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

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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)[1] 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[168]-Pro-Glu and corresponds to a loop containing amino acids important for

catalysis and recognition of peptide substrate (4–6). Lys[168] coordinates to the γ-phosphate of ATP, and Glu[170] 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 *Saccharomyces 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) Arg[1136] of the Ala-Ala-Arg sequence is hydrogen-bonded to the hydroxyl group of Tyr[1162] and makes other contacts to the carboxylate groups of Asp[1132] and Asp[1161] and with the indole ring Trp[1175] (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). Tyr[1162] is an autophosphorylation site and thus represents a substrate structure; Asp[1132] 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 analogous to the function of Lys in the catalytic loop of PSK (13, 16), it has also been proposed to interact with substrate analogous to Glu[170] 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 (RRLIEDAEYAARG) and angiotensin II (DRVYYHPH) were purchased from Sigma; Cdc2-(6–20) (KVEKIGEG-TYGVVYK) was synthesized by the UCSD Peptide Synthesis Facility; Src optimal peptide (AEEEIYGEFEAKKKK) and EGFR optimal peptide (AEEEEYFELVAKKKK) were kindly provided by Dr. Songyang Zhou, Harvard Medical School. Anti-EGFR monoclonal antibody 13A9 was provided by Genetech Inc. (17), anti-Src monoclonal antibody 2–17 was provided by Dr. Tony Hunter, Salk Institute (18), and anti-phosphotyrosine monoclonal antibody PY20 was purchased from Transduction Laboratories (19). [γ-32P]ATP was purchased from ICN.

Site-specific Mutagenesis—pRC/CMV-EGFR and the C-terminal deletion mutants pRC/CMV-c′991 EGFR and pRC/CMV c′957 EGFR were constructed by cloning EGFR or deletion mutant cDNAs from pXER (20) into *Xba*I and *Hin*dIII 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.

[1] The abbreviations used are: PSK, protein-serine kinase; PTK, protein-tyrosine kinase; EGF, epidermal growth factor; EGFR, EGF receptor; InsR, insulin receptor; protein kinase A, cyclic AMP-dependent protein kinase; PCR, polymerase chain reaction; FSBA, 5′-fluorosulfonylbenzoyl adenosine; WT, wild type.
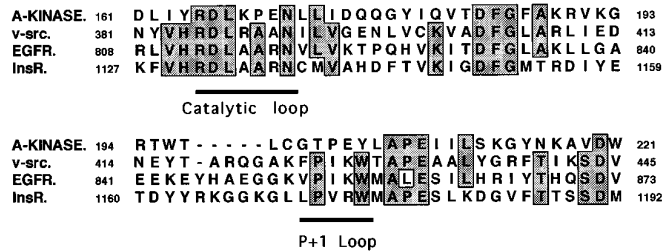
```
A-KINASE. 161 D L I Y R D L K P E N L L I D Q Q G Y I Q V T D F G F A K R V K G 193
v-src.    381 N Y V H R D L R A A N I L V G E N L V C K V A D F G L A R L I E D 413
EGFR.     808 R L V H R D L A A R N V L V K T P Q H V K I T D F G L A K L L G A 840
InsR.    1127 K F V H R D L A A R N C M V A H D F T V K I G D F G M T R D I Y E 1159
```
Catalytic loop

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A-KINASE. 194 R T W T - - - - - L C G T P E Y L A P E I I L S K G Y N K A V D W 221
v-src.    414 N E Y T - A R Q G A K F P I K W T A P E A A L Y G R F T I K S D V 445
EGFR.     841 E E K E Y H A E G G K V P I K W M A L E S I L H R I Y T H Q S D V 873
InsR.    1160 T D Y Y R K G G K G L L P V R W M A P E S L K D G V F T T S S D M 1192
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P+1 Loop

FIG. 1. **Sequence alignment of protein kinase A, v-Src, EGFR, and InsR active sites.** Highly conserved residues are *shaded* in *gray*. The catalytic and P+1 substrate loops of the protein kinases are *underlined*.

Primer 1 (GGTACCATGGAACGCCACATCGTTCGG) and primer 2 (ATATAAGCTTAAATGACAAGGTAGCG) each contained a restriction site for cloning purposes; primer 3 contains the desired mutation. The first PCR reaction mixture contained 100 pmol of primer 2 and 3, 20 ng of pRC/CMV-c′991 EGFR (with EGFR cDNA truncated at nucleotide 3231 (21)), 200 μM dNTP, 10% (v/v) Me$_2$SO, and 2.5 units of *Pfu* polymerase (Stratagene, San Diego, CA). Reaction conditions were: 1.5 min at 95 °C to denature the template DNA, 2 min at 45 °C to allow primer to anneal, and 3 min at 72 °C for DNA extension. This reaction was cycled 30 times, and the DNA fragment containing the mutated base was generated. The first PCR product (0.6 pmol) was gel-purified and incubated with 20 ng of pRC/CMV c′957 EGFR (with EGFR cDNA truncated at nucleotide 3089) and extended to full length with five cycles of PCR. Then primers 1 and 2 were added directly to this second PCR mixture and run for 30 cycles. This reaction amplified the mutated EGFR kinase domain selectively. Mutations were confirmed by DNA sequencing, and the final PCR product was digested with restriction endonucleases *Apa*LI and *Bst*EII and cloned into the plasmid pRC/CMV-EGFR.

v-Src cDNA was cloned into the plasmid pRSET-B (Invitrogen) to introduce a polyhistidine tag at the 5′ end of v-Src cDNA. The polyhistidine-v-Src cDNA was excised from pRSET-v-Src with *Xba*I and *Hin*dIII, and the resulting DNA fragment was gel-purified and recloned into the plasmid pRC/CMV. v-Src mutants were constructed with the Chameleon double-stranded site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Protein Expression and Purification—Human embryonic kidney fibroblasts (293 cells) were grown in Dulbecco's modified Eagle's medium containing 10% calf serum. One day prior to transfection, 1.5×10^5 cells were seeded into each well of a six-well dish. Transfections were carried out using 4 μg of plasmid DNA by the calcium phosphate procedure (22), and cells were harvested 48 h later. Wild-type and mutant EGFR were isolated by immunoprecipitation with the monoclonal anti-EGFR antibody, 13A9. Transfected cells were washed twice with cold phosphate-buffered saline containing 1 mM EDTA and 1 mM EGTA and lysed in buffer containing 20 mM Hepes, pH 7.4, 1 mM EDTA, 6 mM β-mercaptoethanol, 0.05% Triton X-100, 10% glycerol, and 130 mM NaCl. EGFR were selectively precipitated with 13A9-protein G Sepharose (23). Recombinant v-Src and mutants were purified by Ni^{2+}-affinity chromatography. Transfected 293 cells were harvested and homogenized in five volumes of Buffer A (10 mM Hepes, pH 7.8, 10 mM NaCl, 6 mM β-mercaptoethanol, 4 mM benzamidine, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% glycerol) containing 0.1% Triton X-100. The lysate was centrifuged at 115,000 × g for 1 h, NaCl and imidazole were added to the supernatant to a final concentration of 300 mM and 8 mM, respectively, and this was loaded on a 1-ml Ni^{2+}-NTA-agarose column (Qiagen). The column was washed with a linear gradient of imidazole (8−40 mM) in Buffer A, and recombinant v-Src proteins were competitively eluted with 100 mM imidazole in Buffer A.

Western Blot Analysis of Phosphorylated EGFR and v-Src—Transfected 293 cells were stimulated with 30 ng/ml EGF for 10 min at 37 °C. After stimulation, cells were lysed with 200 μl of hot SDS sample buffer and proteins were separated on 8% SDS-polyacrylamide gels and transferred electrophoretically to polyvinylidene difluoride filters. For autophosphorylation analysis, polyvinylidene difluoride filters were incubated with 5% bovine serum albumin in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.02% Tween 20), incubated for 2 h with the anti-phosphotyrosine monoclonal antibody PY-20, and incubated for another hour with peroxidase-conjugated goat anti-mouse antibody. Immunoblots were developed using the ECL system (Amersham Corp.), and multiple
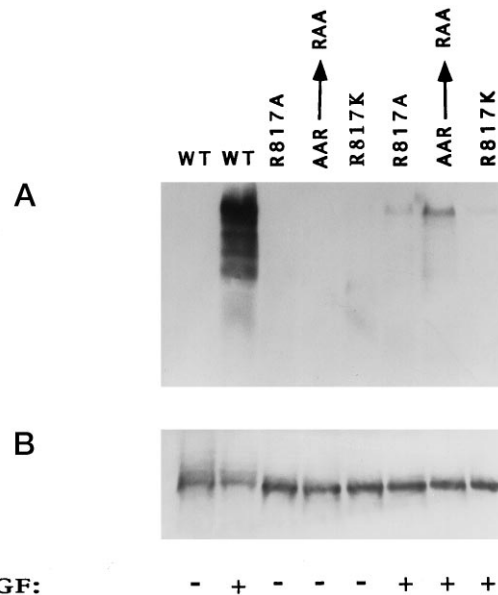

FIG. 2. *In vivo* **autophosphorylation activity of EGFR mutants.** *A*, 293 cells expressing EGFR mutants were treated without or with 30 nM EGFR for 10 min at 37 °C. Immunoaffinity-purified recombinant EGFR or mutants (50 ng) were resolved on an 8% SDS-polyacrylamide gel, and autophosphorylation activity 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.

exposures were obtained to ensure linearity. The autophosphorylation activity of v-Src was determined using a similar protocol but without EGF stimulation. Following the initial immunoblotting analysis, polyvinylidene difluoride filters were incubated with stripping buffer (62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, and 2% SDS) for 30 min at 50 °C. After washing two times with TBST buffer, the filters were reprobed with anti-EGFR polyclonal antibody 1964 that is specific for the EGFR C terminus (24) or with the monoclonal anti-v-Src antibody 2–17. After washing three times with TBST, the filters were incubated with peroxidase-conjugated secondary antibodies and developed with ECL reagents.

ATP Binding to EGFR and v-Src—The ATP-binding activities of EGFR and v-Src were determined using an ATP-binding protein detection kit (Boehringer Mannehim). Affinity-purified EGFR, v-Src, and various mutants were incubated with 1 mM 5′-fluorosulfonylbenzoyl adenosine (FSBA) at room temperature for 30 min. FSBA, an ATP analog labeling reagent, binds covalently to the nucleotide binding sites of kinases (25). FSBA-derivatized proteins were detected by Western blotting techniques using polyclonal anti-FSBA antibody as probe.

Kinase Assays and Kinetic Analysis—Phosphate incorporation into the various peptide substrates was determined using the phosphocellulose paper binding assay (26). Reaction mixtures contained 10 mM Hepes, pH 7.4, 10 mM MnCl$_2$, 100 mM Na$_3$VO$_4$, 10 μM [γ-^{32}P]ATP, 100 ng of purified recombinant protein, and different concentrations of peptide in a final volume of 40 μl. Reactions were carried out for 10 min at room temperature and terminated by the addition of 17 μl of 24% trichloroacetic acid. Bovine serum albumin (100 μg) was added to facilitate precipitation of the kinase domain. After centrifugation, ^{32}P incorporation into the peptide substrate was determined by spotting the supernatant onto phosphocellulose paper disks. The phosphocellulose disks were washed three times in 75 mM phosphoric acid, dried, and radioactivity measured. Kinetic data were analyzed with the program k-cat (BioMetallic, Inc.). The concentration of EGFR and v-Src was determined by quantitative Western blotting. The ECL Western blot signal was measured by densitometry scanning and compared with a standard curve prepared from purified EGFR and v-Src.

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: $Arg^{817} \rightarrow Ala$ (R817A), $Arg^{817} \rightarrow Lys$ (R817K), and a reversal of sequence mutation Ala-Ala-Arg^{817} to Arg-Ala-Ala^{817} (AAR $\rightarrow$ RAA) were prepared.
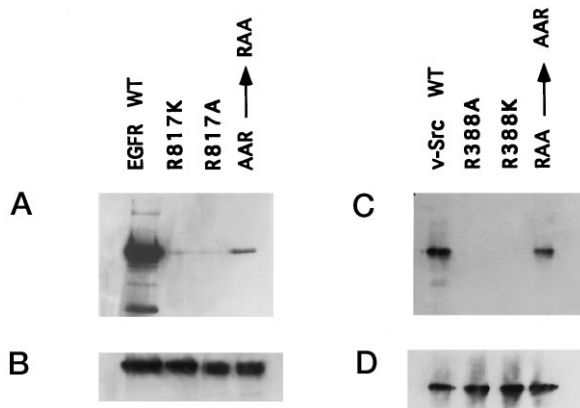

FIG. 3. **ATP binding activity of EGFR and v-Src mutants.** Immunoaffinity-purified recombinant WT or mutant EGFR (2 μg) (*A*) or WT or mutant v-Src (2 μg) (*C*) were incubated with 1 mM FSBA for 30 min at room temperature. The reaction mixtures were resolved on an 8% SDS-polyacrylamide gel, and FSBA binding activity was monitored 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.
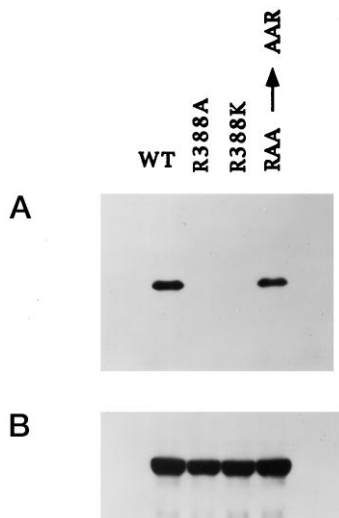

FIG. 4. **Autophosphorylation activity of recombinant v-Src proteins.** *A*, affinity-purified recombinant wild-type or mutant v-Src (50 ng) were resolved on a 10% SDS-polyacrylamide gel. The autophosphorylation activity of recombinant v-Src proteins was monitored by Western blotting using PY-20 antibody. *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.

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 $\cong$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^{817} were due to decreased binding of ATP, wild-type and mutant EGFR were immunoisolated and incubated with FSBA, a suicide inhibitor of ATP. As shown in Fig. 3*A*, R817A and R817K mutant EGFR exhibited severe reductions in FSBA binding. The sequence reversal mutant AAR $\rightarrow$ RAA had greatly reduced but detectable FSBA binding in the range of 1% that of WT EGFR. The reduction in ATP binding of EGFR mutants corresponded closely to the reduction in tyrosine kinase activity, suggesting that Arg^{817} 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^{388} 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. 3*C*, 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ AAR v-Src (Fig. 4), kinetic analysis *in vitro* indicated decreased activities for the RAA$\rightarrow$AAR mutant (Table I). The K_m for ATP for the loop

TABLE I
Kinetic analysis of recombinant WT and mutant v-Src

Substrate	WT		RAA $\rightarrow$ AAR mutant	
	K_m	V_{max}	K_m	V_{max}
	μM	*mol phosphate min^{-1} mol^{-1} v-Src*	μM	*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

^{a} 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_{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_{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

TABLE II
Rate of phosphorylation of various peptides by WT and mutant v-Src and EGFR

	v-Src	vSrc (RAA → AAR)	EGFR	EGFR (AAR → RAA)
Src (1.5 mM)	0.44	0.41	0.38	ND
Angiotensin II (10 mM)	0.17	0.09	0.61	ND
Cdc2-(6–20) (1.75 mM)	6.68	1.34	0.12	ND
Src optimal peptide (250 μM)	1.25	0.28	0.29	ND
EGFR optimal peptide (250 μM)	0.66	0.08	0.35	ND

The kinase reaction was performed as described under "Experimental Procedures." Recombinant protein (100 ng) was used in each assay, and the final concentration of each peptide is stated in the table. Kinase activity was measured as the initial velocity of the phosphorylation reaction and was expressed as pmol of PO_4 incorporated into the substrate peptide per min. ND, not detectable.

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[168] 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[1136] makes a hydrogen bond with the catalytic base Asp[1132], 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.** This homology model was based on the crystallographic coordinates of the InsR kinase domain and has been refined with energy minimization approach. The catalytic base Asp[813] is *green*, Arg[817] is *yellow*, and arginine in the reverse mutant is *white*. Tyr[845] and Trp[856] are *blue*.
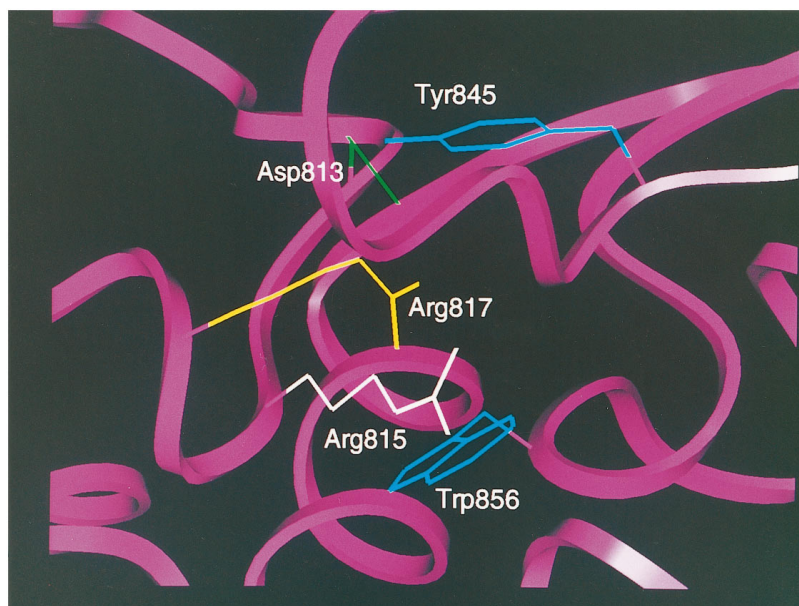
γ-phosphate of ATP is maintained. However, the catalytic efficiency (k_{cat}/K_m) of the reverse mutant of v-Src was about 25-fold lower than WT v-Src using Cdc2-(6–20) and Src optimum peptide substrates. This suggests that, analogous to Arg[1136] interactions in InsR, Arg[388] in v-Src interacts with 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.

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