Drug binding and resistance mechanism of KIT tyrosine kinase revealed by hydrogen/deuterium exchange FTICR mass spectrometry

Hui-Min Zhang,^{1,2} Xiu Yu,³ Michael J. Greig,³ Ketan S. Gajiwala,³ Joe C. Wu,⁴ Wade Diehl,³ Elizabeth A. Lunney,³ Mark R. Emmett,^{1,5*} and Alan G. Marshall^{1,5*}

¹Ion Cyclotron Resonance Program, National High Magnetic Field Laboratory, Florida State University, Tallahassee, Florida 32310-4005

²Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306

³Global Research and Development, Pfizer Inc. La Jolla Laboratories, San Diego, California 92121

⁴Department of Enzymology and Biochemistry, Pfizer Research Technology Center, Cambridge, Massachusetts 02139

⁵Department of Chemistry and Biochemistry, Florida State University, Tallahassee, Florida 32306

Received 22 August 2009; Revised 10 January 2010; Accepted 13 January 2010

DOI: 10.1002/pro.347

Published online 21 January 2010 proteinscience.org

Abstract: Mutations of the receptor tyrosine kinase KIT are linked to certain cancers such as gastrointestinal stromal tumors (GISTs). Biophysical, biochemical, and structural studies have provided insight into the molecular basis of resistance to the KIT inhibitors, imatinib and sunitinib. Here, solution-phase hydrogen/deuterium exchange (HDX) and direct binding mass spectrometry experiments provide a link between static structure models and the dynamic equilibrium of the multiple states of KIT, supporting that sunitinib targets the autoinhibited conformation of WT-KIT. The D816H mutation shifts the KIT conformational equilibrium toward the activated state. The V560D mutant exhibits two low energy conformations: one is more flexible and resembles the D816H mutant shifted toward the activated conformation, and the other is less flexible and resembles the wild-type KIT in the autoinhibited conformation. This result correlates with the V560D mutant exhibiting a sensitivity to sunitinib that is less than for WT KIT but greater than for KIT D816H. These findings support the elucidation of the resistance mechanism for the KIT mutants.

Keywords: hydrogen/deuterium exchange; tyrosine kinase; conformation; mass spectrometry; drug resistance

Abbreviations: ESI, electrospray ionization; FT ICR, Fourier transform ion cyclotron resonance; GISTs, gastrointestinal stromal tumors; HDX, hydrogen/deuterium exchange; JM, juxtamembrane; KID, kinase insertion domain; LC, liquid chromatography; LTQ, linear ion trap; MS, mass spectrometry; PTR, phosphotyrosine; WT, wild type.

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: NIH; Grant number: R01 GM78359; Grant sponsor: NSF Division of Materials Research; Grant number: DMR-0654118; State of Florida.

*Correspondence to: Alan G. Marshall, Ion Cyclotron Resonance Program, National High Magnetic Field Laboratory, Florida State University, 1800 East Paul Dirac Drive, Tallahassee, FL 32310-4005. E-mail: marshall@magnet.fsu.edu or Mark R. Emmett, Ion Cyclotron Resonance Program, National High Magnetic Field Laboratory, Florida State University, 1800 East Paul Dirac Drive, Tallahassee, FL 32310-4005. E-mail: emmett@magnet.fsu.edu

Introduction

KIT is a stem cell factor receptor and is a member of the Type III transmembrane receptor protein tyrosine kinase (RPTK) subfamily.¹ The Type III RPTK family has several domains, including five extracellular immunoglobulin (Ig) domains, a single transmembrane region, an autoinhibitory juxtamembrane domain, and a cytoplasmic kinase domain including a kinase insertion domain (KID).^{2,3} The activity of RPTK is tightly regulated.⁴ KIT is normally in autoinhibited or unactivated state until a ligand binds and induces the receptor oligomerization, resulting in tyrosine autophosphorylation by ATP. However, activating mutations of KIT activate it in the absence of ligand binding, leading to abnormal cell growth and proliferation and thus human cancers, for example, gastrointestinal stromal tumors (GISTs).^{5,6} Most of the primary activating mutations in GIST occur in the juxtamembrane domain (JM domain), for example, V560D [Fig. 1(b)]. The efficacy of some small-molecule tyrosine kinase inhibitors (e.g., sunitinib and imatinib) in GIST patients is reported⁷ to be based on the ability of the drug to inhibit the KIT kinase activity. Imatinib mesylate is currently the first-line treatment for GIST, but many patients show resistance to the drug, either initially or within 2 years of treatment.^{8,9} Sunitinib malate has been used as a second-line treatment for GIST patients who are resistant to imatinib. However, some KIT mutants show secondary resistance to sunitinib, for example, D816H/V in the activation loop (A-loop) of the catalytic domain [Fig. 1(b)].

KIT protein is in equilibrium among various conformations.^{6,10} Without phosphorylation, KIT is in an autoinhibited conformation, in which the autoinhibitory JM domain inserts into the cleft between the N- and C-terminal lobes of KIT and interacts with the C-alpha helix, which also regulates the catalytic activity of the kinase.¹¹ The Asp-Phe-Gly (DFG) motif in the beginning of the activation loop (A-loop) [Fig. 1(b)] is in a "DFG out" conformation, with the Phe811 residue protruding out and preventing ATP binding. Upon phosphorylation of Tyr residues, including Tyr568 and Tyr570 [Fig. 1(b), right], KIT protein equilibrium shifts to the activated conformation, in which the JM domain is released to the solvent. The DFG motif moves to a "DFG in" conformation, with the Phe811 buried, permitting ATP to enter the binding pocket. The unactivated conformation can be any conformation that is intermediate between the autoinhibited and activated conformations. Imatinib binds to an unactivated conformation. We have recently shown that sunitinib binds to the autoinhibited conformation¹²; we have also found that the D816H mutant autoactivates much faster than wild-type (WT) KIT, and its JM domain conformation resembles activated KIT.¹²

Drug resistance is a serious problem for cancer treatment failure. Overexpression or mutation of the drug target is a major contributor to acquired resistance.¹³ Understanding of the mechanism of drug sensitivity change for the clinical mutants is critical to development of new therapies to overcome the resistance. The JM domain mutant V560D is sensitive to both imatinib and sunitinib, but the sensitivity to sunitinib is less than that for the WT KIT.¹² The A-loop mutant D816H is resistant to both imatinib and sunitinib. X-ray crystallography has been used to elucidate the structure of the mutant and WT KITs. However, crystallization of mutant V560D has not been successful. Moreover, the KID [see Fig. 1(a)] is very flexible and had to be deleted in order to obtain crystals for X-ray analysis.¹⁰ Conversely, biochemical measurements were carried out with protein, in which the KID region remained intact, thus presenting the need to determine any structural impact of removing the KID. Solution-phase hydrogen/deuterium exchange monitored by mass spectrometry (HDX MS) has been used to examine the structural basis of KIT activation and drug resistance mechanisms of the mutant KIT proteins. HDX MS also clarified whether or not the KID domain influences the overall structure of KIT and provided a direct relationship between enzymatic assays and crystal structures.¹²

We recently characterized protease type XIII as superior to pepsin, with more overlapping fragments due to its cleavage preference toward the C-terminus of basic amino acids.¹⁴ Therefore, all of the HDX experiments for KIT were performed with protease type XIII digestion. We also successfully reduced back exchange (by ~25%) by suitable choice of HPLC stationary phase and fast gradient conditions.¹⁵ Additionally, solution-phase direct binding analysis by mass spectrometry was performed for activated and unactivated forms of KIT to assess binding of sunitinib and ATP to each state. The solution-phase HDX results, along with the crystal structures, enzymatic assay data, biophysical data, and clinical data provide a fairly comprehensive understanding of the equilibrium states of the unactivated and activated KIT and the effects of mutations on that equilibrium.

Results and Discussion

High sequence coverage of KIT with protease Type XIII digestion

We used protease Type XIII rather than pepsin to digest all of the KIT constructs due to its higher proteolytic efficiency.¹⁴ High sequence coverage (for the proteolytic peptides detected after enzymatic digestion and LC/MS) was achieved for all of the KIT forms. Table I shows the sequence coverage for all of the HDX experiments: WT KIT (with and without

Figure 1. WT KIT primary sequence and KIT conformations. (a) The flexible 60 a.a. KID (in green) must be removed to yield crystals suitable for X-ray diffraction analysis. Here, the V560D mutant contains the KID, whereas the D816H mutant does not. Overall sequence coverage for HDX analysis for each KIT was more than 90%, and included the significant regions, that is, the JM domain (red), the C-alpha helix (cyan) and the A-loop (orange). The fragments denoted by double-headed arrows appear in subsequent figures. (b) Autoinhibited (left) and activated (right) conformations of KIT. The three domains are colored the same as in (a). Two mutation sites V560 and D816, ATP binding motif Asp810-Phe811-Gly812 (DFG), and phosphorylation sites Tyr568 and Tyr570 in JM domain are highlighted in sticks and colored in magenta. In the autoinhibited conformation (left, PDB ID: 1T45⁶), the JM domain is in contact with the C-alpha helix and the A-loop site. The DFG motif is in “DFG out” conformation, in which Phe811 protrudes and prevents ATP binding. In the activated conformation (right, PDB ID: 1PKG¹⁰), Tyr568 and Tyr570 are phosphorylated to PTR568 (phosphotyrosine568) and PTR570 (phosphotyrosine570). The JM domain is released to the solvent and the DFG motif is in “DFG in” position, in which Phe811 is buried away from the ATP binding pocket so that ATP can bind.

Table I. Sequence Coverage from Digestion with Protease Type XIII for all of the KIT Proteins Studied, As Well As the Proteins Bound with Imatinib or Sunitinib

Drug	KIT + KID (%)	KIT – KID (%)	D816H – KID (%)	V560D (%)
None	93	90	91	90
Sunitinib	91	89	92	91
Imatinib	83	87	N/A	N/A

the KID), and two KIT mutants, all in both the apo state and bound with inhibitor. Proteolysis of the KIT proteins, with and without sunitinib bound yielded ~90% sequence coverage. Sequence coverage for imatinib-bound KIT proteins was lower (~85%). HDX comparisons for different KIT forms require identical proteolytic peptides. Sequence coverage for the identical fragments covered most of the significant KIT regions, including the JM domain (542–586), C-alpha helix and preceding loop (626–643), the P-loop (595–601), the gatekeeper region (including Thr670), the kinase active center (including Asp792), and the activation loop (A-loop, 810–837). High sequence coverage is essential for structural and functional comparisons.

Determination of deuterium incorporation

Quenched/digested KIT fragments after H/D exchange were separated over a ProZap C18 column at 0.3 mL/min flow rate. The fast reversed-phase HPLC separation reduces back exchange by ~25% compared to conventional HPLC separations.¹⁵ Data analysis was facilitated by two custom software programs to identify each proteolytic peptide for KIT unexposed to D₂O, to aid in subsequent assignment of proteolytic peptides following hydrogen/deuterium exchange, and to determine the H/D exchange rate distribution from deuterium uptake versus HDX incubation period profiles. For each KIT protein of ~50 kDa mass, with and without bound drug, ~200 fragments were generated (in triplicate, plus controls). Manual data reduction would require several days for each pair of proteins. The software programs reduce the analysis to less than 10 min for each comparison.

The KID has no significant conformational influence on apo-KIT kinase or drug inhibition of KIT kinase

As for other tyrosine kinases within the family, the KIT kinase contains the 60 a.a. KID. Our HDX MS results showed that these residues in KID have relatively high deuterium incorporation, indicating high solvent exposure. The flexible KID interferes with crystallization and was thus deleted for X-ray crystallography.¹⁰ Determining the influence of KID on the conformation and activity of KIT kinase is imperative to directly relate the crystal structure and the biochemical results for KIT kinase. The primary sequence of WT KIT with the KID is illustrated in Figure 1(a), along with functionally significant elements [juxtamembrane (JM) domain, C-alpha helix, KID, and activation loop] highlighted. Figure 1(b) shows the WT KIT crystal structures (PDB ID: 1T45, 1PKG), with the KID deleted and functionally important parts color coded the same as for the primary sequence. Solution-phase HDX MS experiments for two constructs of KIT (the WT KIT kinase

with and without the KID) were performed under the same conditions. Peptides in the three functional elements listed earlier have similar HDX deuterium incorporation profiles, indicating no significant conformational differences for the two KIT constructs with and without the KID.¹²

To determine the effect of the KID on KIT conformation upon binding of drug inhibitors, solution-phase HDX was performed for KIT with and without the KID in the presence and absence of the inhibitors, sunitinib¹² and imatinib. Figure 2 shows that peptides in the JM domain, the loop preceding and the beginning of the C-alpha helix and the A-loop have the same deuterium incorporation rate for both protein constructs with or without the imatinib bound, as is the same for sunitinib binding. The fitted deuterium incorporation profiles and the calculated exchange rate constants are displayed in Figures 4 and 5. The inserts in the figures show the number of fast ($k > 10 \text{ h}^{-1}$), medium ($0.4 < k < 10 \text{ h}^{-1}$), and slow ($k < 0.4 \text{ h}^{-1}$) exchanging hydrogens. The full HDX results for all of the common proteolytic WT KIT peptides with the two bound drugs are presented in Supporting Information Figure 1. In addition, binding of sunitinib and imatinib has no conformational influence on the WT KIT KID itself (data not shown). These results consistently show that the KID has no significant conformational influence on KIT kinase or on binding of the two drugs to KIT.

Solution-phase HDX MS reveals conformation details of the WT KIT

A bar graph showing relative deuterium incorporation level (i.e., number of incorporated deuteriums divided by the total exchangeable amide hydrogens for that peptide) after 2-h exchange for proteolytic peptides from KIT (without KID) is shown in Figure 3. The X-ray structure for WT KIT, which favors the autoinhibitory conformation, is also displayed and is color-coded based on the deuterium incorporation level for each fragment. The highest relative deuterium incorporation levels (and thus the most solvent exposed regions) are consistent with disordered flexible loop regions of the X-ray structure (in red); fast deuterium incorporation is observed for long loops connected to short helices (orange); intermediate and slow incorporation levels are found for loops connected to α -helix or β -sheets (tan and cyan); and the lowest incorporation level was observed for α -helix or β -sheet regions (blue; Fig. 3).

Residues 542–547 and 547–567

More detailed data analysis reveals the folding/conformational details of KIT related to its biological function. For example, peptide 542–547 in the N-terminal of the JM domain is almost completely disordered in solution. The solution structure exhibits

Figure 2. Deuterium incorporation versus H/D exchange period for four proteolytic segments of WT KIT with and without the KID and with imatinib bound. No significant conformational differences are seen in the JM domain (residues 542–586), the C-alpha segment (residues 630–647) or the A-loop (residues 810–837). Thus the KID has no significant conformational influence on WT KIT itself, or on imatinib binding to WT KIT. Inserts: The number of fast ($k > 10 \text{ h}^{-1}$), medium ($0.4 < k < 10 \text{ h}^{-1}$), and slow ($k < 0.4 \text{ h}^{-1}$) exchanging hydrogens has been calculated based on the MEM rate constant calculation and colored the same as for the deuterium uptake profiles.

deuterium incorporation of ~72%, indicating high solvent accessibility. In contrast, the ~20 N-terminal residues 547–567 form a compact hairpin loop that interacts directly with the C-alpha helix in the N-

lobe and with the A-loop in the C-lobe of the kinase. Deuterium incorporation level (~52%) for the 547–567 residues was lower than for residues 542–547. This insertion of the JM domain between the kinase

Figure 3. Deuterium incorporation for WT KIT segments (without the KID). The 3D structure of WT KIT (PDB ID: 1T45⁶) without the KID is color-coded according to relative deuterium incorporation after 2 h of exchange. High deuterium incorporation corresponds to flexible loop regions and low deuterium incorporation correlates to highly folded α -helix and β -sheets of KIT without the KID. Deuterium incorporation levels also reflect interaction and folding states of different regions of KIT.

Figure 4. Deuterium incorporation profiles for four representative segments of WT KIT with and without imatinib (a) and sunitinib (b). Imatinib binding increased solvent accessibility for the JM domain, and reduced solvent accessibility for C-alpha helix region, the ATP binding site and the A-loop. Sunitinib binding stabilized (less deuterium exchange) the JM domain, the loop preceding and the beginning of the C-alpha helix region, the ATP binding site, and the A-loop. Inserts: notation as for Figure 2. (c) Deuterium incorporation levels for all of the resolved segments of the WT KIT with and without bound imatinib or sunitinib were calculated for the 2 h H/D exchange period. KIT ribbon structures (PDB ID: 1T45, IPKG⁶)—Green: no conformational change upon drug binding; Red: increased solvent accessibility upon drug binding; and Blue: reduced solvent accessibility upon drug binding.

Figure 4. (Continued)

N- and C-lobes impedes the A-loop from attaining an active conformation.

Residues 810–828 and 793–798

A-loop residues 810–816 were more solvent-accessible than residues 817–828. Residues Ile817, Lys818, Asn819, and Asp820 form approximately one turn of an α -helix and thus have less solvent accessibility. Another reason for reduced solvent accessibility of regions 817–828 and 819–828 is that the target A-loop Tyr823 inserts into the kinase active center and binds as a pseudosubstrate. The Tyr823 side chain forms hydrogen bonds with Asp792 and Arg796. Conversely, the catalytic loop region, partially covered in the 793–798 fragment, has only 10% deuterium incorporation, in keeping with its very rigid secondary structure, which is highly conserved in protein kinases.

The color coding applied to the 3D structure of KIT (Fig. 3) was derived both from individual protease type XIII digestion products and also from sequence overlaps between those segments. For example, deuterium incorporation levels for 569–582, 569–588, and 574–588 are 31, 30, and 19%. Those values were used to predict deuterium incorporation for segments 570–574 (63%), 575–582 (13%), and 583–588 (28%). For example, for segment 583–588, there are five potentially exchangeable amide hydrogens ($588 - 583 + 1 = 5$). Normally, the N-terminal hydrogen of the peptide exchanges (and back-exchanges) too rapidly to measure with this

method; but 583–588 is not a real peptic fragment, so the N-terminal hydrogen on 583 is measurable; there is no exchangeable amide hydrogen on proline), adding the 11 exchangeable hydrogens in 569–582 gives total of 16 potentially exchangeable hydrogens for 569–588. If the deuterium incorporation percentage in 583–588 is X , then $5X + 11 \times 31\% = 16 \times 30\%$, from which $X = 28\%$. Similar calculations can be applied to obtain deuterium incorporation levels for segments 575–582 (total of seven exchangeable amide hydrogens) and 570–574 (total of four exchangeable amide hydrogens) as 13 and 63%. Data interpretation from proteolytic fragment overlaps increases spatial resolution of the deuterium exchange assignment. In this case, the assignment was localized to a 5 a.a. span of the original 13 a.a. segment. HDX results are consistent with previously reported X-ray structures. In some cases, HDX MS can provide sequence resolution to within one or two amino acids. HDX MS has the potential to determine partial protein conformation even in the absence of X-ray or NMR data.

Effect of imatinib and sunitinib binding on the conformation of the WT KIT

Both imatinib and sunitinib are effective inhibitors of the WT KIT. Upon imatinib binding to the WT KIT, higher solvent accessibility (more fast exchanging hydrogens) is observed for the JM domain (e.g., residues 558–568) [Fig. 4(a)], indicating less

interaction of the JM domain with the kinase domain. The HDX results support the previous crystal structure conclusion that the small JM domain functions to maintain the kinase in an autoinhibited state through insertion into the cleft between the kinase N- (the C-alpha helix) and the C- (A-loop) lobes.⁶ The cocrystal structure of KIT in complex with imatinib revealed that the autoinhibited state of the WT KIT is prevented by imatinib binding, resulting in release of the JM domain from association with the kinase N- and C-lobes. Almost no deuterium incorporation difference was induced by imatinib for segment 542–547 of the JM domain (data not shown), which is already almost completely disordered in the autoinhibited conformation and is not involved in the interaction with the other regions of KIT. Imatinib binding reduces solvent accessibility (fewer fast exchanging deuteriums upon drug binding) of the ATP binding site (793–798), and the A-loop (812–828) of WT KIT. This result is reflective of imatinib forming a tight, stabilizing complex with the protein. Protection of the ATP binding site and the A-loop upon sunitinib binding also correlates with the binding and inhibition of the WT KIT's kinase activity [Fig. 4(b)].¹²

Bar graphs summarize HDX results for all of the assigned proteolytic fragments of WT KIT on binding of imatinib [Fig. 4(c), top panel] and sunitinib [Fig. 4(c), bottom panel]. Changes in solvent accessibility are color-coded on the inset 3D structure of WT KIT. In addition to the three segments [see Fig. 4(a)] that are protected upon imatinib binding, several other segments also showed protection: that is, 760–764 and in the G-helix, 879–882. The functional relevance of those conformational changes is yet to be elucidated. Interestingly, a segment (662–672) containing the gatekeeper residue Thr670 appears not protected upon imatinib binding. This result could be due to any protection afforded by inhibitor binding being balanced out by an increased exposure of the b₄–b₅ loop of the segment.

Sunitinib binding to WT KIT reduces solvent accessibility of the A-loop, and moderately reduces solvent accessibility of the JM domain, the C-alpha helix region, and the ATP binding site [Fig. 4(b)]. By comparison to the binding effect of imatinib, we infer that the JM domain is not released from interacting with the A-loop on sunitinib binding. Sunitinib binding to the ATP binding pocket evidently affects the A-loop as well as the JM domain, which is in contact with the A-loop in the autoinhibited KIT. The ATP binding site (793–798) also exhibited decreased solvent exposure upon sunitinib binding. The reduced solvent exposure overall could be due to sunitinib binding to the ATP binding pocket, interacting with A-loop and stabilizing the autoinhibited form of KIT. In contrast to imatinib, sunitinib binding induces slight protection for segment 662–672 containing the

gatekeeper residue Thr670, further supporting different binding and inhibition mechanisms for the two drugs.

Conformational changes induced by sunitinib binding in the D816H and V560D mutants

In contrast to binding of sunitinib to the WT KIT, binding of sunitinib to the A-loop mutant D816H did not significantly reduce deuterium incorporation in the JM domain, C-alpha helix region, and the A-loop [Fig. 5(a)]. Deuterium incorporation is slightly higher (~0.5 D) for JM fragment 558–568 for both the fast and medium exchanging hydrogens upon sunitinib binding). This result further indicates the different JM domain conformations of the D816H and the WT KIT, and the different effects upon sunitinib binding. As observed for the D816H mutant, binding of sunitinib to the V560D mutant also slightly increased solvent exposure for the JM domain and did not affect the C-alpha helix region [Fig. 5(b)], but did significantly reduce solvent accessibility for segment (657–672) containing the gatekeeper residue Thr670 and the A-loop (817–828). Sunitinib thus might be expected to interact with the gatekeeper residue region and the A-loop upon binding to protein and inhibiting KIT activity. These results indicate that binding of V560D partially resembles both the WT KIT and the D816H mutant, as discussed in the following section.

Conformational differences between the apo WT KIT with the KIT mutants reveals the sunitinib resistance mechanism

Apo WT KIT and clinical mutants, D816H and V560D (V560D was not structurally characterized), were analyzed to determine structural changes due to mutation.¹² Most fragments in the apo D816H mutant are more solvent exposed than in the apo wild type, including the JM domain and the C-alpha helix region.¹² The greater flexibility for the D816H protein suggested by the HDX results may be due to the lack of JM domain binding and formation of the tight autoinhibited structure. The crystal structure complexes of sunitinib with D816H and WT KIT show little structure difference,¹² with the caveat that the JM domain was truncated to obtain the former structure. However, HDX MS exhibits significantly higher solvent accessibility for D816H bound to sunitinib (data not shown), presumably because the X-ray crystal structure reflects the static structure, whereas the HDX embodies dynamics of the complex. The HDX data also show that the solvent exposure of the A-loop region (812–816) of the D816H mutant is similar to that of the wild type.

The most significant changes in HDX rates between the apo wild type and the apo mutants occur in the segment (835–842) located at and

Figure 5. Deuterium incorporation profiles for the D816H and V560D mutants with and without bound sunitinib. (a) Sunitinib binding does not change the conformation of the D816H JM domain (slight increase in deuterium incorporation), C-alpha helix region, and the A-loop. (b) Sunitinib binding does not significantly change the conformation of the JM domain or the C-alpha helix region of V560D, but does stabilize segment (657–672) containing the gatekeeper residue Thr670 and the A-loop. Inserts: notation as for Figure 2.

beyond the C-terminus of the activation loop (Fig. 6). That segment in the D816H mutant is the most solvent-accessible, followed by the V560D mutant

and the WT KIT. The different exposures of segment 835–842 may be due to different conformational sampling of the A-loop for the various KIT proteins.

Figure 6. Deuterium incorporation profiles for proteolytic fragment 835–842 (residues at and beyond the C-terminal of the activation loop) in three forms of KIT: wild type, D816H and V560D. The D816H mutant is the most solvent-exposed, followed by the V560D mutant and the WT KIT. KIT ribbon structure (PDB ID: 1T45) with this segment highlighted in pink.

HDX results for the two segments from the JM domain (569–588) and the C-alpha helix region (624–636) of the three constructs of KIT (WT KIT, V560D, and D816H), reveal specific conformational differences and provide insight into their different drug sensitivities. There are two different kinds of exchange kinetics for HDX MS: EX2 and EX1.^{16,17} Stable proteins that display a single conformation usually follow EX2 exchange kinetics, showing binomial isotopic distributions. Proteins with more than one conformation (e.g., under denaturant or extreme pH conditions), usually exhibit EX1 exchange kinetics, showing bimodal isotopic distributions. As noted above, the WT KIT JM domain inserts into the N- and C-lobes of the kinase A-loop, creating an autoinhibited state. The C-alpha helix is a single α -helix in the N-lobe of KIT kinase and it directly contacts the A-loop DFG motif in the activated conformation of the protein.

Figure 7 displays ESI FTICR mass spectra for two segments: 569–588 from the JM domain and 624–636 from the C-alpha helix and preceding loop after various HDX periods. Both of the D816H mutant fragments follow EX2 H/D exchange kinetics^{16,17} for all exchange periods, and exhibit the highest deuterium incorporation, indicating that each of the two segments has a single conformation and is more solvent-accessible than the same segment in the other two KIT variants. For the WT KIT at short exchange periods (less than 8 min), the two fragments also display EX2 kinetics, namely, a single conformation. After longer (>30 min) HDX incubation periods, the WT KIT shows some EX1 behavior, evidenced by split peaks, presumably because a small fraction of the KIT molecules escape from the autoinhibited conformation. For the V560D mutant, both fragments follow EX1 kinetics from the 2 min to

the 2 h exchange period. These results suggest two conformations for the V560D mutant: one with the autoinhibited conformation with the JM domain and the C-alpha helix region less solvent-exposed than the other. The abundance ratio of the slow and fast exchanging components is $\sim 1:2$ – $1:3$, that is, more molecules move away from the autoinhibited conformation. Because in these cases the isotopic distributions are not completely separated for the WT and V560D fragments, the exchange kinetics could be mixed EX1 and EX2.¹⁸ In any case, there is more than one conformation for the V560D KIT. These results provide a specific conformational explanation for the drug sensitivity of different KIT constructs: that is, the WT KIT is the most sensitive to sunitinib, followed by the V560D mutant,⁷ and D816H KIT is the most resistant to the drug.

Evidence from direct noncovalent binding and enzyme kinetic experiments

Direct binding studies provide further conformational basis for the binding and inhibition of KIT constructs by sunitinib. 25 μ M unactivated KIT (with KID) was incubated with sunitinib and binding was measured by FTICR MS (Fig. 8). 125 μ M

Figure 7. ESI FTICR mass spectra of segment 569–588 in the JM domain (top) and 624–636 in the C-alpha helix region (bottom) after each of several HDX periods (*Interferant peaks). These results correlate with different sensitivities toward sunitinib (see text).

Figure 8. ESI FTICR mass spectra of intact unactivated KIT with and without sunitinib. Top: ATP was added to unactivated KIT (with KID) without sunitinib binding. A significant portion of the KIT is phosphorylated and activated. Bottom: Unactivated KIT (with KID) was incubated with sunitinib and ATP was then added. ATP did not bind to KIT to a significant extent, whereas sunitinib did bind to KIT, implying effective binding and inhibition of unactivated WT KIT by sunitinib.

ATP was then added with and without sunitinib. ATP was observed bound to KIT, but ATP and sunitinib did not bind at the same time to a significant extent. Sunitinib and ATP thus appear to bind to the same site on KIT, so that the binding of sunitinib could inhibit the binding of ATP.

As previously reported,¹² K_d 's for sunitinib inhibition of unactivated WT KIT and the mutant D816H and V560D proteins were all in the range: 4–22 nM. When all the constructs are activated, they show similar resistance to sunitinib binding. These findings are consistent with the binding observed by MS for the intact constructs, indicating that sunitinib does not bind well to the activated form. From a combination of crystallographic, biochemical, and mass spectrometry techniques, a model of the *in vivo* equilibrium states of WT KIT and the D816H and V560D mutants can be inferred (Fig. 9). Clinical resistance to sunitinib arising from the A-loop mutations is most likely due to a shift in equilibrium structure to the activated form of KIT.

Materials and Methods

Materials

Protease type XIII from *Aspergillus saitoi*, HEPES, ethylenediaminetetraacetic acid (EDTA), sodium chloride, Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), deuterium oxide, and formic acid were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade H₂O and acetonitrile were purchased from VWR International (Suwanee, GA). KIT constructs were cloned and purified as previously described.^{10,12}

Hydrogen/deuterium exchange

The entire HDX experiment was automated with a LEAP robot (HTS PAL, Leap Technologies, Carrboro, NC).^{19,20} A 5 μ L stock of c-KIT (20 μ M) was mixed with 45 μ L of 25 mM HEPES, pH 7.5, 250 mM NaCl, 1 mM EDTA, and 0.5 mM TCEP in D₂O to initiate each H/D exchange period. Each KIT kinase was incubated with a three-fold molar excess of the drug for 1 h before HDX MS analysis (KIT was 20 μ M, and imatinib and sunitinib were 63 μ M). The drug was allowed to bind for 1 h before H/D exchange. For the blank control, the initial dilution was made in H₂O. For the zero-time control, quenching of the HDX and protease initiation were achieved simultaneously by addition of 50 μ L of protease solution in 1.0% formic acid. The remaining HDX incubation periods were 0.5, 1, 2, 4, 8, 15, 30, 60, 120, 240, and 480 min, each followed by simultaneous quench and proteolysis. The controls and each of the incubation periods were performed in triplicate. H/D exchange was quenched by 1:1 (v/v) addition of protease Type XIII¹⁴ solution in 1.0% formic acid to decrease the final pH to 2.3–2.5 and to start the 2-min protease digestion. The digested sample was then injected to a liquid chromatograph. The entire HDX process was performed at $\sim$ 0°C controlled by a Huber power water bath (Peter Huber, Offenburg, Germany).

On-line LC ESI FTICR MS/MS

A Jasco HPLC/SFC instrument (Jasco, Easton, MD) was interfaced to the LEAP robot (HTS PAL, Leap

Figure 9. Cartoon representation of the conformational equilibrium model for the WT KIT and the D816H mutant. The WT KIT favors the autoinhibited conformation and can be effectively inhibited by both imatinib and sunitinib. The D816H mutant predominantly adopts the activated state. This conformational change results in reduced sensitivity of the KIT mutants to sunitinib in the presence of mM ATP, because sunitinib preferentially binds to the autoinhibited state. The V560D samples the conformations similar to both the WT and D816H mutant (at $\sim$ 1:2 ratio), explaining its intermediate sensitivity to sunitinib compared to those for either state.

Technologies, Carrboro, NC) to desalt and separate the c-KIT peptides after proteolysis. For LC, the protein digest was injected from a 30 μ L loop to a ProZap Prosphere HP C₁₈ column (Grace Davidson, Deerfield, IL), HR 1.5 μ m particle size, 500 Å pore size, 2.1×10 mm². Peptides were eluted with a rapid gradient from 2% B to 95% B in 1.5 min (A: acetonitrile/H₂O/formic acid, 5/94.5/0.5; B: acetonitrile/H₂O/formic acid, 95/4.5/0.5), at a flow rate of 0.3 mL/min. A postcolumn splitter reduced the LC eluent flow rate to $\sim 1/1000$ of the flow rate for efficient microelectrospray ionization (micro-ESI).²¹

The ionized LC eluent was directed to a custom-built hybrid LTQ 14.5 T FTICR mass spectrometer (ThermoFisher, San Jose, CA).²² Mass spectra were collected from $400 < m/z < 2000$ at high mass resolving power ($m/\Delta m_{50\%} = 200,000$ at m/z 400). The total data acquisition period for each sample was 6 min. External ion accumulation²³ was performed in the linear ion trap with a target ion population of three million charges collected for each FTICR measurement. LTQ-accumulated ions were transferred (~ 1 ms transfer period)²⁴ through three octopole ion guides (2.2 MHz, 250 V_{p-p}) to a capacitively coupled²⁵ closed cylindrical ICR cell (55 mm i.d.) for analysis. The ion accumulation period was typically less than 100 ms during peptide elution and the FTICR time-domain signal acquisition period was 767 ms (i.e., an overall duty cycle of ~ 1 Hz per acquisition). Automatic gain control²⁶ and high magnetic field²⁷ provided excellent external calibration mass accuracy (typically better than 500 ppb rms).

Nano-LC MS/MS of the protease type XIII digested peptides was performed by use of a Nano-LC system (Eksigent, Dublin, CA). The gradient was from 15% B to 40% B at 300 nL/min in 40 min and 40% B to 98% B in 10 min (Solvent A: 97.5% H₂O, 2% methanol, 0.5% formic acid; Solvent B: 2% H₂O, 97.5% methanol, 0.5% formic acid). The column is a 10 cm $\times$ 75 μ m i.d. Pico-frit C₁₈ column with a 15 μ m i.d. tip. The column was packed with 5- μ m particle size Proteoprep II packing (New Objective, Woburn, MA). The eluent was analyzed by positive-ion microelectrospray 14.5 T FTICR MS. Data-dependent MS/MS was performed in the linear ion trap (collisional dissociation) for the three most abundant ion species. The raw data were analyzed by Multiplierz (BLAIS Proteomics center) to generate a peak list. Data were sent to Mascot (Matrix Science, London) with KIT protein (WT and mutant) sequences added to the database for peptide identification. The Mascot searching parameters were set at 2 ppm for parent ion and 0.8 Da for LTQ fragment ions.

HDX data analysis

Data were collected with Xcalibur software (ThermoFisher) and analyzed by an in-house analysis package,²⁸ permitting reliable isotopic distribution and

charge state identification and accurate assignment of the deuterium incorporation for each of the peptide fragments in the spectrum. Deuterium incorporation versus incubation period profiles were fitted a maximum-entropy method²⁹ to provide a distribution of HDX exchange rate constants. A custom Python program automatically generated time-course curves from which deuterium incorporation for identical fragments for two proteins could be compared. The Python software also automatically generated bar graphs for deuterium incorporation for all proteolytic peptides, enabling direct comparison of deuterium incorporation of all of the peptic fragments with a single bar graph.

Inhibitor binding monitored by mass spectrometry of intact KIT constructs

A total of 25 μ M unactivated KIT (with the KID) was incubated with 50 μ M sunitinib in 25 mM ammonium acetate and binding was measured by use of FTICR MS. 125 μ M ATP was then added with and without sunitinib. A separate 10 μ M unactivated KIT sample was activated with 4 mM ATP in Buffer A [50 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 3 μ M Na₃VO₄, 0.1% Brij-35] for 3 h at room temperature. 20 μ M sunitinib was incubated with the 10 μ M activated KIT (after dialysis into 25 mM ammonium acetate).

Conclusions

H/D exchange coupled with FTICR MS data demonstrates no conformational difference between constructs of KIT with and without the KID domain, thereby validating prior X-ray crystallography data for the truncated WT KIT. In addition, HDX studies of drug interactions with KIT reveal different binding and inhibition mechanisms for imatinib and sunitinib. Differences in solvent accessibility between the functionally significant parts of WT KIT and two KIT mutant constructs (D816H and V560D) provide valuable insight into the sensitivity and mechanism of the two drugs. Regions of the D816H mutant are more solvent-accessible than the WT KIT, sampling non-autoinhibited conformations. HDX MS reveals two major conformations for the V560D mutant: one is more solvent-accessible and resembles the D816H mutant, and another is less solvent-accessible and resembles the WT KIT, accounting for its intermediate sensitivity to sunitinib. These dynamic conformation details are not available by X-ray crystallography. In summary, the present results demonstrate that solution-phase HDX with high resolution mass analysis is complementary to X-ray analysis and also provides unique insight into conformational changes not possible with X-ray analysis. HDX MS is a vital biophysical tool to aid in elucidation of drug binding, resistance, and to aid in design of more effective therapeutic compounds.

References

- Yarden Y, Kuang W-J, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessner J, Franke U, Ullrich A (1987) Human proto-oncogene c-Kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J* 6:3341–3351.
- Yarden Y, Escobedo JA, Kuang W-J, Yang-Feng TL, Daniel TO, Tremble PM, Chen EY, Ando ME, Harkins RN, Franke U, Fried VA, Ullrich A, Williams LT (1986) Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* 323:226–232.
- Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. *Cell* 61:203–212.
- Blume-Jensen P, Hunter T (2001) Oncogenic kinase signalling. *Nature* 411:355–365.
- Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Tunio GM, Matsuzawa Y, Kanaura Y, Sino-mura Y, Kitamura Y (1998) Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 279:577–580.
- Mol CD, Dougan DR, Schneider TR, Skene RJ, Kraus ML, Scheibe DN, Snell GP, Zou H, Sang B-C, Wilson KP (2004) Structural basis for the autoinhibition and STI-571 inhibition of c-kit tyrosine kinase. *J Biol Chem* 279:31655–31663.
- Heinrich MC, Maki RG, Corless CL, Antonescu CR, Harlow A, Griffith D, Town A, McKinley A, Ou W-B, Fletcher JA, Fletcher CDM, Huang X, Cohen DP, Baum CM, Demetri GD (2008) Primary and secondary kinase genotypes correlate with the biological and clinical activity of sunitinib in imatinib-resistant gastrointestinal stromal tumor. *J Clin Oncol* 26:5352–5359.
- Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H (2002) Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 347:472–480.
- Verweij J, Casali PG, Zalcberg J, LeCesne A, Reichardt P, Blay JY, Issels R, van Oosterom A, Hogendoorn PC, Van Glabbeke M, Bertulli R, Judson I (2004) Progression-free survival in gastrointestinal stromal tumors with high-dose imatinib: randomised trial. *Lancet* 364:1127–1134.
- Mol CD, Lim KB, Sridhar V, Zou H, Chien EYT, Sang B-C, Nowakowski J, Kassel DB, Cronin CN, McRee DE (2003) Structure of a KIT product complex reveals the basis for kinase transactivation. *J Biol Chem* 278:31461–31464.
- Li S, Covino ND, Stein EG, Till JH, Hubbard SR (2003) Structural and biochemical evidence for an autoinhibitory role for tyrosine 984 in the juxtamembrane region of the insulin receptor. *J Biol Chem* 278:26007–26014.
- Gajiwala KS, Wu JC, Christensen J, Deshmukh GD, Diehl W, DiNitto JP, English JM, Greig MJ, He Y-A, Jacques SL, Lunney EA, McTigue M, Molina D, Quenzer TA, Wells PA, Yu X, Zhang Y, Zou A, Emmett MR, Marshall AG, Zhang H-M, Demetri G (2009) KIT kinase mutants show unique mechanisms of drug resistance to imatinib and sunitinib in gastrointestinal stromal tumor patients. *Proc Natl Acad Sci USA* 106:1542–1547.
- Gottesman MM (2002) Mechanisms of cancer drug resistance. *Annu Rev Med* 53:615–627.
- Zhang H-M, Kazacic S, Schaub TM, Tipton JD, Emmett MR, Marshall AG (2008) Enhanced digestion efficiency, peptide ionization efficiency, and sequence resolution for protein hydrogen/deuterium exchange monitored by FT-ICR mass spectrometry. *Anal Chem* 80:9034–9041.
- Zhang H-M, Bou-Assaf GM, Emmett MR, Marshall AG (2009) Fast reversed-phase liquid chromatography to reduce back exchange and increase throughput in H/D exchange monitored by FT-ICR mass spectrometry. *J Am Soc Mass Spectrom* 20:520–524.
- Smith DL, Deng Y, Zhang Z (1997) probing the non-covalent structure of proteins by amide hydrogen exchange and mass spectrometry. *J Mass Spectrom* 32:135–146.
- Xiao H, Hoerner JK, Eyles SJ, Dobo A, Voigtman E, Melcuk AI, Kaltashov IA (2005) Mapping protein energy landscapes with amide hydrogen exchange and mass spectrometry: I. A. Generalized model for a two-state protein and comparison with experiment. *Protein Sci* 14:543–557.
- Weis DD, Wales TE, Engen JR, Hotchkiss M, Eyck LF (2006) Identification and characterization of EX1 kinetics in H/D exchange mass spectrometry by peak width analysis. *J Am Soc Mass Spectrom* 17:1498–1509.
- Kazacic S, Nilsson CL, Emmett MR, Marshall AG (2005) An automated system for performing hydrogen/deuterium exchange experiments with on-line LC Micro-ESI FT-ICR MS analysis. In: *Proceedings of 5th North American FT-ICR Mass Spectrometry Conference*, Key West, Florida.
- Chalmers MJ, Busby SA, Pascal B, He Y, Hendrickson CL, Marshall AG, Griffin PR (2006) Probing protein ligand interactions by automated hydrogen/deuterium exchange mass spectrometry. *Anal Chem* 78:1005–1014.
- Emmett MR, Caprioli RM (1994) Micro-electrospray mass spectrometry: ultra-high-sensitivity analysis of peptides and proteins. *J Am Soc Mass Spectrom* 5:605–613.
- Schaub TM, Blakney GT, Hendrickson CL, Quinn JP, Senko MW, Marshall AG (2007). LC/MS, proteins, and petroleum: performance characteristics of A 14.5 Tesla LTQ FT-ICR mass spectrometer. In: *Proceedings of 55th American Society of Mass Spectrometry Annual Conference on Mass Spectrometry*. Indianapolis, USA: American Society of Mass Spectrometry.
- Senko MW, Hendrickson CL, Emmett MR, Shi SDH, Marshall AG (1997) External accumulation of ions for enhanced electrospray ionization fourier transform ion cyclotron resonance mass spectrometry. *J Am Soc Mass Spectrom* 8:970–976.
- Wilcox BE, Hendrickson CL, Marshall AG (2002) Improved ion extraction from a linear octopole ion trap: simulation analysis and experimental demonstration. *J Am Soc Mass Spectrom* 13:1304–1312.
- Beu SC, Laude DA (1992) Elimination of axial ejection during excitation with a capacitively coupled open trapped-ion cell for FT-ICR MS. *Anal Chem* 64:177–180.
- Schwartz JC, Senko MW, Syka JEP (2002) A two-dimensional quadrupole ion trap mass spectrometer. *J Am Soc Mass Spectrom* 13:659–669.
- Marshall AG, Guan S (1996) Advantages of high magnetic field for FT-ICR mass spectrometry. *Rapid Commun Mass Spectrom* 10:1819–1823.
- Kazacic S, Emmett MR, Blakney GT, Hendrickson CL, Marshall AG (2010) Automated data reduction for hydrogen/deuterium exchange experiments, enabled by high-resolution fourier transform ion cyclotron resonance mass spectrometry. *J Am Soc Mass Spectrom* 21.
- Zhang Z, Li W, Logan TM, Li M, Marshall AG (1997) Human recombinant [C22A] FK506-binding protein amide hydrogen exchange rates from mass spectrometry match and extend those from NMR. *Protein Sci* 6:2203–2217.