Mutations in human insulin cause an autosomal-dominant syndrome of diabetes and fasting hyperinsulinemia. We demonstrate by residue-specific photo cross-linking that diabetes-associated mutations occur at receptor-binding sites. The studies use para-azido-phenylalanine, introduced at five sites by total protein synthesis. Because two such sites (ValA3 and PheB24) are largely buried in crystal structures of the free hormone, their participation in receptor binding is likely to require a conformational change to expose a hidden functional surface. Our results demonstrate that this surface spans both chains of the insulin molecule and includes sites of rare human mutations that cause diabetes. *Diabetes* 53:1599–1602, 2004

The insulinopathies describe a monogenic form of adult-onset diabetes due to mutations in the insulin gene (1,2). Patients respond normally to exogenous insulin but exhibit fasting mutant hyperinsulinemia due to delayed receptor-mediated clearance of the variant hormone (2). Inheritance is autosomal dominant with variable penetrance. The presence of one wild-type allele permits normal growth and development; homozygous or hemizygous mutations have not been observed and are presumably incompatible with life. Diabetes-associated mutations may either impair binding of the variant insulin to its receptor or perturb subcellular trafficking and processing of the variant proinsulin in the pancreatic β-cell (2). Mutations that impair binding have been identified at three invariant sites: ValA3 → Leu, PheB24 → Ser, and PheB25 → Leu. By analogy to the nomenclature describing abnormal hemoglobin, these are designated insulins Wakayama, Los Angeles, and Chicago, respectively (1). We demonstrate here that these mutations occur at contact sites between insulin and the α-subunit of the insulin receptor.

The structure of insulin is well characterized by crystallography (3) and nuclear magnetic resonance spectroscopy (4,5) (Fig. 1A). Residues A3, B24, and B25 exhibit distinct environments. Whereas PheB25 projects from the surface, ValA3 and PheB24 are engaged in long-range interactions (Fig. 2). ValA3 contacts TyrB26 and ProB28 at an interface between the NH2-terminal A-chain α-helix and COOH-terminal B-chain β-strand (Fig. 2B). PheB24 packs against ValB12, LeuB15, TyrB16, and CysB19 to stabilize the supersecondary structure of the B-chain. In dimers and hexamers, PheB24 and PheB25 also participate in an intermolecular β-sheet, an essential element of insulin’s storage form in the β-cell (3). Whereas considerable evidence indicates that the exposed side chain of PheB25 contacts the insulin receptor (6, including previous photo cross-linking studies [7,8]), the roles of ValA3 and PheB24 have long been the subject of speculation (3,9–13).

To test whether residues A3, B24, and B25 contact the insulin receptor, we have synthesized insulin analogs containing a photo-activatable derivative of phenylalanine (Phe), para-azido-Phe (Pap) (8,14). Pap was chosen based on its rigidity and small size (relative to other photoactivatable moieties), thus limiting the distance range for cross-linking. Modified A- and B-chains were prepared by solid-phase synthesis using the photostable precursor para-α-mono-Phe. To enable efficient detection of cross-linked peptides, the α-amino group of the B-chain was biotinylated (8). The nonstandard side chain was introduced into an engineered insulin monomer (DKP-insulin, which contains three B-chain substitutions: HisB10 → Asp, ProB28 → Lys, and LysB29 → Pro), chosen as a template for its efficiency of synthesis, enhanced receptor binding, and absence of confounding self-association (4). A3, B24, and B25 *para*-α-mono-Phe analogs exhibit respective receptor-binding affinities of 2.0 ± 0.2, 59 ± 2, and 147 ± 3% relative to native insulin (Kd 0.48 nmol/l); the affinity of the biotin adduct of DKP-insulin is 132 ± 5% (assays performed in triplicate). Corresponding analogs were prepared at terminal positions A1 and A21 (ordinarily conserved as Gly and Asn, respectively) at the periphery of insulin’s putative receptor-binding surface (3,15); their relative receptor-binding affinities are 46 ± 5 and 79 ± 22% AsnA21 projects from the surface near GlyB23 and PheB25 (Fig. 2A). Conversion of *para*-α-mono-Phe to Pap in the intact hormones was verified by mass spectrometry.

From the 1Department of Biochemistry, Case Western Reserve School of Medicine, Cleveland, Ohio; the 2Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York University, New York, New York; and the 3Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois.

Address correspondence and reprint requests to Michael A. Weiss, Case Western Reserve University, Department of Biochemistry, 10900 Euclid Ave., SOM Room W427, Cleveland, OH 44106-4935. E-mail: michael.weiss@case.edu.

Received for publication 11 December 2003 and accepted in revised form 25 February 2004.

DDT, dichloroethanol; FullII, second fibronectin-homology domain; ID, insert domain; Pap, *para*-azido-phenylalanine; Phe, phenylalanine.

© 2004 by the American Diabetes Association.
A3, B24, and B25 Pap analogs each exhibit rapid and efficient cross-linking to the ectodomain of the receptor on ultraviolet irradiation (red asterisks in Fig. 1B and C). Efficiency (defined as the probability of photo cross-linking of the Pap derivative once bound to the receptor) is highest for PapA3. Similar results are obtained with the lectin-purified holoreceptor. No covalent complex is observed in the absence of irradiation or in control studies of the para-amino-PheB25 precursor. Photo cross-linking is successively diminished by the addition of native insulin or by higher concentrations of IGF-1 (Fig. 1D). In contrast to the cross-linking at sites of clinical mutation, photo cross-linking of Pap derivatives at A1 and A21 is markedly less efficient (green arrows in lanes 6 and 14 in Fig. 1C; relative to A3, complexes are reduced by 19- and 4-fold, respectively). As a first step in identifying sites of cross-linking in the insulin receptor, we used partial proteolysis with trypsin and chymotrypsin to characterize fragments of the receptor/α-subunit covalently bound to insulin. Analysis of such fragments demonstrates that PapB24 contacts the NH$_2$-terminal L1/β-helix domain, the major hormone-binding region of the receptor (13). By contrast, PapB25 contacts the COOH-terminal region of the α-subunit in accordance with the pioneering study of Kurose et al. (8). Edman sequencing demonstrated that PapB25 cross-links to tryptic peptide 704-718 in the insert-domain tail.

**FIG. 1.** Insulin structure and photo cross-linking. A: Ribbon model based on crystal structure (3) showing sites of clinical mutation (ValA3, PheB24, and PheB25, red) and terminal residues of A-chain (GlyA1 and AsnA21, blue). The A-chain is shown in silver and B-chain in gray or magenta (B26-B30). B and C: Photo cross-linking of Pap-modified insulin analogs (6 kDa) to ectodomain of insulin receptor (290 kDa α,β,γ tetramer; α subunit, 115 kDa; β fragment, 30 kDa). Analysis of photo products (asterisks) by SDS-PAGE and Western blot using NeutrAvidin to detect biotin tag on insulin B-chain (NAv) or polyclonal antiserum to NH$_2$-terminal peptide of the α-subunit (IRα-N; Santa Cruz Biotech). B: Top panel: Photo cross-linking via positions B25 (lane 4) and B24 (lane 8) analyzed after reduction by DTT. Lanes 1–3 and 5–7 indicate control reactions in which either the ectodomain was omitted (lanes 1, 2, 3, and 6) or samples not irradiated (lanes 1, 3, 5, and 7). Middle and bottom panels: Control blots demonstrating that equal amounts of ectodomain (middle panel; with DTT) and insulin (bottom panel; without DTT) were present in each reaction. C: Photo cross-linking via positions B25, A1, A3, and A21 (lanes 2, 6, 10, and 14, respectively) analyzed without reduction. D: Specificity of photo cross-linking is indicated by competition using native insulin (lanes 1–6) or IGF-1 (lanes 7–12). Protein concentrations in successive lanes are in each case 0-, 6-, 20-, 60-, 200-, and 600-fold greater than that of the photoreactive analog (PapB25). Efficiency of photo cross-linking is not affected by the addition of lysozyme or IgG as nonspecific competitors (not shown). In each experiment, the concentration of insulin analog and/or ectodomain was ca. 200 nmol/l in 50 mmol/l HEPES, 0.1% Triton X-100, and 110 mmol/l NaCl (pH 7.4).
hormone-receptor complex, followed by reduction with dithiothreitol (DTT), yields a 34-kDa adduct that, on further digestion, yields a 20-kDa adduct. Following enzymatic deglycosylation, the apparent mass of this fragment is 14 kDa and thus contains about 120 amino acids. The results of Kurose et al. imply that the latter fragment contains the COOH-terminal portion of the α-subunit derived from the second fibronectin-homology domain (FnIII₁) and insert domain (ID) (13).

Pap⁺³, which is not predicted to contact the receptor in a current model based on electron-microscopic image reconstruction (16), cross-links the COOH-terminal to the L1 and cysteine-rich domains. To localize this site more precisely, a second Pap⁺³ derivative was prepared in which the biotin tag was attached at the NH₂-terminus of the A-chain (rather than the NH₂-terminus of the B-chain; see RESEARCH DESIGN AND METHODS). This design facilitates mapping following DTT reduction as above. Limited chymotryptic digestion thus demonstrates that Pap⁺³ cross-links to the same COOH-terminal 34-kDa and 20-kDa adducts as Pap⁻²⁵, i.e., within the FnIII₁-ID-derived tail. We suggest that the A3 binding site (like that of Pap⁻²⁵ [8]) resides within the ID-derived portion, since the FnIII₁ moiety may be deleted in active fragments of the α-subunit (13). Because the L1 domain and the COOH-terminal domain are distant in the sequence of the α-subunit, the present results suggest that these and other sites of photo cross-linking are nearby in the three-dimensional structure of the hormone-receptor complex (13,16). It is not known whether Pap⁺³ and Pap⁻²⁵ cross-link to the same α-subunit or to dimer-related α-subunits within the αβ₂ heterotetramer. Sites of weak cross-linking by Pap⁺¹ and Pap⁺³ derivatives were not characterized.

Photo cross-linking of Pap⁺³ and Pap⁻²⁴ derivatives is of structural interest. Because Val⁺³⁰ and Phe⁻²⁴ are largely buried in crystal structures of insulin (3), it has been unclear whether these residues contact the receptor or serve as structural supports. A possible role for Phe⁻²⁴ in redirecting the main chain of insulin on receptor binding has been proposed based on the unexpectedly high activities of n-aminoo acid substitutions (9). We and others have hypothesized that detachment or reorganization of the COOH-terminal region of the B-chain near B24 exposes the side chains of Phe⁻²⁴ and Val⁺³⁰ and thus enables them to contact the receptor (11–13,17). The present results support (but do not establish) this hypothesis. Although we cannot exclude that Pap-mediated contacts are probe dependent (i.e., not ordinarily made by Phe⁻²⁴ or Val⁺³⁰), a direct interaction would rationalize the exquisite sensitivity of binding at each site to subtle modifications (such as Tyr⁻²⁴, Ala⁺³⁰, Thr⁺³¹, and Leu⁺³⁰) (9,10). A direct interaction is consistent with the structure and function of a truncated insulin analog lacking the COOH-terminal five residues of the B-chain (residues B26–B30) (magenta in Fig. 1A). In the crystal structure of this analog, Val⁺³⁰ is exposed in an otherwise native-like conformation (18). When the new COOH-terminus is amidated, this analog is fully active (19). Conversely, tethering the COOH-terminal segment of the B-chain to the A-chain yields a native-like single-chain analog with essentially no biological activity (17). Furthermore, a conformational change in the B-chain would rationalize the low activity of a "chiral" analog in which the internal side chain of Ile⁻²⁰ (also shielded by Tyr⁻²¹ and Pro⁻²⁶) is substituted by allo-isoleucine (10). This modification does not perturb the structure or stability of insulin but would alter its "hidden" functional surface (20).

In the decades since the crystal structure of insulin was elucidated in 1969 by D. Hodgkin et al. (3), the residues required for its function have been extensively investigated by mutagenesis and chemical modification (3,13). Interpretation of these results is incomplete, however, as such approaches do not generally distinguish between side chains that contact the receptor and those required to stabilize insulin’s active conformation. By exploiting site-
specific photo cross-linking, the present studies strongly suggest that sites of clinical mutations (1,2) are in direct contact with the insulin receptor. A molecular understanding of such contacts, likely to emerge from a crystal structure of the hormone-receptor complex, may enable design of nonpeptide insulin agonists for the treatment of diabetes.

RESEARCH DESIGN AND METHODS
Insulin analogs were synthesized and purified as described (4,8). The relative receptor-binding affinities of penta-, amino-Phe analogs were determined by competitive displacement of $^{125}$I-insulin from a human placental membrane preparation, as previously described (10). Conversion of such analogs to Pap derivatives was effected as described (4). Lectin-purification of the insulin receptor overexpressed in cell line P3-A was performed by the procedure of Yoshimasa et al. (21). Photo cross-linking reactions were performed at high concentrations of hormone and receptor (ca. 200 nM/ml) to enable essentially complete binding of the Papp$^{5}$ analog. Short-wave ultraviolet light (254 nm) generated from a Mineralight Lamp (Model UVG-54; UVP, Upland, CA) was used with an optimum exposure time of 20 s and a distance of 1 cm from the light source. Identification of photo cross-linked receptor domains utilized prior characterization of chymotryptic and tryptic sites (22,23). Western blots used Neutrinivin (Pierce, IL) and a polyclonal anti-receptor antisem that recognizes the NH₂-terminal region of the α subunit (N-20; Santa Cruz Biotech, Santa Cruz, CA). For such mapping studies, analogs contained an amino-caproyl-biotin tag either at the ε-amino group of residue B1 or, in the case of the second Papp$^{5}$ derivative, the ε-amino group of ε-lysine introduced in place of glycine at position A1. Domain-mapping studies are supported by SDPAGE analysis of cross-linked fragments following enzymatic deglycosylation as described (24).

ACKNOWLEDGMENTS
We thank G.D. Smith and C. Yip for kindly providing the receptor ectodomain and D.F. Steiner for a mammalian cell line overexpressing the human insulin receptor. S.H.N. was supported in part by the Diabetes Research & Training Center of the University of Chicago. This work was supported in part by grants from the National Institutes of Health to P.G.K. (DK56673) and M.A.W. (DK40949).

REFERENCES
2. Steiner DF, Tager HS, Chan SJ, Nanjo K, Sanke T, Rubenstein A: Lessons of glycine at position A1. Domain-mapping studies are supported by SDS-PAGE analysis of cross-linked fragments following enzymatic deglycosylation as described (24).