

## Inhibition of Spontaneous Receptor Phosphorylation by Residues in a Putative $\alpha$ -Helix in the KIT Intracellular Juxtamembrane Region\*

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**KIT receptor kinase activity is repressed, prior to stem cell factor binding, by unknown structural constraints. Using site-directed mutagenesis, we examined the role of KIT intracellular juxtamembrane residues Met-552 through Ile-563 in controlling receptor autophosphorylation. Alanine substitution for Tyr-553, Trp-557, Val-559, or Val-560, all sitting along the hydrophobic side of an amphipathic  $\alpha$ -helix (Tyr-553–Ile-563) predicted by the Chou-Fasman algorithm, resulted in substantially increased spontaneous receptor phosphorylation, revealing inhibitory roles for these residues. Alanine substitution for other residues, most of which are on the hydrophilic side of the helix, caused no or slightly increased basal receptor phosphorylation. Converting Tyr-553 or Trp-557 to phenylalanine generated slight or no elevation, respectively, in basal KIT phosphorylation, indicating that the phenyl ring of Tyr-553 and the hydrophobicity of Trp-557 are critical for the inhibition. Although alanine substitution for Lys-558 had no effect on receptor phosphorylation, its substitution with proline produced high spontaneous receptor phosphorylation, suggesting that the predicted  $\alpha$ -helical conformation is involved in the inhibition. A synthetic peptide comprising Tyr-553 through Ile-563 showed circular dichroism spectra characteristic of  $\alpha$ -helix, supporting the structural prediction. Thus, the KIT intracellular juxtamembrane region contains important residues which, in a putative  $\alpha$ -helical conformation, exert inhibitory control on the kinase activity of ligand-unoccupied receptor.**

binding-induced receptor dimerization and autophosphorylation, is well exemplified in the case of KIT (5, 6). Molecular lesions that impair the kinase activity of KIT can lead to a variety of developmental disorders (7), while mutations that constitutively activate KIT (8–10) are associated with the pathogenesis of mastocytosis (10–12) and gastrointestinal stromal tumors (9). These activating mutations can transform cells *in vitro* and confer aggressive behavior to the cells *in vivo* (9, 13).

Prior to SCF binding, the kinase activity of KIT is kept in a repressed state. The structural basis for this repression is unknown. A number of in-frame deletion mutations in the *c-KIT* intracellular juxtamembrane coding region have recently been identified *in situ* in gastrointestinal stromal tumors and in mastocytomas and shown to cause SCF-independent receptor activation (9, 10). While these findings imply that this region is involved in negative control of the receptor kinase activity in the absence of SCF stimulation, the amino acid(s) that play inhibitory roles are not known. This is because deletion mutations are likely to result in conformational changes that are not specifically related to the eliminated residues but are necessary to compensate for the gap left by the ablation.

In this study we examined the role of a series of residues in the KIT intracellular juxtamembrane region in controlling receptor autophosphorylation. Our results reveal important residues in this region that exert inhibitory effects on the receptor kinase activity in the SCF naive state and demonstrate conformational requirements for these residues in repressing autoactivation of the receptor kinase.

### EXPERIMENTAL PROCEDURES

**Materials**—Recombinant human SCF, mouse monoclonal and rabbit polyclonal anti-human KIT antibody (Ab), and wild-type human full-length KIT cDNA were provided by Amgen (Thousand Oaks, CA). Mouse anti-phosphotyrosine (Tyr(P)) monoclonal Ab was purchased from Upstate Biotechnology (Lake Placid, NY).

**cDNA Construction and Transfection**—Single residue substitutions were generated in human KIT cDNA in the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA) using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). COS cells (90% confluent in 10-cm plate) were transfected with 5  $\mu$ g of plasmid using 15  $\mu$ l of LipofectAMINE<sup>TM</sup> (Life Technologies, Gaithersburg, MD) in serum-free medium for 5 h. An equal volume of medium with 20% bovine calf serum was then added, and cells were incubated overnight, followed by 24-h culture in regular medium prior to receptor phosphorylation experiments.

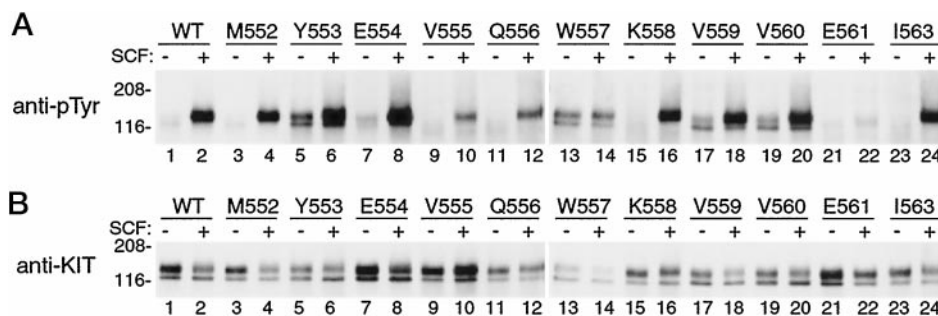
**Immunoprecipitation and Immunoblotting**—For tyrosine phosphorylation assay, cells expressing either wild-type or mutant KITs were serum-starved for 18 h before incubation with, or without, SCF at 200 ng/ml for 10 min at 37 °C. Cells were harvested in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 1 mM sodium orthovanadate. Centrifugation-clared cell lysates were immunoprecipitated for 1.5 h at 4 °C with mouse

KIT, encoded by the protooncogene *c-KIT* (1, 2), is the receptor tyrosine kinase for stem cell factor (SCF)<sup>1</sup> (3). KIT and the receptors for colony-stimulating factor 1 and platelet-derived growth factor define the receptor tyrosine kinase type III subfamily (1, 2, 4). These receptors have in common five immunoglobulin-like motifs in the extracellular domain and a bipartite kinase in the cytoplasmic portion. The current model for activation of receptor tyrosine kinases (4), which involves ligand

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<sup>1</sup> The abbreviations used are: SCF, stem cell factor; Tyr(P), phosphotyrosine; Ab, antibody; CD, circular dichroism.



**FIG. 1. Effect of alanine substitution on KIT phosphorylation.** *A*, anti-Tyr(P) (*pTyr*) blot of immunoprecipitated wild-type and mutant KITs expressed in COS cells treated (+), or not (–), with SCF (200 ng/ml, 37 °C, 10 min) after 18 h of serum starvation shows that basal receptor phosphorylation is highly increased with mutations at Tyr-553, Trp-557, Val-559, or Val-560 (lanes 5, 13, 17, and 19) and slightly increased by mutating Glu-554 (lane 7) in contrast to wild-type KIT (lane 1). Substituting Val-555 or Glu-561 impairs SCF-induced phosphorylation (lanes 10 and 22) compared with wild-type KIT (lane 2). *B*, reprobing the anti-Tyr(P) blot with anti-KIT Ab (after stripping) shows that all the mutants (lanes 3–22) are expressed as two protein products of 145 kDa and 125 kDa corresponding to wild-type KIT (lanes 1 and 2). WT, wild-type KIT. Molecular mass markers are indicated in kDa on the left.

anti-KIT Ab and protein A-agarose. Immunoprecipitates were washed with lysis buffer and heat-eluted in sodium dodecyl sulfate (SDS) sample buffer. Samples were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membrane, blocked by 5% bovine serum albumin in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), and probed with mouse anti-Tyr(P) Ab for 1 h, followed by washing with TBST. Membranes were incubated in TBST with horseradish peroxidase-linked secondary Ab for 45 min and washed, and antigen-Ab complexes were detected using the ECL System (Amersham Pharmacia Biotech). Anti-Tyr(P) blots were stripped in 100 mM  $\beta$ -mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) at 50 °C for 30 min and then reprobed with rabbit anti-KIT Ab.

**Secondary Structure Prediction**—The amino acid sequence of the KIT intracellular juxtamembrane region (Thr-544 through Lys-581) was analyzed by the Chou-Fasman algorithm (14) using MacVector program (Oxford Molecular Group) for prediction of secondary structures. Homology modeling of this region's structure was tried, but experimentally defined three-dimensional structures of proteins in the Brookhaven Protein Data Bank showed too low homology to be used as templates for modeling.

**Circular Dichroism Spectroscopy**—An 11-residue peptide (YEVQWKVVEEI) corresponding to residues 553 to 563 in the KIT intracellular juxtamembrane region was synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University and evaluated by circular dichroism (CD) spectroscopy. CD spectra of the peptide were recorded at 25 °C in a 0.2-cm path length cell on a 62DS Circular Dichroism Spectrometer (Aviv Instruments) running Aviv software. Spectra were recorded as an average of three scans either in the presence or absence of trifluoroethanol in a pH 7.0 buffer containing 10 mM sodium phosphate and 20 mM NaCl with a step size of 1 nm and a 10-s equilibration time from 260 nm to 196 nm. Peptide concentrations were determined by amino acid analysis of a stock solution. The  $\alpha$ -helical content was calculated based on the mean residue ellipticity at 222 nm (15).

## RESULTS AND DISCUSSION

**A Number of Juxtamembrane Residues Are Necessary for Inhibition of Spontaneous KIT Phosphorylation**—To examine which amino acid(s) in the KIT intracellular juxtamembrane region may play inhibitory roles in control of the receptor kinase activity, we generated a series of mutant KITs with single alanine substitutions for residues Met-552 through Ile-563. This region covers most of the mutations identified in gastrointestinal stromal tumors (9) and in mastocytomas (10) and includes a putative  $\alpha$ -helix (Tyr-553 through Ile-563) predicted by the Chou-Fasman algorithm (14). We assessed tyrosine phosphorylation of the mutated receptors in COS cells because these cells express neither KIT nor SCF (16).

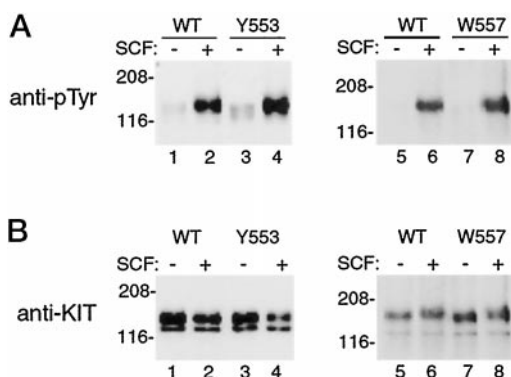
Compared with wild-type receptor which showed a low basal level of spontaneous tyrosine phosphorylation in the absence of SCF stimulation, five mutants displayed elevated spontaneous tyrosine phosphorylation (Fig. 1A). Substantially increased phosphorylation (>10-fold) resulted from removing the side

chain of Tyr-553, Trp-557, Val-559, or Val-560, and slightly increased phosphorylation (~2-fold) was caused by eliminating the side chain of Glu-554 (density-assessed after normalizing the difference in protein expression shown in Fig. 1B). In the presence of SCF, these mutants became more phosphorylated, but the increases varied; the higher the basal level, the less the increase. Of the other six mutants with no enhanced spontaneous phosphorylation, four (M552A, Q556A, K558A, and I563A) underwent SCF-induced phosphorylation in a manner comparable to the wild-type receptor, but two (V555A and E561A) showed mild and severe impairments in ligand-induced phosphorylation, respectively. These results reveal five residues in this region that exert inhibitory effects on spontaneous receptor phosphorylation: Tyr-553, Trp-557, Val-559, Val-560, and to a lesser extent Glu-554.

**The Phenyl Ring of Tyr-553 Is Critical for Inhibition**—To look more closely at the inhibitory role of Tyr-553, we mutated this tyrosine residue to phenylalanine. Removal of the hydroxyl group of Tyr-553 resulted in a slight increase (~2-fold) in basal receptor phosphorylation in comparison with wild-type receptor (Fig. 2). This result, together with the substantially increased spontaneous receptor phosphorylation caused by Y553A substitution (Fig. 1, lane 5), indicates that the phenyl ring of Tyr-553 exerts a major inhibitory effect, while its hydroxyl group plays a relatively minor inhibitory role. Whether this hydroxyl group interacts directly with another structural element or it is subject to phosphorylation is not known and awaits further study.

**The Hydrophobicity of Trp-557 Is Important for Inhibition**—Since the side chain of tryptophan allows both hydrophobic and hydrophilic interactions, the inhibitory effect of Trp-557, as indicated by the high spontaneous phosphorylation of the W557A mutant (Fig. 1, lane 13), could be mediated through either type of interaction. To test whether the amphipathic property of tryptophan is important for the inhibition, we converted Trp-557 to phenylalanine. With a hydrophobic phenyl ring at position 557, the mutant receptor showed no alteration in phosphorylation compared with wild-type receptor (Fig. 2). This result therefore suggests that it is the hydrophobic character of Trp-557 that contributes to repression of spontaneous KIT phosphorylation.

**A Predicted  $\alpha$ -Helical Conformation Is Involved in Inhibition**—The spontaneous phosphorylation pattern displayed by this series of mutant receptors is consistent with the corresponding residues being contained in an amphipathic  $\alpha$ -helix (Tyr-553 through Ile-563) predicted by the Chou-Fasman algorithm (14). Specifically, residues (Tyr-553, Trp-557, Val-559, and Val-560) that exert large inhibitory effects on spontaneous

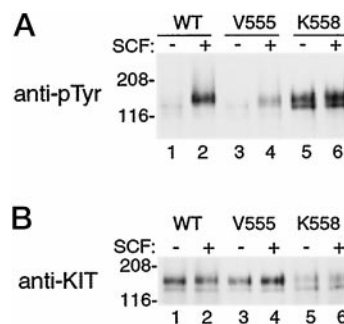


**FIG. 2. Effect of phenylalanine substitution on KIT phosphorylation.** *A*, anti-Tyr(P) blot of immunoprecipitated wild-type and mutant KITs expressed in COS cells treated (+), or not (-), with SCF (200 ng/ml, 37 °C, 10 min) after 18 h of serum starvation shows that substitution for Tyr-553 results in slightly increased basal receptor phosphorylation (lane 3) compared with wild-type KIT (lane 1), and substitution for Trp-557 has no effect on receptor phosphorylation (lanes 7 and 8) compared with wild-type KIT (lanes 5 and 6). *B*, reprobing the anti-Tyr(P) blot with anti-KIT Ab (after stripping) shows that the mutants are expressed as two isoforms of 145 kDa and 125 kDa (lanes 3 and 4 and lanes 7 and 8) corresponding to wild-type KIT (lanes 1 and 2 and lanes 5 and 6). WT, wild-type KIT. Molecular mass markers are indicated in kDa on the left.

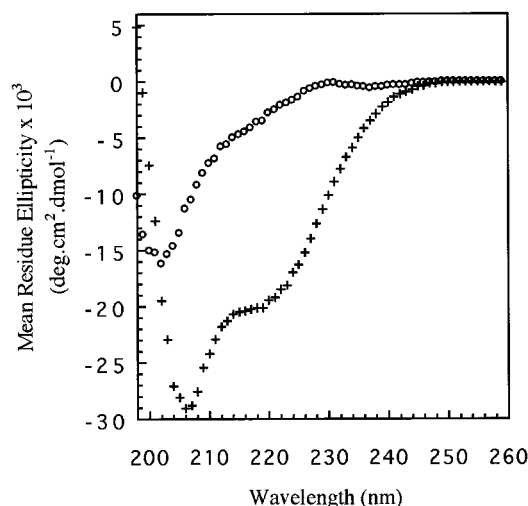
receptor phosphorylation all sit along the hydrophobic side of the predicted helical cylinder (analyzed by plotting the sequential residues along a helix wheel). In contrast, Glu-554, which plays a small inhibitory role, as well as Lys-558 and Glu-561, which are not involved in the repression, all lie on the hydrophilic side of the helix. We did not assess Glu-562 but it is predicted to have at most a minor inhibitory role because of its placement on the hydrophilic side of the helix. In addition, these data demarcate the longitudinal boundaries critical for the inhibition in that Met-552 (adjacent to the beginning of the helix) and Ile-563 (at the end of the helix) are not necessary for the inhibition, even though they both possess large hydrophobic side chains.

To further test whether the predicted  $\alpha$ -helical conformation is involved in the inhibition, we tried to disrupt the helical structure by introducing in this region proline residues that are sterically incompatible with  $\alpha$ -helical conformation. We selected Val-555 and Lys-558 to be replaced by proline, since the alanine substitution assay had demonstrated that their side chains have no inhibitory effects, and they are located within the predicted helix. Therefore, any significantly increased basal receptor phosphorylation resulting from the proline substitution can be specifically ascribed to steric constraints imposed by proline rather than to loss of inhibitory side chain function. Substitution of proline for Lys-558 led to a very high level of constitutive receptor phosphorylation, which was even higher than SCF-induced wild-type receptor phosphorylation (Fig. 3). This result thus suggests that the predicted  $\alpha$ -helical conformation is involved in the repression of spontaneous receptor phosphorylation. Substitution of proline for Val-555 did not produce any increased basal receptor phosphorylation but resulted in a decrease in SCF-induced phosphorylation (Fig. 3), as did substitution of alanine for Val-555 (Fig. 1, lane 10). The lack of autoactivation of the V555P mutant is most likely due to the absence of the side chain of Val-555, which is critical for KIT activation.

*The Tyr-553-Ile-563 Fragment Forms an  $\alpha$ -Helix in Solution*—To examine more closely the predicted  $\alpha$ -helical structure, we measured the CD spectra of an 11-residue peptide synthesized corresponding to Tyr-553 through Ile-563. This peptide displayed negative minima in ellipticity at 208 nm and



**FIG. 3. Effect of proline substitution on KIT phosphorylation.** *A*, anti-Tyr(P) blot of immunoprecipitated wild-type and mutant KITs expressed in COS cells treated (+), or not (-), with SCF (200 ng/ml, 37 °C, 10 min) after 18 h of serum starvation shows that substitution for Lys-558 leads to a high level of constitutive receptor phosphorylation (lane 5), and substitution for Val-555 impairs SCF-induced phosphorylation (lane 4), compared with wild-type KIT (lanes 1 and 2), respectively. *B*, reprobing the anti-Tyr(P) blot with anti-KIT Ab (after stripping) shows that the mutants are expressed as two products of 145 kDa and 125 kDa (lanes 3–6), as is wild-type KIT (lanes 1 and 2). WT, wild-type KIT. Molecular mass markers are indicated in kDa on the left.



**FIG. 4. CD spectra of the Tyr-553-Ile-563 peptide.** The spectra of 0.1 mM solution of an 11-residue peptide synthesized corresponding to KIT Tyr-553 through Ile-563 were recorded in 10 mM sodium phosphate (pH 7.0) and 20 mM NaCl with (+), or without (o), 70% trifluoroethanol.

222 nm, which are characteristic of  $\alpha$ -helical structure (15). The overall helical content of this peptide was 11% in aqueous solution and increased to 76% in the presence of trifluoroethanol (Fig. 4), which stabilizes the  $\alpha$ -helical structure of peptides that have inherent propensity to be  $\alpha$ -helical but are marginally stable in water (17, 18). The fact that this peptide can fold into an  $\alpha$ -helix reinforces the prediction that an  $\alpha$ -helical conformation of the juxtamembrane Tyr-553-Ile-563 region is involved in repression of spontaneous receptor phosphorylation.

The features of the inhibitory site, as we have shown, suggest that it interacts with another structural element for the inhibition. In light of the domain-domain interactions for repressing the kinase activity of the Src and Hck tyrosine kinases (19, 20), we speculate that the KIT juxtamembrane inhibitory site interacts with an epitope of the adjacent kinase domain and, in doing so, affects the kinase activity. Other mechanisms may also contribute to inhibition of KIT autoactivation and remain to be elucidated.

In summary, the present study identifies a number of residues in the KIT intracellular juxtamembrane region that exist in a putative  $\alpha$ -helical conformation and exert inhibitory effects on the kinase activity of SCF-unoccupied receptor. These find-

ings provide a structural basis for understanding why multiple deletion and missense mutations in this region, which have been identified *in situ* in gastrointestinal stromal tumors and mastocytomas, are able to cause constitutive activation of the receptor kinase (9, 10).

## REFERENCES

1. Yarden, Y., Kuang, W. J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T. J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987) *EMBO J.* **6**, 3341–3351
2. Qiu, F., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H., and Besmer, P. (1988) *EMBO J.* **7**, 1003–1011
3. Martin, F. H., Suggs, S. V., Langley, K. E., Lu, H. S., Ting, J., Okino, K. H., Morris, C. F., McNiece, I. K., Jacobsen, F. W., Mendiaz, E. A., Birkett, N. C., Smith, K. A., Johnson, M. J., Parker, V. P., Flores, J. C., Patel, A. C., Fisher, E. F., Erjavec, H. O., Herrera, C. J., Wypych, J., Sachdev, R. K., Pope, J. A., Leslie, I., Wen, D., Lin, C., Cupples, R. L., and Zsebo, K. M. (1990) *Cell* **63**, 203–211
4. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61**, 203–212
5. Blume-Jensen, P., Claesson-Welsh, L., Siegbahn, A., Zsebo, K. M., Westermark, B., and Heldin, C. H. (1991) *EMBO J.* **10**, 4121–4128
6. Lev, S., Yarden, Y., and Givol, D. (1992) *J. Biol. Chem.* **267**, 15970–15977
7. Nocka, K., Tan, J. D., Chiu, E., Chu, T. Y., Ray, P., Traktman, P., and Besmer, P. (1990) *EMBO J.* **9**, 1805–1813
8. Furitsu, T., Tsujimura, T., Tono, T., Ikeda, H., Kitayama, H., Koshimizu, U., Sugahara, H., Butterfield, J. H., Ashman, L. K., Kanayama, Y., Matsuzawa, Y., and Kanakura, Y. (1993) *J. Clin. Invest.* **92**, 1736–1744
9. Hirota, S., Isozaki, K., Moriyama, Y., Hashimoto, K., Nishida, T., Kurata, A., Takeda, M., Tunio, G. M., Matsuzawa, Y., Kanakura, Y., Shinomura, Y., and Kitamura, Y. (1998) *Science* **279**, 577–580
10. Ma, Y., Longley, B. J., Wang, X., Blount, J. L., Langley, K., and Caughey, G. H. (1999) *J. Invest. Dermatol.* **112**, 165–170
11. Nagata, H., Worobec, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y., and Metcalfe, D. D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10560–10564
12. 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
13. Kitayama, H., Kanakura, Y., Furitsu, T., Tsujimura, T., Oritani, K., Ikeda, H., Sugahara, H., Mitsui, H., Kanayama, Y., Kitamura, Y., and Matsuzawa, Y. (1995) *Blood* **85**, 790–798
14. Chou, P. Y., and Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251–276
15. Greenfield, N., and Fasman, G. D. (1969) *Biochemistry* **8**, 4108–4116
16. Reith, A. D., Ellis, C., Lyman, S. D., Anderson, D. M., Williams, D. E., Bernstein, A., and Pawson, T. (1991) *EMBO J.* **10**, 2451–2459
17. Dyson, H. J., Merutka, G., Waltho, J. P., Lerner, R. A., and Wright, P. E. (1992) *J. Mol. Biol.* **226**, 795–817
18. Zhang, M., and Vogel, H. J. (1994) *Biochemistry* **33**, 1163–1171
19. Xu, W., Harrison, S. C., and Eck, M. J. (1997) *Nature* **385**, 595–602
20. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997) *Nature* **385**, 602–609