THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol 258, No 18, Issue of September 25, pp 10973–10980, 1983
Printed in U S A

Characterization of the Insulin Receptor Kinase Purified from Human Placental Membranes*

(Received for publication, February 22, 1983)

Masato Kasuga‡§, Yoko Fujita-Yamaguchi¶, Diana L. Blithe‖, Morris F. White‡, and C. Ronald Kahn‡**

From the ‡*Elliot P.* Joslin Research Laboratory, *Joslin* Diabetes Center and Department of Medicine, *Brigham* and Women's Hospital, Harvard Medical School, Boston, Massachusetts *02215*, the ¶*Department* of Molecular Genetics, City of Hope Research *Institute, Duarte,* California *91010*, the ‖Developmental Endocrinology Branch, National Institutes of Child Health and Human *Development,* Bethesda, *Maryland 20205*, and the §*Third* Department *of* the Internal Medicine, Faculty of Medicine, University of Tokyo, *7-3-1* Hongo, Tokyo, Japan *113*

The insulin receptor purified from human placenta by sequential affinity chromatography on wheat germ agglutinin- and insulin-Sepharose to near homogeneity retained tyrosine-specific protein kinase activity. This purified insulin receptor kinase specifically catalyzed the incorporation of ^{32}P from [γ-^{32}P]ATP into not only the β-subunit of the insulin receptor but also histone H2B, a synthetic peptide which is sequentially similar to the site of tyrosine phosphorylation in pp60^{src} (a gene product of the Rous sarcoma virus) and antibodies to pp60^{src} present in the sera obtained from three rabbits bearing tumors induced by the Rous sarcoma virus. In each case, phosphorylation occurred exclusively on tyrosine residues. Insulin stimulated phosphorylation of these substrates 3- to 5-fold. Kinetic analysis using the synthetic peptide indicated that insulin acted by increasing the V_{max} of peptide phosphorylation from about 3.1 to **9.5 nmol·mg^{-1}** of protein·min^{-1}, whereas the value of the K_m for the peptide, about 1.5 mM, was not significantly changed. This kinase acted weakly on casein, α-S-casein, actin, and a tyrosine-containing peptide analogue of a serine-containing peptide used commonly as a substrate for the cyclic AMP-dependent protein kinases. These data show that the insulin receptor kinase displays specificity toward exogenous substrates similar to the substrate specificity observed for pp60^{src} and the protein kinase activity associated with the receptor for epidermal growth factor. The data suggest that the catalytic sites of these three tyrosine kinases are similar and that insulin activates its receptor kinase by increasing the V_{max}.

We have found that insulin binding to cells stimulates rapidly the phosphorylation of the β-subunit of the insulin receptor (1, 2). In the intact cell, there are multiple sites of phosphorylation; insulin stimulates an increase in phosphoserine and possibly phosphothreonine and the appearance of phosphotyrosine (2). Subsequently, we demonstrated that insulin stimulated the phosphorylation of the solubilized insulin receptor exclusively at tyrosine residues of the β-subunit (3, 4) and that the insulin-sensitve tyrosine kinase remained associated with the insulin receptor after 2500-fold purification (5). Furthermore, antibodies to the insulin receptor precipitate the insulin binding and the kinase activities, results which suggest that both are associated on the same macromolecule (5). Similar findings in several other laboratories using different systems (6–10) and the recent demonstration of ATP affinity labeling of the insulin receptor (11) suggest that the insulin receptor itself is a tyrosine-specific protein kinase which is involved in the insulin-enhanced phosphorylation.

Tyrosine-specific protein kinases have been reported to be associated with the transforming gene product of RNA tumor viruses (12–16), the EGF' receptor (17, 18), and a protein believed to be the receptor for PDGF (19, 20). One of the best studied tyrosine kinases is the gene product of the Rous sarcoma virus referred to as pp60^{src}. pp60^{src} not only phosphorylates itself but also has been shown to phosphorylate antibodies directed at pp60^{src} (12, 14). A synthetic peptide which resembles the site of tyrosine phosphorylation in pp60^{src} has been used as a substrate for other tyrosine kinases (21). Recently the EGF receptor kinase has also been shown to be active on both anti-pp60^{src} and a synthetic peptide resembling the site of phosphorylation in pp60^{src} (22–24). The PDGF-sensitive kinase,[2] on the other hand, does not appear to act on anti-pp60^{src} and phosphorylates weakly (25) or not at all[2] the synthetic peptides resembling the site of phosphorylation in pp60^{src}. In the present report we examine the activity of the purified insulin receptor kinase toward these and other possible artificial substrates.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin (lot QA 246P) was purchased from Elanco and epidermal growth factor was from Collaborative Research. Actin from chicken muscle and casein were obtained from Sigma. Histone H2B was obtained from Boehringer Mannheim, and histone H2B and H1 were from Worthington. Casein-a-S was a generous gift from Dr. E. Bingham at the Eastern Regional Research Agricultural Center. Protein A-Sepharose and protein A-bearing Staphylococcus

* This work has been supported in part by Grants AM31036 and AM29770 from the Institute of Health and Human Development, National Institutes of Health, United States Public Health Service and also by Grant 82R273 from Juvenile Diabetes Foundation International. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom reprint requests should he addressed.

[1] The abbreviations used are: EGF, epidermal growth factor; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid src, the gene responsible for cellular transformation by the Rous sarcoma virus; pp60^{src}, the M_r = 60,000 protein product of src; IgG, immunoglobin *G;* TBR serum, serum from rabbits bearing tumors induced by the Rous sarcoma virus; PDGF, platelet-derived growth factor.

[2] B. Ek and C.-H. Heldin, personal communication.

aureus (Pansorbin) were purchased from Pharmacia and Calbiochem, respectively. All reagents for $NaDodSO_4$-PAGE were from Bio-Rad.

Sera containing autoantibodies to the insulin receptor were obtained from patients with insulin resistance and acanthosis nigricans (26). Sera from rabbits bearing tumors induced by the Rous sarcoma virus, designated TBR-1, TBR-2, and TBR-3 were provided by **Dr.** H. Hanafusa (Rockefeller University, New York), Dr. B. Schaffhausen (Harvard Medical School, Boston, MA) (originally made by Dr. L. B. Chen, Sidney Faber Cancer Institute, Boston, MA), and Dr. J. G. Burr (Massachusetts Institute of Technology, Cambridge, MA), respectively. Other antisera were provided by the following investigators: rabbit anti-insulin receptor serum, Dr. S. Jacobs (Wellcome Research Laboratory, Research Triangle Park, NC); Fujinami sarcoma virus-specific regressing tumor antiserum (anti-FST) (27), Dr. H. Hanafusa; antiserum reactive toward the middle T antigen of polyoma virus, Dr. B. Schaffhausen. The abilities to immunoprecipitate the transforming protein kinase in TBR-1 and anti-FST may be not so much different (16, 27). Each serum sample was incubated at 56 °C for 20 min before use.

Two synthetic peptides resembling sequentially the tyrosyl phosphorylation site of $pp60^{src}$ (28) and a tyrosine-containing peptide used as a substrate for CAMP-dependent protein kinases were generous gifts from Dr. J. E. Casnellie (University of Washington, Seattle, WA) (24). The sequences of these peptides are shown in Fig. 1, ***A-C.***

Insulin receptor was purified about 2500-fold from Triton X-100-solubilized human placental membranes by sequential affinity chromatography on wheat germ agglutinin-Sepharose and insulin-Sepharose, as described previously (5, 29). Insulin binding to this purified receptor yielded a curvilinear Scatchard plot with a binding capacity of about 4700 pmol of insulin per mg of protein. This value represents 82% of the theoretical binding capacity assuming two binding sites in a molecular aggregate of $M_r = 350{,}000$. Protein composition was estimated from amino acid analysis (29).

Phosphorylation Assays—The purified insulin receptor (3 μl of a solution containing 0.1 μg of protein per ml) was incubated with or without hormones for 45 min at 22 °C and pH 7.4 in 50 μl of the standard phosphorylation reaction mixture containing 25 mM HEPES (Sigma), 0.1% Trition X-100 (New England Nuclear), $MgCl_2$ (15 mM), and $MnCl_2$ (2 mM). To this solution, 5 μl of histone (5 mg/ml), casein (5 mg/ml), actin (5 mg/ml), or 3 μl of the desired antiserum were added and incubated for 10 or 30 rnin as indicated in the figure legends. The phosphorylation assay was initiated by adding 25 μM [γ-^{32}P]ATP (10–20 μCi/nmol, New England Nuclear) and incubated for an additional 10 min at 22 "C. The reaction was terminated by adding unlabeled ATP (Sigma) to a final concentration of 2 mM, 60 pl of 2-fold concentrated Laemmli sample buffer (30) containing 100 mM dithiothreitol, and boiling this mixture for 3 min. The samples were analyzed by $NaDodSO_4$-PAGE using a 4% stacking and 7.5 or 10% resolving gels (30). Then the slab gels were stained for 5 rnin with 0.25% Coomassie blue in 50% trichloroacetic acid and incubated for 12 h at 37 "C with 400 ml of 7% acetic acid. Autoradiographs of the phosphorylated proteins were obtained by exposing overnight the dried gels to Kodak X-Omat film (2). Molecular weights were estimated with protein standards (2).

Phosphorylation of immunoadsorbed insulin receptor kinase or IgG from TBR serum was accomplished on protein A-Sepharose. The standard phosphorylation reaction mixture (50 pl) containing 0.1 μM insulin, 3 μl of the insulin receptor kinase, and 3 μl of the indicated antiserum were incubated at 4 °C with 30 μl (packed volume) of protein A-Sepharose. The protein A-Sepharose was agitated every 5 min for 1 h at 4 "C and then separated from the reaction mixture by centrifugation at 10,000 × *g* in a Beckman microfuge B. Assay of phosphorylation in the supernatant was performed as described above. The protein A-Sepharose was washed 3 times with ATP-free reaction buffer and phosphorylation of the adsorbed protein was initiated by suspending the protein A-Sepharose in 50 μl of reaction buffer containing 25 μM [γ-^{32}P]ATP for 10 min at 22 "C. The Sepharose was resuspended every 1 min during this incubation. This reaction was terminated by adding unlabeled ATP to a final concentration of 2 mM, 60 μl of 2-fold concentrated Laemmli sample buffer (30) containing 100 mM dithiothreitol, and boiling this mixture for 3 min. The protein A-Sepharose was sedimented by centrifugation, the proteins in the supernatant were separated by $NaDodSO_4$-PAGE, and an autoradiogram was obtained as described above.

Phosphorylation of the synthetic peptides was performed in 30 μl of a solution containing 25 mM HEPES, pH 7.4, 2 mM $MnCl_2$, 15 mM $MgCl_2$, 0.1% Triton X-100, and 40 μM [γ-^{32}P]ATP (7–8 μCi/nmol). Phosphorylation was initiated by addition of 0.1 pg (1 μl) of insulin receptor kinase in the absence or presence of 0.1 μM insulin and continued for 30 min. The synthetic peptide was then added at the concentrations indicated in the corresponding figure legends. This reaction was terminated after the indicated time interval by the addition of 50 μl of 5% trichloroacetic acid and 20 μl of bovine serum albumin (10 mg/ml) (24). After incubation of this mixture at 0 °C for 30 min, the precipitated protein was sedimented by centrifugation for 5 min in a Beckman microfuge B. A 40-pl aliquot of the supernatant was absorbed onto a square (2 × 2 cm) of phosphocellulose paper (Whatman) and washed exhaustively in acetic acid as previously described (31). Each square of phosphocellulose paper was dried, placed in a vial containing 10 ml of Aquasol (New England Nuclear), and assayed in a liquid scintillation spectrometer. The kinetic parameters for the phosphorylation of these synthetic peptides were determined by using a FORTRAN program described by Cleland (32) that fitted the data to the logarithmic form of the Michaelis-Menten equation by the nonlinear Gauss-Newton least squares method.

A. Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly

B. Leu-Arg-Arg-Ala-Tyr-Leu-Gly

C. Ile-Glu-Asp-Asn-Slu-Tyr-Thr-Ala-Arg-Gln-Gly

FIG. 1. **Sequences of synthetic tyrosine-containing peptides.**

Identification of Phosphoamino Acids—Phosphoamino acids were identified by a modified version (2) of the method of Hunter and Sefton (12). The position of the phosphorylated proteins separated on polyacrylamide gels was determined by autoradiography and this portion of the gel was excised. The protein was eluted from the rehydrated gel fragment by electrophoresis and subjected to acid hydrolysis in 6 N HCl for 2 h at 110 "C. The phosphoamino acids were separated electrophoretically on paper **(2).** Phosphoserine (Sigma), phosphothreonine (Sigma), and phosphotyrosine (a gift from Dr. T. Hunter, Salk Institute, San Diego, CA) were added to all samples immediately before electrophoresis. These standards were identified by reaction with ninhydrin and the radioactivity was located by autoradiography.

Tryptic Peptide Mapping of the *Phosphorylated* Histone—Phosphorylated histone H2B (Worthington) was separated from the reaction mixture by $NaDodSO_4$-PAGE using a 10% polyacrylamide gel. The gel was stained, destained, and dried as described above. Then the phosphorylated histone was located by autoradiography and the corresponding portion of the dried gel was excised and washed for 12 h at 37 °C in 10% methanol. This gel fragment (about 1 cm^2) was dried at 100 °C for 1 h and rehydrated with 2 ml of 50 mM NH_4HCO_3 containing 50 μg/ml of trypsin (Worthington crystalline trypsin-tosylphenylalanyl chloromethyl ketone, 238 units/mg). The digestion was allowed to proceed for 16 h at 37 "C. The supernatant which contained about 90% of the total radioactivity was lyophilized and dissolved in 10 μl of 30% (v/v) formic acid and applied onto cellulose thin layer plates (Schleicher & Schuell, G1440, 20 × 20 cm). These plates were sprayed uniformly with 30% formic acid and electrophoresis was complete after 70 min at 15 °C and 400 V. The plates were rotated 90" and ascending chromatography was accomplished in 1-butanol:acetic acid:pyridine:water (60:12:40:48) for 8 h at 23 °C (33). An autoradiogram was obtained by exposing Kodak X-Omat AR film with a Dupont Cronex Lightening Plus intensifier screen to the dried chromatogram for 4 days at −80 "C.

Immunoprecipitation of *Iodinated* Insulin Receptor—The purified insulin receptor (1 pg) was iodinated by reaction with $Na^{125}I$ (New England Nuclear) and chloramine-T (Eastman) as previously described (34) and was separated from this reaction mixture by affinity chromatography on wheat germ agglutinin-agarose (Vector). The iodinated receptor was eluted from the affinity column with a buffer containing 25 mM HEPES, pH 7.4, 0.1% Triton X-100, and 300 mM *N*-acetyl-D-glucosamine. The insulin receptor (approximately 0.05 pg containing 40,000 cpm) in 50 μl of this elution buffer was incubated for 4 h at 4 °C with 3 μl of serum containing anti-insulin receptor antibodies. Protein A (Pansorbin, 200 μl, 10% w/v) was added and the incubation was continued for 1 h. The Pansorbin was collected by centrifugation for 5 min at 10,000 × g and washed twice with a solution composed of 25 mM HEPES, 1% Triton, and 0.1% $NaDodSO_4$. The precipitated protein was solubilized in Laemmli

buffer, the Pansorbin was removed by centrifugation, and the supernatant was analyzed by $NaDodSO_4$-PAGE followed by autoradiography.

RESULTS

Phosphorylation of Histones and Caseins by the Insulin Receptor Kinase — Incubation of the purified insulin receptor kinase with [γ-^{32}P]ATP in the presence of 2 mM $MnCl_2$ and 15 mM $MgCl_2$ revealed one ^{32}P-labeled polypeptide with a M_r = 95,000 (Fig. 2, lane A). Incubation of this receptor preparation for 45 min with 0.1 μM insulin at 22 °C before addition of [γ-^{32}P]ATP caused a 3- to 5-fold increase in the amount of ^{32}P incorporated into this protein (Fig. 2, lane B). This phosphoprotein was shown previously to correspond to the β-subunit of the insulin receptor based on electrophoretic mobility, recognition by various anti-insulin receptor sera, and the specificity of stimulation by insulin analogues **(5)**.

To test the action of the kinase on other protein substrates, histone H2B (Worthington) was incubated with the insulin receptor kinase for 10 min in the presence of Mn^{2+} and Mg^{2+} followed by the addition of [γ-^{32}P]ATP. Under these conditions, we found phosphorylation of both the β-subunit of the insulin receptor and the added histone (Fig. 2, lane C). Insulin (0.1 μM) stimulated the phosphorylation of both proteins (Fig. 2, *lane* D). However, the data in Table I indicate that histone H2B inhibited by about 50% the phosphorylation of the β-subunit of the insulin receptor kinase.

One-dimensional electrophoresis of acid hydrolysates obtained from phosphorylated histone either without or with insulin stimulation indicated the presence of only phosphotyrosine (data not shown). Phosphopeptides obtained by tryptic digestion for 16 h of the phosphorylated histone were separated on cellulose thin layer plates in two dimensions by electrophoresis and chromatography. This analysis yielded three major phosphopeptides (Fig. ***3A***). Insulin stimulated the phosphorylation of each peptide and caused detectable phosphorylation of several additional peptides (Fig. 3B). These

TABLE I

Insulin stimulation of incorporation of ^{32}P from [γ-^{32}P]ATP into the β-subunit of the insulin receptor and histone H2B

The incorporation of ^{32}P into the indicated proteins was determined by scanning densitometry of the corresponding lane of the autoradiograms shown in Fig. 2.

Incubation system	Radioactivity in substrates (arbitrary units)	
	β-Subunit of insulin receptor	Histone H2B
Insulin receptor (lane A)	5.4	
Insulin receptor + insulin (lane B)	19.0	
Insulin receptor + histone H2B (lane C)	5.2	39.1
Insulin receptor + histone H2B + insulin (lane D)	9.7	115.1

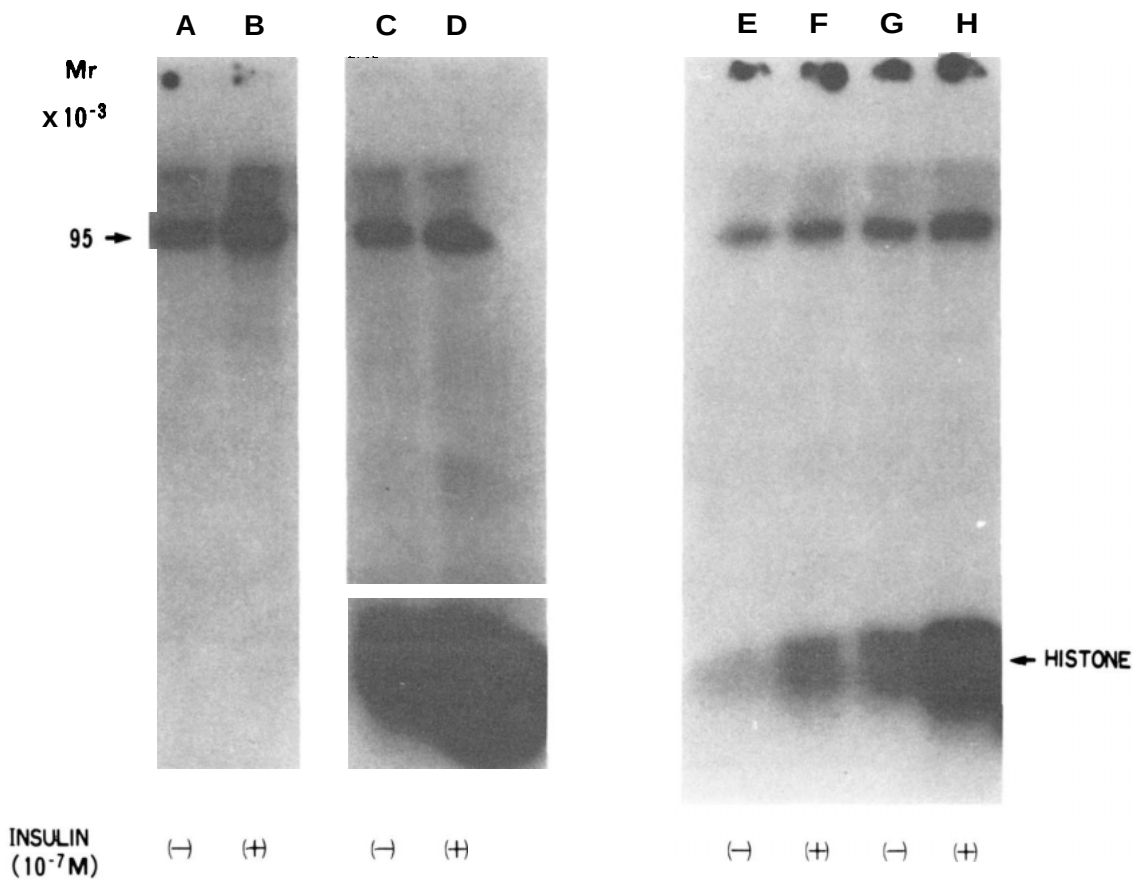

FIG. 2. **Autoradiograms obtained from $NaDodSO_4$-PAGE separation of the β-subunit of the insulin receptor and histone H2B labeled with ^{32}P.** The purified insulin receptor kinase (approximately 0.3 μg) was incubated with [γ-^{32}P]ATP in the standard assay mixture for 10 min at 22 °C in the absence ***(lane A)*** or presence ***(lane B)*** of 0.1 μM insulin. Histone H2B from Worthington ***(lanes C*** and ***D)*** or histone H2B from Boehringer Mannheim ***(lanes E*** and ***F)*** were added to the reaction mixture at a concentration of 0.5 mg/ml in the absence ***(lanes C*** and ***E)*** or presence ***(lanes*** D and ***F)*** of 0.1 μM insulin. The effect of bovine serum albumin (10 μg/ml) and glycerol (10%) added to the reaction mixture on the phosphorylation of histone H2B from Boehringer Mannheim is shown in the absence ***(lane*** C) or presence of 0.1 μM insulin ***(lane H)***.

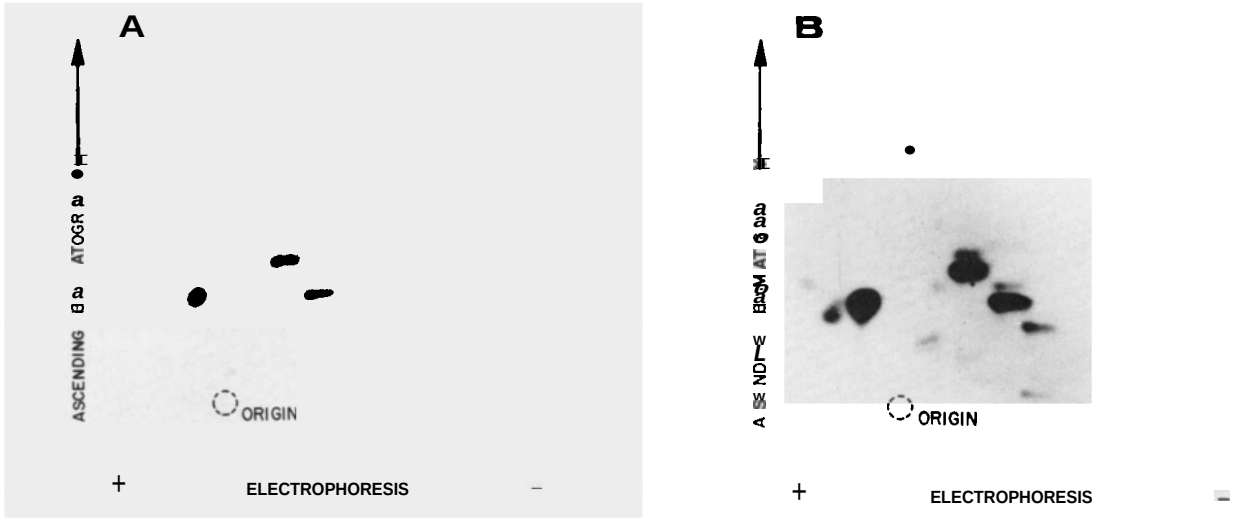

FIG. 3. **Tryptic peptide mapping of histone H2B phosphorylated by the insulin receptor kinase.** Histone **H2B** (Worthington) was phosphorylated as described in Fig. 2 in the absence ***(A)*** or presence (*B*) of 0.1 μM insulin. After separation of the phosphorylated histone by $NaDodSO_4$-PAGE, the protein was digested with trypsin as described under "Experimental Procedures." Electrophoresis corresponds to the horizontal dimension and chromatography to the vertical dimension.

results indicate that although not all tyrosine residues in histone H2B are surrounded by the appropriate sequence of amino acid which promote a rapid reaction with the insulin receptor kinase, there was more than one site of phosphorylation in this histone preparation.

All histones were not equally good substrates for the insulin receptor kinase. Histone H1 (Worthington) which acts as a phosphate acceptor for serine kinases showed no detectable phosphorylation in our system (data not shown). Histone H2B obtained from Boehringer Mannheim was also a poor substrate when compared to the preparation of histone H2B obtained from Worthington (Fig. 2, lanes E and F). However, phosphorylation of histone H2B (Boehringer Mannheim) became more prominent when our reaction mixture was supplemented with bovine serum albumin (10 μg/ml) and glycerol (10%), especially after insulin treatment (Fig. 2, lanes G and *H*). These data may suggest that some factor(s), possibly a phospholipid, present in the bovine serum albumin and glycerol or in the preparation of histone H2B from Worthington enhanced the activity of the insulin receptor kinase. A similar result has been reported for the kinase activity of $pp60^{src}$ (35).

Other exogenous substrates, including actin and casein (both casein-n-S and casein from Sigma) could be phosphorylated by the insulin receptor kinase; however, these proteins were not good substrates when compared to histone H2B (Worthington). Furthermore, insulin (0.1 μM) stimulated the phosphorylation of these exogenous substrates by only about 50% (data not shown).

Phosphorylation of Sera from Rabbits Bearing Tumors Induced by the Rous Sarcoma Virus—Antisera obtained from rabbits bearing tumors induced by the Rous sarcoma virus (TBR sera) contain IgG molecules that react immunologically with $pp60^{src}$ (12, 14). The EGF receptor and the protein kinase, $pp60^{src}$, a gene product of the Rous sarcoma virus, phosphorylate the IgG in this antisera (12, 14, 22, 23). When an aliquot of one such serum (TBR-1) was added to the phosphorylation reaction mixture including the insulin receptor kinase, autoradiograms of the $NaDodSO_4$ gels revealed two phosphoproteins. By molecular weight, these phosphoproteins correspond to the @-subunitof the insulin receptor and the IgG heavy chain of TBR-1 serum (Fig. 4, lane E). Insulin (0.1 μM) stimulated significantly the incorporation of

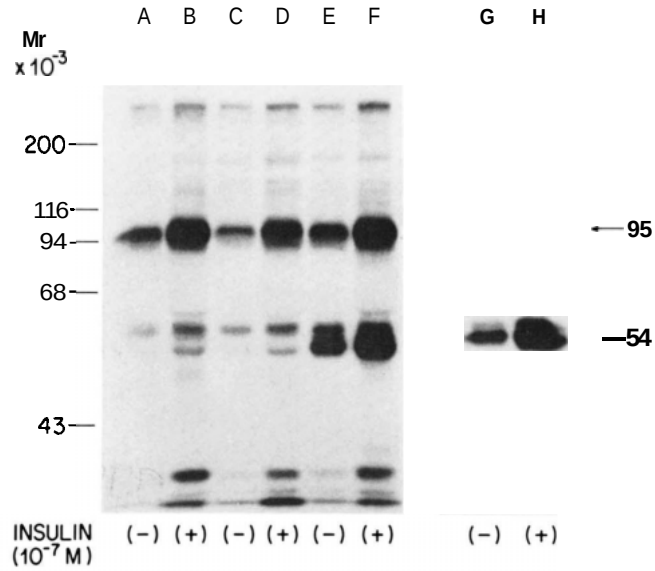

FIG. 4. **Autoradiograms obtained from $NaDodSO_4$-PAGE separations of the phosphorylated β-subunit of the insulin receptor and phosphorylated IgG contained in TBR serum.** The insulin receptor kinase (approximately 0.3 μg) in the standard phosphorylation assay mixture was incubated with 3 μl of normal rabbit serum ***(lanes A*** and *B*), rabbit serum containing antibodies to the insulin receptor ***(lanes C*** and *D*), or **TBR-1** serum ***(lanes*** E and *F*) in the absence ***(lanes A, C, E,*** and *G*) or presence of 0.1 μM insulin ***(lanes*** B, D, F, and H). The experiment shown in ***lanes*** G and ***H*** parallels exactly the conditions in ***lanes*** E and F, respectively, except this autoradiogram represents a $NaDodSO_4$-PAGE separation of phosphoprotein removed from the reaction mixture by binding to staphylococcal protein A.

^{32}P into both proteins (Fig. 4, compare lanes E and F). Only faint bands of phosphoprotein were detected in the region of the IgG heavy chain when normal rabbit serum was substituted for TBR serum (Fig. 4, lanes A and B) and phosphorylation of the insulin receptor was unaltered.

After addition of staphylococcal protein A the phosphorylated IgG heavy chain of TBR-1 serum, but not the @-subunit of the phosphorylated insulin receptor, could be precipitated from this reaction mixture (Fig. 4, lanes G and H). Acid

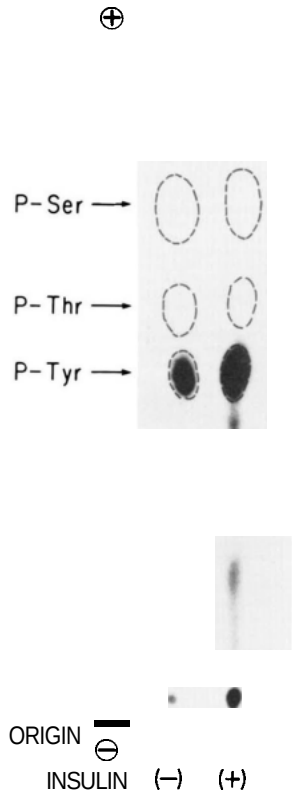

FIG. 5. Identification of phosphoamino acids in the IgG from **TBR-1** serum. IgG in TBR-1 serum separated from the standard phosphorylation reaction mixture by $NaDodSO_4$-PAGE was subjected to partial acid hydrolysis and one-dimensional electrophoresis as described under "Experimental Procedures." The mobility of phosphoserine, phosphothreonine, and phosphotyrosine determined by the addition of standards to all radioactive samples is delineated by the ***broken lines.***

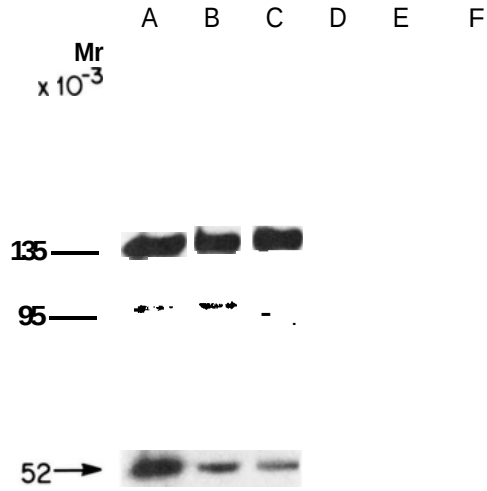

FIG. *6.* $NaDodSO_4$-PAGE separation of the iodinated insulin receptor kinase before and after immunoprecipitation. Fifty μl of the iodinated insulin receptor kinase (approximately 40,000 cpm) were incubated with 3 μl of human ***(lane B)*** or rabbit ***(lane C)*** anti-insulin receptor sera, or TBR-1 ***(lane D)***, TBR-2 ***(lane E)***, or TBR-3 ***(lane F)*** sera and immunoprecipitated with staphylococcal protein A. The iodinated receptor (50 μl) without immunoprecipitation ***(lane A)*** or the immunoprecipitates were analyzed by $NaDodSO_4$-PAGE and autoradiography.

hydrolysates of the phosphoprotein corresponding to the heavy chain of IgG were separated by one-dimensional electrophoresis at pH 3.5. Only phosphotyrosine could be identified after phosphorylation in both the presence and absence of insulin (Fig. 5). The IgG heavy chains in TBR-2 and TBR-3 sera obtained from other sources also proved to be good substrates for phosphorylation, although the degree of phosphorylation was different between preparations (data not shown). The phosphorylation of these IgG molecules was not due to an endogenous protein kinase activity in the serum since no phosphorylation of IgG was detected in the absence of added insulin receptor kinase.

In contrast to the TBR sera, rabbit serum containing antibodies reactive toward the insulin receptor and human sera obtained from three different patients (B-2, B-8, B-9) (26) containing autoantibodies to the insulin receptor (Fig. **4,** ***lanes*** C and ***D),*** as well as normal rabbit and human IgG molecules, were phosphorylated only slightly by the insulin receptor kinase. Insulin produced a slight increase in the phosphorylation of each of these substrates, but none approached the intensity of phosphorylation seen with the TBR sera. The antisera which precipitate the tyrosine kinases associated with Fujinami sarcoma virus-transforming protein and middle T antigen of polyoma virus were also phosphorylated only slightly by the insulin receptor kinase.

The specific phosphorylation of the IgG in TBR sera by the insulin receptor kinase may result from an immunologic reaction between the kinase and the IgG molecules. To test this hypothesis, the iodinated insulin receptor kinase was immunoprecipitated by anti-insulin receptor sera or TBR sera, separated by $NaDodSO_4$-PAGE, and the gel was used to prepare an autoradiogram. In agreement with our previous result (5), iodinated purified receptor showed three peptides of M_r = 135,000, 95,000, and 52,000 on $NaDodSO_4$-PAGE (Fig. 6, ***lane A).*** These proteins correspond to the **a-** and @-subunits of the receptor, respectively, and a protein of M_r = 52,000 which is presumably a degradation product of @-subunits.[3] All three peptides were immunoprecipitated by two different anti-insulin receptor sera (Fig. 6, ***lanes*** B and ***C),*** whereas none of the three different TBR sera successfully precipitated the insulin receptor kinase (Fig. 6, ***lanes*** D–F). These results are consistent with the fact that protein A immunoprecipitated the phosphorylated IgG in TBR-1 serum but did not immunoprecipitate the phosphorylated insulin receptor kinase from the same reaction mixture (Fig. **4,** ***lanes*** G and ***H).***

Anti-insulin Receptor Sera but Not TBR Sera Immunoprecipitate the Insulin Receptor Kinme—The insulin receptor associated with protein A-Sepharose after immunoprecipitation still possessed protein kinase activity as determined by phosphorylation of the @-subunit (Fig. 7, ***lane E).*** To eliminate the possibility that the receptor preparation contained another kinase responsible for phosphorylation of the TBR sera, the receptor kinase was incubated with TBR-1 serum and protein A-Sepharose to produce a precipitate. The precipitate and supernatant were then incubated with [γ-^{32}P]ATP to study phosphorylation. Phosphorylation of the receptor was found in the supernatant only (Fig. 7, ***lane B).*** No phosphorylated IgG was detected in this supernatant or after a phosphorylation reaction was carried out with the washed Sepharose (Fig. 7, ***lanes*** B and ***C,*** respectively). Reconstitution of

[3] **Y.** Fujita-Yamaguchi, unpublished observation.

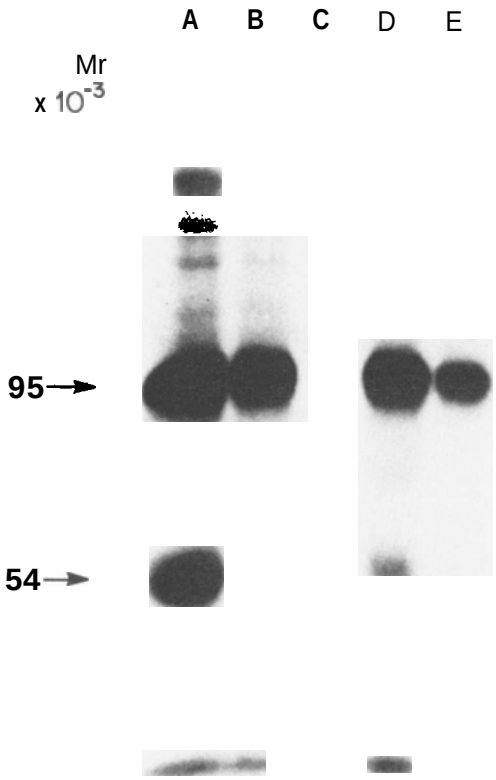

FIG. 7. The effect of immunoadsorption on protein A-Sepharose of the insulin receptor kinase by IgG from TBR-1 serum or anti-insulin receptor rabbit serum. In a reaction mixture containing the insulin receptor kinase, 0.1 μM insulin, and TBR-1 serum, phosphorylation was initiated by adding [γ-^{32}P]ATP before immunoadsorption *(lane A)* or by adding [γ-^{32}P]ATP to both the supernatant *(lane B)* and the precipitate *(lane C)* obtained after incubation of a solution prepared in parallel with protein A-Sepharose as described under "Experimental Procedures." The effect of reconstitution of this supernatant and precipitate before addition of [γ-^{32}P]ATP is shown in *lane* D. *Lane E* shows the autoradiogram obtained when [γ-^{32}P]ATP was added to the immunoprecipitate prepared from a reaction mixture containing 0.1 μM insulin, the insulin receptor kinase, and anti-insulin receptor serum.

the protein A-Sepharose/IgG precipitate with the corresponding supernatant containing the insulin receptor kinase and [γ-^{32}P]ATP resulted in phosphorylation of both the β-subunit of the receptor and the IgG (Fig. 7, lane *D*). These results suggest that the tyrosine kinase activity associated with our preparation of the insulin receptor is due exclusively to the insulin receptor kinase. The decreased phosphorylation measured after reconstitution of the immunoprecipitates (Fig. 7, *lanes D* and E) suggests that the protein A-Sepharose complexes are not fully active as both kinase and substrates or that recovery of these immunoprecipitates is not quantitative.

Phosphorylation of Synthetic Peptides—The synthetic peptide shown in Fig. 1*C* corresponds to the site of tyrosine phosphorylation between amino acid residues **414** to **424** of pp60^{src} **(28).** A mixture of [γ-^{32}P]ATP and a membrane fraction prepared from A-431 cells which contains a large number of EGF receptors phosphorylates this synthetic peptide in an EGF-sensitive reaction **(24).** This peptide sequence has been modified by Casnellie et *al.* (Fig. 1, peptide ***A)*** to enhance its usefulness in kinetic assays of phosphorylation by tyrosine-specific kinases **(21).** The extension of the natural NH_2 terminus to include the leucine and arginine residue and the addition of a second arginine enhances adsorption of the peptide to phosphocellulose paper which allows rapid separation of the phosphopeptide from a reaction mixture **(31).** In addition, the only site available for phosphorylation is a tyrosine residue. This peptide, hereafter referred to as peptide A (Fig. **1,** ***A),*** serves as a good substrate for the EGF-sensitive protein kinase **(24).** Peptide B (Fig. **1,** B), which is a tyrosine analogue of a serine-containing peptide used as a substrate for CAMP-dependent protein kinases (36), is a relatively poor substrate for the EGF-stimulated kinase **(24).**

The time course of phosphorylation of peptide A is shown in Fig. 8. The reaction was linear for at least **3** min under our assay conditions, and insulin stimulated the phosphorylation approximately 3- to 5-fold. Steady state kinetic analysis also requires linear product formation as a function of added enzyme. When 0.05, **0.1,** 0.15, or **0.2** μg of purified receptor kinase was added to the reaction mixture, the phosphorylation of peptide A in 3 min was linearly increased as a function of added receptor kinase both in the absence and presence of insulin (10^{-7} **M).** The initial velocity of phosphorylation of peptide A was measured at a constant ATP concentration of **40** μM in the presence or absence of insulin and analyzed in a Lineweaver-Burk (Fig. 9). The data in Table II show that

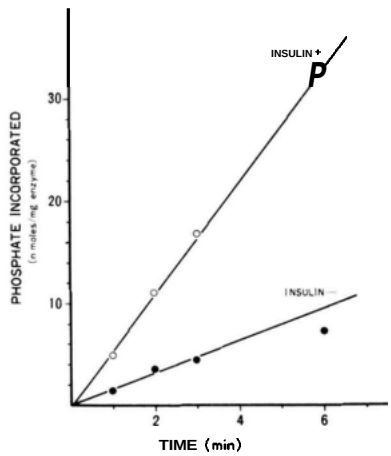

FIG. 8. The time course of phosphorylation of peptide A (1 mM) by the insulin receptor kinase (approximately 3.3 μg/ml) in the absence (O) and presence *(O)* of 0.1 μM insulin.

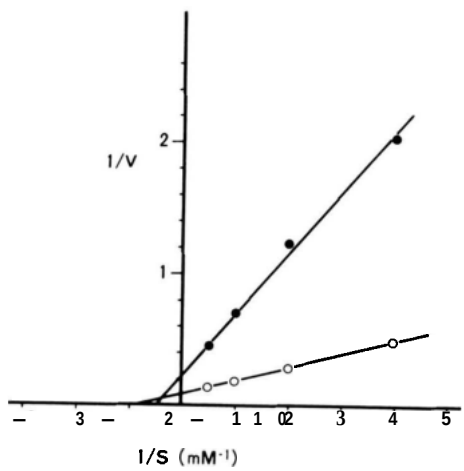

FIG. 9. A Lineweaver-Burk plot of phosphorylation of peptide A by the insulin receptor kinase (approximately 3.3 μg/ml) in the presence *(O)* and absence (O) of 0.1 μM insulin. The initial velocities of phosphorylation were estimated during 3-min incubation as described under "Experimental Procedures" at the following concentrations of synthetic peptide: 0.25, 0.5, 1.0, and 2.0 mM. Values for the kinetic parameters corresponding to these data are provided in Table II.

TABLE II

The kinetic constants for phosphorylation of synthetic peptides by the insulin receptor kinase

The initial velocities of phosphorylation of the indicated synthetic peptides were estimated during a 3-min incubation as described under "Experimental Procedures." The following concentrations (mM) of synthetic peptide were used Experiment 1, 0.25, 0.5, 1.0, 2.0; Experiment 2, 0.66, 1.0, 2.0, 4.0, 8.0; Experiment 3, 0.25, 0.5, 1.0, 2.0. The kinetic parameters ± S.E. were determined by fitting the data to the Michaelis-Menten equation.

Experiment	Substrate	−Insulin		+Insulin	
		K_m	V_{max}	K_m	V_{max}
		mM	nmol ng^{-1} protein min^{-1}	mM	nmol mg^{-1} protein min^{-1}
1	Peptide A	2.1 ± 0.3	4.5 ± 0.5	1.2 ± 0.1	11.8± 0.4
2	Peptide A	1.0 ± 0.2	1.7 ± 0.1	1.4 ± 0.3	7.1 ± 0.8
3	Peptide B	0.22 ± 0.09	0.15 ± 0.02	1.3 ± 0.3	0.41 ± 0.04

binding of insulin to its receptor enhances tyrosine phosphorylation of peptide A by increasing mainly the V_{max} of the reaction with little change in the K_m.

Insulin receptor kinase also catalyzed the phosphorylation of peptide B, but was much less efficient. This peptide contains basic amino acid residues in the vicinity of tyrosine. The V_{max} for phosphorylation of peptide B was more than an order of magnitude lower than the values observed for peptide A in both the presence and absence of insulin, although the K_m was quite similar. This finding is in good agreement with the results for the EGF-stimulated kinase (24).

DISCUSSION

Since our initial observation (1) that insulin stimulates the phosphorylation of its own receptor, additional information has been reported which suggests that the insulin receptor is a protein kinase. This evidence includes the demonstrations that insulin stimulates receptor phosphorylation in a variety of intact (1, 2, 6, 7) and cell-free preparations (3–11). Furthermore, the kinase activity is retained after partial purification of the receptor (4, 7–9) and immunoprecipitation of the receptor (5). The receptor can be labeled specifically with ATP affinity reagents (11, 43, 44). We have shown recently that a preparation of insulin receptors purified to near homogeneity from human placenta also possesses protein kinase activity as measured by insulin-stimulated phosphorylation of the β-subunit of its own receptor (5). In all of the cell-free experiments, insulin-stimulated phosphorylation occurs exclusively on tyrosine residues suggesting that the insulin receptor is a tyrosine-specific protein kinase.

Phosphotyrosine is 3000-fold less abundant in cells than phosphoserine and phosphothreonine combined (12) suggesting that tyrosine kinases and their reactive substrates are relatively rare in cells. Previous studies have shown that several other proteins possess a tyrosine-specific protein kinase activity. These enzymes include a variety of gene products from transformation viruses (12–16, 21), the receptors for EGF (17, 18), and a protein presumed to be the receptor for PDGF (19, 20). The cellular substrates for these kinases remain unknown. Both the pp60^{src} (37) and the EGF receptor (17, 22, 23, 38) have been shown to be active on exogenous substrates including histone, sera containing antibodies to pp60^{src}, and synthetic peptides which resemble the phosphorylation site of pp60^{src} (24). By contrast, preliminary data from two different laboratories' (25) suggest that the PDGF receptor kinase will not catalyze phosphorylation of the pp60^{src} antiserum and phosphorylates the synthetic peptide more slowly than EGF-stimulated kinase. This latter result is due apparently to a lower V_{max} for phosphorylation by the PDGF-stimulated kinase (25).

In this report, we have established that the purified insulin receptor kinase will also catalyze tyrosyl phosphorylation of several protein acceptors. These include histone H2B, IgG in sera from rabbits bearing tumors induced by the Rous sarcoma virus (TBR sera), and a synthetic peptide resembling the phosphorylation site of pp60^{src}. In each case, insulin stimulates the phosphorylation of these proteins at least 3-fold. Insulin-stimulated phosphorylation of the β-subunit of the insulin receptor could result from a conformational change which enhances the interaction between the insulin-receptor complex and another protein kinase. However, since insulin also stimulates directly the phosphorylation of several other substrates using a very highly purified receptor preparation, we favor the notion that binding of insulin to the receptor stimulates the insulin receptor kinase directly.

Even though the number of phosphorylation sites in the IgG molecules may be large, the specificity of phosphorylation of the antibodies to pp60^{src} present in TBR sera is striking. IgG present in three different TBR sera all proved to be good substrates for the receptor kinase, whereas the IgG present in normal rabbit and normal human sera and the four different sera containing IgG antibodies which react specifically with the insulin receptor showed relatively little phosphorylation by the insulin receptor kinase. TBR sera could not immunoprecipitate either the iodinated purified insulin receptor or the associated kinase activity. Although the insulin receptor kinase interacts with anti-pp60^{src} in a manner sufficient to facilitate phosphorylation, the interaction is not sufficient for immunoprecipitation. Our results are very similar to the observation that the EGF receptor kinase can phosphorylate antibodies to pp60^{src} (22, 23).

The insulin receptor kinase, like the EGF and PDGF receptor (24, 25), phosphorylated the synthetic peptide (Fig. 1, peptide A) containing a sequence of amino acids similar to the site of tyrosine phosphorylation in pp60^{src} (28). The K_m for reaction of this peptide with the insulin receptor kinase was about 1.5 mM and this value was not altered by insulin. The K_m for phosphorylation by the EGF- and PDGF-stimulated kinases is about 2 mM (24, 25). Insulin stimulated phosphorylation of peptide A by a 3- to 5-fold increase in the V_{max}. This result is similar to the action of EGF and PDGF. Using 350,000 as the M_r of the insulin receptor kinase (39), the average turnover number for phosphorylation of peptide A in the presence of insulin in our assay system is about 4/min at 22 °C. This turnover number is more than 100-fold lower than the value extrapolated from the kinetic data obtained for the EGF-stimulated phosphorylation of peptide A in membranes from A-431 cells (24). Whereas the insulin receptor kinase phosphorylates casein slowly, pp60^{src} yields a turnover number of about 2/min at 22 °C for phosphorylation of this substrate (40). These kinetic differences may reflect different specificities of these tyrosine kinases or different requirements for optimal activation, such as a requirement for a certain phospholipid environment (35).

Our results suggest that, although the structure of the proteins is quite different and the requirements for optimal activity are probably different, the EGF receptor kinase, the insulin receptor kinase, and the pp60^{src} kinase possess a basic similarity in substrate recognition. Based on limited data, however, the PDGF receptor kinase appears somewhat different in this substrate specificity. This difference may be of some physiologic importance since, although insulin, EGF, and PDGF are regarded as growth factors, insulin and EGF appear to act as "progressive factors" whereas PDGF acts a "competence factor" early in the cell cycle (41). This difference may parallel the differences in their ability to phospho-

rylate exogenous substrates. Also, it is interesting that EGF and insulin cause normal chicken heart mesenchymal cells to proliferate like their Rous sarcoma virus-infected counterparts (42). The effect of PDGF on these cells is unknown. Obviously, it will be important to determine whether the natural substrates for these kinases also differ in a similar manner. If one assumes that the actions of insulin and these other growth factors are transmitted from the hormone to the cell via stimulation of the receptor kinase, it is possible that determination of the endogenous substrates for the insulin receptor kinase may yield a sequence of regulatory proteins coupled by phosphorylation-dephosphorylation reactions. It is also possible, however, that the receptor itself is the most important substrate, and that receptor autophosphorylation is associated with a change in properties of the receptor which are recognized intracellularly and initiate the chain of events in insulin action.

Acknowledgments—We wish to thank Drs. H. Hanafusa, J. S. Burr, S. Jacobs, and B. Schaffhausen for gifts of serum, Dr. J. E. Casnellie for the synthetic peptides, Dr. T. Hunter for phosphotyrosine, Drs. L. J. Pike and C.-H. Heldin for providing unpublished data, and Dr. H. U. Haring for helpful discussions and comments.

REFERENCES

1. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1982) *Science* **215,185–187**
2. Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Haring, H. U., and Kahn, C. R. (1982) *J. Biol. Chem.* **257,9891–9894**
3. Kasuga, M., Zick, Y., Blithe, D. L., Cretta, M., and Kahn, C. R. (1982) *Nature (Lond.)* **298,667–669**
4. Zick, Y., Kasuga, M., Kahn, C. R., and Roth, J. (1983) *J. Biol. Chem.* **258,** 75–80
5. Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., and Kahn, C. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80, 2137–2141**
6. Van Obberghen, E., and Kowalski, A. (1982) *FEBS Lett.* **143, 179–182**
7. Haring, H.-U., Kasuga, M., and Kahn, C. R. (1982) *Biochem. Biophys. Res. Commun.* **108,1538–1545**
8. Petruzzelli, L. M., Ganguly, S., Smith, C. R., Cobb, M. H., Rubbin, C. S., and Rosen. O. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79, 679216796**
9. Machicao, F., Urumow, T., and Wieland. O. H. (1982) *FEBS Lett.* **149,** 96–100
10. Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W., and Osathanondh, R. (1982) *J. Biol. Chem.* **257, 15162–15166**
11. Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., and Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80, 945–949**
12. Hunter, T., and Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77, 1311–1315**
13. Witte, O. N., Dasgupta, A., and Baltimore, D. (1980) *Nature (Lond.)* **283,826–831**
14. Collet, M. S., Purchio, A. F., and Erikson, R. L. (1980) *Nature (Lond.)* **285,167–169**
15. Kawai, S., Yoshida, M., Segawa, K., Sugiyama, H., Ishizaki, R., and Toyoshima, K. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77, 6199–6202**
16. Feldman, R., Hanafusa, T., and Hanafusa, H. (1980) *Cell* **22, 757–765**
17. Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. (1982) *J. Biol. Chem.* **257, 1523–1531**
18. Ushiro, H., and Cohen, S. (1980) *J. Biol. Chem.* **255, 8363–8365**
19. Glenn, K., Bowen-Pope, D. F., and Ross, R. (1982) *J. Biol. Chem.* **257,5172–5176**
20. Ek, B., and Heldin, C.-H. (1982) *J. Biol. Chem.* **257,** 10486–**10492**
21. Casnellie, J. E., Harrison, M. L., Pike, L. J., Hellstrom, K. E., and Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 282–**286**
22. Chinkers, N., and Cohen, S. (1981) *Nature (Lond.)* **290,516–519**
23. Kudlow, J. E., Buss, J. E., and Gill, G. N. (1981) *Nature (Lond.)* **290,519–521**
24. Pike, L. J., Gallis, B., Casnellie, J. E., Bornstein, P., and Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79, 1443–1447**
25. Pike, L. J., Bowen-Pope, D. F., Ross, R., and Krebs, E. G. (1983) *J. Biol. Chem.* **258,9383–9390**
26. Hedo, J. A., Kasuga, M., Van Obberghen, E., Roth, J., and Kahn, C. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,4791–4795**
27. Mathey-Prevot, B., Hanafusa, H., and Kawai, S. (1982) *Cell* **28, 897–906**
28. Smart, J. E., Opperman, H., Czernilofsky, A. P., Purchio, A. S., Erikson, R. L., and Bishop, J. M. (1981) *Proc. Natl. Acad. Sci. U. S.* **A. 78,6013–6017**
29. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., and Itakura, K. (1983) *J. Biol. Chem.* **258,5045–5049**
30. Laemmli, U. K. (1970) *Nature (Load.)* **227,680–685**
31. Glass, D. B., Masaracchia, R. A., Feramisco, J. R., and Kemp, B. E. (1978) *Anal. Biochem.* **87, 566–575**
32. Cleland, W. W. (1979) *Methods Enzymol.* **63, 103–138**
33. Ellis, R. W., De Feo, D., Shih, T. Y., Gonda, M. A., Young, H. A., Tsuchida, N., Lowy, D. R., and Scolnick, E. M. (1981) *Nature (Lond.)* **292,506–511**
34. Jacobs, S., Hazum, E., Schecter, Y., and Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76, 4918–4921**
35. Ito, S., Richert, N. D., and Pastan, I. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,4628–4631**
36. Kemp, B. E., Graves, D. J., Benjamini, E., and Krehs, E. G. (1977) *J. Biol. Chem.* **252,4888–4894**
37. Collet, M. S., and Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2021–2024
38. Carpenter, G., King, L., Jr., and Cohen, S. (1979) *J. Bwl. Chem.* **254,4884–4891**
39. Kasuga, M., Hedo, J. A., Yamada, K. M., and Kahn, C. R. (1982) *J. Biol. Chem.* **257, 10392–10399**
40. Richert, N. D., Blithe, D. L., and Pastan, I. H. (1982) *J. Biol. Chem.* **257,7143–7150**
41. Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., Van Wyk, J. J., and Pledger, W. J. (1979) *Proc. Natl. Acad. Sci. U. S.* **A. 76, 1279–1283**
42. Balk, S. D., Shin, R. P. C., LaFleur, M. M., and Young, L. L. (1982) *Proc. Natl. Acad. Sci. U. S.* **A. 79, 1154–1157**
43. Roth, R. A., and Cassell, D. J. (1983) *Science (Wash. D. C.)* **219, 299–301**
44. Shia, M., and Pilch, P. F. (1983) *Biochemistry* **22, 717–721**