7972 *Biochemistry* **2010,** *49,* 7972–7974
DOI: 10.1021/bi100409w

BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

The Crystal Structure of a Constitutively Active Mutant RON Kinase Suggests an Intramolecular Autophosphorylation Hypothesis

Jing Wang,*[,‡] Stefan Steinbacher,[§] Martin Augustin,[§] Patrick Schreiner,[§] David Epstein,[‡] Mark J. Mulvihill,[‡] and Andrew P. Crew[‡]

[‡]*OSI Pharmaceuticals, Inc., 1 Bioscience Park Drive, Farmingdale, New York 11735, and* [§]*Am Klopferspitz 19D, 82152 Martinsried, Germany*

Received March 16, 2010; Revised Manuscript Received August 16, 2010

ABSTRACT: A complex of RON^{M1254T} with AMP-PNP and Mg^{2+} reveals a substratelike positioning of Tyr1238 as well as likely catalysis-competent placement of the AMP-PNP and Mg^{2+} components and indicates a tendency for cis phosphorylation. The structure shows how the oncogenic mutation may cause the constitutive activation and suggests a mechanistic hypothesis for the autophosphorylation of receptor tyrosine kinases.

Receptor tyrosine kinases (RTKs) are typically composed of an extracellular domain, a transmembrane segment, and an intracellular segment containing a kinase domain (*1*). These proteins are normally activated through a multistep process beginning with extracellular ligand binding, followed by conformational changes and receptor dimerization resulting in the activation of the intracellular kinase, phosphorylation of tyrosine residues, and the subsequent recruitment of downstream signaling proteins (*2*). In addition to being activated by extracellular ligands, RTKs can also undergo constitutive activation resulting from specific mutations (*3*). During either a constitutive or nonconstitutive activation, the induction of the kinase activity is a critical step and is associated, in many RTKs, with the autophosphorylation of specific tyrosine residues located in the activation loop (A-loop) of the kinase domain. The detailed molecular mechanisms by which kinases are activated and regulated are not fully understood. One proposal, primarily based on the crystal structures of the insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) kinases, is that kinases rest in an inactive conformational state where the A-loop occludes access of ATP and substrate to the active site, and phosphorylation of critical tyrosine residues on this loop (*4*, *5*) causes its displacement, thus leading to activation. This proposal allows us to understand why the phosphorylation of certain tyrosine residues appears to control the kinase activity, but it does not address how the key tyrosine residues become phosphorylated either as a result of the mutation or following receptor rearrangement. One particular mechanistic unknown is whether the autophosphorylation event occurs through an intramolecular (cis phosphorylation) process in which a kinase molecule catalyzes the phosphorylation of its own tyrosine residues or an intermolecular process (trans phosphorylation) where a second kinase molecule is involved as has previously been proposed (*5*). The molecular mechanism through which mutation drives constitutive activation is also a poorly understood element of kinase function. A better understanding of these molecular details should enable a more effective discovery of therapeutic treatments against cancers driven by aberrantly activated RTKs (*6*).

Herein, we describe the crystal structure of the kinase domain of the RON (Recepteur d'Origine Nantais) RTK bearing the constitutively activating mutation M1254T. RON is a member of the MET proto-oncogene family and a receptor of macrophage-stimulating protein (*7*). When the ligand binds, RON becomes autophosphorylated (*8*) at Tyr1238 and Tyr1239 in the A-loop (F1227−P1250). The phosphorylation of these regulatory tyrosine residues activates the kinase activity, probably via displacement of the A-loop as previously mentioned, leading to further phosphorylation of Tyr1353 and Tyr1360 in the C-terminal multifunctional docking site (*9*). The latter, after phosphorylation, serves as a docking site for downstream signaling proteins triggering multiple pathways (*10*).

The M1254T mutation corresponds to a change of the wild-type methionine 1254 to threonine in the P + 1 loop (*3*). The same type of mutation was originally identified in the RET kinase in patients with multiple endocrine neoplasia 2B type syndrome, commonly termed the 2B mutation (*11*). This mutation has also been found in the Kit kinase implicated in human mast cell leukemia (*12*) and human mastocytosis (*13*), and in MET in patients with metastatic renal carcinomas (*14*). Introduction of the 2B mutation into RON has been shown to cause constitutive activity resulting in transformed cells with high tumorigenic and metastatic potential in vitro and in vivo (*3*, *15*).

The crystal structure is a complex containing a segment of unphosphorylated RON^{M1254T}, an AMP-PNP molecule, and a Mg^{2+} ion at 2.24 Å resolution, revealing a part of the juxtamembrane region (R1060−V1081), the entire kinase domain, and a part of the C-terminal docking site (S1346−P1357). The overall folding pattern is similar to those of other RTKs adopting the typical bilobal architecture. A homology search of the Protein Data Bank (PDB) identified MET as the closest homologue with a sequence identity of 62%, and the IR and IGF1R as more remote homologues with sequence identities of 38 and 36%, respectively. Structural alignments of the RON structure with the crystal structures of the inactive forms of the MET (PDB entry 2G15), IR (PDB entry 1IRK), and IGF1R (PDB entry 1P4O) resulted in successful overlaps with C^{α} root-mean-square deviations (rmsd's) of 4.7, 7.5, and 8.3 Å, respectively. The alignments with the active forms of the IR (PDB entry 3BU5) and IGF1R (PDB entry 1K3A) were also successful, with rmsd's of 8.3 and 7.7 Å, respectively. The largest deviations between RON and MET are localized in the juxtamembrane segment, phosphate-binding loop (P-loop), helix αC (*16*), A-loop, and P + 1 loop (see Figure S1 of the Supporting Information for details). Relatively

*To whom correspondence should be addressed. E-mail: jwang@osip.com. Phone: (631) 962-0724. Fax: (631) 845-5255.

 Published on Web 08/20/2010

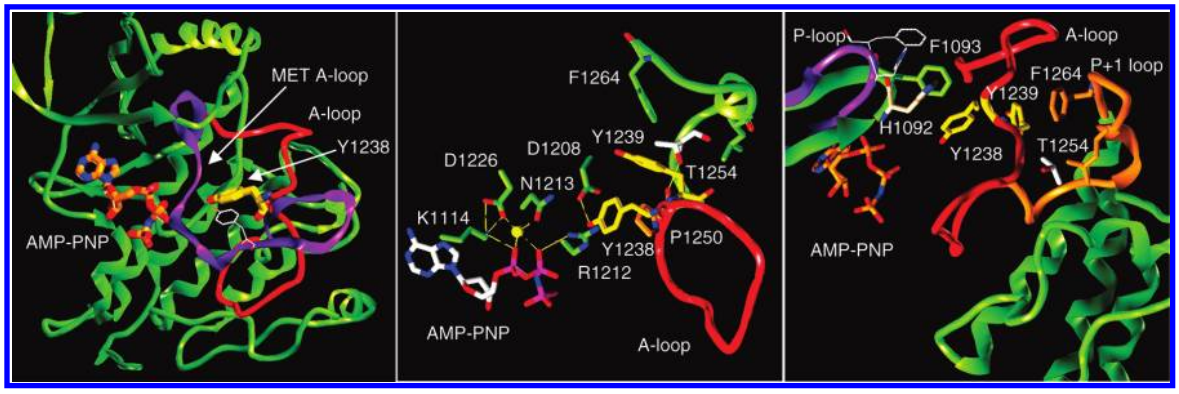

FIGURE 1: Open conformation of the first half of the A-loop of mutant RON (red) enabling AMP-PNP binding in contrast with the A-loop of MET (purple) clashing with AMP-PNP (left). Interactions stabilizing phosphates and Y1238 and location of the T1254-centered cluster relative to Y1238 (middle). Recruitment of the P-loop by Y1238 to chase out the A-loop and protect AMP-PNP (right).

large deviations between RON and the IR or IGF1R also exist in the same regions described above. The most interesting novel feature of this RON structure is that, unlike the other unphosphorylated kinases, the part of its A-loop preceding Tyr1238 adopts an open conformation enabling the binding of the ATP analogue AMP-PNP (Figure 1 Left), qualitatively similar to the phosphorylated or activated kinases (Figure S2 of the Supporting Information).

AMP-PNP exhibits many interactions with RON (Figure 1, middle) that are similar to the interactions found in the known catalysis-competent complexes of the IGF1R and IR. Namely, the adenine group forms two hydrogen bonds with the hinge backbone, and the α- and β-phosphate oxygen atoms form coordinate bonds with Mg^{2+}, which also receives coordinate bonds from Asp1226 of the DFG loop, Asn1213 of the catalytic loop, and two bound water molecules. The lengths of the coordinate bonds range between 1.9 and 2.6 Å, comparable with the lengths between 2.0 and 2.6 Å found in the IR systems (*4*, *17*). In addition, the phosphates are stabilized by ion pairing with Arg1212 (2.87 Å) and Lys1114 (3.25 Å), which also forms an ion pair with Asp1226 (3.12 Å).

Tyr1238 occupies the position usually reserved for the substrate tyrosine. The phenol hydroxyl group of Tyr1238 forms a short 2.65 Å hydrogen bond with a carboxyl oxygen atom of the catalytic Asp1208, apparently allowing abstraction of a proton from the phenol. In comparison, the hydroxyl groups of the substrate tyrosine residues in the active complexes of the IR and IGF1R (PDB entries 3BU5, 1IR3, and 1K3A) also form the same hydrogen bonds with the corresponding distances varying between 2.4 and 2.7 Å. The distance between the phenol oxygen of Tyr1238 and the γ-phosphorus atom of AMP-PNP in the RON system is ~8.75 Å. Although this distance is relatively large in comparison to that in the IR and IGF1R systems (5.1−6.1 Å), a rotation with a torsional angle of 120° around the bond linking the α−β ether oxygen atom and the β-phosphorus atom, which still maintains the number of coordinate bonds with Mg^{2+}, places the γ-phosphorus atom just 4.1 Å from the Tyr1238's phenol oxygen atom (Figure S3 of the Supporting Information).

The characteristics of the crystal structure of mutant RON described above indicate a possible pathway that allows the transfer of the γ-phosphate to Tyr1238 following a similar mechanism previously established by the crystal structures of the wild-type IR and IGF1R (*4*, *5*), except that our structure suggests an intramolecular or cis autophosphorylation of the regulatory tyrosine. A complication in justifying such a mechanism is that helix αC in this structure largely deviates from the classical location (Figure S2 of the Supporting Information) and Glu1131 of helix αC is ~15.7 Å from Lys1114, whereas this pair of residues should form an ion pair according to the classical active forms. However, a recent publication (*18*) showed that the ribosomal S6 kinase 2 is still in an active state despite its helix αC being displaced and disordered and the corresponding ion pair absent. This precedent suggests that the unusual location of helix αC in RON may not impede the cis autophosphorylation.

The mutant Thr1254 appears to play a pivotal role in stabilizing the specific open conformation of the A-loop and the positioning of Tyr1238. Thr1254 is located at the nucleus of an amino acid cluster composed of part of the second half of the A-loop (Y1239 and Pro1250), the P + 1 loop (V1251−F1264, green ribbon shown in the middle panel), and a segment of three residues centered at Asp1208. Thr1254 stabilizes the cluster by providing direct interactions with the member residues, including tight interactions with Tyr1239 (shortest distance being 3.7 Å), a hydrogen bond with a length of 2.70 Å to the backbone of Pro1250, and many favorable contacts (< 5 Å) with Val1251, Leu1259, and Phe1264. The overall stability of the cluster is also enhanced by a strong hydrogen bond between the hydroxyl group of Tyr1239 and the backbone carbonyl group of Arg1207 of the catalytic loop with a distance of 2.65 Å. The Thr1254-based cluster propagates its influence from three aspects. (1) Tyr1239 of the cluster forms π−π interactions with Tyr1238, with the shortest distance being 3.78 Å that stabilizes the substratelike positioning of Tyr1238. (2) Tyr1238 makes subsequent π−π interactions with His1092 and Phe1093 of the P-loop, with the shortest distance being 3.20 Å (right panel). Such contacts do not exist in the inactive form of MET in which the corresponding residues of the P-loop (purple ribbon) are at least 10.2 Å from the similar tyrosine (Tyr1235). Apparently, the P-loop in RON is sequestered by Tyr1238 to chase the first half of the A-loop away from the active site while leaving enough room underneath to accommodate binding of the ATP-like molecule. (3) Some residues (Tyr1239, Tyr1262, Arg1263, and Phe1264) in the Thr1254-centered cluster directly interact with the first half of the A-loop to stabilize the open conformation of the latter. Thus, this crystal structure clearly reveals how the mutant Thr1254 causes a conformational rearrangement enabling positioning of ATP and Tyr1238 onto the active site.

Cis autophosphorylation of Tyr1238 would be the initiating step of the activation process. The phosphorylated tyrosine can move away from the substrate-binding site so that the active site

will be unmasked and become activated or partially activated. Subsequent questions would be whether an activated or partially activated molecule can perform intermolecular phosphorylation of the remaining regulatory tyrosine residues and whether and how Tyr1239 becomes phosphorylated. This structure of mutant RON does not obviously support the intermolecular phosphorylation of Tyr1238 or Tyr1239, or the intramolecular phosphorylation of Tyr1239, because both residues are packed between other residues and their transformable hydroxyl groups are tethered by strong hydrogen bonds. No experimental evidence has been reported to establish whether the autophosphorylation of Tyr1239 of mutant RON can happen. However, for the MET 2B mutant, convincing kinetic and cellular studies (*19*) have demonstrated that only one of the two regulatory tyrosine residues undergoes phosphorylation to achieve the full activation. This seems consistent with the current mutant crystal structure in which only one tyrosine is engaged for phosphorylation.

Changing Thr1254 to the wild-type methionine would eliminate a hydrogen bond and introduce van der Waals repulsions within the cluster (Figure S4 of the Supporting Information), leading to a destabilization of the clustering. It is likely that the flexible loop of the wild-type RON would reorganize into an inactive conformation similar in some extent to the crystal structures of the wild-type MET, IGF1R, and IR in the unphosphorylated state.

On the basis of the crystal structures of the MET (PDB entry 2WGJ), IGF1R (PDB entry 1P4O), and IR (PDB entry 1IRK), the main obstacle to a potential cis phosphorylation within a wild-type system is the fact that the first half of the A-loop occludes the ATP-binding site while a regulatory tyrosine occupies the substrate-binding site suitable for cis phosphorylation (e.g., Figure 1, left), a situation commonly seen in all these systems. A crystallographic study (*20*) indicated that a designed inhibitor can displace the entire A-loop of the IGF1R from the autoinhibitory position and cause a tyrosine in this loop to move onto the active site of an adjacent molecule, suggesting the possibility of trans phosphorylation. However, the possibility of displacing only the first half of the A-loop by ATP similar to the scenario occurring in the current mutant RON structure may exist, and if that happens, a cis phosphorylation to the tyrosine preoccupying the substrate-binding site could follow. Such a cis phosphorylation event could be the initiation step of subsequent phosphorylations, explaining the fact that it is the tyrosine residue that is seen to occupy the substrate-binding site that becomes phosphorylated prior to other tyrosine residues. Tyr1235 in MET, Tyr1135 in the IGF1R, and Tyr1162 in the IR occupy the respective substrate-binding sites in the corresponding crystal structures in the unphosphorylated state, while these residues also acquire phosphorylation earliest in the respective activation processes based on the kinetic studies (*5*, *19*, *21*). Similar to the role of the 2B mutation elucidated above, the ligand binding and/or receptor dimerization may assist ATP in opening the first half of the A-loop leading to ATP binding and autophosphorylation.

In summary, the crystal structure of RON^{M1254T} suggests that the oncogenic mutation may cause the cis phosphorylation of a regulatory tyrosine that could lead to constitutive activation and that a modified version of the mechanism may also control the activation of wild-type RTKs. The potential for this structure-based hypothesis warrants further experimental evaluations.

SUPPORTING INFORMATION AVAILABLE

Protein production, crystallographic methods, more discussion, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wagh, P. K., Peace, B. E., and Waltz, S. E. (2008) *Adv. Cancer Res. 100*, 1–33.
2. Zwick, E., Bange, J., and Ullrich, A. (2001) *Endocr.-Relat. Cancer 8*, 161–173.
3. Yuan, Z., Guan, Y., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. (2004) *Mol. Cell. Biol. 24*, 9390–9400.
4. Hubbard, S. (1997) *EMBO J. 16*, 5573–5581.
5. Favelynukis, S., Till, J. H., Hubbard, S. R., and Miller, W. T. (2001) *Nat. Struct. Biol. 8*, 1058–1063.
6. Wang, M.-H., Lee, W., Luo, Y.-L., Weis, M. T., and Yao, H.-P. (2007) *J. Pathol. 213*, 402–411.
7. Feres, K. J., Ischenko, I., and Hayman, M. J. (2009) *Oncogene 28*, 279–288.
8. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994) *Oncogene 9*, 49–57.
9. Iwama, A., Yamaguchi, N., and Suda, T. (1996) *EMBO J. 15*, 5866–5875.
10. Wang, J., Rajput, A., Kan, J. L. C., Rose, R., Liu, X.-Q., Kuropatwinski, K., Hauser, J., Beko, A., Dominquez, I., Sharratt, E. A., Brattain, L., LeVea, C., Sun, F.-L., Keane, D. M., Gibson, N. W., and Brattain, M. G. (2009) *J. Biol. Chem. 284*, 10912–10922.
11. Hofstra, R. M., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W., van Amstel, H. K., and Romeo, G.; et al. (1994) *Nature 367*, 375–376.
12. Furitsu, T., Tsujimura, T., and Tono, T.; et al. (1993) *J. Clin. Invest. 92*, 1736–1744.
13. Longley, B. J., Tyrrell, L., Lu, S. Z., Ma, Y. S., Langley, K., Ding, T. G., Duffy, T., Jacobs, P., Tang, L. H., and Modlin, I. (1996) *Nat. Genet. 12*, 312–314.
14. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 11445–11450.
15. Germano, S., Barberis, D., Santoro, M. M., Penengo, L., Citri, A., Yarden, Y., and Gaudino, G. (2006) *J. Biol. Chem. 281*, 21710–21719.
16. Huse, M., and Kuriyan, J. (2002) *Cell 109*, 275–282.
17. Wu, J., Tseng, Y. D., Xu, C. F., Neubert, T. A., White, M. F., and Hubbard, S. R. (2008) *Nat. Struct. Mol. Biol. 15*, 251–258.
18. Malakhova, M., Kurinov, I., Liu, K., D'Angelo, I., Shim, J.-H., Steinman, V., Bode, A. M., and Dong, Z. (2009) *PLoS One 4*, 1–11.
19. Chiara, F., Michieli, P., Pugliese, L., and Comoglio, P. M. (2003) *J. Biol. Chem. 278*, 29352–29358.
20. Wu, J., Li, W., Craddock, B. P., Foreman, K. W., Mulvihill, M. J., Ji, Q., Miller, W. T., and Hubbard, S. R. (2008) *EMBO J. 27*, 1985–1994.
21. Wei, L., Hubbard, S. R., Hendrickson, W. A., and Ellis, L. (1995) *J. Biol. Chem. 270*, 8122–8130.