The unoccupied insulin receptor is a structurally symmetric, disulfide-linked dimer, comprising two αβ halves, each with a potential insulin binding α subunit and a kinase active β subunit. In the accompanying paper (Shoelson, S. E., Lee, J., Lynch, C. S., Backer, J. M., and Pilch, P. F. (1993) J. Biol. Chem. 268, 4085–4091), we described the utility of a novel insulin analogue, L-benzoylphenylalanine\textsuperscript{29-},\textsuperscript{29'-}biotin insulin (BBpa insulin)\textsuperscript{1} as a probe for receptor behavior, and we determined that binding and cross-linking of one BBpa insulin molecule could fully stimulate insulin receptor autophosphorylation. Here we use this analogue to determine the symmetry of the autophosphorylation reaction. The αβ half-receptor that does not covalently couple to BBpa insulin incorporates 50% more orthophosphate than the αβ half that becomes coupled to the insulin analogue. Phosphopeptide mapping of each receptor half shows minimal differences in the phosphorylation sites or their relative contribution to the phosphate content of each half. The kinetics of \textsuperscript{32}P incorporation into each receptor half are essentially identical over a 10–20-min time course. Phosphopeptide mapping analysis reveals that the phosphate incorporation patterns do not change between the two αβ half-receptor forms (BBpa insulin-linked and unlinked, respectively) at different time points or concentrations of ATP ranging from 12 to 200 μM. Based on these and other data, we propose a model of insulin receptor activation whereby binding of one insulin molecule can trigger autophosphorylation in an asymmetric fashion.

The insulin receptor is a member of the ligand-activated receptor/tyrosine kinase family of transmembrane signaling proteins. Binding of a hormone/growth factor ligand to the extracellular portion of its corresponding receptor results in allosteric regulation of the receptor’s intrinsic tyrosine kinase activity in the cytoplasm of the cell (Ullrich and Schlessinger, 1990). For the epidermal growth factor and platelet-derived growth factor receptors, ligand-dependent allosteric regulation of kinase activity requires the non-covalent association of two monomers formation to form functional dimers (Ullrich and Schlessinger, 1990). As discussed in the previous paper, the insulin receptor is also a functional dimer, but together with the closely related insulin-like growth factor-1 receptor, it is unusual in that the dimeric structure is maintained in the absence of ligand by a covalent disulfide linkage (the class I disulfide) (Massague and Czech, 1982; Boni-Schnetzler et al., 1986; Sweet et al., 1987). Beyond the requirement for receptor dimerization, the subsequent mechanistic steps by which ligand binding activates kinase activity is largely unknown for the insulin receptor and the other members of the receptor tyrosine kinase family.

Because the insulin holoreceptor is composed of two structurally identical αβ-heterodimers (Massague et al., 1980; Ullrich et al., 1985; Ebina et al., 1986), it should have two insulin-binding sites/holoreceptor. However, most experimental evidence indicates that only one insulin molecule binds with high affinity to one insulin holoreceptor at physiological concentrations of insulin. Three lines of evidence for this conclusion have been reported. First, insulin binding to cells and membranes was shown to exhibit negative co-operativity as determined by Scatchard analysis indicating that binding of one insulin molecule to the receptor made the binding of the second molecule more difficult (DeMeyts et al., 1973). Second and in confirmation of this hypothesis, purified αβ-heterodimers prepared by mild reduction of the class I disulfides show only low affinity binding with a stoichiometry of one insulin/αβ-heterodimer, whereas purified αβ3-holo receptors exhibits negative co-operativity and only one high affinity insulin-binding site (Boni-Schnetzler et al., 1987; Sweet et al., 1987). Third, double probe analysis using two different insulin analogues showed that only one analogue at a time could bind to the receptor with high affinity (Pang and Shafer, 1983, 1984).

In the previous paper (Shoelson et al., 1993), we have described L-benzoylphenylalanine\textsuperscript{29-},\textsuperscript{29'-}biotin insulin (BBpa insulin) and its utility for probing insulin receptor structure-function correlates and receptor trafficking. In particular, we confirmed that only one insulin can be cross-linked to one insulin holoreceptor by means of the gel shift assay. Under the conditions used, nearly all receptors were cross-linked and were separated from uncross-linked receptors by the addition of avidin and the gel shift. The fact that the insulin itself has no obvious intrinsic symmetry (Baker et al., 1988; Hua et al., 1991) but must interact with both αβ halves for high affinity binding raises the possibility that autophosphorylation of the β subunits may be asymmetric with respect to insulin-receptor contact sites. Autophosphorylation of the insulin receptor is essential for the full activation of its protein tyrosine kinase activity toward exogenous substrates. There-

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\textsuperscript{1} The abbreviations used are: BBpa insulin, L-benzoylphenylalanine\textsuperscript{29-},\textsuperscript{29'-}biotin insulin; HPLC, high pressure liquid chromatography; TPCK, N\textsuperscript{-}tosylphenylalaninechloromethyl ketone; WGA-agarose, wheat germ agglutinin-agarose.
fore, if autophosphorylation is an asymmetric process, receptor halves may differ in their interactions with cellular signal molecules. We show here that BBpa insulin can be used to study receptor symmetry under conditions where only those receptors covalently linked to this ligand become phosphor-ylated.

EXPERIMENTAL PROCEDURES

Preparation of Insulin Receptors—The NIH 3T3 cells (line 1502) transfected with human insulin receptor cDNA were generously provided by Drs. Takashi Kadowaki and Simeon Taylor (National Institutes of Health, Bethesda, MD). Succinylavadin and insulin receptors were prepared as described in the previous paper (Shoeleson et al., 1983). All experiments were performed in the presence of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 nM leupeptin, 50 mM TLU aproatin, 1 mM pepstatin, 1 mM 1,10-phenanthroline, 2.5 mM benzamidine-HCl, and 2 mM EDTA). Gel Shift Assay—WGA-agarose-purified insulin receptors were incubated in the presence or the absence of 10⁻⁷ M BBpa insulin overnight at 4 °C unless indicated otherwise and were photolyzed on ice with a 200-Watt UV visible lamp (Oriel Corp.) with a 340-nm cut off filter. Receptors were then autophosphorylated in a solution typically containing 50 μM ATP, 10 mM MgCl₂, and 8 mM MnCl₂ in the presence of [γ-³²P]ATP (1 μCi/ml) at room temperature. The reactions were stopped by adding 60 mM EDTA. The preparations of αβ-heterodimeric receptors gel shift assay were performed as previously described (Shoeleson et al., 1993). Briefly for the gel shift assay, phosphorylated αβ₂⁻holoreceptors or αβ⁻heterodimeric receptors (Boni-Schneitzler et al., 1986) were incubated with a 30:1 molar ratio of succinylavadin to insulin in Laemmli (1970) sample buffer containing 0.6% SDS (Pang and Shafar, 1985). In some cases, biotin was added in large excess over the concentration of succinylavadin, to block the reaction of succinylavadin and the biotinyi group of the insulin analogue (BBpa insulin). After the insulin receptor was separated on a 3–10% gradient SDS-polyacrylamide gel, it was analyzed by autoradiography. The gels were stained, destained, and dried. The insulin receptor was then visualized by autoradiography. The corresponding region was excised from the gels and the radioactivity was determined as Cerenkov counts/minute with 30% efficiency. Phosphopeptide Mapping Analysis—Phosphopeptide mapping analysis was achieved by using an HPLC system as described in White et al. (1988). Briefly, the fixed and dried gel fragments containing the insulin receptor were washed with 20% (v/v) methanol for 12 h at 37 °C. After drying with a Savant Speed-vac, the gel fragments were rehydrated in a 2 ml of 50 mM NH₄HCO₃, pH 8.0, containing 100 μg of TPCK-treated trypsin. After incubation for 8 h at 57 °C, an additional 100 μg of trypsin was added, and another incubation for 16 h. The phosphopeptides were separated by a C₄-reverse-phase column with a Beckman HPLC system. For the mobile phase, 20 mM phosphate buffer, pH 3.0, was used. Peptides were eluted by a linear gradient of acetonitrile from 5 to 25% during an 85-min interval at a flow rate of 1.1 ml/min. The fractions (1.0 ml) were collected and counted as Cerenkov radiation. Protein Assay—Protein amounts were determined by the Bio-Rad protein assay solution using bovine serum albumin as a standard. The insulin binding assay was performed as previously reported (O’Hare and Pilch, 1988). Polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

RESULTS

In the accompanying article, we have shown that one molecule of BBpa insulin could be cross-linked to an αβ⁻heterotrameric receptor, and this complex was fully capable of autophosphorylation. While it was not clear whether one or both β subunits is undergoing phosphorylation under these conditions, the covalent linkage of BBpa insulin allowed us to address this question. There are three possible ways that autophosphorylation might occur that can readily be distin-

guished by a gel shift assay (Fig. 1). The first possibility is that only the β subunit on the receptor half to which insulin is cross-linked becomes autophosphorylated (Fig. 1A). The second way is that autophosphorylation and covalent coupling of insulin occurs exclusively on different αβ-heterodimers (Fig. 1B). The last way is that autophosphorylation may occur on both β subunits, regardless of where insulin is bound and cross-linked (Fig. 1C). These models would be differentiated by the ³²P autoradiogram patterns of the αβ⁻heterodimeric receptors in the gel shift assay as depicted in the idealized gels of Fig. 1.

The gel shift assay of the photocoupled and autophosphorylated insulin receptors was performed as shown in Fig. 2. Notably, all of the ³²P-labeled αβ⁻β-receptors were shifted to the higher molecular weight form in the presence of succinylavadin (Fig. 2, lane 4). This is consistent with the fact that only those receptors covalently linked to the BBpa insulin could be autophosphorylated. In other words, the photocoupling efficiency of the BBpa insulin was 100% in terms of phosphorylation activity, although the total efficiency of cross-linking of BBpa insulin is normally about 70% as reported in the companion paper (Shoeleson et al., 1993). Following mild reduction, the phosphorylated αβ⁻heterodimeric receptors migrated as two distinct forms in the presence of succinylavadin, shifted and non-shifted (Fig. 2, lane 8). These data agree with model C of Fig. 1 where autophosphorylation occurs on both β subunits of the insulin receptor. Biotin could inhibit the effects of succinylavadin without the loss of radioactivity (Fig. 2, lanes 5 and 9). Although the autophosphorylation occurred on both αβ⁻heterodimeric receptors, the degree of phosphorylation was not equal. The shifted αβ⁻ half-receptors, those covalently linked to BBpa insulin, accounted for 40% of the radioactive phosphate and the non-shifted αβ⁻ receptors contained the other 60% (Fig. 2, lane 8). There was no loss of phosphate upon reduction of holoreceptors into αβ⁻heterodimers, and the 60-40 ratio of non-shifted to shifted species was observed under all experimental conditions (see below) over dozens of experiments.

To determine if there were any effects of UV irradiation on autophosphorylation activity and the autophosphorylation patterns, the conditions for photolysis were examined. There was no difference in the amount of ³²P incorporation nor the amount of shifting whether photolysis was performed before or after autophosphorylation (data not shown). Without photolysis, no receptors were shifted in the presence of succinylavadin. We confirmed that autophosphorylation of the insulin holoreceptor is an intramolecular mechanism (Shia et al., 1983; White et al., 1984) under the assay conditions employed for photocoupling and autophosphorylation of BBpa-linked receptor. Also, the relative amount of ³²P that was shifted by succinylavadin remained around 40%, with no significant difference over the range of insulin receptor concentration examined (data not shown).

Thus, the consistent result of quantitatively asymmetric receptor autophosphorylation could be explained if different tyrosine residues were reactive on each β subunit. Insulin receptor autophosphorylation sites can be separated into two clusters of tyrosine residues: three at positions 1146, 1150, and 1151 (numbering of Ulrich et al., 1985), and the other two within the COOH terminus at residues 1316 and 1322 (White et al., 1988; Tavare and Denton, 1988; Tornquist et al., 1988). The 60-40 ratio of non-shifted to shifted receptor halves could be explained by the predominant use of one site exclusively on one receptor half. Thus, we determined the phosphorylation sites of each receptor half following photocoupling of BBpa insulin, autophosphorylation, and separa-
Fig. 1. Protocol for assessing the symmetry of autophosphorylation of the insulin receptor using a gel shift assay. The WGA-agarose-purified insulin receptor was incubated with BBpa insulin, cross-linked by UV visible light, then autophosphorylated with \([\gamma-32P]ATP\). Holoreceptor or reduced half-receptors were subjected to SDS-polyacrylamide gel electrophoresis in the presence or absence of avidin. Shifted and non-shifted receptor species were detected by a \(32P\) autoradiogram. Depicted are the three hypothetical possibilities for receptor autophosphorylation.

A comparison of HPLC profiles from the insulin holoreceptor stimulated by native insulin with that stimulated by BBpa insulin revealed no differences in the phosphorylation sites or in the distribution of radioactivity in each peptide (data not shown). We were surprised that only quantitative asymmetry was seen between insulin-coupled and -uncoupled receptor halves, and we surmised that time of autophosphorylation and/or ATP concentrations might influence these results.

We therefore examined peptide mapping profiles for shifted and non-shifted receptors as a function of these parameters. The kinetics of incorporation of \(32P\) into the \(\alpha\beta\)-receptor and into each \(\alpha\beta\)-heterodimeric half-receptor were essentially identical, and we observed no significant difference in the relative amount of \(32P\) that was shifted by succinylavidin at any time point (data not shown). We performed phosphopeptide mapping analysis by HPLC after 1, 3, 10, and 20 min of autophosphorylation, and the data showed no significant difference in the phosphorylation sites nor in the relative amount of \(32P\) incorporation in each site (Fig. 4).

Finally, we investigated autophosphorylation patterns at 12.5, 50, and 200 mM ATP. At all concentrations, the degree of shift in the \(\alpha\beta\) half-receptor was maintained at 40% (data not shown). Phosphopeptide mapping revealed that there was no difference in phosphorylation sites between shifted and
**SUCC-AVIDIN**

**BIOTIN**

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**Asymmetric Insulin Receptor Autophosphorylation**

1 2 3 4 5 6 7 8 9 10

αβ●

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**Fig. 2. Autoradiogram of 32P-labeled insulin receptor.** The WGA-agarose-purified receptor was incubated in the absence (lanes 1 and 10) or presence (lanes 2-9) of 10−5 M BBpa insulin and cross-linked as described under “Experimental Procedures.” Autophosphorylation was performed using 50 μM [γ-32P]ATP, and this reaction was stopped after 3 min with 60 mM EDTA. The αβ● receptor (lanes 1-5) or the dihydrotritol-reduced αβ half-receptor (lanes 6-10) were incubated in the absence (lanes 1-3, 6, 7, 9 and 10) or presence (lanes 4, 5, 8, and 9) of succinylavidin. To compete with the biotinyl group of BBpa insulin, exogenous biotin was added (lanes 3, 5, 7, and 9). The samples were separated in a 3-10% gradient gel and visualized by autoradiography.

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**Discussion**

We wish to determine the mechanism by which insulin binding to the insulin receptor a subunit can transduce a signal to β subunit(s) resulting in autophosphorylation and activation of the receptor’s tyrosine kinase activity toward exogenous substrates. Insulin binding occurs toward the amino terminus of the α subunit with the first 400 amino acids being necessary but not sufficient for this binding (Kjeldsen et al., 1991; Waugh et al., 1989; DeMeurys et al., 1990; Yip et al., 1990; Schumacher et al., 1991; Zhang and Roth, 1991). A conformational change occurs upon ligand binding that alters the interface between each α and also, between each β subunit (Schenker and Kohanski, 1988; Waugh and Pilch, 1989; Perlmutter et al., 1989; Baron et al., 1990). Autophosphorylation then occurs, most probably by a “trans” mechanism where one αβ half-receptor initially phosphorylates the other (Treadway et al., 1991; Frattali et al., 1992). Hybrid receptors composed of a kinase inactive αβ-heterodimer and a COOH terminus truncated αβ-heterodimer were shown to undergo insulin-stimulated autophosphorylation that could only occur by a trans mechanism, although a certain amount of autophosphorylation was attributed to a cis mechanism. Interestingly, this hybrid receptor, composed of a kinase inactive half and a kinase active half, was unable to mediate insulin-stimulated exogenous protein tyrosine kinase activity consistent with a dominant negative effect for receptor possessing one normal and one kinase defective half (Frattali et al., 1992). Other studies have been performed consistent with a cis mechanism of autophosphorylation. When the expressed cytosolic portion of the β subunit monomer was tested, autophosphorylation occurred at the same sites as in the native receptor with one group concluding that this was a cis phenomenon occurring in monomers (Herrera et al., 1988; Villaalba et al., 1989) and another concluding that oligomerization was required (Cobb et al., 1989). Another approach to this issue used trypsin-activated and -truncated receptors which contained the entire β subunit and part of the α subunit near its COOH terminal and showed a cis autophosphorylation mechanism was occurring (Shoelson et al., 1991). The kinetics of autophosphorylation for the truncated receptors, however, do not match that of the insulin holoreceptor. Thus, all of the constructs described in this paragraph exhibit some abnormal biochemical properties as compared to native insulin holoreceptor.

For this reason, we have used BBpa insulin to study autophosphorylation within intact insulin holoreceptors with the following criteria in mind: 1) the analogue must bind to the insulin receptor without changing the receptor’s structural and functional characteristics. 2) It must cross-link to insulin receptors with high specificity and efficiency so the receptor-ligand complex is amenable to conventional biochemical techniques. 3) The insulin analogue must have a reporter group which would allow one to easily follow the covalently cross-linked insulin-insulin receptor complex. BBpa insulin does in fact meet all these criteria. As reported in the previous paper, the 60-100% cross-linking efficiency of the analogue to insulin receptors is exceptional with 55% or less being achieved by previously described techniques (Shoelson et al., 1993). Also, the biotin-avidin interaction allowed the desired easy determination of ligand-linked receptor and receptor half. Appropriate controls revealed that the biotin-avidin interaction was stoichiometric, and the cross-linking conditions were without effect on receptor structure and function.

We show for the first time that insulin-induced autophosphorylation is asymmetric with 40% of the receptor-incorporated phosphate being found on the same half to which BBpa insulin is photocoupled (Fig. 2). However, somewhat unexpectedly, the specific tyrosine residues phosphorylated are nearly identical on each receptor half as determined by phosphopeptide mapping analysis using HPLC (Fig. 3) differing only in the position of pY1 (see text under “Results”). In addition, at the various time points of autophosphorylation or at various concentrations of ATP, neither the degree of distribution of 32P into each αβ half-receptor nor the phosphorylation sites in each half changes with these parameters (Figs. 4 and 5). We had originally postulated that if insulin binding was asymmetric, this might induce asymmetry in the kinase domains such that different tyrosine residues might be phosphorylated on the half-receptor covalently coupled to BBpa insulin as compared to the other half, but this does not appear to be the case.

To explain our results, we propose the working models shown in Fig. 6. The insulin-binding site is depicted as being between the αβ halves because as previously discussed, high affinity insulin binding of one insulin molecule requires both halves of the receptor, i.e. specific contact of insulin with both αβ-heterodimers. We propose that ligand receptor contact will consist of a predominant interaction with one receptor half (the right side αβ in Fig. 6) and a less complete contact with the other (left) half receptor since a second insulin can bind to the complex with the low affinity characteristic of insulin binding to an individual αβ-insulin receptor half (Boni-Schntzler et al., 1987; Sweet et al., 1987). The recently described three-dimensional structure for the extracellular domain of the human growth hormone receptor (human growth hormone-binding protein) (de Vos et al., 1992) provides a general precedent for the participation of two receptor subunits in binding one molecule of hormone. Like the insulin receptor, the human growth hormone-binding protein is composed of two symmetrical subunits that bind only one ligand molecule with high affinity, albeit with non-cooperative kinetic interactions (Cunningham et al., 1991). For the insulin receptor, ligand binding is proposed to trigger autophosphor-
FIG. 3. Phosphopeptide mapping analysis of shifted and non-shifted αβ half-insulin receptor. A, insulin receptor was phosphorylated with 50 μM [γ-32P]ATP for 5 min and separated as described under “Experimental Procedures.” The gel fragments corresponding to the shifted and non-shifted receptor halves were excised, rehydrated, and digested extensively with TPCK-treated trypsin prior to separation of phosphopeptides by a C18 reverse-phase HPLC as described under “Experimental Procedures.” The pY1 peak is a tri-phosphorylated peptide, pY4 and pY5 are bis-phosphopeptide forms from the “tri-tyrosine” residues. pY2 and pY3 are the peptides from the COOH terminus region. B, the amount of 32P radioactivity in each peak from the shifted (closed) and non-shifted (hatched) αβ half-receptors were added up and set to 100% for each half. The contribution of each peak to the total for each half was then calculated and these results are an average ± S.E. of five HPLC phosphopeptide maps and are presented in B. The percentage shifted and non-shifted for each phosphopeptide is shown in C where the radioactivity in each phosphopeptide was set to 100%.
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Fig. 4. Phosphopeptide map of shifted and non-shifted αβ half-receptors as a function of autophosphorylation time. Conditions were as exactly as described in Fig. 3 from HPLC profiles, the proportion of each peptide was calculated as the percentage of the total counts/minute in each αβ half-receptor, and were compared between the shifted (closed) and non-shifted (open) receptor species. Panel A shows the peptides from the tri-tyrosine region and panel B shows the peptides from the COOH terminus region.

at least two possible ways. The first is that there may be sufficient flexibility in the covalently coupled insulin molecule such that it can interact with and trigger autophosphorylation via either receptor half. The carboxyl terminus of the insulin B chain is known to be flexible and moves upon binding of insulin to its receptor (Hua et al., 1991). The second possibility is that the site of covalent linkage of the insulin derivative may be very close to α-α contact sites with 60% of receptors becoming coupled to half, and the rest, the other half, thus leading to the observed distribution of phosphate following autophosphorylation.

Model A can hypothetically be experimentally distinguished from models B and C because in the former case, all ligand-bound receptors would be phosphorylated on both half receptors whereas this would not be the case for the latter models. However, the biotin group on BBpa insulin is not accessible
Fig. 6. Possible models for asymmetric insulin receptor autophosphorylation. Insulin (the shaded oval) is shown to contact both α subunits in an asymmetric fashionresulting in high affinity binding. This binding triggers autophosphorylation (horizontal bars) in trans to the major contact/photocoupling site (step 1) followed immediately by a second transphosphorylation which occurs to a lesser extent (model A). In models B and C, transactivation is occurring either on the opposite half of the receptor to the site of photocoupled insulin (B) or on the same half (C) and no second step occurs.

to avidin when bound to unladen receptor (data not shown). We are currently further exploiting the type of technology presented here for BβA insulin, and we are synthesizing additional insulin derivatives in order to gain a further understanding of insulin receptor structure-function correlates regarding receptor autophosphorylation and their physiological consequences.

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