Insulin-binding Peptide

DESIGN AND CHARACTERIZATION*

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The design and characterization of a six-amino acid-containing peptide that binds insulin is described. The amino acid sequence of the insulin-binding peptide (IBP) was determined from the strand of DNA complementary to the strand of DNA coding for the insulin molecule in the domain of the insulin monomer believed to interact with the insulin receptor. The IBP (Cys-Val-Glu-Glu-Ala-Ser) binds specifically to insulin in a saturable manner with a $K_d$ of 3 nM. This binding process is time dependent and slightly temperature dependent, and the peptide appears to interact with insulin near the carboxyl terminus of the B-chain of insulin. Incubation of insulin with the peptide decreases insulin binding to the insulin receptor by 50%, with no effect on the affinity of insulin for the receptor and no effect on cellular insulin-stimulated deoxyglucose uptake. A polyclonal antibody produced against the IBP will inhibit specific insulin binding to intact cells by approximately 50%, with no effects on insulin-stimulated glucose uptake.

From this data, we suggest that there are at least two domains of the insulin molecule through which it interacts with its receptor, the "binding region" of insulin, which is the domain blocked by the IBP, and the "message region" of insulin, through which insulin not only binds to the receptor, but also generates the cellular signal.

A study was recently published in which a peptide was designed to mimic the ACTH receptor (1). The amino acid sequence of this peptide was determined by the nucleotide sequence of the strand of DNA complementary to the strand of DNA coding for the ACTH (1-24) molecule. The results of this study indicated that this "complementary" peptide bound to ACTH, and antibody generated against the complementary peptide appeared to interact with the ACTH receptor. In an attempt to generate an antibody against the insulin-binding domain of the insulin receptor, we decided to devise and synthesize a peptide according to the method described above (1). However, instead of synthesizing a peptide complementary to the whole insulin molecule, we restricted our search to only those residues of the insulin monomer which 1) are in a domain reported to interact with the insulin receptor (2-4) and 2) are in a linear sequence in the primary amino acid sequence of the insulin molecule. This report describes the design and characterization of this insulin-binding peptide.

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MATERIALS AND METHODS

Design of the Insulin-binding Peptide—The various domains of the insulin molecule have been described by Blundell and Wood (5), and the sequence of the complementary strand of DNA (reading from the 5' to the 3' end) is shown in Fig. 1. Reading this strand of DNA from the 3' to the 5' direction (as would the RNA polymerase) would result in the generation of the messenger RNA species shown in Fig. 1, which codes for the polypeptide Cys-Val-Glu-Glu-Ala-Ser. This peptide was synthesized by OCS Laboratories, Inc. (Denton, TX).

Binding of Insulin to the Peptide—The IBP was immobilized in the wells of flexible, U-bottom 96-well polystyrene microtiter plates (Falcon, Becton Dickinson Labware). To each well was added 100 ml of peptide, 5 mg/ml in 0.1 M sodium carbonate, pH 9.6, and the peptide was allowed to adsorb to the well overnight at room temperature. The wells were washed two times with PBS containing 0.1% bovine serum albumin (Fraction V, Sigma) and then incubated with 32P-insulin in PBS, 0.1% bovine serum albumin for 3 h at room temperature. The wells were washed three times with PBS, 0.1% bovine serum albumin, and then recollected from the plate and counted in a gamma counter. Total binding of insulin to the IBP was determined with iodinated insulin alone. Nonspecific binding was determined in the presence of radiolabeled insulin plus 3 mM unlabeled insulin. Specific binding was calculated as the difference between total and nonspecific binding. All values are reported as specific binding, unless otherwise indicated. All determinations (both total and nonspecific) were performed in triplicate.

Insulin was iodinated by the method of chloramine T, as previously described (6).

In some experiments, the immobilized IBP was incubated with 10 mM N-ethylemaleimide (NEM, Aldrich) before the addition of 125I-insulin to the wells.

The specificity of the binding of IBP to insulin was determined by incubating iodinated calcitonin, parathyroid hormone, or atrial natriuretic peptide (kindly supplied by Dr. D. Scott Linthicum, University of Texas Medical School at Houston) with immobilized IBP. Further specificity was investigated by coincubating immobilized IBP with 125I-insulin and unlabelled proinsulin, desoctapeptide insulin (gifts of Dr. R. Chance, Lilly), insulin A-chain or insulin B-chain (Sigma). The specificity of the binding of iodinated insulin to immobilized peptides was determined by immobilizing peptides of various sizes in the microtiter wells, as described above. The peptides utilized were provided by Dr. D. Scott Linthicum, and have the following designations and amino acid sequences: S28, TTHYQKG; S29, AGHHRPQDEG; S30, YG61PKAGQHRPQDEG; S31, THYXQPQKAGQHRPQDEG; EAE, FSWGAGEQQR.

Binding data were analyzed and $K_d$ values determined through the use of the EBDA and LIGAND computer programs described by Munson and Rodbard (7).

The Generation and Characterization of Anti-IBP Antibodies—Three mg of hemocyanin (Behring Diagnostics) and 10 mg of IBP were dissolved in a total volume of 0.9 ml of PBS. After the addition

1 The abbreviations used are: IBP, insulin-binding peptide; NEM, N-ethylemaleimide; PBS, phosphate-buffered saline.
of 100 μl of 0.5% glutaraldehyde, the solution was stirred overnight at room temperature. The mixture was then diluted to a total volume of 3 ml with PBS, resulting in a stock solution which was 1 mg/ml in carrier protein. The initial immunization of the rabbits was performed with complete Freund's adjuvant, containing 250 μg of immunogen (250 μl of stock), 250 μl of PBS, and 500 μl of complete Freund's adjuvant. Each rabbit was injected subcutaneously on the back, at five separate sites; 200 μl injected at each site. One month after the initial injection, the animal was boosted with incomplete adjuvant. Two weeks after the boost, the rabbits were bled from the ear vein to determine the titer of antibody against IBP. Boosting of the animals into the insulin-binding peptide (IBP) sequence.

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The method for transforming the amino acid sequence of the receptor-binding domain of the insulin molecule into the insulin-binding peptide (IBP) sequence. The details are described under “Materials and Methods."

Fig. 1. The method for transforming the amino acid sequence of the receptor-binding domain of the insulin molecule into the insulin-binding peptide (IBP) sequence. The details are described under “Materials and Methods."

RESULTS

The time course and the temperature dependence of the binding of insulin to immobilized IBP is shown in Fig. 2. At 25°C, the binding attains an equilibrium level within 1.5 h, with a half-time of approximately 20 min. At 4°C, the binding process is slightly slower, requiring approximately 2 h to attain an equilibrium level of binding.

As demonstrated in Fig. 3A, the specific binding of insulin to IBP is a concentration-dependent, saturable process. Plotting of the binding isotherm data according to the method of Scatchard (10), as in Fig 3B, generates a linear plot, with a Kd of 3 nM.

We had some concern that this high affinity interaction between IBP and insulin was a result of thiol exchange between the cysteine sulfhydryl group of IBP and a thiol group on insulin. To eliminate this possibility, IBP was immobilized in the microtiter wells and then incubated with 10 mM NEM to alkylate the sulfhydryl group of IBP. Blocking with ovalbumin and incubation with 125I-insulin was performed as described previously. No change in the binding of insulin to IBP was observed as a result of incubation with NEM (data not shown). A number of variations of this experimental format were also performed: IBP was dissolved in a buffer containing NEM, such that alkylation was achieved before immobilization of the peptide on plastic; NEM was incubated with IBP after the peptide was immobilized, but the alkylation agent was maintained in the buffers throughout the remainder of the experiment, including the incubation with 125I-insulin. The results of these various protocols were the same. Sulfhydryl alkylation of IBP has no effect on the binding of insulin to the peptide.
The binding of insulin to IBP appears to be a specific process. As shown in Fig. 4, the specific binding of the low molecular weight polypeptide hormones calcitonin, parathyroid hormone, and atrial natriuretic peptide is less than 1% of the level of the specific binding of insulin to IBP. The binding of insulin to various immobilized peptides, as shown in Fig. 5, demonstrates the specificity of the interaction between IBP and insulin.

In theory, IBP should be interacting with residues 22-27 of the insulin B-chain. If this is occurring, insulin analogs or subunits which contain these residues should compete with intact 125I-insulin for binding to IBP. That is, insulin B-chain, proinsulin, and unlabeled insulin should compete with 125I-insulin for binding to immobilized IBP. Insulin A-chain and desoctapeptide insulin (which lacks the carboxyl-terminal eight amino acids of the B-chain), should not inhibit the binding of 125I-insulin to IBP. As shown in Fig. 6, proinsulin, insulin B-chain, and unlabeled insulin all compete equally effectively with 125I-insulin for binding to IBP. The IC50 is approximately 50 nM. Desoctapeptide insulin and insulin A-chain had no effect on 125I-insulin binding to IBP. These results are consistent with the interpretation that IBP binds to insulin in the vicinity of the eight carboxyl-terminal residues of insulin B-chain.

The data presented in Figs. 2-6 indicate that the binding of insulin to immobilized IBP is a high affinity, saturable,
time- and temperature-dependent interaction, with a high degree of specificity.

The data presented above address the interaction of insulin with immobilized "solid-phase" IBP. The interaction of insulin with solid-phase IBP might be expected to be different from the interaction of insulin with IBP that is free in solution. In an attempt to elucidate the relative affinity of insulin for "solution-phase" IBP compared to solid-phase IBP, $^{125}$I-insulin was preincubated with various concentrations of soluble IBP before the addition of the $^{125}$I-insulin-IBP mixture to immobilized IBP. In theory, the solution-phase IBP would bind to the $^{125}$I-insulin and prevent the subsequent binding of $^{125}$I-insulin to immobilized IBP. Solution-phase IBP is competing with solid-phase IBP for binding to $^{125}$I-insulin. The results of this experiment are shown in Fig. 6. Solution-phase IBP inhibits the binding of $^{125}$I-insulin to solid-phase IBP with an IC$_{50}$ of 5 nM. This result indicates that the solution-phase affinity of IBP for insulin is similar to the solid-phase affinity of IBP for insulin.

The above data indicate that insulin and IBP interact with a high affinity. Since IBP was designed to act as a receptor analog, the next question to be addressed is whether IBP inhibits the binding of insulin to bona fide insulin receptor. In the experiment shown in Fig. 7, $^{125}$I-insulin was incubated with intact 3T3-1 adipocytes in the presence of increasing concentrations of either unlabeled insulin or unlabeled IBP. In the experiment shown in Fig. 7, unlabeled insulin effectively competed with $^{125}$I-insulin for binding to the cellular insulin receptor, but IBP inhibited insulin binding by approximately 30%. Repetition of this experiment demonstrated that IBP could never inhibit insulin binding by more than 50%. Maximal inhibition reproducibly occurred at approximately 20 nM IBP.

In order to determine if the inhibition of insulin binding to receptor induced by IBP, as shown in Fig. 7, is due to change in the affinity of insulin for the receptor or a change in the number of insulin molecules capable of binding to the receptor, insulin-binding isotherms were constructed in the absence or presence of a constant, high (250 nM) concentration of IBP. At this concentration of IBP, the peptide is in large excess over insulin, and based on the solution phase interaction between IBP and insulin shown in Fig. 6, the binding sites for IBP on insulin should be saturated. The binding isotherms are shown in Fig. 8. Plotting of the data according to the method of Scatchard (10) generated the following results: Without IBP, $K_d = 4.3$ nM; $B_{max} = 0.46$ nM. With IBP, $K_d = 3.1$ nM; $B_{max} = 0.23$ nM.
nm IBP for 3 h to optimize the interaction between peptide and insulin. These solutions were then added to 3T3-L1 cells, and the assay was continued as described above. No effect of the peptide could be detected on either basal (without insulin) uptake, or insulin-stimulated uptake (data not shown), under conditions where 50% inhibition of insulin binding could be demonstrated.

The injection of hemocyanin-coupled IBP into rabbits resulted in the generation of antiserum that reacts with immobilized IBP. As shown in Fig. 9, antiserum from early bled animals demonstrated a signal at dilutions as great as 1:400, compared to preimmune serum. Since this “dot-blot” assay is based on the binding of the antibody to protein A, the antibody would appear to be of an IgG class.

The effect of anti-IBP antibody on the binding of insulin to the insulin receptor in intact cell monolayers is shown in Fig. 10. Increasing concentrations of antiserum result in increasing inhibition of specific insulin binding to cells, such that at a 1:25 dilution of antiserum, insulin binding is inhibited by approximately 50%. Even utilizing undiluted serum, no more than 50% inhibition of insulin binding to intact cells could be demonstrated (data not shown). Corresponding dilutions of preimmune serum have no effect on insulin binding to the cells (data not shown). This antiserum was unable to immunoadsorb 125I-insulin (data not shown).

The effect of anti-IBP antiserum on cellular basal and insulin-stimulated deoxyglucose uptake is demonstrated in Fig. 11. At a 1:50 dilution of antiserum, where insulin binding to the cells is inhibited by 45%, no effect on either basal or insulin-stimulated uptake could be detected. That is, the antiserum itself did not stimulate deoxyglucose uptake nor did the antiserum inhibit the insulin-stimulated uptake of radiolabel. Treatment of the cells with preimmune sera or normal rabbit IgG was also without effect on cellular deoxyglucose uptake (data not shown).

Attempts to immunoadsorb insulin receptor out of a detergent extract of metabolically labeled cells with the anti-IBP antiserum were unsuccessful. No specific bands of radiolabeled protein could be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography when comparing immune versus preimmune antiserum. These experiments were performed under conditions which result in the clean and quantitative immunoadsorption of radiolabeled insulin receptor with an antibody directed against the β subunit of the insulin receptor, and an antibody directed against phosphorylated tyrosine residues (data not shown). In addition, Western blot analysis of the insulin receptor with the anti-IBP antiserum and 125I-protein A did not reveal the presence of any immunoreactive bands.

**DISCUSSION**

The methodology utilized in the design of the insulin-binding peptide described in this report was initially outlined by Bost et al. (1). This technique was based on the initial observation of Blalock and Smith (11) that for a given codon coding for a hydrophilic amino acid, the corresponding codon on the complementary strand of DNA will code for a hydro-
phobic amino acid. In this way, two peptides synthesized from complementary strands of DNA would be "negative images" of each other, when the amino terminus of one peptide is lined up with the carboxyl terminus of the other peptide; hydrophobic regions of one peptide would be aligned with hydrophilic regions of the other peptide. For peptides long enough to have secondary and tertiary structure, one could envision how this might occur. A string of hydrophobic residues on one peptide might form a hydrophobic pocket. The complementary peptide would contain a string of hydrophilic residues in the corresponding region, which could form a solvent-accessible protrusion. The hydrophobic pocket might then interact with the hydrophilic region. While this scenario is highly speculative for describing the interaction of large peptides, it could clearly not be the case for describing the interaction between insulin and the insulin-binding peptide described in this report. The insulin-binding peptide is only 6 amino acid residues long. A single turn of an α helix requires approximately 3.6 residues. While the valine, glutamic acid, and alanine residues, which comprise 66% of IBP, are all believed to strongly participate in the formation of an α helix (12), the peptide could, at most, generate a single helical turn in solution. Therefore, while IBP could have a small degree of secondary structure, there is no potential for tertiary structure. The questions remains, then, as to the nature of the interaction between IBP and insulin. The solution-phase conformation of IBP and the nature of its interaction with insulin is currently under investigation with spectroscopic techniques. In addition, the interaction of IBP with synthetic fragments of insulin B-chain are also under investigation to ascertain the degree to which the intact insulin molecule stabilizes the interaction between insulin and IBP.

When immobilized in the bottom of a microtitre well, IBP interacts with insulin with a high affinity and an apparent high degree of specificity, an interaction characteristic of the binding of insulin to the insulin receptor. In addition, in order for this interaction to occur, the insulin derivative was required to contain that region of the molecule against which the peptide was designed (i.e. B-chain sequences 22–27). As such, neither insulin A-chain nor desoctapeptide insulin would inhibit insulin binding to IBP (Fig. 6). According to the hypothesis of Bost et al. (1), IBP should be considered a receptor "mimic." In many respects, this is the case. Insulin binding to bona fide insulin receptor is not inhibited by insulin B-chain or proinsulin, in contrast to the situation with insulin binding to IBP. However, the binding of insulin to IBP is remarkably high in affinity and specificity. A search for sequence homology between IBP and the insulin receptor was therefore performed to confirm any regions of identity. This search identified two regions, each with a 66% homology (4 of 6 residues). These homologous receptor sequences were found in the insulin-binding subunit, in the region postulated by Rutter et al. (13) to be the insulin-binding domain. We have synthesized one of these receptor fragments, and find that this receptor peptide also binds insulin with high affinity.

There is currently much controversy in the literature over the amino acid residues of the insulin molecule which interact with the receptor. Early work (3) indicated that the carboxyl terminus of the insulin B-chain was responsible for both binding activity and biological activity. More recent work (4) demonstrated that desoctapeptide insulin, which is lacking the terminal eight amino acids of the B-chain, would bind to the insulin receptor, but three orders of magnitude greater concentrations of ligand were required, compared to native insulin. It appeared clear, therefore, that this region of the insulin B-chain was involved in the binding interaction between insulin and the receptor, and that the binding domain map proposed by Blundell and Wood (2) might have some validity. The data presented here also support this region of the insulin molecule as important in binding to the receptor.

The finding that IBP would inhibit insulin binding to the receptor by 50% but have no effect on insulin action was consistent with the finding that the antiserum generated against the peptide would inhibit insulin binding by 50% but have no effect on glucose uptake. Regarding this discrepancy between insulin binding and insulin action, some data have been recently published indicating that insulin binding activity can be modulated independently of biological activity. Cutfield et al. (14) have demonstrated that the carboxyl terminus of insulin A-chain is involved in insulin bioactivity but not binding activity. More recently, in a series of papers (15–17), Chu et al. demonstrated that modification of A19 aspartate led to a very slight reduction in binding activity but a drastic decrease in bioactivity. The conclusion from their studies was that the amide bond between residues 20 and 21 of insulin A-chain is part of the "message region" of the insulin molecule, and is quite distinct from the "binding region." Along these lines, then, IBP would appear to interact with the insulin molecule in a binding region, and not a message region. However, IBP and anti-IBP antiserum only inhibit 50% of the total insulin binding capacity. Therefore, another binding region may exist on insulin and on the receptor which has an affinity equal to the IBP-inhibitable binding region and is able to generate full biological response. Validation of this speculation awaits further study.

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