Mutational Analysis of the Nucleotide Binding Site of the Epidermal Growth Factor Receptor and v-Src Protein-tyrosine Kinases*

(Received for publication, March 19, 1996, and in revised form, June 26, 1996)

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Tyrosine kinases differ from serine/threonine kinases in sequences located at the active site where ATP and substrate bind. In the structure of cyclic AMP-dependent protein kinase, the catalytic loop contains the sequence Lys-Pro-Glu where the Lys residue contacts the γ-phosphate of ATP and the Glu residue contacts a basic residue located in the peptide substrate. In tyrosine kinases, the analogous sequence is Ala-Ala-Arg in the receptor tyrosine kinase subfamily and Arg-Ala-Ala in the Src tyrosine kinase subfamily. To deduce the role of these residues in tyrosine kinase function, site-directed mutations were prepared in the epidermal growth factor receptor (EGFR) and in v-Src and effects on ATP binding and kinase activity were determined. Changing Arg to either Lys or Ala dramatically reduced activity of both tyrosine kinases and this correlated with loss of ATP binding. Changing the orientation of this sequence impaired activity of EGFR to a greater extent than that of v-Src but did not change substrate specificity of the two enzymes. These results support the hypothesis that Arg functions to coordinate the γ-phosphate of ATP. Analysis of sequence inversions in the catalytic loop indicate that the active site of v-Src exhibits greater flexibility than that of EGFR.

Protein kinases are divided into two major classes based on specificity for substrate hydroxyl-amino acid: serine/threonine (PSK) and tyrosine (PTK) (1, 2). Sequence homologies within the core domains of all protein kinases and three-dimensional structures available for several members (3–11) indicate a similar overall conformation. However, amino acids assigned to the catalytic loop and to the P+1 peptide binding loop regions differ between PSK and PTK and these differences distinguish the two major classes (see Fig. 1). Two subfamilies of PTK are distinguished by sequences located in the catalytic loop. The receptor tyrosine kinase subfamily contains Ala-Ala-Arg in the catalytic loop, while the Src subfamily contains this sequence in the reverse orientation Arg-Ala-Ala (1, 2). The analogous position in the PSK protein kinase A is Lys-Pro-Glu and corresponds to a loop containing amino acids important for catalysis and recognition of peptide substrate (4–6). Lys coordinates to the γ-phosphate of ATP, and Glu coordinates to a positively charged amino acid located at P-2 in the peptide substrate. Replacement of either residue with Ala markedly impaired catalytic activity of the Scaeccharomyces cerevisiae protein kinase A (12).

The Arg residue in the catalytic loop of PTK is predicted to function analogously to Lys in PSK coordinating the γ-phosphate of ATP (13). In the crystal structure of the tyrosine kinase domain of the insulin receptor (InsR) Arg1136 of the Ala-Ala-Arg sequence is hydrogen-bonded to the hydroxyl group of Tyr1162 and makes other contacts to the carbohydrate groups of Asp1132 and Asp1161 and with the indole ring Tyr1175 (11). Because ATP was not present in this structure, the relation of the Arg residue in the catalytic loop to ATP could not be compared to residues of the catalytic loop in the ternary complex of protein kinase A that contains ATP and a peptide inhibitor (5). Tyr1162 is an autophosphorylation site and thus represents a substrate structure; Asp1132 is the proposed catalytic base. The essential function of Arg was demonstrated by a Glu substitution in the human PTK Bruton’s tyrosine kinase that resulted in X-linked agammaglobulinemia (14, 15).

Although it appears likely that Arg in the catalytic loop of PTK contacts the γ-phosphate of ATP analogously to the function of Lys in the catalytic loop of PSK (13, 16), it has also been proposed to interact with substrate analogues to Glu709 in protein kinase A. In the present studies, we have investigated the catalytic loop of PTK using site-directed mutagenesis to change Arg to Lys or Arg to Ala. Additionally, the orientation of this sequence in the catalytic loop of EGFR and v-Src has been reversed to examine the specificity of sequence that distinguishes the two PTK subfamilies. Results indicate that mutation of Arg dramatically reduced ATP binding and catalytic activity of both PTKs. Reversing the orientation of the Ala-Ala-Arg sequence severely impaired EGFR activity, but reversing the Arg-Ala-Ala sequence had lesser effects on v-Src, suggesting greater flexibility in the active site of v-Src.

EXPERIMENTAL PROCEDURES

Materials—Src peptide (RRLIEADAAYARG) and angiotensin II (DRVYIHYPH) were purchased from Sigma; Cdc2-(6–20) (KVEKIGEGTYGVYVK) was synthesized by the UCSD Peptide Synthesis Facility; Src optimal peptide (AEEEEYGPEFAKKK) and EGFR optimal peptide (AEEEEEYLFFIARKKK) were kindly provided by Dr. Songyang Zhou, Harvard Medical School. Anti-EGFR monoclonal antibody 13A9 was provided by Dr. Tony Hunter, Salk Institute (18), and anti-phosphotyrosine monoclonal antibody PY20 was purchased from Transduction Laboratories (19).

Site-specific Mutagenesis—PCR/CMV-EGFR and the C-terminal deletion mutants pRC/CMV-c991 EGFR and pRC/CMV-c987 EGFR were constructed by cloning EGFR or deletion mutant cDNAs from pXER (20) into XbaI and HindIII sites of a pRC/CMV vector (Invitrogen) that was modified to contain a polylinker region derived from pBSK+ (Stratagene). A polymerase chain reaction (PCR) protocol was used to prepare site-specific mutations. Three oligonucleotide primers were employed.

* These studies were supported in part by National Institutes of Health Grant DK51349. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Postdoctoral Fellowship 1FB-0319 from the California Breast Cancer Research Program.

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The abbreviations used are: PSK, protein-serine kinase; PTK, protein-tyrosine kinase; EGF, epidermal growth factor; EGFR, EGFR receptor; InsR, insulin receptor; protein kinase A, cyclic AMP-dependent protein kinase; PCR, polymerase chain reaction; FSBA, 5'-fluorosulfonylbenzoyl adenosine; WT, wild type.
The catalytic and P concentration of 300 mM and 8 mM, respectively, and this was loaded on for 1 h, NaCl and imidazole were added to the supernatant to a final in Buffer A.

Western Blot Analysis of Phosphorylated EGFR and v-Src—

**FIG. 2.** In vivo autophosphorylation activity of EGFR mutants. A, 293 cells expressing EGFR mutants were treated without or with 30 nM EGFR for 10 min at 37°C. Immunofinity-purified recombinant EGFR or mutants (50 ng) were resolved on an 8% SDS-polyacrylamide gel, and autophosphorylation activity was measured by Western blotting using PY-20 anti-phosphotyrosine antibody. The EGFR form is indicated above each lane, B, the same filter as in panel A was stripped and reprobed with anti-EGFR polyclonal antibody 1964. The intensity of the band for EGFR in each lane was determined by the program SigmaScan to ensure equivalent loading.

**RESULTS**

Effects of Changes in the Catalytic Loop of EGFR—Because human embryonic kidney 293 cells express high levels of introduced gene products and little endogenous EGFR (27–29), they...
provide a useful system for analysis of the activities of mutant EGFR. To examine the features of the catalytic loop required for kinase activity two point mutations: Arg→Ala (R817A), Arg→Lys (R817K), and a reversal of sequence mutation Ala-Ala-Arg to Arg-Ala-Ala (AAR→RAA) were prepared. Activity was first measured in vivo as EGF-dependent autophosphorylation. As shown in Fig. 2, EGF strongly stimulated autophosphorylation of WT EGFR. In contrast, mutations that changed the Arg residue to either Ala or Lys (R817A, R817K) abolished kinase activity. Retaining an Arg residue but reversing its position in the catalytic loop sequence to resemble that found in v-Src greatly impaired EGFR kinase activity to the range of ±1% of ligand-activated WT EGFR. Autophosphorylation of EGFR was not detected in untransfected 293 cells, indicating that measured activities reflected those of transfected EGFR (data not shown). In an immunocomplex kinase assay using a variety of peptide substrates, no activity of the EGFR mutants could be detected (see Table II).

To determine whether the severe reductions in tyrosine kinase activity exhibited by mutations of Arg were due to decreased binding of ATP, wild-type and mutant EGFR were immunoprecipitated and incubated with FSBA, a suicide inhibitor of ATP. As shown in Fig. 3A, R817A and R817K mutant EGFR exhibited severe reductions in FSBA binding. The sequence reversal mutant AAR→RAA had greatly reduced but detectable FSBA binding in the range of 1% of WT EGFR. The reduction in ATP binding of EGFR mutants corresponded closely to the reduction in tyrosine kinase activity, suggesting that Arg is essential for ATP binding.

*Effect of Changes in the Catalytic Loop of v-Src—Analysis of mutations in the catalytic loop of v-Src revealed that mutation of Arg to Ala (R388A) or to Lys (R388K) abolished tyrosine kinase activity measured as enzyme autophosphorylation (Fig. 4). As observed for EGFR, an Arg residue in the catalytic loop was necessary for enzymatic activity. However, reversing the orientation of the catalytic loop to that found in EGFR had only a small effect on v-Src tyrosine kinase activity. ATP binding activity paralleled tyrosine kinase activity. As shown in Fig. 3C, the R388A and R388K v-Src mutants did not bind detectable amounts of the ATP analog FSBA, whereas FSBA binding by the loop sequence reversal mutant RAA→AAR approached that of wild-type v-Src. In v-Src, as in EGFR, an Arg residue in the catalytic loop is essential for ATP binding and enzymatic activity. Positioning of the Arg residue in the catalytic loop is, however, much more constrained in EGFR than in v-Src.

The kinase activities of wild-type and mutant EGFR and v-Src were further analyzed using in vitro kinase assays. Immunoprecipitated EGFR had a $K_m$ for AII peptide of 2.2 mM. The $V_{max}$ value for EGFR was 1.7 mol of phosphate min$^{-1}$ mol$^{-1}$ EGFR using AII peptide as a substrate. These data are comparable with previously reported kinetic parameters for EGFR tyrosine kinase activity (30). The activity of EGFR mutants was below the sensitivity limit of the in vitro kinase assay and could not be determined. Use of Mg$^{2+}$ in place of Mn$^{2+}$ did not reveal tyrosine kinase activity of mutant EGFR.

v-Src and the reverse orientation mutant RAA→AAR both showed detectable kinase activity in vitro as well as in vivo. Although the extent of autophosphorylation in vivo appeared similar for wild-type and RAA→AAR v-Src (Fig. 4), kinetic analysis in vitro indicated decreased activities for the RAA→AAR mutant (Table I). The $K_m$ for ATP for the loop

![Fig. 3. ATP binding activity of EGFR and v-Src mutants. Immunoprecipitation of recombinant WT or mutant EGFR (2 µg) (A) or WT or mutant v-Src (2 µg) (C) were incubated with 1 mM FSBA for 30 min at room temperature. The reaction mixtures were resolved on an 8% SDS-polyacrylamide gel, and FSBA binding activity was detected by Western blotting using an anti-FSBA antibody. The same filters as in panels A and C were stripped and reprobed with anti-EGFR polyclonal antibody 1964 (B) or anti-Src monoclonal antibody 2–17 (D), respectively. The intensity of the bands in each lane was determined by the program SigmaScan to ensure equivalent loading.](image)

![Fig. 4. Autophosphorylation activity of recombinant v-Src proteins. A, affinity-purified recombinant wild-type or mutant v-Src (50 ng) were resolved on a 10% SDS-polyacrylamide gel. The autophosphorylation activity of recombinant v-Src proteins was monitored by Western blotting using PY-20 antibody. B, the same filter as in panel A was stripped and reprobed with anti-Src monoclonal antibody 2–17. The intensity of the band for v-Src in each lane was determined by the program SigmaScan to ensure equivalent loading.](image)

### Table I

**Kinetic analysis of recombinant WT and mutant v-Src**

| Substrate          | WT        | RAA → AAR mutant |
|--------------------|-----------|------------------|
|                    | $K_m$ µM  | $V_{max}$ mol phosphate min$^{-1}$ mol$^{-1}$ v-Src | $K_m$ µM  | $V_{max}$ mol phosphate min$^{-1}$ mol$^{-1}$ v-Src |
| Cdc2-(6–20) peptide | 170 ± 20 | 3.75 ± 0.43       | 840 ± 102 | 0.79 ± 0.09       |
| Src optimal peptide | 32.6 ± 2.8 | 0.48 ± 0.03     | 256.2 ± 30 | 0.14 ± 0.02     |
| ATP                | 3.21 ± 0.24 | 3.4 ± 0.42      | 5.48 ± 0.49 | 0.21 ± 0.03      |

* Apparent kinetic constants were determined at 25 °C in the presence of 10 mM MnCl$_2$ and 10 µM ATP as described under “Experimental Procedures.” Values are averages of three independent experiments ± standard deviation.
sequence reversal mutant of v-Src was approximately 2-fold higher than that of wild-type v-Src, and the $V_{\text{max}}$ was significantly reduced. The $K_m$ for both the Cdc2-(6–20) and Src optimal peptide substrates were 5-fold or more higher for the reverse mutant compared to wild-type v-Src. The catalytic efficiency $K_{\text{cat}}/K_m$ was correspondingly less for the RAA → AAR loop sequence reversal mutant v-Src.

**Comparison of Substrate Preferences for Mutant EGFR and v-Src**—Using selection from highly degenerate peptide libraries, Songyang et al. (31) deduced optimal tyrosine-containing peptide substrates for several PTKs. The crystal structure of InsR suggested that the Arg residue corresponding to EGFR Arg$^{817}$ interacted with a Tyr residue that is autophosphorylated in the activated InsR (11). The Arg in the catalytic loop may thus coordinate to both ATP and the peptide substrate. The sequence of the catalytic loop in EGFR and v-Src thus had the potential to alter substrate specificity. To determine whether sequence orientation in the catalytic loop affected substrate specificity, a variety of peptide substrates were tested using wild-type EGFR and v-Src and the catalytic loop sequence reversal mutants of each. As shown in Table II, while the loop reversal mutant of v-Src was in general less active than wild-type v-Src, in no case did the mutant have altered substrate preference. Specifically, no preference for the EGFR optimal peptide was acquired. Reversal of the loop sequence in EGFR reduced activity to undetectable levels, and no preference for v-Src kinase preferred substrates was acquired.

**DISCUSSION**

Mutation of the Arg residue in the catalytic loop of either EGFR or v-Src abolished ATP binding and phosphotransfer activity. These results support the proposal that, analogous to Lys$^{1126}$ in protein kinase A, Arg coordinates to the γ-phosphate of ATP providing charge neutralization. Neither Ala, which eliminated the side chain beyond the β-carbon without imposing severe constraints on conformation (32), nor Lys, which retains the ability to neutralize charge, could replace Arg. Although homology modeling of EGFR suggested that the amino group of lysine should be able to interact with the γ-phosphate of ATP (13), Arg is the conserved residue in the catalytic loop of the extended PTK family (1, 2). This suggests that the δ-guanidinium group of Arg interacts with additional residues, as observed in the InsR tyrosine kinase structure (11). In InsR Arg$^{1126}$ makes a hydrogen bond with the catalytic base Asp$^{1123}$ whereas in protein kinase A the catalytic base was hydrogen-bonded to the Thr$^{201}$. A need to simultaneously interact with additional residues such as the catalytic base and residues in the P+1 site can only be provided by Arg, which is proposed to play an essential role in both ATP binding and catalysis.

Reversing the orientation of the catalytic loop had differential effects on members of the two subfamilies of PTK. Arg is located 4 and 2 residues from the catalytic base in WT EGFR and v-Src, respectively (1). Reversal of the orientation of the sequence in the catalytic loop in EGFR seriously impaired both ATP binding and catalytic activities. In an attempt to understand these effects, a homology model of the active site of EGFR was prepared using the coordinates of the InsR kinase core. As shown in Fig. 5, reversing the orientation of the loop resulted in the δ-guanidinium group pointing away from the catalytic base Asp$^{813}$. This orientation also disrupted interaction with the P+1 site. Because ATP was not present in the InsR structure, these changes that result from catalytic loop sequence reversal do not address additional effects on coordination to ATP.

The orientation of this sequence has less effect on v-Src. ATP binding was only slightly reduced as assessed with FSBA binding, and the $K_m$ for ATP of RAA → AAR v-Src was increased only about 2-fold. This suggests that Arg coordination to the

![Fig. 5. Homology model of the active site of EGFR kinase domain.](https://www.jbc.org/content/early/2018/07/18/jbc.M118.002721/Fig5.png)
\(\gamma\)-phosphate of ATP is maintained. However, the catalytic efficiency \(k_{cat}/K_m\) of the reverse mutant of v-Src was about 25-fold lower than WT v-Src using Cdc2-(6–20) and Src optimum peptide substrates. This suggests that, analogous to Arg\(^{1136}\) interactions in InsR, Arg\(^{\text{G12/22}}\) in v-Src interacts with the catalytic base and P\(^1\) site. Reversal of sequence in the catalytic loop likely impairs these functions of the Arg residue. The flexibility at the active site of v-Src appears greater than that of EGFR and is consistent with the observation that Src tyrosine kinase can phosphorylate a wide spectrum of peptide substrates including substrates with different chirality and chain length of alcohol (33).

No change in peptide substrate preference was observed with catalytic loop sequence reversals. Neither PTK acquired the peptide substrate preference of the other. These results suggest that the orientation of the catalytic loop is not a major structural determinant of substrate preference. Rather these sequences are essential to ATP binding and catalysis.

Acknowledgments—We thank Daniel Knighton for homology modeling of the EGFR kinase domain, Chia-ping Chang for the pRC/CMV EGFR constructs, Mark Kamps for v-Src cDNA, Tony Hunter for the engineering of the EGFR kinase domain, Chia-ping Chang for the pRC/CMV cat/585–596

References

1. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) Science 241, 42–52
2. Hanks, S. K. & Hunter, T. (1995) PASE J. 9, 576–596
3. Knighton, D. R., Zheng, J. H., Ten-Eyck, L., Ashford, V. A., Xuong, N. H., Taylor, S. S. & Sowadski, J. M. (1991) Science 253, 407–414
4. Knighton, D. R., Zheng, J. H., Ten-Eyck, L., Xuong, N. H., Taylor, S. S. & Sowadski, J. M. (1991) Science 253, 414–420
5. Zheng, J., Knighton, D. R., Ten-Eyck, L., Karlsson, H., Xuong, N., Taylor, S. S. & Sowadski, J. M. (1993) Biochemistry 32, 2154–2161
6. Rossemeyer, D., Engle, R. A., Kinzel, V., Penasingh, H. & Huber, R. (1993) EMBO J. 12, 849–859
7. Zhang, F., Strand, A., Robbins, D., Cobb, M. H. & Goldsmith, E. J. (1994) Nature 367, 794–711
8. Hu, S. H., Parker, M. W., Lei, J. Y., Wilce, M. C. J., Benian, G. M. & Kemp, B. E. (1994) Nature 369, 581–584
9. De Bondt, H. L., Rosenblatt, J., Jancarik, J., Jones, H. D., Morgan, D. O. & Kim, S.-H. H. (1993) Nature 363, 595–602
10. Xu, R. M., Carmel, G., Sweet, R. M., Kuret, J. & Cheng, X. (1995) EMBO J. 14, 1015–1023
11. Hubbard, S. R., Wei, L., Ellis, L. & Hendrickson, W. A. (1994) Nature 372, 746–754
12. Gibbons, C. S. & Zoller, M. J. (1991) J. Biol. Chem. 266, 8923–8931
13. Knighton, D. R., Cadena, D. L., Zheng, J., Ten-Eyck, L., Taylor, S. S., Sowadski, J. M. & Gill, G. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5503–5507
14. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flinter, F., Hammarstrom, L., Kinnon, C., Levin, R., Bobrow, M., Smith, C. T. E. & Bentley, D. R. (1993) Nature 361, 226–233
15. Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, M., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., Jenkins, N. A. & Witte, O. N. (1993) Science 258, 358–361
16. Vihinen, M., Vorechovsky, I., Maniai, H. S., Ochs, H. D., Zhu, Q., Vorechovsky, I., Webster, A. D., Notarangelo, D. L., Nilsson, L., Sowadski, J. M. & Smith, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12863–12867
17. Winkler, M. E., O’Connor, L., Winget, M. & Fendly, B. (1989) Biochemistry 28, 6373–6378
18. Rigaudy, P., Simon, S., Hunter, T., Sollazzo, M., Billetta, R., Zanetti, M. & Eckhart, W. (1994) DNA Cell Biol. 13, 585–591
19. Glenney, J. R., Jr., Zokas, L. & Kamps, M. P. (1988) J. Immunol. Methods 109, 277–285
20. Chen, W. S., Lazar, C. S., Land, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N. & Rosenfeld, M. G. (1989) Cell 59, 33–43
21. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tan, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) Nature 310, 418–425
22. Chen, C. & Okayama, H. (1987) Mol. Cell Biol. 7, 2745–2752
23. Nebrowsk, A., Wiley, H. S. & Gill, G. N. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8719–8723
24. Wedegaertner, P. B. & Gill, G. N. (1992) Arch. Biochem. Biophys. 292, 273–280
25. Burrow, S. A. & Staros, J. V. (1985) Methods Enzymol. 109, 816–827
26. Weber, W., Bertics, P. J. & Gill, G. N. (1984) J. Biol. Chem. 259, 14631–14636
27. Paborsky, L. R., Fendly, B. M., Fisher, K. L., Lawn, R. M., Marks, B. J., McCray, G., Tate, K. M., Vehar, G. A. & Gorman, C. M. (1990) Protein Eng. 3, 547–553
28. Cachianes, G., Ho, C., Weber, B. F., Williams, S. R., Goeddel, D. V. & Leung, D. W. (1993) Biotechniques 15, 255–259
29. Herbst, R., Lammers, R., Schlessinger, J. & Ullrich, A. (1991) J. Biol. Chem. 266, 19908–19916
30. Wedegaertner, P. B. & Gill, G. N. (1988) J. Biol. Chem. 263, 11346–11353
31. Songyang, Z., Carraway, K. L., Eck, M. J., Harrison, S. C., Fedman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J. & Cantley, L. (1995) Nature 373, 536–539
32. Wells, J. A. (1991) Methods Enzymol. 202, 390–411
33. Lee, E. L., Niu, J. & Lawrence, D. S. (1995) J. Biol. Chem. 270, 5375–5380
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J. Biol. Chem. 1996, 271:22619-22623.
doi: 10.1074/jbc.271.37.22619

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