Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2137–2141, April 1983
Biochemistry

Tyrosine-specific protein kinase activity is associated with the purified insulin receptor

(human placental membranes/insulin affinity column/immunoprecipitation/anti-insulin receptor antibody/phosphorylation)

MASATO KASUGA*†, YOKO FUJITA-YAMAGUCHI‡, DIANA L. BLITHE§, AND C. RONALD KAHN*

*Elliott P. Joslin Research Laboratory, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02215; ‡Department of Molecular Genetics, City of Hope Research Institute, Duarte, California 91010; and §Developmental Endocrinology Branch, National Institute of Child Health and Human Development, Bethesda, Maryland 20205

Communicated by Eugene Braunwald, January 3, 1983

ABSTRACT **Highly purified human placental insulin receptors were obtained by sequential affinity chromatography on wheat germ agglutinin and insulin-agarose. The preparation had an insulin binding capacity of 4,700 pmol/mg of protein approaching theoretical purity. The purified receptor revealed three major bands of M_r 135,000, 95,000, and 52,000 in NaDodSO$_4$/polyacrylamide gel electrophoresis after reduction by dithiothreitol. All three bands were immunoprecipitated by anti-insulin-receptor antibodies. When this preparation was incubated with [γ-^{32}P]ATP in the presence of $MnCl_2$ (2 mM) and analyzed in NaDodSO$_4$/acrylamide gel electrophoresis, only the M_r 95,000 band was labeled. Preincubation with several concentrations of insulin increased the ^{32}P incorporation into this peptide in dose-dependent fashion, whereas insulin-like growth factors were ≈2% as potent and epidermal growth factor had little or no effect, consistent with their known affinities for the insulin receptor. Insulin stimulation of phosphorylation of the M_r 95,000 subunit of the receptor was observed also in immunoprecipitates of this highly purified insulin receptor by anti-insulin-receptor antibodies. Phosphoamino acid determination revealed only phosphotyrosine in both the basal and insulin-stimulated states. These data suggest that a tyrosine-specific protein kinase activity is closely associated with insulin receptor, and this may be important in the signal transmission required for insulin action.**

Polypeptide hormones and other related biologically important molecules bind to specific membrane receptors in target cells initiating a wide spectrum of biological effects. The mechanisms by which the initial interaction between these substances and their receptors results in a transfer of information remains poorly understood. We have found that insulin stimulates the phosphorylation of its own receptor (1, 2) and also that this phenomenon can be reproduced in a solubilized insulin receptor system (3, 4). From these results, we proposed the phosphorylation of insulin receptor may be an early step in insulin action. Phosphorylation of receptor proteins or proteins presumably related to the receptor also has been reported for epidermal growth factor (EGF) (5), platelet-derived growth factor (6, 7), acetylcholine (8), and immunoglobulin E (9, 10), suggesting that receptor phosphorylation may be an important general mechanism for transmission of the information of a ligand into the cell.

In intact cells, the phosphorylation of the insulin receptor involved multiple sites including both phosphotyrosine and phosphoserine (2), whereas in the solubilized fraction only tyrosine is phosphorylated (3). These results suggest that a tyrosine-specific protein kinase is involved in the insulin-stimulated phosphorylation of its own receptor and that tyrosine phosphorylation may be the primary phosphorylation that occurs upon insulin binding. We report here that insulin-sensitive tyrosine phosphorylation activity still exists after 2,500-fold purification of insulin receptor. The activity is precipitated by an antibody to the insulin receptor and retained in the immunoprecipitate. These data suggest that a tyrosine-specific protein kinase activity is associated with the purified insulin receptor and is probably intrinsic to the receptor itself.

MATERIALS AND METHODS

Materials. Porcine insulin (lot QA 246p) was purchased from Elanco (Indianapolis, IN), insulin-like growth factor (IGF) I and II were gifts of R. E. Humbel (Zurich), EGF was obtained from Collaborative Research (Waltham, MA), and Na^{125}I and [γ-^{32}P]-ATP were from New England Nuclear. Aprotinin, phenylmethylsulfonyl fluoride and *N*-acetyl-D-glucosamine were obtained from Sigma. All reagents for NaDodSO$_4$/polyacrylamide gel electrophoresis were from Bio-Rad.

Purification of Insulin Receptor. The insulin receptor with full binding activity was purified by a method developed by Fujita-Yamaguchi *et al.* and will be published in detail elsewhere (11). Briefly, wheat germ agglutinin (WGA) was coupled to cyanogen bromide-activated Sepharose 4B by the method of Porath *et al.* (12) to a concentration of 6.2 mg of WGA per ml of gel. Insulin-Sepharose was prepared as described by Cuatrecasas (13). The final amount of insulin was 0.24 mg/ml of gel.

Solubilized insulin receptors were obtained by Triton X-100 extraction of a crude microsomal membrane prepared from normal human full-term placenta. From a clear supernatant, obtained by centrifugation at 100,000 × *g* for 90 min at 4°C, the insulin receptor was first partially purified by affinity chromatography on a column containing 50 ml of WGA-Sepharose and eluted by 50 mM Tris·HCl buffer (pH 7.4) containing 0.1% Triton X-100 and 0.3 M *N*-acetylglucosamine. The active fractions were pooled and applied to a column containing 30 ml of insulin-Sepharose, and the purified receptor was eluted by 50 mM acetate buffer (pH 5.0) containing 0.1% Triton X-100 and 1 M NaCl. Fractions containing insulin receptor were neutralized and concentrated. The protein concentration of the sample was calculated from amino acid analysis data.

Standard Phosphorylation Assay with NaDodSO$_4$/Acrylamide Gel Electrophoresis. Purified insulin receptor from human placental membranes (10 μl; 1–2 μg of protein) was incubated without or with hormones for 45 min at room temperature in 25 mM Hepes buffer (pH 7.4) containing 0.1% Triton

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Abbreviations: IGF, insulin-like growth factor; EGF, epidermal growth factor; WGA, wheat germ agglutinin.

† Present address: Third Dept. of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan.

X-100 and $MnCl_2$ (2 mM) in a final volume of 50 μl. The mixture was then chilled on ice for 10 min, the phosphorylation assay was initiated by adding 10 μl of [γ-^{32}P]ATP (final concentration, 5 μM; 10–20 μCi/nmol; 1 Ci = 3.7×10^{10} Bq). The reaction was continued for 30 min at 4°C and stopped by adding unlabeled ATP (2 mM) and 60 μl of twice concentrated $NaDodSO_4$ gel sample buffer (2) and boiled at 100°C for 3 min.

The samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Laemmli (14) with 4% stacking and 7.5% resolving gels. After electrophoresis, the slab gels were stained with 0.25% Coomassie blue in 50% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed with Kodak X-Omat film overnight (15). M_rs were calculated by using standard proteins as described (1). Silver staining of gels was performed by the methods of Morrissey (16).

Identification of Phosphoamino Acids. The phosphoamino acids were analyzed by a modification of the method of Hunter and Sefton (17). The phosphorylated bands of interest were localized by autoradiography and excised from $NaDodSO_4$ gels, and the proteins were eluted by electrophoresis, lyophilized, subjected to acid hydrolysis in 6 M HCl for 2 hr at 108°C, and the phosphoamino acids were separated by two-dimensional paper electrophoresis (pH 2.0 and pH 3.5) as described (2, 3). Samples of phosphoserine (Sigma), phosphothreonine (Sigma), and phosphotyrosine (a gift from T. Hunter, Salk Institute) were added to all radioactive samples analyzed. The standards were located by ninhydrin; the radioactive material was located by autoradiography.

Phosphorylation Assay in the Immunoprecipitate. Purified insulin receptor (10 μl; 1–2 μg) was incubated with 1 μl of rabbit serum containing antibodies to insulin receptor (a gift from S. Jacobs, Wellcome) (18) in 100 μl of 25 mM Hepes containing 0.1% Triton X-100 (pH 7.4) for 2 hr at 4°C. Antigen–antibody complexes were incubated with prewashed staphylococcal protein-A [Pansorbin, Calbiochem; 150 μl of a 10% (wt/vol) suspension] for 1 hr at 4°C, followed by centrifugation at 10,000 × *g* for 5 min. The precipitates were washed twice with 1% Triton X-100/0.1% $NaDodSO_4$ and twice with 0.1% Triton X-100/25 mM Hepes, pH 7.4, as described for the immunoprecipitation method (15). The pellet was resuspended in 500 μl of 25 mM Hepes (pH 7.4) containing 0.1% Triton X-100 and 2 mM $MnCl_2$ and incubated with or without insulin at 100 nM for 30 min at room temperature. The mixture was then chilled on ice for 10 min, and phosphorylation assay was initiated by adding [γ-^{32}P]ATP (final concentration, 5 μM; 10–20 μCi/nmol). The reaction was continued for 30 min at 4°C and terminated by adding NaF, sodium pyrophosphate, EDTA, and unlabeled ATP (final concentrations, 50 mM, 10 mM, 5 mM, and 2 mM, respectively), followed by centrifugation. The pellets were washed once with Hepes buffer containing 0.1% Triton and resuspended in $NaDodSO_4$ gel sample buffer (2) and boiled 3 min. Staphylococcal protein-A was removed by centrifugation, and the samples were analyzed by $NaDodSO_4$/acrylamide gel electrophoresis as described above.

RESULTS

Phosphorylation of Purified Receptor. Human placental insulin receptor was purified 2,500-fold with an overall yield of 40%. The receptor was eluted from an insulin-Sepharose column under mild conditions at 4°C in the absence of urea so that it retained full insulin binding activity. The purified receptor showed a curvilinear Scatchard plot with maximum insulin binding capacity of 28 μg (4,700 pmol) per mg of protein. Direct Coomassie blue staining of the receptor analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction revealed one major and one minor polypeptide chain of M_rs 135,000 and 95,000, which correspond to the α and β subunits of the insulin receptor (Fig. 1, lane A). Furthermore, as discussed in the original paper (11), a variable amount of a M_r 52,000 band was found also in silver staining, which we believe to be a degradation product of the receptor (Fig. 1, lane B); the M_r of this protein was originally estimated to be 60,000 (11). When this highly purified insulin receptor was labeled by using Na^{125}I with chloramine T as described (19) and analyzed in $NaDodSO_4$/polyacrylamide gel electrophoresis after reduction and autoradiography, the same three major bands (M_rs 135,000, 95,000, and 52,000) were found (Fig. 1, lane C). Addition of antibody to the insulin receptor resulted in precipitation of all of the three major bands (Fig. 1, lane D), whereas no radioactive bands were precipitated by control serum (Fig. 1, lane E).

The highly purified insulin receptor was phosphorylated and analyzed as described in Fig. 2. The resulting autoradiogram revealed one ^{32}P-labeled polypeptide of M_r 95,000 in the position of the β subunit of the insulin receptor (Fig. 2, lane A). Preincubation of the receptor preparation with 100 nM insulin for 45 min at room temperature prior to addition of the [γ-^{32}P]-ATP produced a 3- to 5-fold increase in the amount of ^{32}P incorporation into this band (Fig. 2, lane B). Incorporation of ^{32}P was markedly inhibited when the specific activity of the [γ-^{32}P]-ATP was reduced 99.9%; thus, it was unlikely that some labeled contaminant was the ^{32}P source rather than ATP.

To confirm that the M_r 95,000 phosphoprotein was the β subunit of the insulin receptor, the samples were immunoprecipitated by serum containing autoantibodies to insulin receptor from a patient (B-2) with insulin resistance and acanthosis nigricans as described (15). Analysis of the immunoprecipitates revealed the M_r 95,000 phosphoprotein (Fig. 2, lanes C and D), whereas the supernatant remaining after immunoprecipitation showed only an indistinct band of higher M_r and a trace amount of M_r 95,000 phosphoprotein. Similar results were obtained before and after insulin stimulation (Fig. 2, lanes E and F).

In the native insulin receptor, the receptor subunits are

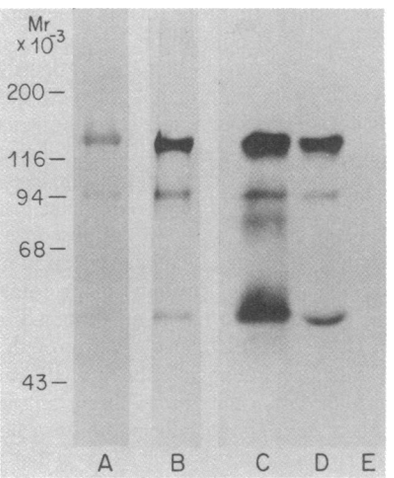

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of highly purified human placental insulin receptor. Lanes: A, Coomassie blue staining of 40 μl (≈7 μg) of purified receptor; B, silver staining of 30 μl (≈4 μg) of purified receptor; C–E, autoradiographs of purified insulin receptor preparations labeled with ^{125}I (chloramine-T method). ^{125}I-Labeled receptors (lane C) were immunoprecipitated by serum containing anti-insulin-receptor autoantibodies (B-2) (lane D) or control serum (lane E). $NaDodSO_4$/polyacrylamide gel electrophoresis was performed after reduction with 100 mM dithiothreitol. Note that three different purified preparations were used for these experiments.

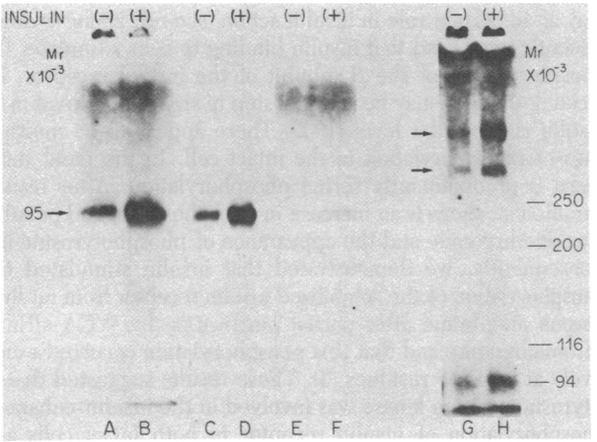

FIG. 2. Autoradiograms showing the incorporation of the ^{32}P from [γ-^{32}P]ATP into highly purified insulin receptor. Highly purified insulin receptor from human placental membranes (1–2 μg) was incubated with [γ-^{32}P]ATP and $MnCl_2$ (2 mM) for 30 min at 4°C without (lane A) or with (lane B) preincubation with 100 nM insulin. The phosphorylation reactions were terminated by adding $NaDodSO_4$ gel buffer (2) and boiling for 3 min. (*Left*) After reduction of disulfide bonds with dithiothreitol (100 mM), these samples were analyzed by 7.5% $NaDodSO_4$/acrylamide gel electrophoresis and autoradiography. The same samples were immunoprecipitated by anti-insulin-receptor antibodies. Immunoprecipitates (lanes C and D) and their supernatants (lanes E and F) were analyzed in the same $NaDodSO_4$/acrylamide gel after reduction and autoradiography. (*Right*) After *in vitro* phosphorylation, the same samples were analyzed by 5% $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography without reduction of disulfide bonds (lane G, basal; lane H, 100 nM insulin stimulation).

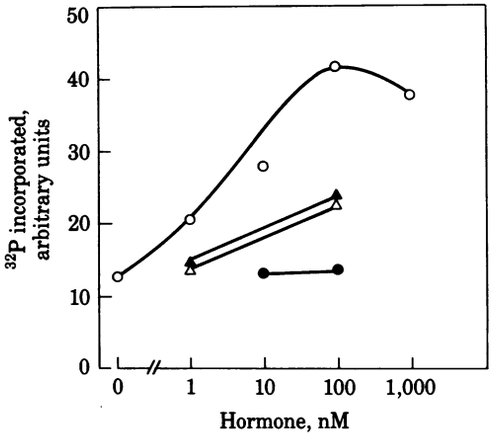

FIG. 3. Dose–response and specificity of insulin receptor phosphorylation. Highly purified human placental insulin receptor (1–2 μg) was incubated with porcine insulin (○), IGFs I (▲) and II (△), or EGF (●) for 45 min at room temperature. The phosphorylation reaction was then initiated by adding Mn^{2+} and [γ-^{32}P]ATP and was allowed to proceed for 30 min at 4°C. Termination of the reaction and analysis were as in Fig. 2 *Left*. The resulting autoradiographs were scanned in a densitometer, and the peak areas corresponding to the M_r 95,000 band were calculated and expressed in arbitrary units of ^{32}P incorporation.

thought to be linked by disulfide bonds to form high M_r oligomers (19, 20). When the same samples were analyzed without reduction of disulfide bonds, three bands were observed (Fig. 2, lane G). Two bands had M_rs of >300,000. These bands are similar to those found when the receptor is labeled by [^{35}S]-methionine and analyzed without reduction (21). In addition, there was one phosphoprotein of M_r 94,000, which may correspond to "free" β subunits (21). After incubation with insulin (100 nM), the phosphorylation of all three bands was increased approximately 3- to 5-fold.

Dose–Response, Specificity, and Identification of the Phosphoamino Acids. We and others have reported that, in addition to insulin receptors, membranes may contain two types of receptors for the IGFs (22–24). Type I IGF receptors (*i*) have a higher affinity for IGF I than do IGF II and insulin, (*ii*) appear to be similar in structure to the insulin receptor (22–24), and (*iii*) crossreact to a varying extent with some anti-insulin-receptor antibodies¶ (25). Type II IGF receptors, on the other hand, have a higher affinity for IGF II than for IGF I but little or no affinity for insulin and have a subunit structure quite distinct from that of either the insulin or type I IGF receptor (22, 24).

To determine the specificity of this receptor, the highly purified insulin receptor was incubated with several concentrations of insulin (1–100 nM), IGFs I and II, and EGF for 45 min at room temperature, and the phosphorylation was carried out as described above. Some stimulation of the phosphorylation of the β subunit of insulin receptor was observed at 1 nM insulin, and maximal stimulation was achieved with an insulin concentration of 100 nM (Fig. 3). IGF I at 100 nM also had some stimulatory effect on the phosphorylation of the M_r 95,000 protein; however, the extent of the effect was much less than that of 100 nM insulin (Fig. 3), suggesting that it was the β subunit of the insulin receptor, not the β subunit of the type I IGF receptor, that was being phosphorylated. IGF II had an effect similar to that of IGF I on the phosphorylation of this protein

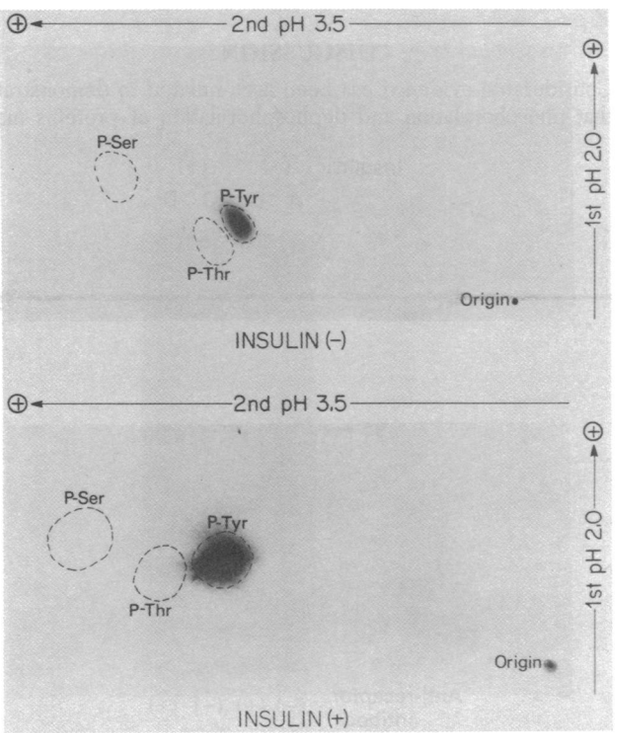

FIG. 4. Identification of phosphoamino acids in the β subunit of highly purified insulin receptor. The phosphorylation of highly purified insulin receptor was conducted without or with stimulation by 100 nM insulin as described in the legend to Fig. 2. The M_r 95,000 bands, localized by autoradiography, were excised from the gel, and the phosphoamino acids were determined. The standards were located by ninhydrin and are delineated by the broken lines. The radioactive material was located by autoradiography.

¶ Kasuga, M. & Rechler, M. M. (1982) Program of the 64th Annual Meeting of the Endocrine Society, June 14–16, San Francisco, p. 180 (abstr. 405).

(Fig. 3). EGF, which was reported to enhance the phosphorylation of its own receptor through a receptor/kinase system (5), had no effect on the phosphorylation of insulin receptor.

To determine the amino acids that were phosphorylated in the M_r 95,000 phosphoprotein of the highly purified receptor, the ^{32}P-labeled phosphoprotein was cut from the gel and partially hydrolyzed in 6 M HCl for 2 hr at 108°C, and the resulting hydrolysates were separated by two-dimensional electrophoresis. By this analysis, only phosphotyrosine was found in both basal and 100 nM insulin-stimulated material, although the insulin-stimulated sample had approximately 3 times more radioactivity (Fig. 4).

Phosphorylation in the Immunoprecipitates of Highly Purified Receptor. As a further attempt to determine the closeness of association of the insulin receptors with the tyrosine-specific protein kinase activity, the highly purified insulin receptor was immunoprecipitated by control serum or serum containing anti-insulin-receptor antibodies and staphylococcal protein A to determine whether the antibody precipitates the kinase activity. The immunoprecipitate was washed with 0.1% Na-DodSO$_4$ or Triton X-100, or both. These immunoprecipitates were incubated with [γ-^{32}P]ATP in the presence of 2 mM $MnCl_2$. Immunoprecipitation by control serum revealed no radioactive band; however, immunoprecipitation by antibodies to receptor revealed one distinct ^{32}P-labeled band of M_r 95,000, suggesting that antibodies to insulin receptor precipitated both receptor and protein kinase activity (Fig. 5, lanes A and B). Incubation of the washed immunoprecipitates with 100 nM insulin also resulted in stimulation of the incorporation of ^{32}P into M_r 95,000 phosphoprotein (Fig. 5, lane D). No phosphorylation of anti-receptor IgG was observed in either basal or insulin-stimulated states.

DISCUSSION

Considerable evidence has been accumulated to demonstrate that phosphorylation and dephosphorylation of proteins may play an important role in insulin action (see ref. 26 for review). Recently, we found that insulin binding to cells stimulates the phosphorylation of the β subunit of the insulin receptor, indicating that this may be an early step in signal transmission by insulin at a cellular level (1, 2). There appear to be multiple sites of phosphorylation in the intact cell. In the basal state, there is predominantly serine phosphorylation. After insulin stimulation, there is 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 from rat liver plasma membrane after partial purification by WGA affinity chromatography and that this phosphorylation occurred exclusively at tyrosine residues (3). These results suggested that (*i*) a tyrosine protein kinase was involved in the insulin-enhanced phosphorylation of insulin receptor in both intact cells and "broken" cell systems and (*ii*) this existed in the plasma membrane and was eluted from a lectin affinity column. In the present study, we have attempted to determine if the tyrosine-specific protein kinase is associated with the insulin receptor itself.

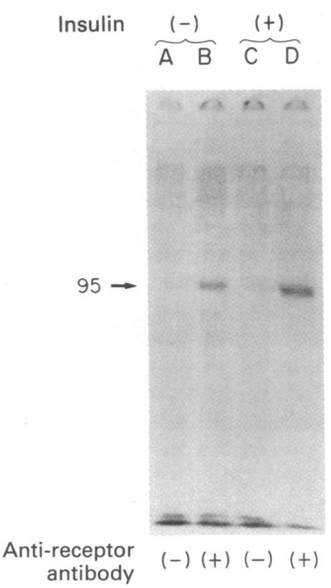

FIG. 5. Phosphorylation of highly purified insulin receptor in the immunoprecipitates. Highly purified insulin receptor (5 μl, 0.5–1 μg) was incubated with serum containing antibodies to the insulin receptor (lanes B and D) or control serum (lanes A and C). Then the IgG was precipitated with protein A. The phosphorylation reactions were initiated by adding [γ-^{32}P]ATP into the immunoprecipitates without (lanes A and B) or with (lanes C and D) preincubation with 100 nM insulin and analyzed. M_r is shown $\times 10^{-3}$.

Recently, one of the authors (Y.F.-Y.) was able to develop a method of obtaining highly purified insulin receptor while preserving a high insulin binding capacity (11). Based on a M_r of 300,000–350,000 for the insulin–receptor complex ($\alpha_2\beta_2$ heterodimer), the purified insulin receptor can bind more than one ^{125}I-labeled insulin molecule per complex. Insulin binding activity of this preparation is 4,700 pmol/mg of protein, approximately 5- to 10-fold higher than previously reported (27–29). The purity of our sample was shown also by Coomassie blue and silver staining and by iodination with Na^{125}I and autoradiography. These methods revealed three major bands of M_rs 135,000, 95,000, and 52,000. The former two bands may be the authentic α and β subunits of insulin receptor, respectively (21). The third major component of M_r 52,000 also appears to be a component of the insulin receptor or, more likely, a degradation product of the α or β subunits, as this component was immunoprecipitated also by several antibodies to insulin receptor.

This highly purified insulin receptor retains a tyrosine-specific protein kinase activity and demonstrates phosphorylation that is hormonally sensitive. Phosphorylation shows proper specificity for the insulin receptor, and the dose–response correlates well with binding properties of the receptor (11). Furthermore, after specific immunoprecipitation by antibody to the insulin receptor, both the highly purified insulin receptor and solubilized, partially purified receptor from other cells still retain protein kinase activity. These results indicate that tyrosine-specific protein kinase activity is closely associated with, and perhaps intrinsic to, the insulin receptor. Despite this very close association, it is still possible that there is a very small amount of contamination by a protein kinase that is bound tightly to its substrate (the receptor), and is not separated from the receptor during the different steps in purification. Thus, absolute conclusions about whether the insulin receptor itself is the protein kinase should be postponed until the insulin receptor has been chemically synthesized or cloned.

Another interesting finding is that the heavy chain of immunoglobulin G against the insulin receptor did not serve as a substrate of the insulin-sensitive tyrosine protein kinase, at least, under our experimental conditions (Fig. 5). In the EGF receptor/kinase system, antibodies against the EGF receptor also are not a substrate (30, 31); however, the antibodies against the transforming protein kinase pp60^{src} of Rous sarcoma virus can be phosphorylated (30, 32). Recently, we (unpublished data) and Petruzzelli *et al.* (33) have shown that partially purified insulin receptor will induce a tyrosine phosphorylation of other

substrates in an insulin-dependent fashion, indicating that under some conditions the insulin receptor/kinase system can indeed act on other substrates.

Phosphorylation of tyrosine is rare in normal cells (17). Tyrosine phosphorylation of specific proteins has been observed after cellular transformation by RNA tumor viruses (17, 34–37) or after stimulation of cell growth by EGF (38), platelet-derived growth factor (6), and human transforming growth factor (39). Tyrosine-specific protein kinase activity has been reported to be closely associated with the transforming gene product (35–37) and also the EGF receptor (5, 31). As in the present experiments, these kinases have been shown to be active in immunoprecipitates and retain activity after partial purification (31, 35–37). These results have attracted considerable interest in the role of tyrosine-specific protein kinases in the control of cell growth and transformation. Here, we have reported that tyrosine-specific protein kinase activity is closely associated with the insulin receptor. This finding raised the possibility that some or even all of the insulin actions may be transmitted from the insulin receptor through activation of an intrinsic tyrosine/protein kinase system. Whether insulin can induce tyrosine phosphorylation of some other cellular proteins, as previously reported for the Rous sarcoma virus-transforming gene product and EGF (40), remains to be determined.

Note Added in Proof. Since submission of this manuscript, several groups have confirmed and extended our observations concerning the phosphorylation of the insulin receptor. These are presented in refs. 33 and 41–44.

We thank Drs. H. U. Häring, Y. Zick, and K. M. Yamada for helpful discussions and comments and Dr. Ora Rosen for making unpublished data available. This work was supported in part by the Juvenile Diabetes Foundation and the National Institutes of Health (Grants AM 31036 and AM 29770). M.K. is a Capps Scholar from Brigham and Women's Hospital and Harvard Medical School.

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