Characterization of Insulin-mediated Phosphorylation of the Insulin Receptor in a Cell-free System*

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Insulin stimulates phosphorylation of both α- and β-subunits of its own receptor in a cell-free system. A solubilized lectin-purified preparation of insulin receptors from rat liver membranes was preincubated with or without insulin at 4 °C and labeled for 10 min with Mn[γ-32P]ATP; the receptor subunits were isolated by specific immunoprecipitation with anti-receptor antibodies, followed by gel electrophoresis in sodium dodecyl sulfate. In gels run under reduced conditions, two bands (M₀ = 135,000 and 95,000) were selectively labeled. These correspond exactly to the position of the α- and β-subunits of the insulin receptor. Labeling of the M₀ = 95,000 band was approximately 5-fold that of the M₀ = 135,000 band. No labeled bands were detected when identical samples were immunoprecipitated in control serum. Phosphorylation of the receptor subunits required the presence of the divalent cation Mn⁺⁺ or Co⁺⁺; other cations such as Mg⁺⁺, Cr⁺⁺, Ca⁺⁺, and Zn⁺⁺ were ineffective. [γ-32P]ATP served as the ³²P donor, whereas [γ-32P]GTP was ineffective. Phosphorylation of both subunits was stimulated 4-6-fold after a 50-min exposure to 10⁻⁷ M porcine insulin. Insulin-stimulated phosphorylation was half-maximal after 5 min of incubation with 10⁻⁷ M insulin or after 18 h with 3 × 10⁻¹⁰ M hormone. The enhanced phosphorylation was specific for insulin and its analogs; guinea pig insulin was about 2% as potent as porcine insulin, whereas epidermal growth factor, adrenocorticotropic hormone, and glucagon, as well as cAMP, were ineffective. The rapidity and specificity of this reaction, as well as the presence of all necessary components in the plasma membrane, suggest that insulin-mediated receptor phosphorylation is one of the earliest biochemical steps following insulin binding.

In a target cell, insulin bound to its receptor ultimately leads to a large array of molecular events (1–4). While the binding of insulin to the receptor as well as some of the metabolic and growth-promoting effects of the hormone are well characterized, the biochemical events that link these early and late events are unclear. Among the intermediate steps noted are both phosphorylation and dephosphorylation of several cell proteins (2, 3, 5), as well as generation of a peptide-like soluble second messenger (6, 7).

It has been previously demonstrated that EGF (8) and acetylcholine (9) stimulate the phosphorylation of their own receptors. Recently, we showed that insulin stimulates phosphorylation of its own receptor in intact cells (10) as well as in a cell-free system (11, 12). In the intact cell, this stimulation resulted from both the increase in the content of phosphoserine and the appearance of phosphotyrosine (13); whereas, in the broken cells, there was an increase in phosphotyrosine only (11). Phosphorylations of tyrosine groups in proteins are known to be involved in both virus transformation (14) and growth stimulation with polypeptide growth factors. For example, EGF stimulates the phosphorylation of a tyrosine residue of its own receptor (15), and platelet-derived growth factor induces the phosphorylation of tyrosine residues of membrane proteins (16).

In order to gain a better insight into the mode of insulin's action to stimulate phosphorylation of the insulin receptor, we have further characterized the effect using solubilized lectin-purified rat liver membranes. We find that (a) in vitro binding of insulin to a soluble receptor in the presence of Mn[γ-32P]ATP stimulates incorporation of ³²P into both M₀ = 135,000 (α) and M₀ = 95,000 (β) subunits of the insulin receptor; (b) all of the elements required for the reaction appear to be present in the plasma membrane; and (c) the dose, time, and specificity of the reaction are compatible with the conditions needed to induce the early metabolic effects of the hormone.

EXPERIMENTAL PROCEDURES

RESULTS

Effect of Insulin on Receptor Phosphorylation—As previously described (11, 12), incubation of the partially purified insulin receptors for 10 min at 4 °C in the presence of Mn[γ-³²P]ATP followed by specific immunoprecipitation of the receptor with anti-receptor antibodies revealed two major ³²P-labeled polypeptides with molecular weights of 135K and 95K.

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1 Portions of this paper (including "Experimental Procedures," Figs. 1, 3–5, 8, and 9, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9590 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1560, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: 135K which indicates the polypeptide of M₀ = 135,000, for example; EGF, epidermal growth factor; FMSF, phenylmethylsulfonyl fluoride; HEPES, 4(2-hydroxyethyl)-1-piperazinethanesulfonic acid; ACTH, adrenocorticotropic hormone; NaN₃, sodium dodecyl sulfate.
receptor antibodies and whose phosphorylation was stimulated by insulin. These were not characterized during this study.

In our previous studies, we found that when insulin was added to cell-free preparations of 293 cells, a time-dependent increase in receptor phosphorylation occurred (14). In this system, insulin was added during a period of receptor phosphorylation, and the presence of insulin was suggested by the fact that the phosphorylation was reduced 2000-fold (Fig. 4). Furthermore, a linear decrease in the phosphorylation of insulin receptor, as determined previously with receptor subunits labeled by various techniques (17, 18). No 32P labeling of these bands was detected when identical phosphorylated samples (treated with or without insulin) were immunoprecipitated with anti-receptor antibodies (B-2, 2000).

The precipitates were adsorbed to Protein A, washed, and subjected to electrophoresis in 7.5% polyacrylamide slab gel in the presence of 0.1% sodium dodecyl sulfate. The gel was stained, destained, dried, and autoradiographed as described under "Experimental Procedures."

Effect of Duration of Preincubation with Insulin on the Extent of Receptor Phosphorylation—In our previous studies (11), preincubation of receptors with insulin was for 18 h. As seen in Fig. 3, insulin (10^{-7} M) stimulated incorporation of 32P into both the 135K and 95K subunits of the receptor after as little as 1 min of preincubation at 4 °C, with half-maximal stimulation by 4 min; maximal phosphorylation was approached with a 30-min preincubation, and the effect of the hormone was changed little when the preincubation with insulin was extended for as long as 18 h at 4 °C. In a separate experiment (data not shown), insulin (or buffer) and [γ-32P]ATP were added simultaneously. In the presence of insulin, phosphorylation of the receptor was observed to be slightly lower than that of other membrane proteins (19); the 32P content of receptor reached half-maximal levels by 5 min and leveled off after 10 min but showed no fall even after 180 min (data not shown). The reaction was linear for only 3 min, possibly because it was impossible to ensure that the reaction was conducted under saturating concentrations of the substrate (i.e. dephosphorylation of receptor).

Characterization of the Nucleotides and Cations—When we measured the rate of [γ-32P]ATP hydrolysis during the phosphorylation reaction, we found that only 20% of the total [γ-32P]ATP was released as free 32P within the initial 10 min of the reaction, both in the presence and absence of insulin (Fig. 5). Therefore, depletion of ATP during the first 10 min of the reaction did not seem to limit the extent of phosphorylation. These findings also suggest that the enhanced receptor phosphorylation in the presence of insulin was not a result of inhibition of an ATPase activity by insulin. The reason for the sharp leveling off of the time curve (Fig. 5) is not clear yet. Incorporation of 32P into the receptor subunits was markedly inhibited when the specific activity of the [γ-32P]ATP was reduced 2000-fold (Fig. 2, lane G). Furthermore, a linear increase in 32P incorporation occurred when we used increasing concentrations of [γ-32P]ATP at a constant specific activity. These findings argue strongly against the possibility that some labeled contaminant was the 32P source rather than ATP. When we compared [γ-32P]ATP and [γ-32P]GTP as phosphate donors, it could be demonstrated (Fig. 2) that, in both basal and insulin-stimulated states, [γ-32P]GTP was not utilized as a 32P donor. This is in contrast to the EGF-mediated phosphorylation of EGF receptors where GTP can substitute for ATP (19).
As with the EGF-stimulated phosphorylation of the EGF receptor (19), Mn2+ is the most potent cation in stimulating the phosphorylation of the insulin receptor, with Co2+ being considerably less potent (Fig. 6). In contrast to the EGF (19) and acetylcholine (9) systems, Mg2+ (even at 20 mM) was totally ineffective, as were Zn2+, Ca2+, and Cr3+.

**The Dose Response of the Insulin-mediated Phosphorylation**—The effect of insulin on receptor phosphorylation was dose-dependent (Fig. 7). Following an 18-h preincubation of hormone with the receptor, half-maximal stimulation occurred with 3 $\times$ 10^{-10} M insulin, a concentration which is within the physiological range of insulin concentrations in vivo. Shortening the duration of the preincubation from 18 to 1 h at 4 °C shifted the dose response curve 20-fold to the right. This shift of the dose response curve may be explained by the slow binding of insulin to its receptor at 4 °C; as seen in Figure 8, approximately 10% of the amount of insulin bound to the receptor by 24 h is bound after 1 h of preincubation.

Note that receptor phosphorylation is stimulated at insulin concentrations which are more than 1 order of magnitude less than those required for occupancy of the receptor. Half-maximal binding (18 h, 4 °C) occurred in the presence of 60–100 ng/ml (1–1.6 $\times$ 10^{-8} M) of insulin (Fig. 1), whereas half-maximal phosphorylation (under similar conditions) occurred after preincubation with 2–3 ng/ml (3–5 $\times$ 10^{-10} M) of insulin (Fig. 7). A remarkably similar phenomenon, which has been attributed to “spare receptors”, is associated with biological responses to insulin (20, 21) or other hormones (22).

**Hormonal Specificity for Receptor Phosphorylation**—The effect of insulin on the phosphorylation of both receptor subunits was specific for insulin (Figs. 7 and 9). cAMP, as well as other hormones, including EGF, had no effect on the phosphorylation of the insulin receptor. Guinea pig insulin, which is 2% as potent metabolically as pork insulin (23), was 2% as potent in stimulating receptor phosphorylation (Fig. 7). Furthermore, guinea pig insulin manifested the same time- and dose-dependent shifts as pork insulin. That guinea pig insulin at 10^{-8} M did not inhibit the stimulatory effect of 10^{-7} M pork insulin when both were preincubated together with the receptor (Fig. 7) suggests that effects of guinea pig insulin, as in other systems, are related solely to its reduced affinity for the insulin receptor. Desoctapeptide insulin, which has a potency that is 1% or less than that of pork insulin in stimulating glucose metabolism (23) and in competing for the binding of 125I-insulin to the receptors, produced only minimal stimulation of phosphorylation (Fig. 7). Similarly, multiplication stimulating activity (an insulin-like growth factor (23)) had only minimal effect (11). Thus, the specificity for the phosphorylation was consistent with the affinity of the hormone for the insulin receptor.

**DISCUSSION**

Insulin-regulated protein phosphorylation has been studied extensively in several cell-free systems (24–27). For example, insulin causes dephosphorylation of the a-subunit of mitochondrial pyruvate dehydrogenase in a mixture of mitochondrial and plasma membrane (26). Direct addition of insulin and [γ-32P]ATP to rat liver plasma membranes triggers cAMP-dependent phosphorylation of three peripheral membrane proteins including the low Ke CAMP phosphodiesterase (25). In a cell-free system derived from 3T3-L1 adipocytes, insulin stimulates 32P incorporation from [γ-32P]ATP into the ribosomal protein S6 (27).

In this study, using detergent-solubilized partially purified insulin receptors, we show that insulin markedly stimulates phosphorylation of its own receptor. Our results correlate with results of previous studies using intact cells (10). Receptor phosphorylation is rapid, specific, and occurs at concentrations of insulin comparable to those in the circulation in vivo. All of the necessary components of this reaction appear to be present in the plasma membrane. These findings lead us to hypothesize that receptor phosphorylation may be an early step in insulin action.
The phosphorylation reaction described in this report has several features common to other phosphorylation systems. For example, there are many similarities between the insulin-stimulated and EGF-stimulated phosphorylations of their respective receptors (8, 19): (a) occurrence at 4°C with soluble receptor preparations; (b) incorporation of $^{32}P$ into tyrosine residues; (c) independence from cAMP; (d) requirement for divalent cations, with Mn$^{2+}$ being the most effective, Ca$^{2+}$ being much less potent, and Ca$^{2+}$ being ineffective; and (e) occurrence without detectable effect of the hormone on the rate of ATP hydrolysis. However, note that these two systems differ in their receptor substrates, receptor binding sites, and some key features of their phosphorylation machinery. The cation and nucleotide requirements for the insulin system seem to be narrower since GTP and Mg$^{2+}$ were ineffective as substrates in the insulin-stimulated receptor phosphorylation but were effective in the EGF system. Thus, it seems that EGF and insulin utilize, at least in part, different enzymatic machineries to mediate the phosphorylation of their own receptors.

The possibility must therefore be entertained that an early event following insulin binding is a specific phosphorylation of a tyrosine residue of the insulin receptor. This reaction is catalyzed by a protein kinase(s), presumably distinct from the better known EGF-, Ca$^{2+}$-, or CAMP-dependent protein kinases. Whether the same or different kinases phosphorylate the $\alpha$- and $\beta$-subunits of the insulin receptor is still unclear.

Since maximal phosphorylation occurs at relatively low receptor occupancy, one could speculate that, in the solubilized preparation, both the free and insulin-bound receptors serve as substrates for the putative insulin-stimulated protein kinase. In the intact cells, however, this might not necessarily be the case, and the relative locations of the receptors and kinase(s) in the membrane will determine the availability of free receptors for phosphorylation.

Although phosphorylation of proteins as means of regulating their function is a well known phenomenon (28, 29), no data are yet available on the functional implications of the phosphorylation of the insulin receptor. Among several possibilities, phosphorylation could alter the affinity of insulin for its binding site, trigger aggregation and internalization of the receptor subunits, or play a role in coupling insulin binding to insulin bioactivity.

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Additional references are found on p. 80.
Insulin Receptor Phosphorylation

Sodium insulin receptors were purified from the liver of diabetic rats. The insulin receptors were phosphorylated with insulin and the phosphorylation was monitored using a radiolabeled insulin binding assay. The time course of insulin receptor phosphorylation was determined by incubating the receptor with insulin for various times and measuring the amount of insulin bound to the receptor at each time point. The results showed that the insulin receptor phosphorylation increased with time and reached a peak at 15 minutes. The phosphorylation was then dropped and reached a steady state level after 1 hour. The time course of insulin receptor phosphorylation was consistent with the known insulin receptor signaling pathway.
Insulin Receptor Phosphorylation

Figure 8. Kinetics of insulin binding to homogenized receptors. Purified receptors (about 50 µg) were incubated with or without insulin (4 µCi/ml) in a 150 µl solution, pH 5.5, of Buffer I that contained a tracer amount of [125I]-insulin (50-60 pg). At the indicated times the receptor and receptor-bound insulin were precipitated (3b) and the fraction of [125I]-insulin specifically bound to the receptors was determined as in Figure 1.

Figure 9. Receptor phosphorylation in stimulated specifically by active mammalian insulin. Purified receptors were incubated with the indicated stimulus (100 µM for 15 minutes at 37°C) before being phosphorylated as in Figure 2. The IP of the 95K and 135K band was determined as in Figure 3 and presented in arbitrary units.

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