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Nonphosphorylatable Substrate Analogs Selectively Block Autophosphorylation and Activation of the Insulin Receptor, Epidermal Growth Factor Receptor, and pp60$^{v\text{-}src}$ Kinases*

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The receptors for insulin and epidermal growth factor undergo tyrosine autophosphorylation in response to ligand stimulation, while pp60$^{v\text{-}src}$ is an unregulated tyrosine kinase. In this report we show that each of the kinases phosphorylates an exogenous peptide that corresponds to the insulin proreceptor sequence 1142–1153. When the kinases were pre-phosphorylated, saturable Michaelis-Menten kinetics were observed. However, when the kinases had not been pre-phosphorylated biphasic kinetics were observed; at progressively higher substrate concentrations (>K_m) less substrate phosphorylation was seen. Furthermore, when the kinases had not been pre-phosphorylated kinase autophosphorylation was inhibited at high substrate concentrations. On this basis we postulated that the substrate inhibition of substrate phosphorylation resulted directly from substrate inhibition of kinase autophosphorylation. To test this we designed additional peptides to function specifically as inhibitors of the kinases. Each of the 3 tyrosine residues within the substrate sequence were replaced either by 4-methoxyphenylalanine or phenylalanine, residues structurally similar to tyrosine but unable to accept phosphoryl transfer. Both analogs inhibited insulin and epidermal growth factor receptor autophosphorylation, whereas only the Phe-substituted analog inhibited pp60$^{v\text{-}src}$ phosphorylation. These data suggest that autophosphorylation of tyrosine residues near the kinase active site is a generalized mechanism for tyrosine kinase activation and that activation can be selectively blocked by substrates and nonphosphorylatable analogs.

Tyrosine phosphorylation is believed to play an important role in both normal cellular growth and the uncontrolled growth that follows malignant transformation of cells. Several growth factor receptors and various viral transforming proteins and their cellular homologs catalyze phosphorylation of tyrosine residues within their own sequences as well as in other proteins (1, 2). For the insulin receptor, autophosphorylation enhances kinase activity toward exogenous substrates (3, 4). Relations between autophosphorylation and kinase activity for the EGF[1] receptor and pp60^{src} have been debated. Substrate inhibition studies suggest that kinase phosphorylation is important for activation of the EGF receptor (5). On the other hand, autophosphorylation of an immobilized EGF receptor in the absence of EGF (6) or pretreatment of the receptor with EGF and ATPγS (7) does not activate the kinase toward exogenous substrates. Autophosphorylation of pp60$^{v\text{-}src}$ has been shown to activate the substrate kinase (8), but substitution of the phosphorylated residue (Tyr^{416}) by phenylalanine does not effect kinase activity or transforming potential (9, 10). More recent studies with pp60$^{c\text{-}src}$, however, suggest that Tyr^{416} is necessary for these activities (11, 12).

In the current study we evaluated the relation between autophosphorylation and kinase activation using a peptide corresponding to the insulin proreceptor sequence 1142–1153 (13, 14).[2] This peptide was phosphorylated by pp60$^{v\text{-}src}$ and the EGF receptor, as well as by the insulin receptor (15, 16), with a relatively low value for K_m. We found that at high concentrations the peptide inhibited both kinase and substrate phosphorylations. Based on these findings we designed nonphosphorylatable analogs of this peptide in which each of the 3 tyrosine residues were substituted either by phenylalanine or 4-methoxyphenylalanine (Mpa). The substrate and nonphosphorylatable analogs were used to investigate possible mechanisms involved in activation and inhibition of each of the kinases.

MATERIALS AND METHODS

The Insulin Receptor Kinase—WGA-purified insulin receptor was prepared from cultured Fao hepatoma cells as described previously (17). In all cases the receptor was incubated with 100 nM insulin and 5 mM $MnCl_2$ for 1 h at 4 °C prior to combination with other reactants. For substrate phosphorylation reactions receptor mixtures were split; one-half was pre-phosphorylated with 25 μM ATP for 10 min at 22 °C and the other half was used without pre-phosphorylation. Substrate phosphorylations were initiated by addition of phosphorylated receptor (10 μl) to aliquots (20 μl) of the peptides (1.5 times the indicated concentrations) containing 25 μM [γ-^{32}P]ATP, 5 mM $MnCl_2$, and 100 nM insulin. For substrate kinase reactions catalyzed by unphosphor-

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[1] The abbreviations used are: EGF, epidermal growth factor; Fao, a sixth generation subclone of the H4-Reuber hepatoma cell line that is azaguanine- and ouabain-resistant; Mpa, 3-methoxyphenylalanine; WGA, wheat germ agglutinin; ATPγS, adenosine 5'-*O*-(thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

[2] The numbering sequence described by Ullrich *et al.* (13) was used in this paper. To compare to the sequence published by Ebina *et al.* (14), add 12 to the residue number (for β-subunit residues only).

TABLE I

Structures of the synthetic peptide substrate, [Tyr]peptide 1142–1153, and nonphosphorylatable substrate analogs

The substrate and analogs are each 11-amino acid peptide-amides; analogs were substituted at each of the tyrosine positions by phenylalanine or 4-methoxyphenylalanine.

Peptide	Sequence
[Tyr]1142–1153	Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys-NH_2
Mpa analog	Arg-Asp-Ile-Mpa-Glu-Thr-Asp-Mpa-Mpa-Arg-Lys-NH_2
Phe analog	Arg-Asp-Ile-Phe-Glu-Thr-Asp-Phe-Phe-Arg-Lys-NH_2

ylated receptor, 10 µl of the peptide(s) and 10 µl of receptor were combined prior to initiation of the phosphorylation reaction with 10 µl of 75 µM [γ-^{32}P]ATP. Final reaction conditions were thus identical: 100 nM insulin, 25 µM [γ-^{32}P]ATP (25 µCi), 5 mM $MnCl_2$, 10 µl (6 µg) of WGA-purified insulin receptor, peptides at the indicated concentrations, 50 mM HEPES, and 0.05% Triton X-100 in 30 µl at pH 7.4. In each case, substrate phosphorylations were allowed to proceed for 2 min at 22 °C and terminated with 50 µl of 5% trichloroacetic acid.

Autophosphorylation reactions were conducted in the absence and presence of varying concentrations of the substrate ([Tyr]peptide 1142–1153) and nonphosphorylatable analogs ([Phe]peptide 1142–1153 and [Mpa]peptide 1142–1153). Following incubation with insulin, 10-µl aliquots (6 µg of protein) of WGA-purified insulin receptor were combined with 15-µl aliquots of the peptides at twice the indicated concentrations. All reaction solutions contained 5 mM $MnCl_2$. Autophosphorylation reactions were initiated with 5 µl of 150 µM [γ-^{32}P]ATP, allowed to proceed for 2 min at 22 °C, and terminated with 30 µl of 2-fold concentrated $NaDodSO_4$-PAGE sample buffer (18).

The EGF Receptor Kinase—Vesiculated membranes from A431 cells were prepared as described by Lin *et al.* (19), solubilized in 0.5% Triton X-100, diluted 5-fold with 50 mM HEPES buffer, and incubated with 100 nM EGF for 1 h at 4 °C. Receptor and substrate phosphorylation reactions were conducted with 50 µM ATP (final concentration); all other conditions were identical to those described above for the insulin receptor.

pp60$^{v\text{-}src}$ Kinase Reactions—Purified pp60$^{v\text{-}src}$, isolated from cells transfected with the Rous sarcoma virus, was the generous gift of R. L. Erickson, Harvard University, Boston, MA. Phosphorylation reactions were conducted with 10 µM ATP in the presence of 10 mM $MnCl_2$. Additional conditions were as described above, without addition of either insulin or EGF.

Substrate Kinase Assay—The peptides (Table I) were prepared by solid-phase peptide synthesis and purified by reverse-phase high performance liquid chromatography. 4-Methoxyphenylalanine (*N-tert*-butoxycarbonyl-*O*-methyltyrosine) was purchased from Bachem. Desired compositions were verified by amino acid analysis. Phosphorylation reactions (30 µl) were allowed to proceed for 2 min, terminated with 50 µl of 5% trichloroacetic acid and 20 µl of 1% bovine serum albumin, and the mixtures were incubated on ice for 30 min. Mixtures were centrifuged for 5 min at 8000 × *G*, and aliquots of the supernatant solutions were spotted onto 2-cm^2 pieces of phosphocellulose paper (Whatman, P81). The papers were washed extensively in 75 mM phosphoric acid, rinsed briefly with acetone, and allowed to air dry (20, 21). Incorporation was determined by Cerenkov radiation or scintillation counting.

Photoaffinity Labeling with 8-N_3-[α-^{32}P]ATP—Each of the kinases were incubated at 4 °C for 30 min in the presence and absence of 10 mM [Tyr]peptide 1142–1153 and [Mpa]peptide 1142–1153 prior to addition of the ATP analog. 8-N_3-[α-^{32}P]ATP (ICN Radiochemicals, 320 µl, 170 µM, 250 µCi) was dried *in vacuo*, dissolved in 25 µl of 50 mM HEPES buffer, pH 7.4, and 5-µl aliquots (50 µCi) were added to 50 µl of each kinase/substrate mixture, all in the dark. The ATP analog (100 µM final concentration) was photoactivated by irradiation at a distance of 6 cm from the 254 nm ultraviolet source (0.16 A, Model UVG-54, Ultraviolet Products, Inc., San Gabriel, CA) for 5 min at 4 °C; control samples were kept in the dark. All samples were maintained at 4 °C and diluted to a final volume of 200 µl with 50 mM HEPES, 0.1% Triton X-100, 0.1% bovine serum albumin, pH 7.4. Kinases were immunoprecipitated following incubation with specific polyclonal antibodies for 24 h and Pansorbin (Behring Diagnostics) for an additional 30 min, all at 4 °C in the dark. Precipitated forms were washed vigorously with excess buffer and the photoaffinity labeled proteins were separated by $NaDodSO_4$-PAGE on 7.5% resolving gels.

Polyacrylamide Gel Electrophoresis—Autophosphorylation reactions were terminated by addition of sample buffer containing 100 mM dithiothreitol and boiled for 5 min (18). To prevent possible artifacts related to variable amounts of the peptides in the gels, peptides were added to all samples to give an equivalent amount in the final sample for $NaDodSO_4$-PAGE. Phosphorylated proteins were separated on 7.5% $NaDodSO_4$-polyacrylamide resolving gels, their positions were determined by autoradiography of the fixed and dried gels, and incorporation was determined by Cerenkov counting of excised fragments.

RESULTS

The insulin and EGF receptors undergo autophosphorylation on tyrosine residues in response to ligand stimulation, while the gene product of the Rous sarcoma virus, pp60$^{v\text{-}src}$, is autophosphorylated on tyrosine in the absence of ligand. Each of these autokinases have been shown to catalyze phosphorylation of additional natural and synthetic polypeptides on tyrosine residues (1, 2). For the insulin receptor autophosphorylation occurs within at least two domains, the carboxyl terminus and a cluster of tyrosine residues at positions 1146, 1150, and 1151 (16, 22–26). A synthetic peptide with a sequence corresponding to this region of the receptor, referred to as [Tyr]peptide 1142–1153 (Table I), is phosphorylated by the insulin receptor (Fig. 1 and Refs. 15 and 16). When the insulin receptor, the EGF receptor, and pp60$^{v\text{-}src}$ were autophosphorylated prior to addition of this substrate, saturable Michaelis-Menten type kinetics for peptide phosphorylations were observed (Fig. 1, *solid lines*). Values for K_m ranged from 0.15 to 2.0 mM (Table II).[3]

Inhibition of substrate phosphorylation at high substrate concentrations has previously been shown for the insulin (27, 28) and EGF (5) receptors. To determine whether substrate inhibition occurs with [Tyr]peptide 1142–1153 the kinases and varying concentrations of substrate were combined prior to ATP addition, and the results obtained were compared to those following kinase pre-phosphorylation. When the ligand-stimulated receptors and pp60$^{v\text{-}src}$ were not pre-phosphorylated, a biphasic curve for peptide phosphorylation was observed (Fig. 1, *dashed lines*). At lower substrate concentrations ($<K_m$) substrate phosphorylation increased with increasing concentration. However, at higher peptide concentrations the amount of peptide phosphorylation actually decreased. Values of K_m and $K_{i(app)}$ for substrate inhibition of substrate phosphorylation are compared in Table II.

To probe the mechanism of substrate inhibition the effect of the substrate on kinase autophosphorylation was examined. Phosphorylations of the insulin receptor, EGF receptor, and pp60$^{v\text{-}src}$ were inhibited in the presence of increasing concentrations of [Tyr]peptide 1142–1153 (Fig. 2, *solid lines*). Values of $K_{i(app)}$ for substrate inhibition of kinase autophosphorylation, determined as the substrate concentration that resulted in half-maximal autophosphorylation, ranged from 0.4 to 3 mM (Table II). Based on these findings, we hypothesized that

[3] All phosphorylations with insulin and EGF receptors were conducted in the presence of saturating concentrations of insulin and EGF, respectively.

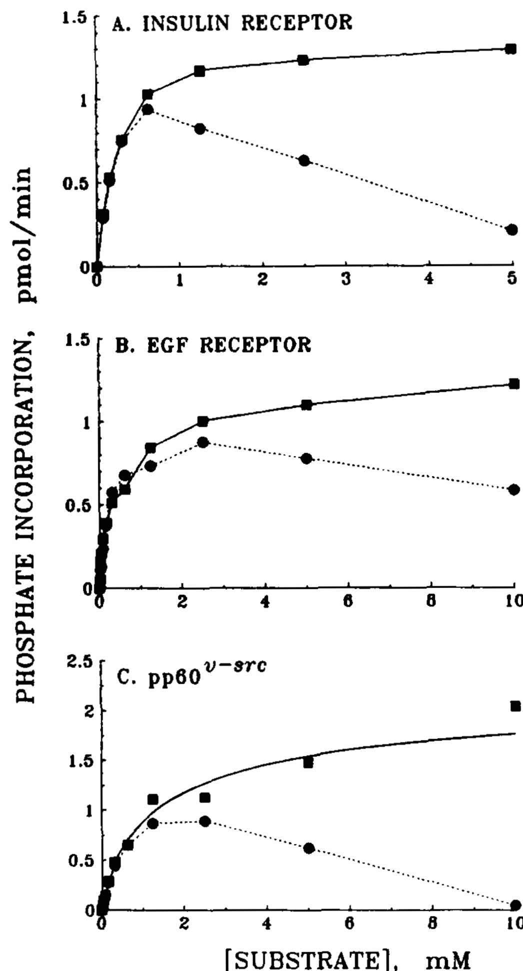

FIG. 1. **Insulin receptor-, EGF receptor-, and pp60^{v-src}-catalyzed substrate phosphorylation.** Substrate ([Tyr]peptide 1142–1153) phosphorylations were catalyzed by the insulin receptor (*A*), the EGF receptor (*B*), and pp60^{v-src} (*C*). In each case the kinases were either pre-phosphorylated (■) or unphosphorylated (●) prior to combination with the substrate and ATP. Obvious saturation was observed in additional experiments for substrate phosphorylation catalyzed by pre-phosphorylated pp60^{v-src}.

substrate inhibition of substrate phosphorylation resulted directly from substrate inhibition of kinase autophosphorylation.

Although depletion of ATP has been proposed as the mechanism of substrate inhibition (5), linear initial velocities are observed during all 2- or 3-min phosphorylation reactions under conditions described here. Thus, it seemed that simple depletion of ATP could not account for the observed inhibition. At high concentrations, however, substrates could bind to the catalytic active site of the kinase and block autophosphorylation. To test this nonphosphorylatable peptide analogs were designed to function as inhibitors of autophosphorylation. Using the substrate [Tyr]peptide 1142–1153 as prototype, analogs were prepared in which all 3 tyrosine residues within the sequence were replaced either by phenylalanine or 4-methoxyphenylalanine (Table I), residues with structural similarities to tyrosine but unable to accept kinase-catalyzed phosphoryl transfer from ATP.[4]

As expected, neither substrate analog was phosphorylated by any of the kinases (data not shown). Both of the nonphosphorylatable analogs were inhibitors of insulin and EGF receptor autophosphorylation, however, with potencies for inhibition nearly identical to those of the parent peptide (Fig. 2, *A* and *B*, Table II). By contrast, pp60^{v-src} autophosphorylation was not inhibited by the 4-methoxyphenylalanine-substituted analog over the entire concentration range tested and was inhibited by the phenylalanine-substituted analog only at high concentrations (Fig. 2*C* and Table II). Limited solubility prevented use of the peptides at higher concentrations than those shown.

Because the nonphosphorylatable substrate analogs were inhibitors of autophosphorylation they could be used in conjunction with substrates to probe the relationship of autophosphorylation to kinase activation further. In each case the analogs inhibited substrate phosphorylations whether the kinases had been pre-phosphorylated or not (Table II). Data for EGF receptor-catalyzed substrate phosphorylations, conducted in the presence of varying concentrations of [Mpa] peptide 1142–1153, are shown in Fig. 3. When the receptor was pre-phosphorylated the Mpa analog was a weak inhibitor of substrate phosphorylation (Fig. 3*A*). Double-reciprocal plots were linear and intersected at the ordinate, suggesting that inhibition was competitive (Fig. 3*C*). By contrast, inhibition of substrate phosphorylation was much more striking when the EGF receptor had not been pre-phosphorylated (compare Fig. 3, *A* and *B*). In this case the double-reciprocal plot did not intersect at the ordinate (Fig. 3*D*) and the data were best approximated by a mixed inhibitory mechanism

TABLE II

K_m and $K_{i(app)}$ values for substrate phosphorylation reactions catalyzed by the kinases

Data were analyzed using the nonlinear least squares method described by Cleland (29) and are expressed in millimolar units ± SE.

	Insulin receptor	EGF receptor	pp60^{v-src}
Substrate phosphorylation[a]			
Pre-phosphorylated, K_m (mM)	0.15 ± 0.07	0.3 ± 0.05	2.0 ± 0.7
Nonphosphorylated, K_i (mM)	0.7 ± 0.3	16 ± 4	1.7 ± 0.6
Values of $K_{i(app)}$ for peptide inhibition of kinase phosphorylation (mM)[b]			
[Tyr]Peptide 1142–1153	1.0	3.0	0.4
[Phe]Peptide 1142–1153	2.0	2.5	5
[Mpa]Peptide 1142–1153	1.3	5.0	>20
Values of K_i for analog inhibition of substrate phosphorylation (mM)[c]			
Pre-phosphorylated			
Competitive		14 ± 7.6	4.0 ± 0.8
Mixed, K_i (slope)	8.6 ± 2.4		
Mixed, K_i (intercept)	5.8 ± 2.4		
Nonphosphorylated			
Mixed, K_i (slope)	4.9 ± 2.9	0.7 ± 0.4	1.4 ± 0.5
Mixed, K_i (intercept)	2.6 ± 0.4	1.8 ± 1.2	3.0 ± 2.1

[a] K_m values were from substrate phosphorylation experiments with pre-phosphorylated kinases; values of K_i were from similar experiments with nonphosphorylated kinases (Fig. 1).

[b] $K_{i(app)}$ values for inhibition of kinase phosphorylation by the substrate and nonphosphorylatable analogs were determined as the peptide concentration yielding half-maximal autophosphorylation (Fig. 2).

[c] Phosphorylations of [Tyr]peptide 1142–1153 (substrate) were conducted in the presence of varying concentrations of nonphosphorylatable substrate analog ([Mpa]peptide 1142–1153 with the insulin and EGF receptors, [Phe]peptide 1142–1153 with pp60^{v-src}) as shown in Fig. 3. Data were analyzed statistically (29) and by inspection; all statistical analyses are presented.

[4] Phenylalanine and 3-methoxyphenylalanine both contain β-phenyl groups, but differ from tyrosine at the *para* position of the ring. 3-Methoxyphenylalanine contains a methyl ether in place of the phenolic hydroxyl whereas phenylalanine is unsubstituted.

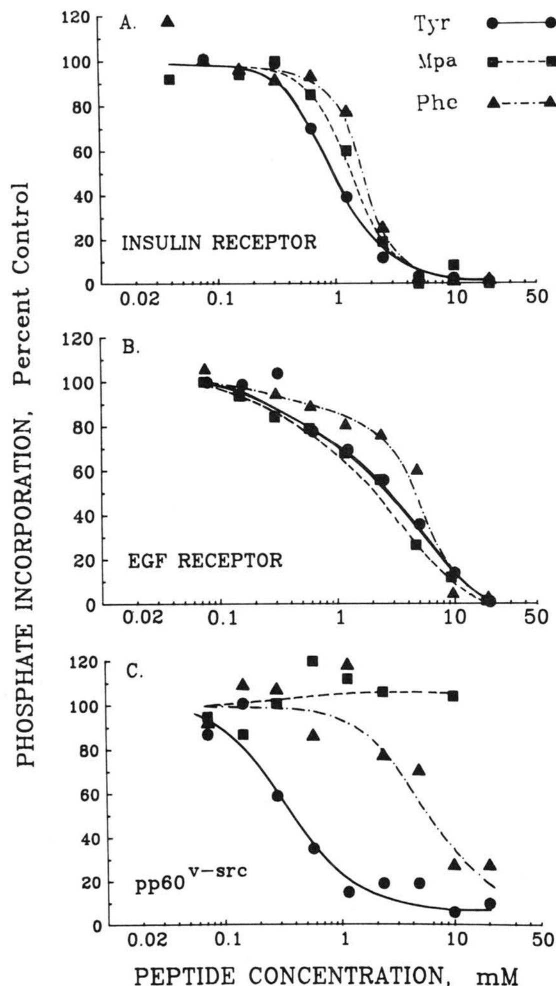

FIG. 2. **Inhibition of insulin receptor, EGF receptor, and pp60$^{v\text{-}src}$ autophosphorylation by the substrate and nonphosphorylatable substrate analogs.** Autophosphorylations of the insulin receptor (*A*), the EGF receptor (*B*), and pp60$^{v\text{-}src}$ (*C*) were conducted in the presence of varying concentrations of substrate (●, [Tyr]peptide 1142–1153) or analogs of the substrate substituted at tyrosine positions by either phenylalanine (▲, [Phe]peptide 1142–1153) or 4-methoxyphenylalanine (■, [Mpa]peptide 1142–1153). Autophosphorylation reactions were terminated with NaDodSO$_4$-PAGE sample buffer and phosphorylated proteins were separated on 7.5% polyacrylamide gels under reducing conditions (18). Positions of phosphorylated proteins were determined by autoradiography and quantified either by Cerenkov counting of excised gel fragments or scanning of the autoradiograms. The amount of autophosphorylation is displayed as a percentage of maximal incorporation.

(containing competitive (slope effect) and uncompetitive (intercept effect) components) (30–32). The value of $K_{i(\text{app})}$ for inhibition of phosphorylation catalyzed by the pre-phosphorylated EGF receptor was 14 mM; slope and intercept components to $K_{i(\text{app})}$ for inhibition of the nonphosphorylated receptor were 0.7 and 1.8 mM, respectively (Table II).

Similar experiments were performed with pre-phosphorylated and nonphosphorylated insulin receptor and pp60$^{v\text{-}src}$ preparations. In each case the analogs inhibited substrate phosphorylations more potently when the kinases had not been pre-phosphorylated, although the differences were less striking than for the EGF receptor (Table II). For pre-phosphorylated pp60$^{v\text{-}src}$ (like the EGF receptor) inhibition appeared to be competitive; data were better approximated by a mixed mechanism for the pre-phosphorylated insulin receptor. Inhibition was of the mixed type when the kinases had not been pre-phosphorylated. Autophosphorylation of the

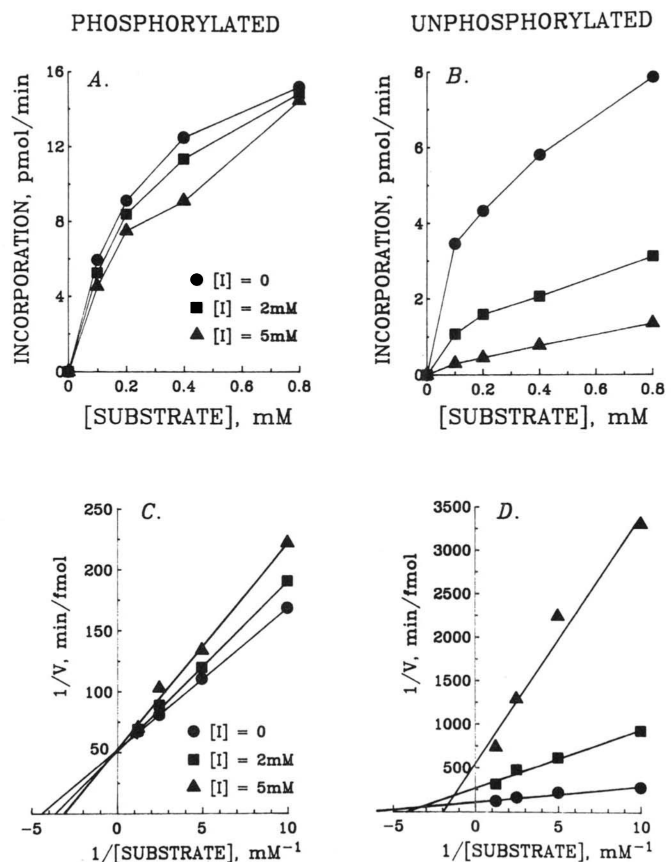

FIG. 3. **Inhibition of the EGF receptor kinase by the Mpa-substituted substrate analog.** *A* shows the effect of 0 (●), 2 mM (■), and 5 mM (▲) inhibitor (Mpa analog, Table I) on pre-phosphorylated EGF receptor-catalyzed substrate ([Tyr]peptide 1142–1153) phosphorylations. *B*, the EGF receptor was not phosphorylated prior to substrate phosphorylations. *C* and *D*, data from *A* and *B*, respectively, replotted as double reciprocals. Final concentrations of all reactants were identical for reactions with pre-phosphorylated and unphosphorylated receptor. Reactions were conducted for 2.0 min (over which period they were linear) and terminated by adding 50 µl of 5% trichloroacetic acid. Phosphate incorporation was determined by the phosphocellulose adsorption method (20, 21).

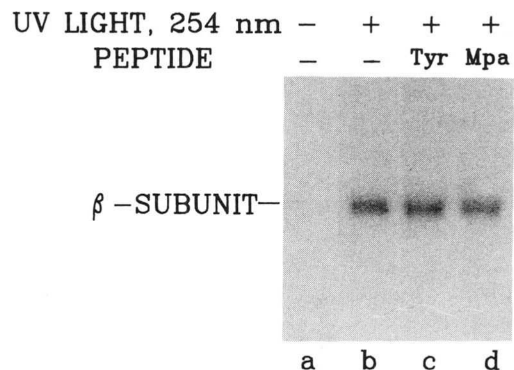

FIG. 4. **Photoaffinity labeling of the insulin receptor with 8-N$_3$-[α-^{32}P]ATP in the presence of [Tyr]peptides 1142–1153 and [Mpa]peptide 1142–1153.** WGA-purified insulin receptor was incubated without peptides (*lanes a* and *b*) or with 10 mM [Tyr] peptide 1142–1153 (*lane c*) or 10 mM [Mpa]peptide 1142–1153 (*lane d*) prior to addition of 50 µCi of 8-N$_3$-[α-^{32}P]ATP. Immediately after addition of the photoactivatable ATP analog samples were either exposed to ultraviolet irradiation (254 nm) for 5 min (*lanes b–d*) or kept in the dark (*lane a*). All samples were then immunoprecipitated and separated by gel electrophoresis.

kinases occurs during substrate phosphorylation reactions catalyzed by nonphosphorylated kinases, however. As a result each kinase may be present in multiple activation states whose concentrations are changing during the time course of the reaction. Therefore, the mechanism of inhibition may be more complex for nonphosphorylated kinases and uninterpretable by classic means. Inhibition is greater (K_i is lower) when the kinases are nonphosphorylated, however, suggesting that inhibition of autophosphorylation blocks kinase activation, and this in turn inhibits substrate phosphorylation.

The catalytic domains for each of the kinases can be separated into sites for ATP binding and substrate binding. Substrate (or nonphosphorylatable analog) binding might block ATP binding and thereby prevent autophosphorylation. On the other hand, substrate binding may prevent autophosphorylation by blocking the transfer of phosphate from bound ATP to tyrosine residues within the substrate binding site. To distinguish between these possibilities the kinases were reacted with the photoaffinity ATP analog 8-N_3-[α-^{32}P]ATP in the presence of [Tyr]peptide 1142–1153 and [Mpa]peptide 1142–1153. Cross-linking of the ATP analog to the insulin receptor was not inhibited by either of these peptides (Fig. 4), indicating that ATP binding to the catalytic domain was not appreciably affected by substrate binding. We have not yet been able to isolate photoaffinity labeled forms of the EGF receptor or pp60$^{v\text{-}src}$.

DISCUSSION

Based upon the similarities exhibited by the kinases for (*a*) substrate inhibition of substrate phosphorylation, (*b*) substrate inhibition of receptor autophosphorylation, (*c*) inhibition of kinase autophosphorylation by the nonphosphorylatable analogs, and (*d*) inhibition of substrate phosphorylation by the nonphosphorylatable analogs, we propose a generalized mechanism for activation of these three tyrosine kinases (Fig. 5). In this mechanism, autophosphorylation of tyrosine residues within or surrounding the catalytic active site enhances the rate of substrate phosphorylation by the kinase. Binding of substrate blocks autophosphorylation, and this in turn inhibits kinase activation. In a similar fashion, binding of a nonphosphorylatable substrate analog blocks autophosphorylation and prevents kinase activation.

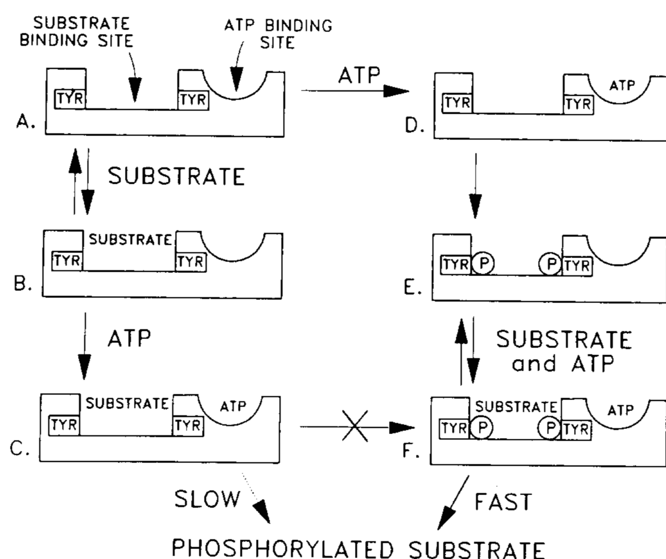

FIG. 5. **Model for tyrosine kinase catalytic domains.** The *semicircular site* represents the ATP binding domain and the *rectangular site* represents the substrate binding pocket (*A*). One or more tyrosine residues are located near the substrate binding region. Substrate binding (*B*) prior to ATP binding (*C*) blocks ATP-dependent autophosphorylation (*F*). On the other hand, when ATP binding (*D*) precedes substrate binding kinase autophosphorylation (*E*) freely occurs. Substrate binding to the autophosphorylated (activated) kinase (*F*) results in an enhanced rate of substrate phosphorylation.

While this simplified mechanism explains most of our data some aspects are as yet incompletely understood. For example, from the model one would expect similar values of $K_{i(\mathrm{app})}$ for substrate or analog inhibition of substrate versus kinase phosphorylation. The differences (*e.g.* compare K_i values for substrate inhibition of EGF receptor and pp60$^{v\text{-}src}$ catalyzed substrate phosphorylation and autophosphorylation, and nonphosphorylatable analog inhibition of kinase *versus* substrate phosphorylation in Table II) may be related either to (*a*) multiple kinase autophosphorylation sites with different K_i values for inhibition, (*b*) different methods used for determining K_i values, or (*c*) inherent differences in mechanism. Therefore, we are using the model as a framework for understanding activation of each of the kinases and we plan to build on this framework following additional studies.

Whether substrate binding precedes ATP binding as suggested for the EGF receptor (33), or the order is random as suggested for the insulin receptor (34), substrate (or analog) binding to the kinase active site prior to autophosphorylation inhibits kinase activation. For the insulin receptor we determined that substrate binding does not interfere with ATP binding (Fig. 4), and on this basis we concluded that bound substrate (or analog) prevents phosphoryl transfer from receptor-bound ATP to tyrosine residues within the substrate binding portion of the kinase active site. Unfortunately, this last point cannot yet be extrapolated to the EGF receptor or pp60$^{v\text{-}src}$.

For pp60$^{v\text{-}src}$, Tyr^{416} is the major site of autophosphorylation and the region surrounding this residue is thought to comprise the active site for substrate phosphorylation. Carboxyl-terminal (Tyr^{527}) phosphorylation also occurs in pp60$^{c\text{-}src}$ but this appears to be inhibitory rather than activating (35, 36). Tyrosine phosphorylation in the insulin receptor can also be separated into at least two major regions (34). The 1150 region (including tyrosines 1146, 1150, and 1151) of the insulin receptor is homologous with the region of pp60$^{v\text{-}src}$ surrounding Tyr^{416}. Multiple phosphorylations in the 1150 region activate the insulin receptor kinase *in vitro* and are necessary for insulin-mediated bioeffects *in vivo* (16, 22–26, 37–39). Phosphorylation also occurs at residues 1316 and 1322 in the carboxyl terminus of the insulin receptor and perhaps near the transmembrane domain, but the significance of phosphorylation at these sites is unclear (16, 22–26, 37). Data presented here suggest that Tyr^{416} of pp60$^{v\text{-}src}$ and tyrosine residues 1146, 1150, and 1151 of the insulin receptor are in spatial proximity to the substrate binding portions of the respective kinase catalytic active sites.

Sequencing data suggest that all phosphorylation sites in the EGF receptor lie in the carboxyl-terminal domain (Tyr^{1068}, Tyr^{1148}, and Tyr^{1173}) (40). However autophosphorylation sites remain following substitution of each of these tyrosine residues by phenylalanine, suggesting that additional tyrosine residues are located elsewhere in the molecule (41, 42). Based on sequence homologies and similar mechanisms of activation exhibited by these kinases (Ref. 5 and this study) we would predict that the region of the EGF receptor surrounding Tyr^{845} is catalytically involved in substrate phosphorylation and undergoes activation by autophosphorylation.

The physiologic relevance of substrate inhibition of tyrosine kinases is unknown. High concentrations of naturally occurring endogenous substrates could attenuate cellular kinase

activities, and this could be a mechanism for regulation of kinase action. We have now shown that nonphosphorylatable peptides or proteins with sequences related to substrates (pseudosubstrate sequences) could act specifically as kinase inhibitors. Such pseudosubstrate sequences may be found within endogenous inhibitors or tyrosine kinases themselves, as has been suggested for serine kinases (43, 44). In this regard a search for potential inhibitory domains within tyrosine kinase sequences may yield interesting information.

Despite the remarkable similarity of these tyrosine kinases regarding their mechanism of activation, one difference between the receptor kinases and pp60$^{v\text{-}src}$ is particularly noteworthy. For the EGF and insulin receptors, inhibition of autophosphorylation by the substrate and its nonphosphorylatable analogs showed nearly identical concentration dependencies. By contrast, the concentration of phenylalanine-substituted analog required to inhibit pp60$^{v\text{-}src}$ phosphorylation was 10–20-fold higher, and the 4-methoxyphenylalanine-substituted analog showed essentially no inhibition of pp60$^{v\text{-}src}$ autophosphorylation. These data suggest that the opportunity exists to develop differential inhibitors of receptor and oncogene product tyrosine kinases which could provide new therapeutic modalities in some neoplastic disorders, as well as in disorders of growth and metabolism.

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