Intermolecular Phosphorylation between Insulin Holoreceptors Does Not Stimulate Substrate Kinase Activity

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We photocoupled benzoylphenylalanine$^{25}$, B29-biotin insulin (BBpa-insulin) to native insulin receptors to obtain a uniform receptor population with covalently bound, non-dissociable ligand. We employed BBpa-insulin-bound and autophosphorylated (activated) receptor to phosphorylate substrate insulin receptor under conditions where the substrate receptor never interacts with insulin. The substrate receptor becomes phosphorylated in this inter-receptor fashion and reaches a phosphorylation state 50% of the maximal obtainable by autophosphorylation. However, this phosphorylation does not activate the substrate receptor to any measurable degree. We conclude that intermolecular phosphorylation of the insulin holoreceptors is unlikely to be of physiological significance.

Insulin-dependent insulin receptor autophosphorylation on tyrosine residues enhances the receptor's exogenous tyrosine kinase activity toward endogenous or exogenous substrates (Kasuga et al., 1983; Roth and Cassell, 1983; Shia and Pilch, 1983; Van Obberghen et al., 1983; Nemenoff et al., 1984; Rosen et al., 1983; Zick et al., 1983; Yu and Czech, 1984) (also reviewed in Lee and Pilch (1994)). The cytoplasmic kinase domain of the insulin receptor has seven documented autophosphorylation sites (Ullrich et al., 1985; Ebina et al., 1985; Torngqvist et al., 1988; Tavare and Denton, 1988; White et al., 1988), and there are two such domains within this functionally dimeric protein. There is consensus that autophosphorylation of the "triphosphorylation site," tyrosines 1146, 1150, and 1151 (for the receptor splice variant lacking exon 11 (Ullrich et al., 1985)), is critical for activation of the receptor (White et al., 1988; Murakami and Rosen, 1991; Wilden et al., 1992; Lee et al., 1993) whereas the role of phosphorylation of other receptor tyrosines is less clear (see Lee and Pilch (1994)). A number of studies have convincingly demonstrated that in transfected cells, insulin receptor phosphorylation can occur by an inter-receptor (intermolecular) mechanism (Lammers et al., 1990; Ballotti et al., 1989; Tartare et al., 1991; Accili et al., 1991; Taouis et al., 1994). However, by necessity, all such studies have employed as substrate receptors mutant or chimeric receptors that are unable to undergo ligand-dependent kinase activation. Nevertheless, in some recent studies, data were obtained that support the notion that such inter-receptor phosphorylation is capable of activating the substrate insulin receptors as kinases toward exogenous substrates (Accili et al., 1991; Taouis et al., 1994). Interpretation of these data is complicated because the supporting experiments were performed in cells and in the presence of ligand. Thus, a cascade of events could lead to receptor activation rather than simple intermolecular phosphorylation of one holoreceptor by another (see "Discussion").

We have recently described the synthesis of BBpa-insulin, an insulin analog that can be covalently coupled to receptor with exceptional efficiency and that is a full agonist for insulin receptor autophosphorylation and insulin action (Shoelson et al., 1993; Lee et al., 1993). Thus, the BBpa-insulin-insulin receptor complex can be used to phosphorylate substrate receptor under conditions where this is the only biochemical event that takes place. Moreover, the covalent linkage of insulin receptor and the hormone analog prevent any complications based on receptor-ligand dynamics. Thus, we observe that native receptors phosphorylated as substrates of BBpa-insulin-linked receptor exhibit no change in exogenous kinase activity from that of the basal, non-insulin-stimulated, non-phosphorylated state.

**EXPERIMENTAL PROCEDURES**

The Insulin Receptor—The insulin receptor was purified from NIH-3T3 cells transfected with human insulin receptor cDNA using wheat germ agglutinin (WGA)-agarose chromatography (Lee et al., 1993). The WGA-agarose-bound insulin receptor was eluted with 0.3 M N-acetylglucosamine in 30 mM HEPES, 0.1% Triton X-100, and 0.02% NaN$_3$ (HTA) with protease inhibitors. The purified receptor was stored at -80 °C with 10% glycerol. The insulin receptor was cross-linked with 10$^7$ M BBpa-insulin under UV light with a >340-nm cut-off filter as described previously (Lee et al., 1993). The autophosphorylation of the receptor was performed in the presence of 50 µM ATP, 10 mM MgCl$_2$, and 8 mM MnCl$_2$. After the indicated time, the reaction was stopped by adding EDTA to the final concentration of 67 mM.

Streptavidin-Agarose-immobilized Phosphorylation Assay (Immobilized Bead Assay)—The insulin receptor was incubated and cross-linked with 10$^7$ M BBpa-insulin for 60 min on ice with a 340-nm cut-off filter. The BBpa-insulin-insulin receptor complex was separated from free BBpa-insulin by spin columns containing Bio-Gel P6DG. The eluant from the spin column was incubated with streptavidin-agarose at a 100-fold molar excess of streptavidin over the original insulin concentration. After a 2-h incubation, the insulin receptor coupled to streptavidin-agarose was isolated by centrifugation. Then, the insulin receptor to be used as a substrate was added to the pelleted agarose beads containing the BBpa-insulin cross-linked receptor. Phosphorylation of the receptor was initiated by addition of 50 µM $^32$P-ATP, 10 mM MgCl$_2$, and 8 mM MnCl$_2$ at room temperature with continuous agitation. At the indicated time, the mixture was centrifuged and the supernatant was subjected to SDS-polyacrylamide gel electrophoresis.

1 The abbreviations used are: BBpa-insulin, benzoylphenylalanine$^{25}$, B29-biotin insulin; IR$_k$, kinase insulin receptor; IR$_s$, substrate insulin receptor; WGA, wheat germ agglutinin; IGF, insulin-like growth factor.

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For measurement of the receptor's exogenous tyrosine kinase activity, the insulin receptor was phosphorylated as described above using cold ATP. Then streptavidin-agarose was pelleted by centrifugation, and the supernatant was assayed for tyrosine kinase activity using poly(Glu:Tyr) (4:1) as a substrate (see below).

In Solution Assay—The insulin receptor was cross-linked with BBpa-insulin as above. Then free BBpa-insulin was removed by Superose 12 desalting chromatography with a fast protein liquid chromatography system. The protein content of fractions was determined and the protein peak at the void volume was pooled. This pool was used as the kinase active receptor (insulin receptor kinase, IRK), for the phosphorylation of substrate receptor. The substrate insulin receptor (IR⁻), without any exposure to insulin, was also passed through a Superose 12 desalting column, and the fractions containing the insulin receptor were pooled and combined with IRK for the inter-receptor phosphorylation assay.

Exogenous Tyrosine Kinase Assay—The exogenous kinase activity of the insulin receptor was examined using poly(Glu: Tyr) (4:1) as a phosphate acceptor. The insulin receptor was preincubated with or without insulin or BBpa-insulin overnight at 4°C and then cross-linked with insulin analog as described above. The insulin receptor was incubated with 50 μM cold ATP containing 10 mM MgCl₂ and 8 mM MnCl₂ for the indicated time at room temperature in order to fully activate the receptor toward reporter substrates. Poly(Glu:Tyr) (4:1) was then added at a final concentration of 0.3 μM. The reaction was stopped by addition of 67 mM EDTA (pH 7.4), and aliquots of the reaction mixture were spotted on 3 × 3-cm Whatman No. 3MM filter paper. The filter paper was washed with 10% (w/v) ice-cold trichloroacetic acid in 10 mM sodium pyrophosphate to remove free 32P. After several washes, the filter paper was dried and counted for 32P as Cerenkov counts/min.

General Assays—Insulin binding assays were performed as previously reported (O'Hare and Pilch, 1989) using polyethylene glycol precipitation. Polyclonal anti insulin receptor was produced by A. Amersham Corp. Insulin was iodinated with Na125I by the lactoperoxidase method (Jorgensen and Larsen, 1980). Monoiodo-A14 insulin was purified by high pressure liquid chromatography and stored at -20°C in the presence of trace amounts of bovine serum albumin and aprotinin. The antibody used for detection of the insulin receptor (R1064) was generated against C-terminal sequenced deduced from insulin receptor (Shoelson et al., 1993). The anti-phosphotyrosine monoclonal antibody (4G10) was purchased from E. Y. Laboratories. γ-[32P]ATP and 125I-protein A were acquired from Amersham Corp. The reagents for cell culture were purchased from Life Technologies, WGA-agarose was obtained from E. Y. Laboratories. γ-[32P]ATP and 125I-protein A were acquired from Amersham Corp. Insulin was iodinated with Na125I by the lactoperoxidase method (Jorgensen and Larsen, 1980). Monoiodo-A14 insulin was purified by high pressure liquid chromatography and stored at -20°C in the presence of trace amounts of bovine serum albumin and aprotinin. The antibody used for detection of the insulin receptor (R1064) was generated against the C-terminus sequence deduced from insulin receptor (Shoelson et al., 1993). The anti-phosphotyrosine monoclonal antibody (4G10) was purchased from UBI. The NIH-3T3 cells (1502) transfected with human insulin receptor cDNA were generously provided by Drs. Takashi Kadowaki and Simon Taylor at NIH (Bethesda, MD). Protease inhibitors included in receptor preparations were 1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 50 trypsin inhibitory milliunits of aprotinin, 1 μM pepstatin, 1 μM 1.5-methylene hydrochloride, and 2 mM EDTA and were purchased from Boehringer Mannheim.

RESULTS

As described in previous reports (Shoelson et al., 1993; Lee et al., 1993), BBpa-insulin, in addition to the benzophenone group at Phe25 for the photoactivatable cross-linker, has a biotin attached to Lys29 that can react with avidin derivatives. Therefore, BBpa-insulin cross-linked to insulin receptor can be separated from the unliganded form of the insulin receptor by streptavidin-agarose. We used this property to determine the effects of insulin receptor phosphorylation occurring by an inter-receptor phosphorylation mechanism. In other words, we used autophosphorylated, streptavidin-immobilized BBpa-linked receptor as an active kinase to phosphorylate unliganded native receptor as a substrate. We then determined the effects of this phosphorylation on the kinase activity of the substrate receptor. Our protocol is shown schematically in Fig. 1, and the results are shown in Fig. 2, a typical autoradiogram of 32P-labeled receptors. Before immobilizing the BBpa-insulin-insulin receptor complex to streptavidin-agarose, excess and non-bound BBpa-insulin was removed by desalting chromatography using spin columns. As shown in Fig. 2A, there is no leakage of immobilized receptor from the beads, and the beads themselves do not alter the basal level of receptor autophosphorylation (compare Fig. 2B and 2D). Insulin receptor phosphorylated as a substrate (Fig. 2C) shows greater 32P incorporation than the basal autophosphorylation state (Fig. 2, B and D) but less than the maximal autophosphorylation state achieved with BBpa-insulin activation (Fig. 2E).

As shown in Fig. 3, top panel, the phosphorylation state of the substrate receptor is 3 times higher than basal but only half the maximal level of autophosphorylated receptor. Nevertheless, numerous published studies have shown a tight correlation with receptor phosphorylation state and exogenous kinase activity (see Lee and Pilch (1994) for a review), and thus it...
might be expected that the higher phosphorylation state of the substrate receptor as compared with basal would result in partial activation of its exogenous kinase activity. However, the substrate receptor (Fig. 3C) did not show any increased tyrosine kinase activity compared with the basal state (Fig. 3B and D). When insulin was added to the receptors from conditions of Fig. 3B, C, and D, all insulin receptors exhibited the same degree of phosphorylation and exogenous kinase activity, suggesting that inter-receptor phosphorylation does not prevent the subsequent full activation of kinase activity by insulin (data not shown). The maximal degree of insulin-stimulated exogenous tyrosine kinase activity was 2.5-fold in the experiment of Fig. 3 (D and E). This is a relatively low value that results from the fact that the immobilized bead assay does not enable us to determine the tyrosine kinase activity at the early time points of autophosphorylation. In order to see a substantial 32P incorporation into substrate receptor, 30 min of phosphorylation was employed in Fig. 3. At this time basal exogenous kinase activity has considerably increased, whereas the activated insulin receptor has already reached its maximal activity, thus resulting in diminished -fold stimulation by insulin. To assess the tyrosine kinase activity of the insulin receptor phosphorylated by the inter-receptor mechanism at the early time points, we employed a solution assay where we mixed activated and non-activated receptor and subsequently measured both autophosphorylation and exogenous kinase activity for the combined receptors.

The active IRK was first photocoupled to BBpa-insulin, and excess ligand was removed as described previously. Phosphorylation was then allowed to proceed for IRK, for IRK, and for mixtures of the two. We show a time course from such an experiment in Fig. 4, where we used a higher amount of substrate receptor than that of kinase active receptor to test intermolecular phosphorylation under the same conditions as in the experiment of Figs. 2 and 3. Therefore, the total 32P incorporation due to basal phosphorylation of IRK is higher than that of the activated IRK. The interaction between the two receptor groups was determined by comparing the sum of the separate results for IRK and IRK by themselves with that from the mixture of IRK and IRK. If there is stimulation of either autophosphorylation or exogenous kinase activity by intermolecular interaction, the results from the mixture of IRK and IRK will be greater than the sum of the results from each group separately. However, if there is no interaction between insulin receptors, then these should be the same. To quantitate these data, the bands corresponding to the insulin receptor from Fig. 4 were cut and counted, and the results are shown in Fig. 4B. At each time point, 32P incorporation into the mixture of IRK and IRK was significantly higher (70-90%) than the sum of the two measured separately, thus indicating that inter-receptor
phosphorylation was taking place, consistent with the previous data of Figs. 2 and 3. We next measured the exogenous kinase activity of the receptor from the various conditions by determining the $^{32}$P incorporation into the synthetic peptide substrate for 5 min as shown in Fig. 4C. The exogenous kinase activity of $I_{R_b}$ is higher than that of $I_{R_k}$ because there is 9 times more $I_{R_b}$ in the assay. After normalizing of tyrosine kinase activity for the amount of the receptor, insulin stimulates the exogenous tyrosine kinase activity of the receptor 7-15-fold over the basal state under these conditions (data not shown). In any case, when the kinase activity of the mixture of $I_{R_b}$ and $I_{R_s}$ is compared with the sum of kinase activity from $I_{R_b}$ and $I_{R_s}$, there is no difference, although at the 10-min time point, there is slightly less kinase activity for the mixture, which we attribute to experimental deviation. Thus, under the conditions of these experiments, the phosphorylation of substrate receptor to a level 2–3-fold over basal is without effect on the kinase activity of this species.

The data from Figs. 2–4 confirm that the interinsulin receptor phosphorylation can occur in vitro, but it is apparently contradictory to the results from our previous study where no phosphorylation of substrate receptor was seen in mixtures of BBpa-insulin-labeled and unlabeled receptor (Lee et al., 1993). However, the major difference from our prior report in the current study is the relative amounts of activated and substrate receptors where the latter was high compared with the former. In other words, the amount of $I_{R_b}$ in the previous studies may have been well below its $K_m$ as a substrate for inter-receptor phosphorylation. This possibility was examined assaying for inter-receptor phosphorylation with various relative amounts of the $I_{R_b}$ and $I_{R_s}$ while maintaining the concentration of the total insulin receptor the same. The results in Fig. 5 clearly show that inter-receptor phosphorylation is dependent on the concentration of substrate receptor and will not occur to detectable levels at low substrate receptor levels.

DISCUSSION

This paper studies the consequences with respect to the tyrosine kinase activity of an insulin receptor phosphorylated as a substrate when one insulin-activated holoreceptor phosphorylates another. The use of BBpa-insulin has allowed us to design a series of experiments to determine if inter-receptor phosphorylation is a possible mechanism for activation of the substrate receptor. Under our experimental conditions, we can separate or clearly distinguish the kinase active receptor covalently coupled to the insulin analog from the substrate receptor. IRS is higher than that of IRK because there is 9–15-fold more IRS in the assay. After normalization of tyrosine kinase activity for the amount of the receptor, insulin receptor was maintained the same as in the reaction mixtures. Proteins were separated in a 3–10% gradient gel, and the insulin receptor was visualized by autoradiography. The autoradiograms were scanned, and the percentage degree of inter-receptor phosphorylation was calculated as $\{\text{result}_{IRS} + \text{result}_{IRK}\} / \{\text{result}_{IRK} + \text{result}_{IRS}\} \times 100$. These results are average of four experiments.

Fig. 5. Inter-receptor phosphorylation of the substrate insulin receptor is dependent on the relative amount of the activated kinase receptor. The insulin receptor preparation and in solution assay were performed as described in the legend of Fig. 4. The relative amount of $I_{R_b}$ and $I_{R_s}$ was changed, but total concentration of the insulin receptor was maintained the same as in the reaction mixtures. Proteins were separated in a 3–10% gradient gel, and the insulin receptor was visualized by autoradiography. The autoradiograms were scanned, and the percentage degree of inter-receptor phosphorylation was calculated as $\{\text{result}_{IRS} + \text{result}_{IRK}\} / \{\text{result}_{IRK} + \text{result}_{IRS}\} \times 100$. These results are average of four experiments.

contradiction to our present results (Accili et al., 1991). Accili et al. (1991) have characterized a Phe$^{382}$ → Val mutant receptor that has normal insulin binding but markedly decreased insulin-stimulated autophosphorylation and exogenous kinase activity (Accili et al., 1989; Quon et al., 1992). When this mutant is mixed with wild type receptor, it becomes phosphorylated and the mixture of the two receptors possesses the exogenous kinase activity one would expect of the similar amount of wild type receptor. One difference between this study and ours is that Accili et al. (1989, 1991) could not exclude insulin from interacting with the substrate receptor, the Val$^{382}$ mutant, which has normal insulin binding. We postulate that insulin binding induces a conformational change in the mutant receptor that either directly allows its phosphorylation as a substrate on the residues critical for kinase activation or in combination with its phosphorylation on other tyrosine residues, it is able to undergo the requisite activation step. In other words, two independent activation mechanisms, insulin binding to the $\alpha$ subunit and phosphorylation of $\beta$ subunit, may enable full activation of the Val$^{382}$ mutant. These observations are supported by the experimental evidence that insulin binding to the insulin receptor as well as phosphorylation of the receptor result in conformational changes that are important and necessary for activation of tyrosine kinase activity (Schenker and Kohanski, 1988; Waugh and Pilch, 1989; Perlman et al., 1989; Baron et al., 1990; Maddux and Goldfine, 1991; Baron et al., 1992). An alternative explanation for the discrepancy between the results of Accili et al. (1991) and our own is that the experimental conditions of our assay do not faithfully mimic the conditions in cells, and thus, different sets of phosphor-
rosine residues are affected. As noted above, we are unable to
directly map these residues in the substrate receptor. In any
case, we think this is a less likely explanation since a very large
body of published data documents the identical behavior of
autophosphorylation and receptor activation whether meas-
ured in cells or with isolated insulin receptors (reviewed by Lee
and Pilch (1994)). Two reports have appeared demonstrating
that phosphorylation of the IGF-1 receptor by the insulin re-
ceptor (Tartare et al., 1991) and by Src (Petersen et al., 1994)
resulted in increased exogenous tyrosine kinase activity of the
substrate IGF-1 receptor. Our data concern only homomeric
interactions of the insulin receptor, and it is possible that the
IGF-1 receptor can serve as a substrate for Src and the insulin
receptor such that it can become activated as a kinase.
In conclusion, the fully phosphorylated and kinase active
insulin receptor can phosphorylate other insulin receptors, but
because this does not result in stimulation of exogenous tyro-
sine kinase activity in the substrate receptor, this process does
not appear to be physiologically significant.

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