Substrate Specificity and Kinetic Mechanism of Human Placental Insulin Receptor/Kinase

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Received August 6, 1986; Revised Manuscript Received October 17, 1986

ABSTRACT: The insulin receptor has been shown to be a protein kinase which phosphorylates its substrates on tyrosine residues. To examine the acceptor specificity of affinity-purified insulin receptor/kinase, hydroxyamino acid containing analogues of the synthetic peptide substrate Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly were prepared. Substitution of serine, threonine, or D-tyrosine for L-tyrosine completely ablated the acceptor activity of the synthetic peptides. These peptides, along with a phenylalanine-containing analogue, did serve as competitive inhibitors of the insulin receptor/kinase with apparent K_i values in the range of 2–4 mM. These data suggest that the insulin receptor/kinase is specific for tyrosine residues in its acceptor substrate and imply that serine phosphate or threonine phosphate present in receptor is due to phosphorylation by other protein kinases. The kinetics of the phosphorylation of the L-tyrosine-containing peptide were examined by using prephosphorylated insulin receptor/kinase. Prephosphorylation of the receptor was necessary to maximally activate the kinase and to linearize the initial velocity of the peptide phosphorylation reaction. The data obtained rule out a ping-pong mechanism and are consistent with a random-order rapid-equilibrium mechanism for the phosphorylation of this peptide substrate. Additional experiments demonstrated that the autophosphorylated insulin receptor was not able to transfer the preincorporated phosphate to the synthetic peptide substrate. Thus, the insulin receptor/kinase catalyzes the reaction via a mechanism that does not involve transfer of phosphate from a phosphotyrosine-containing enzyme intermediate.

Insulin has been shown to elicit its numerous biological effects by interacting with a specific cell-surface receptor [for a review, see Goldfine (1981)]. Work from a number of laboratories has demonstrated that the insulin receptor is a tetramer composed of two α subunits with a molecular weight of about 135 000 and two β subunits with a molecular weight of about 95 000. The tetrameric structure is held together by disulfide bonds (Jacobs et al., 1979; Massagué et al., 1980; Pilch & Czech, 1980).

Kasuga et al. (1982a) first demonstrated that the insulin receptor was associated with a tyrosine protein kinase activity. Since that time, a number of approaches have been utilized to demonstrate that the insulin receptor is itself the tyrosine protein kinase. We (Pike et al., 1986) and others (Petruzelli et al., 1984) have shown that insulin-stimulated kinase activity and insulin binding activity copurify following chromatography on wheat germ lectin–agarose and insulin–agarose columns. In addition, the kinase can be precipitated with antibodies directed against the insulin receptor (Kasuga et al., 1983; Zick et al., 1983). Furthermore, the 95-kilodalton (kDa)[1] subunit of the insulin receptor can be affinity labeled with azido-ATP (Roth & Cassel, 1983; Shia & Pilch, 1983) or oxidized ATP (Van Obberghen et al., 1983), indicating that the receptor possesses a binding site for this nucleotide as would be expected if it were a protein kinase. Finally, a cDNA for the insulin receptor has been cloned, and the translated amino acid sequence indicates that the insulin receptor is closely related to the EGF receptor and pp60^{src}, known protein tyrosine kinases, and is also homologous with protein serine kinases, including the cAMP-dependent protein kinase (Ullrich et al., 1985).

Until recently, most studies of the insulin receptor have focused on its capacity to recognize and bind insulin and have not examined the enzymatic properties of the kinase activity in detail. For example, the kinase is known to phosphorylate tyrosine residues which are preceded by acidic amino acids (Stadtmauer & Rosen, 1983, 1986), but the specificity of the enzyme for the hydroxyamino acid acceptor has not been established. Furthermore, it has been shown that the insulin receptor/kinase is autophosphorylated and that this autophosphorylation stimulates the tyrosine kinase activity of the receptor (Rosen et al., 1983). However, no kinetic analyses have been performed to determine whether autophosphorylation produces a required intermediate for the phosphorylation of exogenous substrates. Because the tyrosine kinase activity of this receptor probably mediates many of the intracellular effects of insulin, a clarification of the properties of this enzyme is necessary to the understanding of the process of signal transduction.

In this report, we use synthetic peptides to demonstrate that affinity-purified insulin receptor/kinase shows an essentially absolute specificity for tyrosine as the phosphate acceptor. We also present data suggesting that the phosphorylation of peptide substrates occurs via a random-order rapid-equilibrium mechanism and rule out the possibility that an autophosphorylated tyrosine serves as a phosphate donor in the phosphorylation of exogenous substrates.

Experimental Procedures

Materials. Affinity-purified insulin receptors were prepared as described by Pike et al. (1986). Porcine insulin was pur-

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[1] Abbreviations: App(NH)p, 5′-adenylyl imidodiphosphate; EGF, epidermal growth factor; kDa, kilodalton(s); DTT, dithiothreitol.

0006-2960/87/0426-1428$01.50/0

chased from Sigma. [γ-^{32}P]ATP was from New England Nuclear. App(NH)p was purchased from Boehringer Mannheim. Phosphocellulose paper was from Whatman. The substrate peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Tyr*-Ala-Ala-Arg-Gly (L-tyrosine peptide) and the analogue peptides Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Ser*-Ala-Ala-Arg-Gly (serine peptide), Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Thr*-Ala-Ala-Arg-Gly (threonine peptide), Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-D-*Tyr*-Ala-Ala-Arg-Gly (D-tyrosine peptide), and Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Phe*-Ala-Ala-Arg-Gly (phenylalanine peptide) were synthesized on an Applied Biosystems peptide synthesizer and purified by ion-exchange and reverse-phase chromatography by high-pressure liquid chromatography. The term "peptide" will be used to indicate the L-tyrosine-containing peptide, unless otherwise stated.

Peptide Phosphorylation Assays. Insulin receptor/kinase assays were performed in a total volume of 18 μL and contained (in final concentrations) 20 mM imidazole, pH 7.2, 85 mM NaCl, 5% glycerol, 0.05% Triton X-100, 200 μM ATP, [γ-^{32}P]ATP [(2–4) × 10^6 cpm/assay, ~3000 Ci/mmol], 12 mM $MgCl_2$, 2 mM $MnCl_2$, 100 μM sodium orthovanadate, 20 mM *p*-nitrophenyl phosphate, 2 mM DTT, 2 mM peptide (when used), 30 nM insulin (when used), and 25–50 ng of affinity-purified insulin receptor. Assays were started by the addition of a 3-μL aliquot of ATP mix which contained unlabeled ATP, [γ-^{32}P]ATP, metal ions, *p*-nitrophenyl phosphate, and sodium orthovanadate. Due to possible redox reactions between the DTT, Mn^{2+}, and vanadate, the exact concentrations of these reagents may change over the course of the assay. Assays were incubated for 15 min at 30 °C, and the reactions were stopped by the addition of 30 μL of 30% acetic acid. A 32-μL aliquot of the stopped reaction mixture was spotted on a square of phosphocellulose paper and washed 3 times for 2 min in 75 mM phosphoric acid. The papers were then dried, placed into vials with 5 mL of scintillation cocktail, and counted for ^{32}P in an LKB 1217 Rackbeta liquid scintillation counter. Background counts due to nonspecific retention of ^{32}P on the filter paper were corrected for by subtracting the cpm bound to phosphocellulose paper in assays performed in the absence of peptide from the cpm bound in the assays where peptide was included.

In assays where the receptor/kinase was prephosphorylated, affinity-purified insulin receptors were incubated with all assay components, except for peptide and [γ-^{32}P]ATP, for 90 min at 4 °C. Peptide phosphorylation assays were started by the addition of a 3-μL aliquot of peptide and [γ-^{32}P]ATP and carried out as described above.

Data Analysis. Modeling of the kinetic data to and error analysis of both the linearized and nonlinearized versions of the Michaelis–Menten equation were performed by using the fit function routine of RS1 (BBN Software Products Corp.) run on a VAX 11/785.

Results

Hydroxyamino Acid Specificity of the Insulin Receptor/Kinase. The synthetic peptide Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly has previously been shown to be a substrate for the insulin receptor kinase (Stadtmauer & Rosen, 1983; Pike et al., 1984). To determine the acceptor amino acid specificity of the insulin receptor/kinase, hydroxyamino acid substituted analogues of the L-tyrosine peptide were synthesized and tested as substrates in the insulin receptor/kinase assay. Table I shows a comparison of the phosphate acceptor activity of the analogue peptides, both in the presence and in the absence of added insulin. The L-tyrosine peptide was a good phosphate acceptor, and insulin stimulated an increase in ^{32}P incorporation into the peptide. The other hydroxyamino acid containing peptides showed no incorporation of ^{32}P, above control assays performed in the absence of peptide.

Table I: Use of Hydroxyamino Acid Containing Peptides as Substrates for the Insulin Receptor/Kinase[a]

peptide	cpm of ^{32}P incorporated into peptide	
	−insulin	+insulin
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Tyr*-Ala-Ala-Arg-Gly	67630 ± 4260	85707 ± 831
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Ser*-Ala-Ala-Arg-Gly	2228 ± 283	2415 ± 470
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Thr*-Ala-Ala-Arg-Gly	2532 ± 228	2326 ± 264
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-D-*Tyr*-Ala-Ala-Arg-Gly	2864 ± 146	2773 ± 317
background	2787 ± 17	2796 ± 194

[a] The phosphorylation of the peptides by affinity-purified insulin receptor/kinase was examined at a peptide concentration of 2 mM. The values represent the mean ± standard deviation of triplicate determinations. Background values represent the results of assays done in the absence of added peptide.

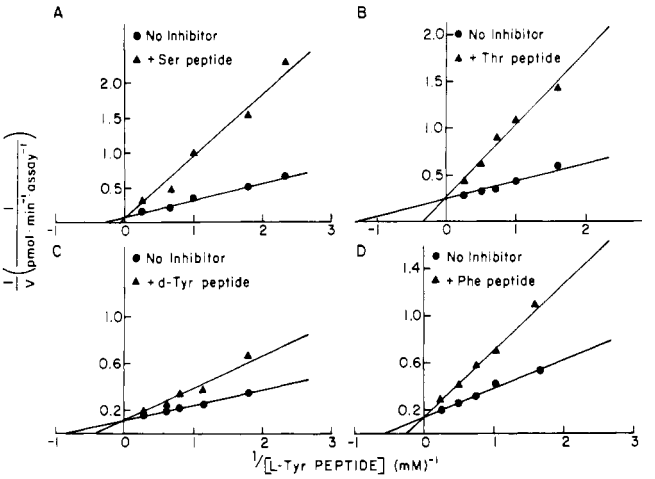

FIGURE 1: Lineweaver–Burk plots of the inhibition of insulin receptor/kinase activity by peptide analogues. Insulin receptor/kinase activity was assayed by using varying concentrations of L-tyrosine peptide in the presence or absence of a fixed concentration of the different peptide analogues. Values represent the mean of triplicate determinations. Each analogue was analyzed in a separate experiment. Panel A, activity in the absence or presence of 6 mM serine peptide. Panel B, activity in the absence or presence of 9 mM threonine peptide. Panel C, activity in the absence or presence of 4 mM D-tyrosine peptide. Panel D, activity in the absence or presence of 3 mM phenylalanine peptide.

Competitive Inhibitors of the Insulin Receptor/Kinase. Since the peptide analogues did not appear to act as substrates for the insulin receptor/kinase, the ability of the hydroxyamino acid substituted analogues, as well as a phenylalanine-substituted analogue, to act as inhibitors of the kinase was investigated. Figure 1 shows the Lineweaver–Burk plots for the inhibition of the insulin receptor/kinase by the peptide analogues. In each case, the plots are linear and intersect on the *y* axis, indicating that all of the peptide analogues are competitive inhibitors with respect to the L-tyrosine peptide. The apparent K_i values of the inhibitors were calculated from the Lineweaver–Burk plots and are given in Table II. All of the apparent K_i values are in the range of 2–4 mM. These values are close to the apparent K_m value of the L-tyrosine peptide, which was determined to be 2 mM.

The above experiments were carried out with insulin receptor/kinase that had not been prephosphorylated prior to

Table II: Apparent K_i Values of Peptide Inhibitors of the Insulin Receptor/Kinase[a]

inhibitor	K_i (mM)
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Ser*-Ala-Ala-Arg-Gly	2.2 ± 0.1
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Thr*-Ala-Ala-Arg-Gly	3.6 ± 0.3
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-D-*Tyr*-Ala-Ala-Arg-Gly	3.5 ± 0.1
Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-*Phe*-Ala-Ala-Arg-Gly	3.4 ± 0.1

[a] Apparent K_i values for the inhibition of insulin receptor/kinase activity by peptide analogues with respect to the L-tyrosine-containing peptide were calculated from Lineweaver–Burk plots. All assays were performed in duplicate. The values represent the mean ± the standard error from three separate experiments.

Table III: Φ Parameters for Phosphorylation of the Synthetic Peptide by the Insulin Receptor/Kinase[a]

Φ parameter	value	units
Φ_0	0.53 ± 0.01	$pmol^{-1}$·min·assay
Φ_1	0.23 ± 0.005	mM·$pmol^{-1}$·min·assay
Φ_2	8.63 ± 0.21	μM·$pmol^{-1}$·min·assay
Φ_{12}	3.45 ± 0.13	μM·mM·$pmol^{-1}$·min·assay

[a] Φ parameter values were calculated from the secondary plots of initial rate data in Figure 2. Φ_0 is the intercept of the plot of primary intercepts vs. 1/[ATP]. Φ_2 is the slope of this plot. Φ_1 is the intercept of the plot of primary slopes vs. 1/[ATP], and Φ_{12} is the slope of this plot. Values and standard deviations were determined by linear regression analyses of the lines in Figure 2B,C.

the addition of the peptide substrate. To determine the effects of insulin receptor autophosphorylation on the K_m of the L-tyrosine peptide, initial rate studies using prephosphorylated insulin receptor/kinase were performed. The insulin receptors were phosphorylated for 90 min at 4 °C prior to the addition of peptide. This protocol resulted in maximal activation of the insulin receptor/kinase with respect to its ability to phosphorylate the L-tyrosine peptide in an insulin-independent fashion (data not shown). Under these conditions, the initial velocity of the reaction is linear (Rosen et al., 1983; Pike et al., 1986), and the L-tyrosine peptide is the only substrate for the insulin receptor/kinase in the subsequent assay. The kinetic parameters obtained from these experiments therefore do not include any contribution from the autophosphorylation reaction. Under these circumstances, the K_m for the L-tyrosine peptide was found to be 420 μM.

To simplify the interpretation of the initial rate and inhibitor studies of the insulin receptor/kinase, all subsequent experiments were carried out with prephosphorylated receptors.

Kinetic Mechanism of the Insulin Receptor/Kinase. There are several general equations describing two substrate kinetic mechanisms. We chose to use the Dalziel Φ parameter nomenclature (Dalziel, 1957), which takes the general form:

$$e/V = \Phi_0 + \Phi_1/A + \Phi_2/B + \Phi_{12}/AB \quad (1)$$

where e is the total enzyme concentration, V is the initial rate, and A and B are the initial substrate concentrations. The Φ parameter values can be determined graphically from secondary plots of the initial rate measurements and provide information on the kinetic mechanism utilized by the enzyme.

Figure 2A shows a Lineweaver–Burk plot of insulin receptor/kinase velocity as a function of L-tyrosine peptide concentration at five fixed concentrations of ATP. This analysis generates a series of intersecting lines which converge at the x axis. Panels B and C of Figure 2 show the secondary plots of the intercepts vs. 1/[ATP] and the slopes vs. 1/[ATP], respectively. The Φ parameter values were determined from these secondary plots and are given in Table III.

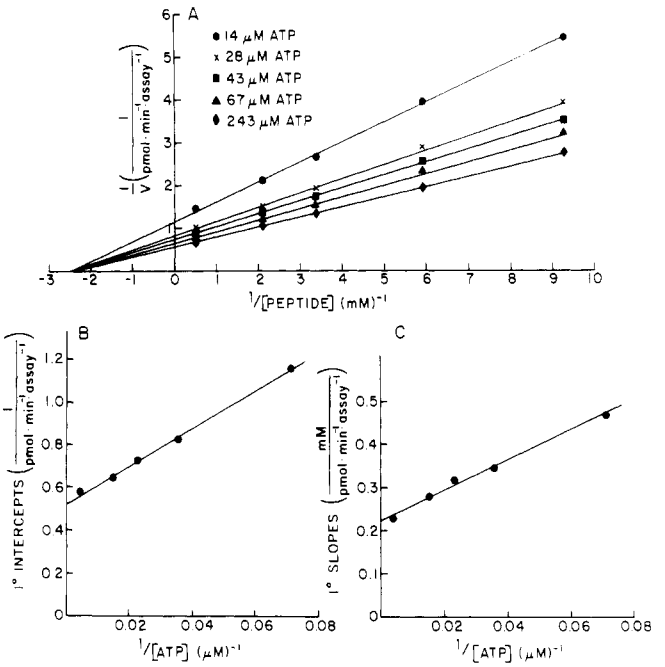

FIGURE 2: Lineweaver–Burk plot and secondary plots of insulin receptor/kinase activity as a function of L-tyrosine peptide. Insulin receptors were prephosphorylated with ATP concentrations ranging from 15 to 250 μM as described under Experimental Procedures. Peptide was added in concentrations from 0.1 to 2 mM. The concentration of ATP and peptide was determined spectrophotometrically on each dilution by using ϵ_{259} = 15 400 for ATP and ϵ_{276} = 1350 for tyrosine in the peptide. Each point represents the mean of triplicate determinations. Panel A, Lineweaver–Burk plot of $1/V$ vs. 1/[L-Tyr peptide]. Panel B, secondary plot of intercepts vs. 1/[ATP]. Panel C, secondary plot of slopes vs. 1/[ATP].

The Φ parameters may be used to calculate the values of V_{max} and the K_m for peptide and ATP at an infinite concentration of the second substrate.

$$V_{max} = 1/\Phi_0 = 1.9 \text{ pmol min}^{-1} \text{ assay}^{-1} = 76 \text{ nmol min}^{-1} \text{ (mg of protein)}^{-1} \quad (2)$$

$$K_m(\text{L-tyrosine peptide}) = \Phi_1/\Phi_0 = 420\ \mu M \quad (3)$$

$$K_m(\text{ATP}) = \Phi_2/\Phi_0 = 17\ \mu M \quad (4)$$

These K_m and V_{max} values were also determined by computer modeling of the data to the nonlinear form of the Michaelis–Menten equation. The values obtained from this analysis were in good agreement with the values determined by analysis of the linear reciprocal plots: V_{max} = 1.8 ± 0.04 pmol min^{-1} $assay^{-1}$; K_m(L-tyrosine peptide) = 412 ± 23 μM; K_m(ATP) = 15.6 ± 1.5 μM.

Inhibitor studies using analogues of both the donor and acceptor substrates were also performed. The serine peptide was used as the analogue inhibitor of the L-tyrosine peptide substrate, and App(NH)p was utilized as the inhibitor of ATP. The serine peptide was selected for use in these studies because it showed the best K_i value among the inhibitory peptides tested and it also exhibited the best solubility.

Figure 3 shows Lineweaver–Burk plots for the inhibition of insulin receptor/kinase activity by App(NH)p with respect to L-tyrosine peptide (Figure 3A) and ATP (Figure 3B). The Lineweaver–Burk plot for App(NH)p inhibition vs. the L-tyrosine-containing peptide shows straight lines which intersect on the x axis. This pattern is consistent with classical noncompetitive inhibition. Analysis of these data by computer modeling to the appropriate nonlinear form of the Michaelis–Menten equation yielded K' and V_{max} values that were

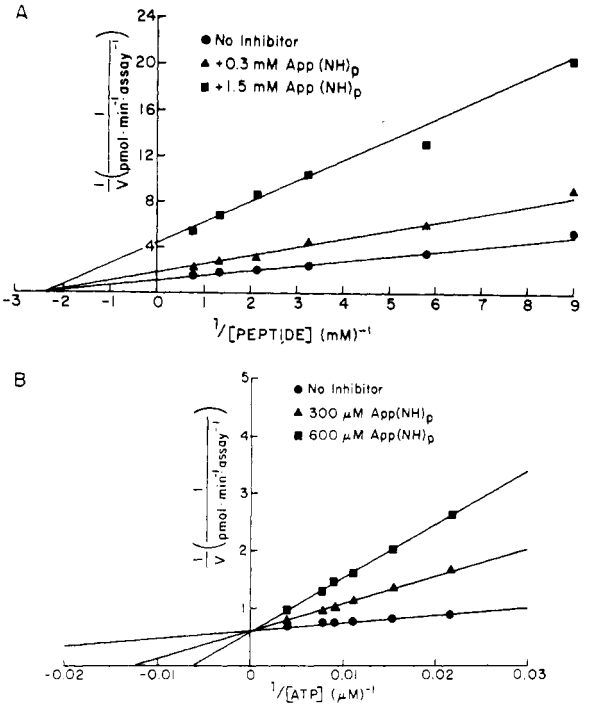

FIGURE 3: Lineweaver–Burk plots of the inhibition of insulin receptor/kinase activity by App(NH)p. Insulin receptors were prephosphorylated and peptide phosphorylation assays carried out as described under Experimental Procedures. Values represent the mean of triplicate determinations. Panel A, inhibition of the insulin receptor/kinase with respect to L-tyrosine peptide. Panel B, inhibition of the insulin receptor/kinase with respect to ATP.

consistent with the interpretation of noncompetitive inhibition [e.g., V_{max} values decreased significantly in the presence of App(NH)p while the K_m or K' values were not significantly different]. The Lineweaver–Burk plot for App(NH)p vs. ATP exhibits intersecting lines which converge on the y axis. This pattern is indicative of competitive inhibition and yields a K_i value of 100 ± 2 μM for App(NH)p. Computer fitting of these data to the nonlinear form of the Michaelis–Menten equation yielded values for the kinetic constants which were consistent with competitive inhibition and provided a value of 90 ± 8 μM for the K_i for App(NH)p.

Figure 4 shows Lineweaver–Burk plots of the inhibition of insulin receptor/kinase activity by the serine peptide with respect to ATP (Figure 4A) and L-tyrosine peptide (Figure 4B). The inhibition pattern exhibited by the serine peptide against ATP shows straight lines intersecting off the y axis and is consistent with mixed type or noncompetitive inhibition. As expected from previous experiments (Figure 1), the serine peptide exhibited strictly competitive inhibition when tested against the L-tyrosine peptide. Under these conditions, the K_i for the serine peptide was 512 ± 18 μM. The interpretation of these inhibition patterns was supported by the parameters obtained from computer modeling of the data to the nonlinear form of the Michaelis–Menten equation. The K_i for the serine peptide calculated from this analysis was 588 ± 36 μM.

To determine whether prephosphorylated insulin receptor/kinase could serve as a phosphate donor in a subsequent incubation with the L-tyrosine peptide, an aliquot of the kinase was autophosphorylated for 90 min at 4 °C, and the unreacted [^{32}P]ATP was removed by gel filtration. The ^{32}P-phoshorylated receptor/kinase was then incubated for 10 min at 30 °C in the absence of any additions or in the presence of the L-tyrosine peptide, unlabeled ATP, or both. The reaction mixture was then spotted on a cellulose thin-layer plate and analyzed by high-voltage electrophoresis. As shown in Figure

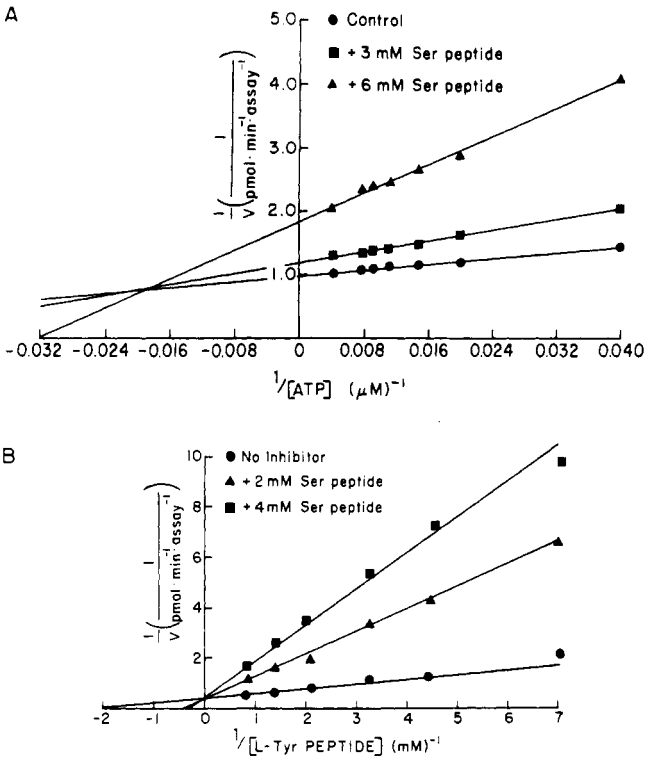

FIGURE 4: Lineweaver–Burk plots of the inhibition of insulin receptor/kinase activity by serine peptide. Insulin receptors were prephosphorylated and peptide phosphorylation assays carried out as described under Experimental Procedures. Values represent the mean of triplicate determinations. Panel A, inhibition of the insulin receptor/kinase with respect to ATP. Panel B, inhibition of the insulin receptor/kinase with respect to L-tyrosine peptide.

5, this protocol did not result in the production of ^{32}P-phosphopeptide under any of the conditions examined, suggesting that prephosphorylated receptor was incapable of transferring its $^{32}PO_4^{3-}$ to the L-tyrosine peptide. In a control incubation, the prephosphorylated insulin receptor/kinase was able to incorporate approximately 18 000 cpm of ^{32}P into the L-tyrosine peptide in 10 min at 30 °C under standard assay conditions, indicating that the kinase was still active after the gel filtration procedure.

The electrophoretic analysis detected some negatively charged contaminants of the insulin receptor/kinase preparation. These contaminants were determined to be ATP and PO_4^{3-} by further analysis in a thin-layer chromatography system. Since the presence of a small amount of [^{32}P]ATP would be expected to give a false positive result, this minor contamination does not alter the interpretation of the negative findings of the experiment shown in Figure 5.

Discussion

The L-tyrosine peptide Arg-Arg-Lys-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly has previously been shown to act as a substrate for the insulin receptor/kinase (Stadtmauer & Rosen, 1983; Pike et al., 1984). The data presented here demonstrate that when serine, threonine, or D-tyrosine is substituted for L-tyrosine in this synthetic peptide, these hydroxyamino acids are unable to act as phosphate acceptors for the insulin receptor/kinase. This suggests that the insulin receptor/kinase is specific for L-tyrosine at the site of phosphorylation. The selectivity of the insulin receptor/kinase for tyrosine residues makes it unlikely that the serine phosphate or threonine phosphate present in the receptor (Kasuga et al., 1982b) is the result of autophosphorylation. Instead, these phosphates are probably added by other protein kinases that

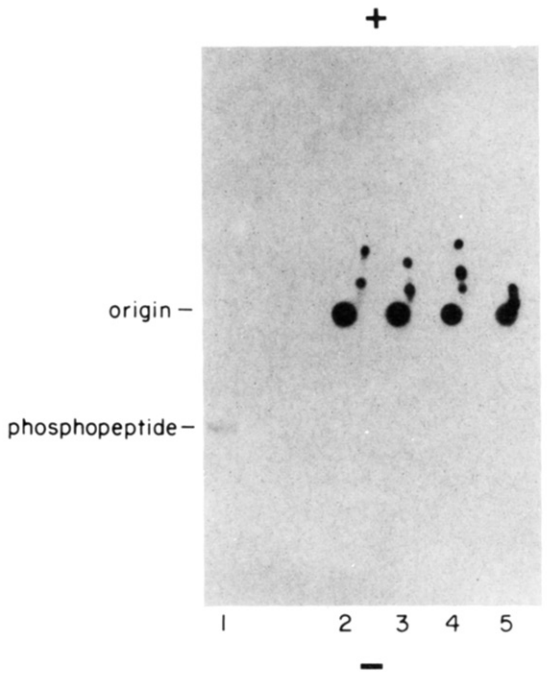

FIGURE 5: Autoradiogram of electrophoretic analysis of reaction mixtures containing prephosphorylated insulin receptor/kinase. Approximately 200 ng of insulin receptor/kinase was phosphorylated in the presence of 20 μM [^{32}P]ATP (50 000 cpm/pmol) for 90 min at 4 °C. The reaction mixture was then applied to a small Sephadex G150 column (0.7 × 5 cm) equilibrated in 40 mM imidazole, 250 mM NaCl, 10% glycerol, and 0.1% Triton X-100. The void volume was collected and used as a source of phosphorylated insulin receptor/kinase. This preparation was then incubated in the absence of additions (lane 2), in the presence of 2 mM L-tyrosine peptide (lane 3), in the presence of 200 μM unlabeled ATP (lane 4), or in the presence of both peptide and ATP (lane 5) for 10 min at 30 °C. The reactions were stopped by spotting on a cellulose thin-layer plate which was subjected to high-voltage electrophoresis at pH 1.9 for 30 min at 500 V. Lane 1 contained 400 cpm of phosphopeptide. Lanes 2–5 contained 2000 cpm of phosphorylated insulin receptor/kinase.

may regulate the function of the insulin receptor. Any effect of insulin on such phosphorylations is most likely due to hormone-induced changes in the conformation of the receptor.

Similar results were reported recently by Weinmaster and Pawson (1986), who used site-directed mutagenesis to examine the hydroxyamino acid specificity of the FSV P130$^{gag\text{-}fps}$ protein, a related protein tyrosine kinase encoded by a retroviral oncogene. In those studies, the tyrosine residue at the major site of autophosphorylation in P130$^{gag\text{-}fps}$ was changed to a serine, threonine, or glycine residue. The mutant proteins were unable to autophosphorylate this altered site. However, they also appeared to have reduced activity against an exogenous substrate, suggesting that the changes may have had an effect on the catalytic properties of the enzyme. The present studies in which analogues of a defined sequence were used to study the hydroxyamino acid specificity of the insulin receptor/kinase avoid the interpretive problems that can arise when the enzyme itself is altered.

The fact that the tyrosine kinases appear to phosphorylate exclusively on tyrosine residues contrasts with the hydroxyamino acid specificity of several other protein kinases. For example, casein kinase II (Hathaway & Traugh, 1982) and protein kinase C (Iwasa et al., 1980) appear to efficiently phosphorylate both serine and threonine residues. In addition, the cAMP-dependent protein kinase shows a strong preference for serine in its substrates but will also phosphorylate threonine residues with a K_m value that is 40-fold greater and a V_{max} that is 4-fold lower (Kemp et al., 1977).

All four peptide analogues used in these studies were shown to be competitive inhibitors of the L-tyrosine peptide substrate in the insulin receptor/kinase assay. The apparent K_i values of the inhibitors, calculated from Lineweaver–Burk plots using receptors which had not been prephosphorylated, were close to the K_m of the peptide under the same conditions. Similarly, the true K_i for the serine peptide was close to the true K_m for the L-tyrosine peptide determined on prephosphorylated insulin receptors. This indicates that the analogues are able to bind to the enzyme with almost the same affinity as the L-tyrosine substrate itself. Thus, their inability to serve as substrates is not related to a reduced affinity for the enzyme. Since phosphorylation does not take place when the analogues are bound to the kinase, this suggests that the structure of the active site is such that only tyrosine in the L configuration is held in a position appropriate for catalysis.

The apparent K_m for the L-tyrosine peptide when the receptors were not prephosphorylated prior to the peptide phosphorylation assay was determined to be 2 mM. This value dropped to 420 μM when the receptors were prephosphorylated prior to the assay. This difference in the K_m values may be due to the nonlinear kinetics of peptide phosphorylation observed using receptors which are not prephosphorylated (Rosen et al., 1983; Pike et al., 1986). It is also possible that the substrate specificity of the insulin receptor/kinase is altered by autophosphorylation, so that the receptor has a higher affinity for exogenous substrates.

Studies to elucidate the kinetic mechanism of the prephosphorylated insulin receptor/kinase were also performed. Initial rate studies were carried out by using five different concentrations of peptide at five different concentrations of ATP (Figure 2). Substituting the Dalziel Φ parameters generated from the secondary plots into the general form of the equation (eq 1) yields an expression that is consistent with either a compulsory-order steady-state mechanism or a random-order rapid-equilibrium mechanism (Dalziel, 1957). Specifically, the results of these studies eliminate the possibility of a ping-pong mechanism since Φ_{12} was not equal to zero (i.e., the double reciprocal plots did not show parallel lines). In addition, the results also exclude a compulsory-order rapid-equilibrium mechanism since Φ_1 was not equal to zero.

Studies of inhibition patterns caused by analogue inhibitors of the substrates were performed to distinguish between a compulsory-order steady-state mechanism and a random-order rapid-equilibrium mechanism. The peptide analogue containing the serine substitution and the nonhydrolyzable ATP analogue App(NH)p were used in these experiments. For either the compulsory-order steady-state mechanism or the random-order rapid-equilibrium mechanism, an inhibitor should show competitive inhibition with respect to the substrate of which it is an analogue. However, the inhibition patterns with respect to the other substrate differ between the two mechanisms. In a compulsory-order steady-state mechanism, an analogue of substrate B, the second substrate to bind, should be uncompetitive with respect to substrate A, the first substrate to bind. Furthermore, an analogue of substrate A should be noncompeitive with respect to substrate B. By contrast, in a random-order rapid-equilibrium mechanism, the inhibition should be noncompetitive in both cases.

In our studies, the serine peptide was found to be a competitive inhibitor with respect to the L-tyrosine peptide, while App(NH)p was found to be competitive with respect to ATP, consistent with these compounds being analogues of the L-tyrosine peptide and ATP, respectively. The serine peptide was found to be a noncompetitive inhibitor with respect to

ATP. Similarly, App(NH)p was shown to be noncompetitive with respect to L-tyrosine peptide. These results rule out a compulsory-order steady-state mechanism but are consistent with a random-order rapid-equilibrium mechanism for the insulin receptor/kinase. It should be noted that although the double reciprocal plots in these studies (Figure 2) appeared to be linear, this apparent lack of curvature cannot reliably rule out a random-order steady-state mechanism. Thus, the kinetic data are indicative of a mechanism involving a random order of addition of substrates and are consistent with an equilibrium mechanism but do not preclude the possibility of a steady-state mechanism.

Several other kinases have been shown to have a random order of substrate addition, including hexokinase (Fromm & Zewe, 1962), pyruvate kinase (Reynard et al., 1961), and phosphorylase kinase (Tabatabai & Graves, 1978). However, our findings contrast with those of Erneux et al. (1983), who reported that the closely related EGF receptor/kinase from A431 cells catalyzed the phosphorylation of a synthetic peptide by a compulsory-order steady-state mechanism. The reason for this discrepancy is unclear. Since the studies of Erneux et al. (1983) were performed on membrane preparations containing EGF receptors, it is possible that contaminating enzymes such as ATPases or proteases may have affected some of their inhibition patterns leading to the apparent difference in mechanism. It is also possible that although the insulin receptor and the EGF receptor are homologous, they catalyze their phosphorylation reactions via different mechanisms.

These kinetic studies definitely rule out a ping-pong or enzyme-substitution mechanism and suggest that the insulin receptor/kinase catalyzes its reaction via a random-order rapid-equilibrium mechanism. The findings suggest that an autophosphorylated tyrosine residue present on the receptor does not serve as a phosphate donor in the phosphorylation of exogenous substrates. The data shown in Figure 5, demonstrating that the autophosphorylated kinase cannot transfer preincorporated ^{32}P to the L-tyrosine peptide, further substantiate this point. The random-order rapid-equilibrium mechanism is similar to that of other kinases that phosphorylate small molecules and proteins. Thus, although the insulin receptor/kinase exhibits both hormone binding and protein kinase activities, and is involved in the regulation of specialized functions within the cell, its enzymatic properties are clearly similar to those of other less complex kinases.

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