies the active site cleft, and have been assigned a variety of roles [36]. The mobile tip of this conserved loop (the so-called phosphate binding motif) is known to make contact with all three parts of ATP whenever nucleotide is present [37]. The wt-Flt-3 protein could potentially use the interaction between S618 and D835 to further fasten the protein in its inactive form. By "gluing" the C-lobe (D835) to the N-lobe (S618), the gate-closed form of the protein would be further stabilized. Although examples of non-covalent interactions between the N-lobe and C-lobe are scarce in the literature, a recent instance can be found in PDK1, where multiple interactions between Ser241 (in the A-loop) and residues from helix-C in the N-lobe (as well as other residues in the C-lobe) were observed [38].

In summary, three different and dynamically interchangeable binding modes are predicted for Asp835 in wt-Flt-3 to regulate the accessibility of the binding site: (i) binding of D835 to both S618 and R834/A833, which ensures full preservation of the inactive form of the receptor, (ii) binding to only one of these two anchoring points (either S618 or R834/A833), which still preserves the gate-closed form of the protein; or (iii) binding to none. Upon mutation of Asp835, binding modes (i) and (ii) are no longer accessible, resulting in a mutant kinase less able to preserve the gate-closed conformation. We propose that this destabilization of the inactive state is the basis for the constitutive activation of the protein upon substitution of D835. With only mode (iii) in place, the equilibrium in the mutant is systematically shifted towards a more gate-open conformation, causing hyperactivation of the protein.

The above proposed modeling-based hypothesis is consistent with all experimental point mutations in Table 1. To further challenge the present assumption, the key D residue was subsequently substituted in our models by each of the 19 possible amino acid replacements. For each of these point mutations at position D835, the side chain of the substituting residue was automatically generated from a database of building blocks and its geometry manually adjusted. Repositioning of the new side chains was done locally, with null to very little adjustment of the protein backbone being required. Only one residue type out of 19 was identified as capable of making the same interactions claimed here for D835, and that was Asn. Given the similarity to an Asp (both in size and in polar nature), an Asn would still be able to make two H-bond interactions with the NH backbone of R834 and A833. In light of this, and assuming that our modeling-based hypothesis for the role of D835 is valid, then a D to N mutation would be expected to make the protein quite insensitive to constitutive activation (or at least less sensitive to constitutive activation than other mutations). This is indeed in good agreement with reported experimental facts [6,10]. Thus, the D to N mutation is precisely the least frequently occurring substitution (Table 1) [6]. Most importantly, the D to N mutation is the only substitution that needs to be combined with additional mutations in order to be effective [10], implying that such a D to N mutation alone may not be sufficient to cause constitutive activation of the protein.

In a recent study [32], Asp802 of the c-FMS receptor (the residue analogous to Asp816 in c-Kit and Asp835 in Flt-3) was replaced with the other 19 amino acids and all mutants were analyzed in haemopoietic cells as well as fibroblasts. The D802N mutation was found to be the only substitution that rendered the receptor neutral (as with Asp, the normal amino acid at residue 802 in wt-M-CSF). All other mutants were found to behave differently than the wild type [32].

To further examine the role played by D835 as well as point mutations at this position in Flt-3, we performed molecular dynamics simulations on a set of mutant structures and compared them to the wild-type structure. We made an initial assumption that each mutant is still able to adopt a gate-closed conformation and has the overall fold of the A-loop as in the inactive conformation of the wild-type protein. To characterize the structural perturbation caused by replacing D with different residues, we analyzed the backbone torsion angles for residues 829–851 (A-loop) and 615–624 (Gly-rich loop) for both the wild-type protein and mutants during MD simulations. We also monitored the RMSD of the main chain backbone atoms of these two segments along the simulations. Our MD simulations revealed that most mutations cause a rather large conformational alteration of the A-loop and, in some cases, to the vicinal Gly-rich loop region as well. The fluctuations of the torsion phi and psi angles in the mutants are much larger then those in the wild-type (up to 6–7 times larger for X = Tyr, Phe). Also, the backbone RMSD values for the mutants and the wild-type compared with the starting structures are 5–7 Å and 1–2 Å, respectively. These changes suggest that the loop becomes more flexible upon mutation. Not only is the ability to form optimal hydrogen bonds with key neighboring residues greatly affected upon mutation, but the new residues not always match the size and polarity requirements, ultimately causing a destabilization of the folded state of the protein.

Is the above hypothesis formulated for Flt-3 also applicable to c-Kit? The second half of the split tyrosine kinase domain of c-Kit is highly homologous to the same domain in Flt-3 (53% sequence identity). In particular, the 11-residue fragment KICDFGLARDI around the "DFG" motif of the A-loop is identical in both protein sequences. Our models reveal that the side chain of D816 in c-Kit is making the same interaction as seen in Flt-3, i.e. H-bonds between the carboxylic acid group of this Asp residue and the NH backbone

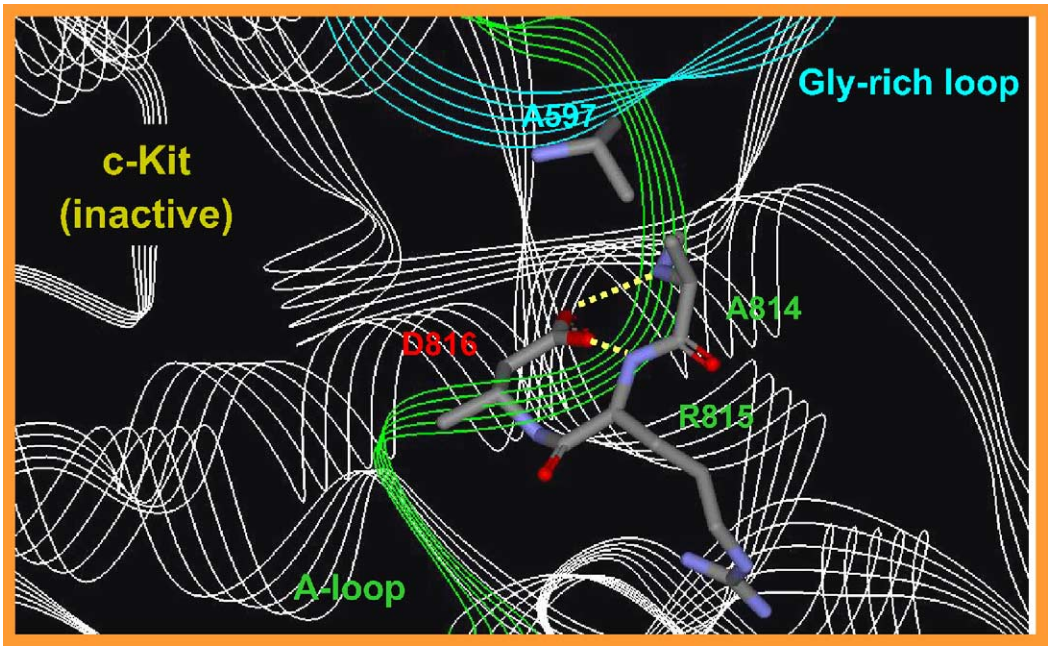

Fig. 3. Ribbon diagram of the inactive conformational state of c-Kit around the cleft region between the two kinase lobes. Dotted lines indicate H-bond interactions identified in the vicinity of residue D816 based on the homology model. Only key neighboring residues are highlighted; A597 from the Gly-rich loop (blue ribbon), and A814 and R815, from the A-loop (green ribbon). Atoms are colored as in Fig. 2.

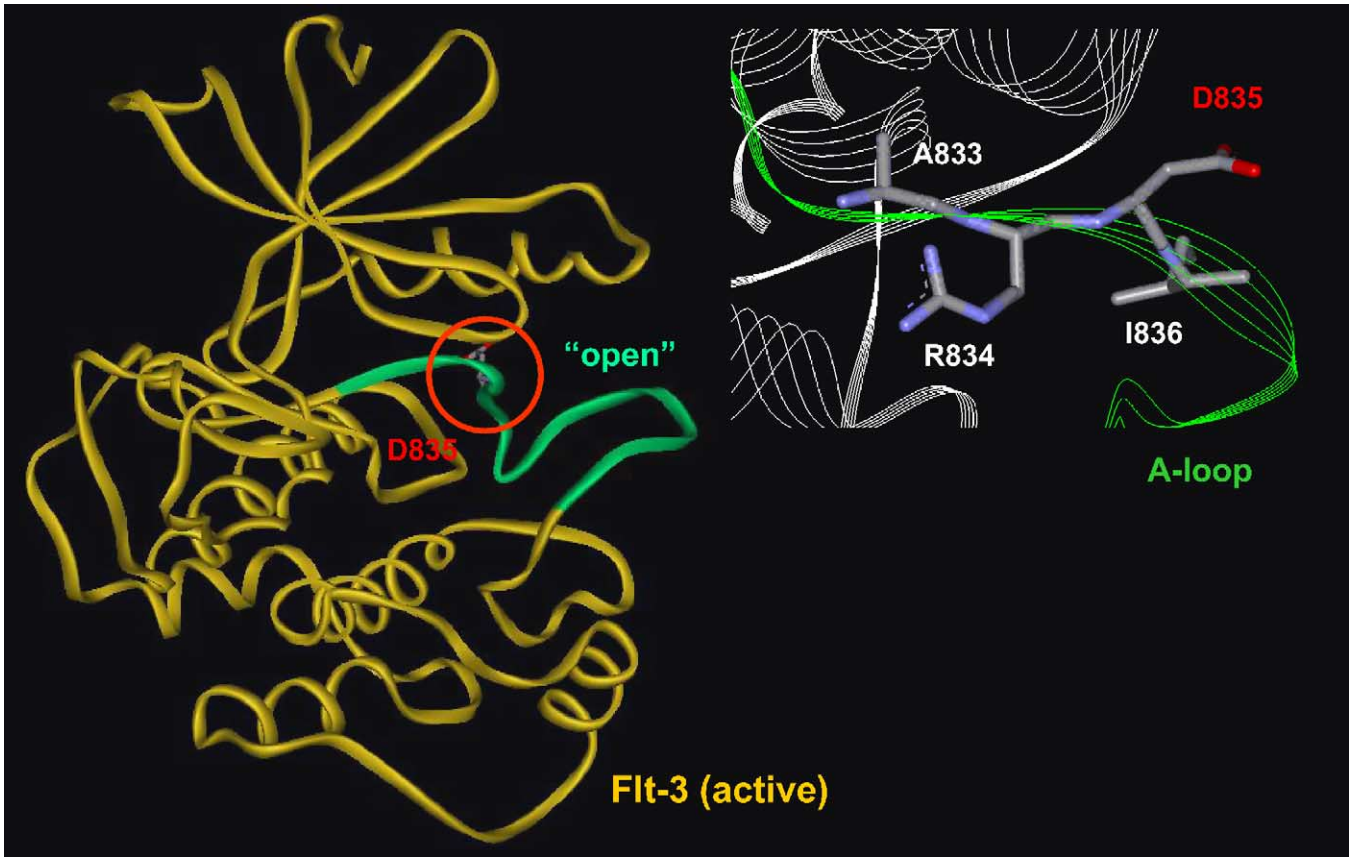

Fig. 4. Ribbon diagram of the active conformational state of Flt-3. View of the region around D835 based on homology models. In the gate-open conformation of the A-loop, D835 points away from the cleft. Only neighboring residues are highlighted. Atoms are colored as in Fig. 2.

of the two preceding amino acids, R815 and A814 in c-Kit (Fig. 3). Unlike in Flt-3, however, in c-Kit there is no room for additional interactions with the Gly-rich loop. The residue in Flt-3 responsible for providing the polar group, S618, corresponds to an Ala in the Gly-rich loop of c-Kit, A597. Ala lacks both the right size and the polar nature required to interact with D816 in the activation loop. As seen from Fig. 3, the only driving force in c-Kit for stabilization of the inactive form is the two H-bond interactions found within the A-loop.

The results presented so far would indicate that mutation of the key Asp residue, D835/Flt-3 or D816/c-Kit, is instrumental in destabilizing the inactive state of each of these two proteins. Homology models for the active conformational state of the target proteins were also inspected. The relevance of the key Asp residue here is different than in the inactive conformational state of the protein. As can be seen from Fig. 4, D835 in Flt-3 does not interact with other protein residues nearby. Instead, it points towards solvent. No stabilizing or destabilizing role is therefore identified for D835 in the gate-open conformation of Flt-3. Similar conclusions can be drawn for c-Kit (data not shown). Mutation of the Asp residue is expected to have negligible impact on the stability (or instability) of the active form of these two proteins.

Overall, we propose that mutations of the above discussed key aspartic residue in Flt-3, D835, as well as in c-Kit, D816, (Table 1) decrease the ties that maintain the A-loop folded inwards. Upon mutation, the interactions made by the key aspartic residue are no longer in place. Thus, the D835Y mutation in Flt-3 (and all other D835X listed in Table 1) makes the kinase unable to form the H-bonds that help lock the A-loop in a gate-closed conformation. When D is mutated to Y or to any of the other residues listed in Table 1, the forces that maintain the two vicinal residues preceding D (R and A) bound together would no longer exist. The A-loop therefore becomes less constrained and is more likely to fold outwards. As a result, the equilibrium is expected to be displaced towards the active form of the protein. That open conformation of the active site is precisely the one known to be capable of accommodating ATP and enabling transphosphorylation of the A-loop [13].

Adoption of the inactive conformation of the A-loop in Abl kinase has been suggested to be indispensable for small-molecule inhibitor binding [25]. To prove that hypothesis, a mutation destabilizing the inactive conformation

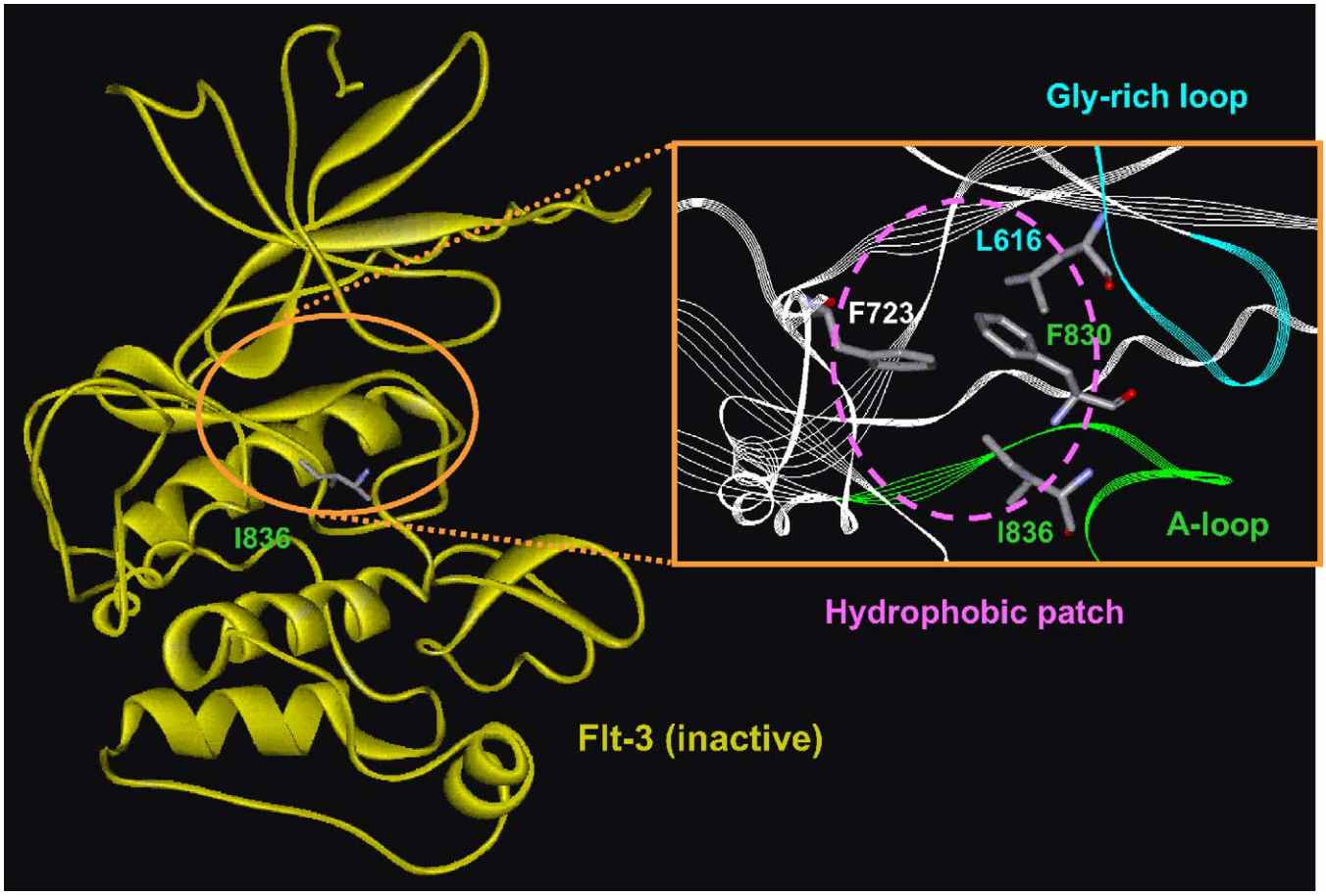

Fig. 5. Hydrophobic patch identified in the binding pocket of Flt-3 in its inactive conformational state. Residues L616, F723, F830 and I836 are the major contributors to the make-up of this non-polar region. Atoms are colored as in Fig. 2.

of Abl (D276S/E279S) was introduced [39]. Such a double mutation disrupted key intramolecular interactions of the A-loop of inhibited Abl with other regions of the enzyme, resulting in the destabilization of the A-loop and, ultimately, the hyperactivation of Abl. The experiment confirmed therefore that the decrease in sensitivity to small-molecule inhibitors experienced by Abl was due to destabilization of the inactive conformation of that kinase [39]. The gate-closed conformation of the A-loop in kinases may play, in general, a more important role than what has been captured in the literature so far. Additional kinase studies are needed to further evaluate this assumption. Experiments on Flt-3 are currently in progress to check the validity of the mechanistic assumption presented herein.

3.2. Deletion

A similar destabilization of the inactive form of the protein is predicted to occur upon deletion of I836 in Flt-3. Inspection of the wt-Flt-3 model around I836 reveals the existence of a hydrophobic patch that encompasses the side chains of several non-polar residues (Fig. 5), including residues from the ATP binding site (F723), from the Gly-rich loop (L616) and from the A-loop (F830 and I836). Although these residues are not close to each other in the protein sequence, the natural folding of the kinase (tertiary structure) places them in the same spatial region. By clustering these non-polar residues together in the center of the protein, unfavorable exposure of their side chains to polar regions and to solvent-exposed areas in the periphery is avoided—a favorable scenario for the protein. The stability of the gate-closed conformation of the kinase is linked to the existence of this cluster of residues buried within the nucleotide binding pocket, and in particular, to the presence of I836 as part of it. Thus, in the absence of nucleotide, residue I836 located in the A-loop is proposed to make key hydrophobic contacts with the other non-polar residues highlighted in Fig. 5. Such interactions would help maintain the A-loop "glued" to the core of the protein (folded state), making I836 a crucial player in the cluster of hydrophobic residues. Upon deletion of I836, the forces that maintain the A-loop folded inwards would be significantly reduced. The residues in the immediate vicinity of I836, R–D–M–S (N − 2, N − 1, N + 1 and N + 2, respectively), which in the mutant are expected to fill in the gap left by I836, are globally more polar in nature than I836. They would be unable to fully regenerate the hydrophobic environment necessary to anchor the A-loop in its closed form. As a result, the loop is expected to shift towards the open form (reactivation of the protein).

No deletion of the analogous residue in c-Kit, I817, has been reported so far in the literature. Based on the present in-silico work, we predict that such a deletion would have very little effect (if any at all) in c-Kit. Our model for c-Kit shows that the analogous hydrophobic patch in this protein (Fig. 6) is significantly smaller than in Flt-3 (Fig. 5). The hydrophobic residue that "faces" I836 in Flt-3 (F723) happens to point outwards in c-Kit (Y675). This difference in spatial disposition is due to a different rearrangement of the

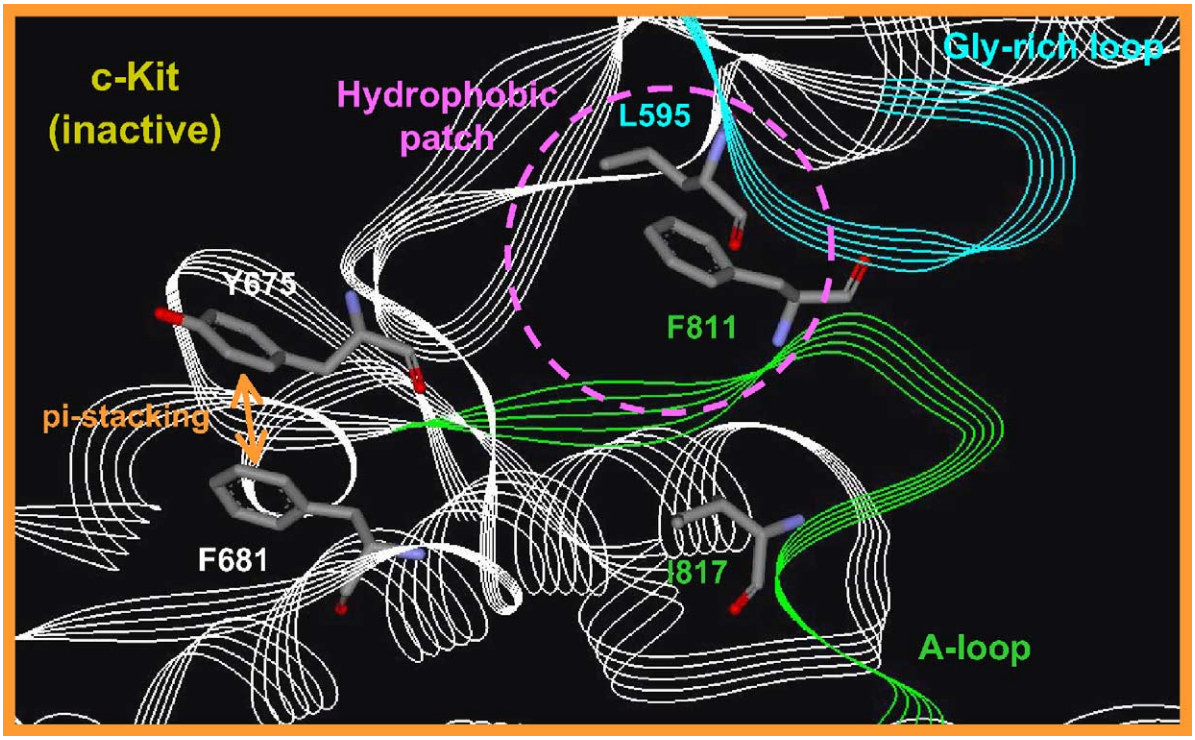

Fig. 6. Small hydrophobic patch identified in the binding pocket of c-Kit in its inactive conformational state. Only residues L595 and F811 are found to contribute to the make-up of this non-polar region. Atoms are colored as in Fig. 2.

protein loop immediately after the nucleotide binding site in c-Kit as compared to Flt-3, based on our homology models. The particular folding of the loop in this region of c-Kit makes Y675 point away from the nucleotide binding site. In addition, Y675 is further stabilized through a π-stacking interaction with the phenyl ring of F681, also pointing away from the nucleotide binding site (Fig. 6). As a result, Y675 is unable to take part of the inner hydrophobic cluster of non-polar residues (a cluster made only by L595 and F811 in c-Kit). Overall, the net effect is a lack of hydrophobic residues in c-Kit capable of pairing with I817, which renders this A-loop residue unable to play the same role as I836 in Flt-3. (Note: Inspection of the now available crystal structure of c-Kit [17] reveals that Y675 does point away from the nucleotide binding site, and that a π-stacking interaction with the phenyl ring of F681 is indeed possible. This reinforces the validity of the assumptions made with our model for c-Kit and described herein).

3.3. Insertion

The results recently reported by Spiekermann et al.[9] on a Flt-3/840GS mutant clearly indicate that such a mutant is hyperphosphorylated on tyrosine residues, inducing interleukin-3-independent growth in Ba/F3 cells. The reported in vitro data underlines the pathophysiologic role of this 6-bp mutant for the leukemic phenotype in patients with AML. To the best of our knowledge, however, the exact mechanism of kinase activation by this mutation has not been yet investigated, and remains unknown.

Our homology models for the catalytic domain of Flt-3 provide a possible explanation for the observed activation. In the inactive conformation of the wt-protein, the A-loop residue where the 6-bp insertion has been reported to take place, N841 (right after position 840), is herein proposed to make a H-bond interaction with an Arg in the catalytic loop, R815 (Fig. 7). Such an interaction would help "glue" the A-loop to the inner part (core) of the protein. Upon insertion of Gly-Ser between S840 and N841, the protein backbone is expected to undergo local adjustments that would inevitably alter the current location of both N841 and S840 in the wt, placing the former residue away from R815. Therefore, the forces that maintain the A-loop in a gate-closed conformation are expected to become weaker in the mutant. The inserted Gly-Ser pair would lack the ability to hydrogen-bond R815. When residues in the immediate vicinity of the point of insertion (amino acids within an 8.0-Å radius sphere) were considered, none was found to be a suitable candidate

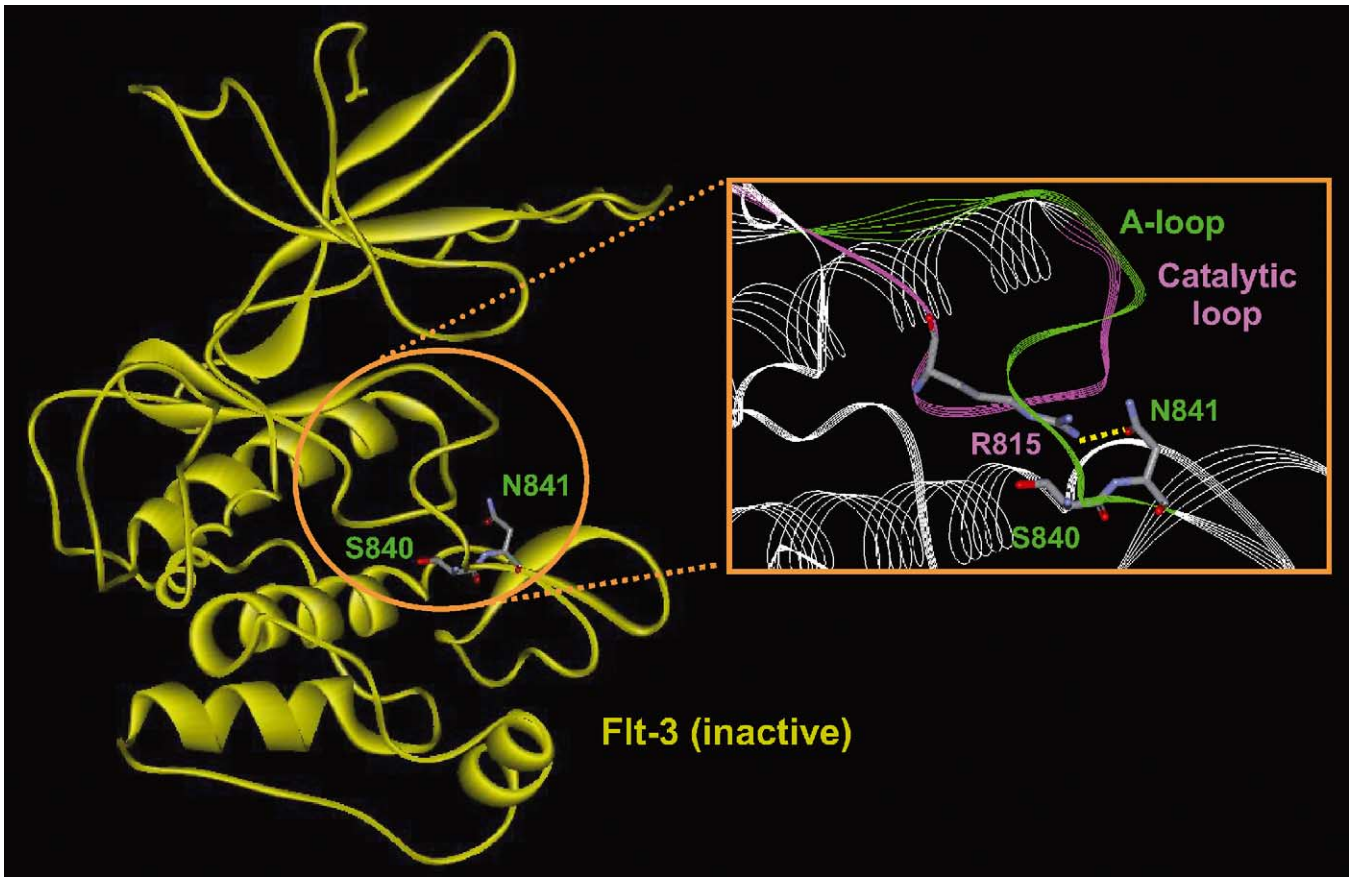

Fig. 7. Ribbon diagram of the inactive conformational state of Flt-3. Zoomed area: A dotted line indicates the hydrogen-bond interaction between the side-chain of N841 (amino acid immediately following S840; where, the 6-bp insertion takes place in the A-loop), and the side-chain of residue R815 (located in the catalytic loop). Atoms are colored as in Fig. 2.

to replace the original role of N841 in the wt. This new scenario would render the mutant more unstable in its inactive conformation, and result again in an activation of the protein.

In his original work, Spiekermann et al.[9] suggested that Flt-3/840GS is probably a rare mutation. Interestingly, our homology models for the two studied proteins reveal that such an insertion would have a similar effect on the kinase activation of c-Kit. Residue N822 of c-Kit, which is the amino acid analogous to N841 of Flt-3, is also involved in a H-bond interaction with an Arg located in the catalytic loop, R796. Like in the Flt-3 case, such an interaction is predicted to have a role in "gluing" the A-loop to the inner part of the catalytic domain of c-Kit. Upon insertion of a non-polar residue (or polar, but lacking the right size) at position 822 of c-Kit, the wt-N822 would no longer be able to help maintain the A-loop in a gate-closed conformation, causing activation of c-Kit.

3.4. Impact of activating mutations on current small-molecule inhibitors

A final question not addressed in any of the studies reporting these activating mutations is, whether such mutations have the potential to affect anticancer agents currently being used or developed for the treatment of hematopoietic diseases. Since 1994, the main thrust in the development of protein tyrosine kinase inhibitors has been toward the generation of ATP-mimics [40]. Currently, all Flt-3 and c-Kit inhibitors in clinical development or heading toward the clinic are competitive inhibitors of ATP [11]. Careful inspection of the homology models presented herein reveals that, neither D835 nor I836 are likely to occupy the same spatial region used by known small molecule inhibitors that target the ATP binding site of Flt-3, c-Kit or similar kinases, neither fully nor partially. Moreover, none of the mutated residues seem to be even making contacts with existing small-molecule inhibitors (docking models not shown). Therefore, the studied mutations are predicted not to exclude existing inhibitors that are ATP-competitive.

4. Conclusions

Protein homology models for the second half of the split kinase domain of Flt-3 and c-Kit were generated to study a number of mutations that have been recently associated with unfavorable clinical responses in AML patients. Most of the experimental studies published so far have focused on the clinical relevance (i.e. prevalence of these activating mutations and the prognostic impact they might have in patients) [6–10]. In contrast, very little has been devoted to the study of the molecular mechanism that might be responsible for the activation of Flt-3 and c-Kit. The present work offers a detailed structure–function analysis at the molecular level for the mutated residues. Previously, insights into the role of activating mutations in Flt-3 (and c-Kit) were obtained only indirectly, in most cases by analogy (or similarity) with other non-receptor and receptor tyrosine kinases [41]. Substitution mutations of critical residues in the A-loop have been proposed to allow the loop to fold out of the active site in the kinase domain, allowing access to ATP and substrates, as suggested for example in a recent study on IGF-1R [42]. In that study, intermolecular autophosphorylation of three specified residues in the A-loop of IGF-1R was found to stabilize the loop in a conformation that facilitates catalysis [42]. Similarly, the reported mutations in Flt-3 have been suggested to cause constitutive activation by triggering the A-loop into an active conformation [9]. The present study reveals that constitutive activation of Flt-3 is not the result of direct stabilization of the active conformation of the protein. Instead, the reported mutations in Flt-3 and c-Kit are found here to weaken the critical forces that maintain the A-loop folded inwards, the main conclusion of the present investigation. Analysis of our homology models reveals that interactions are weakened either by disabling the particular fold of the Asp-Arg-Ala in the A-loop reminiscent of an Asx turn (D835X/D816X point mutations), by reducing the cluster of hydrophobic residues (I836 deletion) or by promoting detachment of the A-loop from the inner core of the kinase (840GS insertion). None of the observed mutations are found to stabilize the active state directly. The reason why the equilibrium is shifted towards the gate-open conformation of the protein is because the mutations critically destabilize the inactive conformational state of the kinase.

A second contribution from the present study is the modeling-based prediction that none of the reported mutations in the A-loop will have the ability to interfere with current small-molecule kinase inhibitors (mainly, by competing for the same spatial region). The investigated activating mutations are different from the resistance mutations observed for example in BCR-ABL [45], where the altered amino acids are residues that do directly contact the kinase inhibitors. This notwithstanding, one may expect resistance to develop to Flt-3 inhibitors and drugs as well, as has been observed with Gleevec therapy of chronic myelogenous leukemia blast crisis [45]. In some cases, resistance may be due to enhanced degradation or cellular export of drug, but it is also clear that other point mutations near the ATP binding site can also affect resistance. In anticipation of this problem, we are currently investigating additional point mutations in the catalytic domain of Flt-3 that would have the potential to interfere with small molecule inhibitors (to be submitted). Such modeling-based predictions may be useful for the future development of novel small molecule drugs directed at the treatment of leukemia.

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