Autophosphorylation Activates the Soluble Cytoplasmic Domain of the Insulin Receptor in an Intermolecular Reaction*

Melanie H. Cobb‡, Bi-Ching Sang, Roberto Gonzalez§, Elizabeth Goldsmith†, and Leland Ellis§
From the Departments of Pharmacology and *Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041 and the †Howard Hughes Medical Institute, Dallas, Texas 75235-9050

The cytoplasmic protein-tyrosine kinase domain of the insulin receptor (residues 959–1355) has been expressed as a soluble protein in Sf9 insect cells via a Baculovirus expression vector (Ellis, L., Levitan, A., Cobb, M. H., and Ramos, P. (1988) J. Virol. 62, 1634–1639). The purified protein is a homomer as judged by its behavior in sucrose gradients and on gel filtration in the presence or absence of protamine. The initial rate of autophosphorylation using 3 mM MgCl₂ is increased 20–30-fold by protamine. A maximum of 4–5 mol of phosphate are incorporated per mol of enzyme. The activity of the enzyme as a function of phosphorylation state was studied for three substrates: a synthetic dodecapeptide derived from the sequence of the major autophosphorylation site in the insulin receptor, poly(Glu,Tyr), 4:1, and histone 2B. Autophosphorylation of the protein to a stoichiometry of 4–5 mol of phosphate/mol increases its enzymatic activity as much as 200-fold; a 30-fold increase in activity occurs upon addition of 1 mol of phosphate/mol. The activities of unphosphorylated enzyme with the three substrates are 3.4, 2.3, and 0.44 nmol/min/mg, respectively. The activities of the autophosphorylated enzyme with the three substrates are 175, 274, and 45 nmol/min/mg, respectively. Exposure of the autophosphorylated enzyme to ADP results in a loss of phosphate from the enzyme which is associated with a decrease in enzymatic activity.

Autophosphorylation of the kinase in the presence or absence of protamine displays a marked dependence on enzyme concentration. Furthermore, the rate of autophosphorylation decreases as the viscosity of the solution increases. Taken together, these data suggest that phosphorylation occurs via an intermolecular reaction.

The insulin receptor is a heterotetrameric (α₂β₂), transmembrane protein consisting of two α-subunits on the outside of the cell that bind insulin, and two β-subunits that span the membrane and terminate in the cytoplasm (1, 2). The cytoplasmic domain of each β-subunit contains a tyrosine-specific protein kinase activity (1, 2) that catalyzes its own phosphorylation, as well as that of a number of protein and peptide substrates (3–6). Receptor autophosphorylation, believed to regulate enzymatic activity (5, 6), occurs on at least 5 tyrosine residues in vivo (7–9). In vitro self-phosphorylation of the receptor appears to be intramolecular (10) and has been correlated with changes in both its specific activity towards exogenous substrates and its reactivity with antibodies to cytoplasmic epitopes (11).

In a previous study (12) it was shown that a protein-tyrosine kinase comprised of residues 959–1355 of the human insulin receptor can be synthesized in Sf9 cells by the use of a Baculovirus expression vector. This soluble protein, the cytoplasmic domain of the receptor, has protein-tyrosine kinase activity and interacts with a panel of monoclonal antibodies that require the native conformation of the cytoplasmic domain for recognition (12, 13).

To examine the enzymatic properties of this independent domain of the insulin receptor, we have measured the rate of phosphorylation of exogenous substrates as a function of the phosphorylation state of the soluble kinase domain and the dependence of the rate of kinase autophosphorylation on its concentration.

MATERIALS AND METHODS

Sf9 cells and Baculovirus vectors, generously provided by Dr. Max Summers and colleagues (Texas A & M), were used and maintained as described (14). The soluble insulin receptor protein kinase expressed in Sf9 cells (12) was purified by chromatography on DEAE-cellulose. Mono Q, and Superose 12. The receptor consisted of one band of M₆, = 48,000 on a SDS-PA gel stained with Coomassie Blue (Fig. 1A). As assessed by 31P NMR spectroscopy, the kinase contained 0.35 mol or less of phosphotyrosine/mol. The intact human insulin receptor was partially purified from human placenta on succinylated wheat germ agglutinin-agarose (modified from Ref. 10). A dodecapeptide based on the sequence of a major insulin receptor autophosphorylation site (4, 7–9), RRRDIYETDYYRK (designated Y3), was synthesized by Clive Slaughter (University of Texas Southwestern). Histone 2B was isolated as described (15). [γ-32P]ATP was synthesized by the method of Johnson and Walseth (16). Poly(Glu,Tyr) in a 4:1 ratio (P-0276) and protamine (P-3880) were from Sigma. Other reagents were obtained from common commercial sources.

Protein was measured with Amido Black using bovine serum albumin as standard (17) and by absorbance at 280 nm. The extinction coefficient of a 0.1% solution of soluble receptor is 1.0; this value was confirmed by N-terminal analysis and was within 5% of that obtained with Amido Black. Phosphoamino acid analysis was as described (5). The Kₘ for ATP was obtained using the Enzfitter program, Elsevier Biosoft.

Protein Kinase Reactions—Protein kinase reactions included 10 mM Hepes, pH 8, either 3 mM MgCl₂ or 3 mM MnCl₂ plus 3 mM MgCl₂, 100 μg/ml bovine serum albumin, protamine, and ATP as indicated, and soluble receptor protein kinase. Exogenous substrates were included at concentrations and for times stated in the figure legends. Aliquots of reactions were terminated with 5 × concentrated

* This work was supported by Grant DK 40511 from the National Institutes of Health, the Howard Hughes Medical Institute, and a postdoctoral fellowship (to B.-C. S.) from the Juvenile Diabetes Foundation. 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 correspondence should be addressed.

† The abbreviations used are: SDS-PA, sodium dodecyl sulfate-polyacrylamide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
‡ B. Levine, B. Clack, and L. Ellis, unpublished.
sample buffer for electrophoresis on SDS-PA gels in order to analyze receptor autophosphorylation and incorporation of $^{32}$P into histone, if it was present. Receptor and histone bands were excised from the gels. Poly(Glu,Tyr) was collected from kinase reactions by precipitation with 1 ml of 10% trichloroacetic acid. Peptide Y3 was separated from radioactive ATP by adsorption to P-81 paper. Incorporation of $^{32}$P into bands excised from gels, acid precipitates, and P-81 paper onto which peptide had been adsorbed was measured by liquid scintillation counting.

RESULTS

The oligomeric state of the purified soluble insulin receptor kinase (Fig. 1A) was assessed by sucrose gradient centrifugation and gel filtration chromatography. Profiles of sucrose gradients (Fig. 1C) indicate that the kinase activity migrates at a position consistent with a monomer of $M_r = 48,000$. The enzyme elutes from Ultragel AcA 44 after alkaline phosphatase counting. Onto which peptide had been adsorbed was measured by liquid scintillation counting and gel filtration chromatography. Profiles of sucrose from radioactive ATP by adsorption to P-81 paper. Incorporation of ATP into bands excised from gels, acid precipitates, and P-81 paper consistent with a monomeric enzyme.

Incubation of the purified enzyme with ATP and Mg$^{2+}$ plus Mn$^{2+}$ results in the slow incorporation of phosphate into the enzyme, which reaches 0.5–1 mol of phosphate/mol of enzyme after 9 h (Fig. 2; see final paragraph of “Results”). The specific activity prior to autophosphorylation using the substrate poly(Glu,Tyr) is 8.4 nmol/min/mg. After 3 h of phosphorylation it has increased to 37.6 nmol/min/mg, a 4.48-fold increase in activity. If the unphosphorylated enzyme is assayed with Mg$^{2+}$ only, the specific activity is 0.5% of that in the presence of both cations.

The addition of the polybasic protein protamine to the reaction dramatically increases the rate of autophosphorylation. Protamine stimulates the phosphorylation reaction by 5–30-fold, maximally between 20 and 300 $\mu$g/ml, in agreement with the findings of Rosen and Lebwohl (18). If protamine is included in assays, MnCl$_2$ is not required for maximal activity. In the presence (or absence) of protamine the amino acid phosphorylated is exclusively tyrosine (Fig. 1B). Protamine itself is not a substrate for the receptor kinase, nor does it appear to cause aggregation of the enzyme. The sedimentation profile of the kinase is indistinguishable whether protamine is incorporated into the gradient solutions (Fig. 1C) or mixed with the kinase prior to centrifugation.

Phosphorylation of the soluble receptor in the presence of 3 mM MgCl$_2$ and protamine results in the incorporation of as many as 5 mol of phosphate/mol of enzyme. During the time course of autophosphorylation shown in Fig. 3, 3.75 mol of phosphate are incorporated per mol of enzyme. Autophosphorylation is associated with a large increase in enzymatic activity. The kinase activity of the most highly phosphorylated enzyme using 60 $\mu$M ATP and peptide Y3 as substrate is 173 nmol/min/mg, 51-fold greater than the 3.4 nmol/min/mg observed for the unphosphorylated enzyme. On the other hand, incubation of the enzyme with MgCl$_2$ and protamine for 60 min in the absence of ATP, so that autophosphorylation does not occur, has no effect on enzyme activity (after 60 min the activity is only 22% of that at zero time). Of the 51-fold activation, a 30-fold stimulation is observed upon the addition of the first mole of phosphate per mol of enzyme (Fig. 3, inset). Activity with the substrates poly(Glu,Tyr) and histone 2B is also increased by autophosphorylation. The specific activity of the kinase with poly(Glu,Tyr) ranges from 2.3 nmol/min/mg in its unphosphorylated state, to 274 nmol/ min/mg, for enzyme with 4.5 mol of phosphate/mol (Fig. 4), indicating a 117-fold increase in activity due to autophosphorylation. This corresponds to a turnover number of approximately 13.2 min$^{-1}$. In some experiments the extent of activation caused by autophosphorylation measured as activity with poly(Glu,Tyr) was as great as 200-fold. The specific activity of the kinase with a third substrate, H$_2$b, ranges from 0.44 nmol/min/mg, in its unphosphorylated state, to 45 nmol/ min/mg, for enzyme with 5.3 mol of phosphate/mol (not shown). Indicating a 101-fold increase in activity due to autophosphorylation.

The specific activity of the soluble enzyme has been measu-
Autophosphorylation of the Soluble Insulin Receptor Kinase

18703

FIG. 2. Correlation of stoichiometry of phosphorylation of the soluble receptor with its enzyme activity. Soluble receptor was autophosphorylated using 250 μM ATP, 3 mM MgCl₂, and 3 mM MnCl₂. At the indicated times aliquots were withdrawn to measure phosphate incorporation into the kinase in mol/mol (○) and enzyme activity in a 10-min assay with 2 mg/ml poly(Glu,Tyr) (●).

FIG. 3. Correlation of stoichiometry of phosphorylation of the soluble receptor with its enzyme activity. Soluble receptor was autophosphorylated using 60 μM ATP, 3 mM MgCl₂, and 30 μg/ml protamine. At the indicated times aliquots were withdrawn to measure phosphate incorporation into the kinase in mol/mol (●) and enzyme activity in a 10-min assay with 250 μM peptide Y3 (○). In the inset the same data are replotted as enzyme activity (nmol/min/mg) versus stoichiometry of autophosphorylation (mol/mol).

Incubation of the autophosphorylated receptor with 10.8 mM ADP results in the time-dependent dephosphorylation of the receptor (Fig. 5). Receptor containing 4.55 mol of phosphate/mol loses 2.6 mol of phosphate in 30 min (Fig. 5A), whereas receptor containing 2.8 mol of phosphate/mol has lost 1.4 mol 30 min after ADP addition (not shown). Fig. 5B shows data for 5 different experiments relating the kinase activity to the phosphate content of the receptor. The kinase activity of the receptor decreases as the receptor is dephosphorylated. Furthermore, enzyme with a given phosphate content has approximately the same specific activity whether that stoichiometry is achieved during phosphorylation or de-
phosphorylation reactions. All assays in Fig. 5A contain the same concentration of ADP indicating that its presence does not account for activity differences. Thus, activation of the enzyme appears to be reversed by its dephosphorylation.

Another correlation between phosphorylation state and specific enzyme activity of the receptor kinase comes from the observation that exogenous substrates, peptide Y3 (Fig. 6), histone, and poly(Glu,Tyr), inhibit the autophosphorylation of the soluble receptor. If enzyme is allowed to autophosphorylate prior to the addition of substrate (Fig. 6, top), its activity increases with increasing concentrations of peptide Y3 until saturation is reached. Phosphate incorporated into the receptor remains relatively unchanged (the largest change is a 35% increase at 0.1 mM peptide Y3) as peptide Y3 concentration is increased. On the other hand, if peptide Y3 is added at the beginning of the autophosphorylation reaction (Fig. 6, bottom), peptide kinase activity increases with increasing concentrations of Y5 up to 0.2 mM and then decreases sharply. Autophosphorylation is maximal in the absence of peptide Y3 and declines in proportion to Y3 concentration. Conditions that reduce autophosphorylation also reduce enzyme activity with exogenous substrates. Qualitatively similar results have been observed using the intact receptor (8), although approximately 10-fold higher concentrations of peptide substrate were required.

To determine directly whether the autophosphorylation reaction is intramolecular or intermolecular, the rate of autophosphorylation has been measured as a function of enzyme concentration. The data of Fig. 7 demonstrate that the amount of phosphate incorporated per mol of enzyme in 15 min is dependent on the amount of enzyme in the reaction. Similar data have been obtained at 1 h, 30 min, and at 5 min. The dependence on enzyme concentration is evident at all ATP concentrations tested (60–500 μM), both in the presence and the absence of protamine (Fig. 7, inset). Furthermore, the concentration dependence is observed both for the first mole of phosphate incorporated per mol of enzyme and for additional phosphate incorporation. This indicates that even after the autophosphorylation reaction has begun, subsequent phosphorylation still depends on enzyme concentration. Autophosphorylation, either under conditions selected to examine its initial rate (with only 0.8% of 1 mol of phosphate incorporated per mol of enzyme (Fig. 7, inset) or in the presence of protamine for a 5-min incubation) or after the reaction has progressed to a significant extent (1.25 mol/mol in Fig. 7 or up to 4 mol/mol), displays a concentration dependence.

As a further test of the possibility that autophosphorylation is intermolecular, the effect of the viscosity of the reaction on autophosphorylation has been examined. Increasing volumes of glycerol have been included in autophosphorylation reactions containing constant amounts of soluble receptor or native receptor. As glycerol concentration increases, the rate of autophosphorylation of the native receptor remains relatively unchanged. On the other hand, the rate of autophosphorylation of the soluble receptor decreases (Fig. 8) by approximately half as the glycerol concentration is increased to 10%. To ensure that the decreased rate is not due to a specific effect of glycerol, autophosphorylation in the presence of mannitol, sucrose, and ethylene glycol at concentrations producing relative viscosities equal to that of 10% glycerol have also been examined. In all three cases the rate of autophosphorylation of soluble receptor is reduced (54, 65, and 46%, respectively, of the control incorporation).

DISCUSSION

The properties of the soluble enzyme described here are similar to the native receptor. Assuming that the tetrameric
The receptor contains two catalytically active β-subunits, the specific activity of the phosphorylated soluble kinase with histone 2B is between two reported estimates of the specific activity for the native receptor. The activity is 3-fold higher than the value published for a similar soluble enzyme by Herrera et al. (21; see below). The $K_\text{m}$ for ATP is also close to that reported for the intact enzyme (20).

Like the intact insulin receptor, the soluble enzyme is regulated by autophosphorylation. The incorporation of 4–5 mol of phosphate into the purified receptor in vitro results in more than a 50-fold increase in enzymatic activity. Activity increases continuously as phosphate is added; however, particularly in the case of the peptide substrate, the largest increase is observed upon addition of the first mole of phosphate to the enzyme. Studies of the intact receptor indicate that phosphate is rapidly incorporated into two to three sites in the receptor that cannot be kinetically distinguished (7–9). As assessed by two-dimensional tryptic phosphopeptide mapping, the tyrosine autophosphorylation sites of this soluble enzyme are comparable to those phosphorylated on the native insulin receptor. From labeling kinetics, no correlation can be made between activation of the kinase and incorporation of phosphate into one specific site. As a result, the physical significance, if any, of the observation noted above is not known.

The extent of activation of the enzyme that occurs following autophosphorylation, from 51–200-fold, is probably an underestimate of the actual enhancement induced by phosphorylation. Because some autophosphorylation has occurred during the time for the kinase assay and there is a small amount of phosphate already on some preparations (see "Materials and Methods"), the activity attributed to the unphosphorylated receptor comes from receptor with a small but finite amount of tyrosine phosphate. If the enzyme contains only 0.025 mol of phosphate/mol (e.g., 5 mol on 0.5% of the enzyme) and we assume that autophosphorylation causes a 200-fold increase in activity, then 50% of the detected activity would come from the 0.5% of the enzyme that is phosphorylated. In some experiments no activity could be detected with the unphosphorylated enzyme suggesting that in cases in which activity is detected it may derive from a small amount of phosphoenzyme. A more detailed evaluation of the activity of the enzyme lacking phosphate should clarify the actual extent of activation.

As noted by others (18), protamine enhances the autophosphorylation rate of the soluble kinase. The mechanism of protamine activation appears to be different from activation by autophosphorylation. The stimulatory effect of protamine does not appear to be due to activation of a distinct kinase for the following reasons. First, protamine has the same effect on the purified soluble receptor as it does on less pure preparations of it. There is no change in the magnitude of the stimulatory effect as the soluble receptor kinase is purified, suggesting that a contaminating enzyme would have to be enriched in proportion to the receptor kinase during purification. Second, the amino acid phosphorylated in the purified soluble receptor in the presence or absence of protamine is tyrosine, ruling out involvement of a serine/threonine kinase. Third, protamine also enhances the activity of the heterotetrameric receptor isolated from a different source (human placenta) by a different procedure (18).

Studies of the autophosphorylation of the intact, tetrameric receptor indicate that its phosphorylation occurs intramolecularly (5, 6; see below). Autophosphorylation of the cytoplasmic domain of the insulin receptor is dependent on enzyme concentration over a wide range of conditions, and the autophosphorylation reaction decreases as the solution viscosity increases. Thus, our data suggest that the autophosphorylation reaction is intermolecular for the soluble kinase domain, both for the first mole of phosphate incorporated and for subsequent phosphate incorporation. Further support for this conclusion is our observation that the soluble receptor phosphorylates the native insulin receptor (to be reported elsewhere). In a recent report (21) it was claimed that a similarly engineered soluble catalytic domain (residues 980–1355) displayed intramolecular autophosphorylation. However, because no data were shown, neither the origin nor the significance of the discrepancy can be determined.

The concentration of enzyme at which a significant rate of autophosphorylation is detected is in the range of 0.5–1 μM. However, because the enzyme and the substrate concentrations cannot be varied independently, it is not possible, without additional information, to predict the $K_\text{m}$ for the binding of two molecules of the kinase.

With respect to the intact receptor there are two ways that intramolecular autophosphorylation could be catalyzed. Each β-subunit of the tetramer might phosphorylate itself (intrachain) or each β-subunit of the tetramer might phosphorylate the other β-subunit (interchain). No studies have yet succeeded in resolving the question of which of these mechanisms is employed or if intrachain and interchain autophosphorylation can both occur (22). Because the monomeric soluble kinase catalyzes the reaction intermolecularly, we suggest that autophosphorylation of the intact receptor may occur by an interchain mechanism.

Acknowledgments—We would like to thank Elliott Ross and Marc Gregory, E. Goldsmith, L. Ellis, and M. H. Cobb, manuscript in preparation.

---

Footnotes:

1. One estimate is 5.2 nmol/min/mg (10) or 0.9 nmol/min/nmol. This value is probably an underestimate because the specific activity of our lectin-purified native receptor is 2–5 nmol/min/mg. From the work of a second group, the value for lectin-purified receptor is 4.6 nmol/min with histone 2B (19). These authors report a 13-fold purification of receptor on insulin-Sepharose using a peptide substrate (20). Extrapolating to histone 2B the purified enzyme would have an activity of 60 nmol/min or 10 nmol/min/nmol of catalytic site. Our value is 3 nmol/min/mg.

2. J. Tavaré, B. Clack, and L. Ellis, unpublished data.

---
Autophosphorylation of the Soluble Insulin Receptor Kinase

Mumby for helpful discussion, Clive Slaughter and Lynn DeOgny for synthesis of the Y3 peptide, Joseph Behan and Jill Gregory for excellent technical assistance, Alla Levitan for technical assistance in the early phase of this work, Beatrice Clack for some preparations of the soluble receptor, and Jo Hicks for preparation of the manuscript.

REFERENCES

1. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., and Rutter, W. J. (1985) Cell 40, 747-758
2. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) Nature 313, 756-761
3. Kasuga, M., Karlsson, F. A., and Kahn, C. R. (1981) Science 212, 185-187
4. Avruch, J., Nemenoff, R., Blackshear, P., Pierce, M., and Otsanathanon, R. (1982) J. Biol. Chem. 257, 15162-15166
5. Rosen, O. M., Herrera, R., Olowe, Y., Petruzzelli, L. M., and Cobb, M. H. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3237-3240
6. Yu, K.-T., and Czech, M. P. (1984) J. Biol. Chem. 259, 5277-5286
7. Tornqvist, H., Gunsalus, J., Nemenoff, R., Frackelton, R., Pierce, M., and Avruch, J. (1986) J. Biol. Chem. 261, 350-359
8. White, M., Shoelson, S., Keutmann, H., and Kahn, C. R. (1988) J. Biol. Chem. 263, 2969-2980
9. Tavare, J., O’Brien, R., Siddle, K., and Denton, R. (1988) Biochem. J. 253, 783-788
10. Petruzzelli, L., Herrera, R., and Rosen, O. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 3327-3331
11. Herrera, R., Petruzzelli, L., Thomas, N., Bramson, N., and Kaiser, T. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7899-7903
12. Ellis, L., Levitan, A., Cobb, M. H., and Ramos, P. (1988) J. Virol. 62, 1634-1639
13. Morgan, D. O., and Roth, R. A. (1986) Biochemistry 25, 1364-1371
14. Summers, M. D., and Smith, G. E. (1987) Texas Agricultural Experimental Station Bulletin No. 1555, College Station, TX
15. Johns, E. W. (1964) Biochem. J. 92, 55-59
16. Johnson, R. A., and Walseth, T. F. (1979) Adv. Cyclic Nucleotide Res. 10, 135-168
17. Schaffner, W., and Weissman, C. (1973) Anal. Biochem. 56, 502-504
18. Rosen, O., and Lebwohl, D. (1988) FEBS Lett. 231, 397-401
19. Pike, L. J., Eakes, A. T., and Krebs, E. G. (1986) J. Biol. Chem. 261, 3782-3789
20. Pike, L. J., Kuenzel, E. A., Casnellie, J. E., and Krebs, E. G. (1984) J. Biol. Chem. 259, 9913-9921
21. Herrera, R., Lebwohl, P., Garcia de Herreros, A., Kallen, R., and Rosen, O. M. (1988) J. Biol. Chem. 263, 5560-5568
22. Bosi-Schnetzler, M., Kaligian, A., DelVecchio, R., and Pilch, P. F. (1988) J. Biol. Chem. 263, 6822-6828
23. Weast, R. C. (ed) (1986) Handbook of Chemistry and Physics, 66th Ed, pp. D-229, D-232, D-237, D-262, CRC Press, Inc., Boca Raton, FL