A recent affinity labeling study has suggested that amino acids 704–717 of the C terminus of the insulin receptor represent a contact site for insulin. To determine whether these amino acids are part of a ligand binding site, we have performed alanine-scanning mutagenesis of this region. Mutant cDNAs encoding recombinant secreted receptors were transiently expressed in 293 EBNA cells, and their insulin binding properties were evaluated. Of the 14 residues in this region only 4 amino acids, Asp-707, Val-712, Pro-716, and Arg-717, could be mutated to alanine without compromising insulin binding. The reduction in affinity resulting from the individual mutation of the remaining amino acids varied from an increase in $K_d$ to 3.69 $\times 10^{-9}$ M (Asn-711) to greater than $10^{-3}$ M (Thr-704, Phe-705, Glu-706, and His-710); the $K_d$ of native secreted recombinant receptor is $0.56 \times 10^{-9}$ M.

Insulin initiates signal transduction in target cells by binding to a specific cell surface receptor, which is a member of the growth factor receptor tyrosine kinase superfamily of proteins (1). Ligand binding leads to the activation of the receptor's tyrosine kinase activity and the initiation of intracellular signaling. Mutational studies of receptor signaling and the elucidation of the structure of the receptor's tyrosine kinase catalytic domain suggest that kinase activation is effected by intramolecular transphosphorylation of the constituent tyrosine kinase catalytic domains of the receptor heterotetramer (2, 3). The molecular details of the mechanism by which insulin binding initiates the transphosphorylation event remain obscure and will require an understanding of the molecular organization of the extracellular domain of the receptor and the molecular basis of insulin binding.

The insulin receptor is composed of two disulfide-linked heterodimers, each of which is composed in turn of a 135-kDa $\alpha$ subunit (entirely extracellular) linked by a disulfide bond to a 95-kDa $\beta$ subunit, which has an extracellular domain, a single $\alpha$ helical transmembrane domain, and an intracellular domain containing the tyrosine kinase catalytic activity (1). While the tertiary structure of the extracellular domain has not been elucidated, the presence of several characteristic structural motifs can be predicted from inspection of the deduced amino acid sequence (4, 5). The $\alpha$ subunit contains a cysteine-rich domain homologous to that of the epidermal growth factor receptor (4, 5), and there are also two fibronectin type III repeats; the first is composed of the C terminus of the $\alpha$ subunit and the N terminus of the $\beta$ subunit, and the second is composed of the C terminus of the extracellular region of the $\beta$ subunit (6).

This proposal has received support from recent experimental observations (8–12). We have recently performed alanine-scanning mutagenesis of the L1 domain of the insulin receptor and have shown it to contain a ligand binding domain composed of 14 amino acids organized in four discontinuous peptide segments (13). However, it is unlikely that this is the complete insulin binding site, as secreted recombinant receptors with deletions N-terminal to the C terminus of the $\alpha$ subunit fail to bind insulin (14). Further, mutant receptors which are not proteolytically cleaved into mature $\alpha$ and $\beta$ subunits only bind insulin with low affinity (15). Recently Kurose et al. (16), using affinity labeling techniques, identified a novel insulin contact site just proximal to the C terminus of the $\alpha$ subunit between threonine 704 and lysine 718. In the present study we have performed alanine-scanning mutagenesis of this region of the receptor and demonstrated that it is a major determinant of insulin binding.

MATERIALS AND METHODS

General—All molecular biological procedures, including agarose gel electrophoresis, restriction enzyme digestion, ligation, bacterial transformation, and DNA sequencing, were performed by standard methods (17). All oligonucleotides were purchased from DNAGen (Malvern, PA). Restriction and DNA modifying enzymes were from New England Biolabs, Inc. (Beverly, MA). Porcine insulin was purchased from BiosPacific (Emeryville, CA), and insulin radiolabeled at tyrosine A14 of the A chain ([125I-Tyr-A14]insulin) was from Amersham Corp. Protease inhibitors were from Boehringer Mannheim. 293 EBNA cells were purchased from Invitrogen (San Diego, CA). Medium for tissue culture was from Mediatech (Herndon, VA), and serum was from Sigma. Anti-insulin receptor monoclonal antibody 18–44 (18) was generously provided by Dr. M. Soos and K. Siddle (University of Cambridge, U.K.).

The abbreviation used is: [125I-Tyr-A14]insulin, insulin radiolabeled at tyrosine 14 of the A polypeptide chain.
A polyclonal antibody directed toward the C terminus of the insulin receptor α subunit (amino acids 657–670) was from Upstate Biotechnology, Inc. (Lake Placid, NY), and affinity-purified goat anti-mouse IgG was from Pierce.

Oligonucleotide-directed Mutagenesis—Oligonucleotide-directed mutagenesis was performed by the method of Kunkel et al. (19). A DNA encoding a recombinant secreted receptor (20) subcloned into the phagegend ptZ18U was used as the template for mutagenesis. Where possible, restriction sites were deleted or introduced with the specific mutations in order to permit enrichment of mutants by restriction selection or purification and to facilitate the screening of mutants. All amino acids in the affinity-labeled peptide identified by Kurase et al. (16) (Thr-704–Lys-718) were mutated to alanine with the exception of Lys-718, since this residue is encoded by the alternatively spliced exon 11 of the insulin receptor gene (21) and, hence, was not considered likely to contribute to an insulin binding site.

Expression of Mutant Insulin Receptor cDNAs—The mutant insulin receptor cDNAs encoding a secreted receptor extracellular domain were subcloned into the expression vector pCDE. This is a modification of pCEP4 (Invitrogen) from which the coding region for the Epstein-Barr virus nuclear antigen has been deleted; this vector thus contains the receptor cDNAs encoding a secreted receptor extracellular domain. These constructs were expressed in 293 EBNA cells (an adenovirus-transformed human kidney cell line expressing Epstein-Barr virus nuclear antigen) by transfection with 2 μg of miniprep DNA using the commercially available lipofection reagent Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s directions. For analysis of transient expression, medium and cells were harvested 1 week after transfection. Conditioned medium was concentrated prior to assay using Centrprep 100 centrifugal concentrators (Amicon, Inc., Beverly, MA).

We chose to utilize the extracellular domain for these experiments as it is expressed in large amounts by this expression system and insulin only binds to a single homogeneous population of binding sites in this protein (8, 13), thus simplifying the analysis of binding data. Further, it has been demonstrated that affinity changes by mutations of this form of receptor show good correlation with those observed in the membrane-bound receptor as a consequence of the same mutation (8, 9, 13, 22).

Beverly, MA).

Insulin Binding Assays—Soluble insulin receptor binding assays were performed using a microtiter plate antibody capture assay. Microtiter plates (Immuno 4, Dynal, Inc., Great Neck, NY) were incubated with affinity-purified goat anti-mouse IgG (50 μg/well of 20 μg/ml solution in 0.2 M sodium carbonate, pH 9.4) for 2 h at room temperature. After washing and blocking for 15 min with 250 μl of SuperblockTM (Pierce), wells were then incubated overnight at 4°C with a 1:100 dilution in SuperblockTM of crude asotes of anti-insulin receptor monoclonal antibody (18, 23). After washing with phosphate-buffered saline, wells were incubated for 4 h at 4°C with soluble receptor, diluted to give 10–20% [125I-Tyr-A14]insulin binding in the absence of added unlabeled insulin under assay conditions. After washing with buffer (0.15 M sodium chloride, 20 mM Heps, pH 7.8, 0.1% bovine serum albumin (w/v), 0.025% Triton X-100 (v/v), and 0.02% sodium azide (w/v)), wells were incubated for 48 h at 4°C with [125I-Tyr-A14]insulin (12 pm) and varying concentrations of unlabeled insulin in 100 μl of binding buffer (137.5 mM sodium chloride, 10 mM magnesium sulfate, 20 mM Heps, pH 7.8, 0.5% bovine serum albumin (w/v), 0.025% Triton X-100 (v/v), and 0.02% sodium azide (w/v)). To terminate the assay, wells were aspirated and washed three times with 300 μl of ice-cold wash buffer and then counted.

Insulin binding data were analyzed by the LIGAND program (24) in order to obtain the Kd of the expressed protein. Transfection and binding assays were repeated at least once to confirm the Kd of each mutant. Each result is the mean of two experiments.

Immunoblotting—Immunoblotting of the insulin receptor α subunit in conditioned medium and detergent lysates of transfected cells was performed according to standard methods (25) using an antibody directed toward the C terminus of the α subunit. Blots were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

RESULTS

Mutant receptor cDNAs were transiently expressed in 293 EBNA cells. To evaluate expression and post-translational processing of the receptor, conditioned medium from transfected cells and detergent lysates of the cells were analyzed by Western blotting with an antibody directed toward the C terminus of the α subunit of the insulin receptor. In conditioned medium, insulin receptor α subunit, M, 135,000, was detectable in all transfections (Fig. 1). In detergent lysates of cells, a protein of M, 160,000, corresponding to the predicted mobility of the secreted receptor precursor (20, 26, 27), was expressed in all transfected cells (Fig. 1). The M, 135,000 band observed in the cell lysates probably represents a nonspecifically reacting protein as it is detectable in equivalent amounts in transfected and nontransfected 293 EBNA cells (data not shown). For each transfection the amount of detectable α subunit relative to precursor was similar (Fig. 1), suggesting that the mutations did not cause any major perturbation of post-translational processing of the receptor.

As in our previous study (13), insulin binding to recombinant insulin receptor extracellular domain secreted by transiently transfected 293 EBNA cells displayed simple kinetics with a linear Scatchard plot (data not shown). However, analysis with the LIGAND program (24) indicated a single population of binding sites with a Kd of 0.56 ± 0.02 × 10⁻⁹ M (mean ± S.E., n = 6) (26). This result is in contrast to our previous study (13) in which we obtained a Kd of 1.41 ± 0.09 × 10⁻⁹ M (mean ± S.E., n = 6) due to the modification of the assay conditions (28). Since studies utilizing alanine-scanning mutagenesis have demonstrated that meaningful changes in affinity produced by a single alanine substitution range from 2- to 100-fold (29), in the experiments described below we regarded any mutant with a greater than 2-fold increase in Kd (i.e. Kd greater than 1.2 × 10⁻⁹ M) as demonstrating a significant disruption of insulin-receptor interactions.

The effects of alanine mutations of amino acids in the region between threonine 704 and lysine 718 on the dissociation constant (Kd) of the secreted receptor for insulin are shown in Table I. Mutation of Asp-707, Val-712, Pro-716, and Arg-717 appeared to be without deleterious effect on insulin binding; in fact mutation of Asp-707, Val-712, and Pro-716 produced small increases in affinity for insulin (Kd = 0.28, 0.31, and 0.37 × 10⁻⁹ M, respectively). Mutation of all other amino acids in this region produced marked perturbations of insulin binding, with Kd values of the mutants varying from 3.69 × 10⁻⁹ M for mutation of Asn-711 to levels too high to be determined accurately by our assay method (Kd > 10⁻⁶ M) for Thr-704, Phe-705, Glu-706, His-710, and Val-713 (Table I). These data are represented graphically in Fig. 2 as ratios of the Kd of the mutant to
acids 704 and 717 of the insulin receptor binding.

Dissociation constants for the binding of insulin to alanine mutants of the recombinant secreted insulin receptor

293 EBNA cells were transfected with cDNAs encoding alanine mutants of the recombinant secreted insulin receptor prepared by oligonucleotide-directed mutagenesis. One-week post-transfection-conditioned medium from the cells was harvested, and the expression and insulin binding of the mutants was examined as described under "Materials and Methods." The dissociation constant was determined by computer analysis of this data with the LIGAND program (24). The dissociation constant of the native recombinant secreted insulin receptor, determined under these conditions, was 0.56 ± 0.02 × 10−9 M (mean ± S.E., n = 6). The mutants are designated by the amino acids mutated to alanine, using the single letter code, followed by the numbers indicating their positions in the sequence of the insulin receptor (4, 5). Thus T704A is a mutant in which Thr-704 of the receptor has been mutated to alanine. — designates mutant receptors with affinities too low to be accurately determined by the methods employed in this study. Each dissociation constant is the mean of two determinations.

Table I

| Mutant | Dissociation constant
|--------|------------------------|
|        | M × 10−10              |
| T704A  | —                      |
| F705A  | —                      |
| E706A  | —                      |
| D707A  | 0.28                   |
| Y708A  | 104.0                  |
| L709A  | 84.4                   |
| H710A  | —                      |
| N711A  | 36.9                   |
| V712A  | 0.31                   |
| F713A  | —                      |
| V715A  | 6.13                   |
| P716A  | 0.37                   |
| R717A  | 1.0                    |

The equivalent region of the insulin-like growth factor-1 receptor (amino acids 690–704) is highly conserved (34) and thus may play a similar role in ligand binding. There are only two nonconservative substitutions, Val-690 for Thr-704, Tyr-708, and Phe-715) play a prominent role in insulin binding.

Several lines of evidence suggest that the decreases in affinity observed as a consequence of these mutations, with the possible exception of Thr-704 (see below), are probably direct effects on ligand receptor interactions rather than the consequences of misfolding of the mutant proteins. Previous analyses of protein structure and function have shown that the effects of alanine mutants tend to be localized and nondisruptive of global protein structure (29). In the case of the growth hormone-growth hormone receptor interactions, crystallographic studies have confirmed the involvement of determinants identified by scanning mutagenesis in hormone receptor interactions (30).

While we have not been able to accurately determine the affinity of all mutants in either this study or our previous study because of technical limitations of our assay procedure, it appears that the amino acids in this region make as great if not greater contribution to the free energy of insulin binding than do those in the L1 domain (13). In the L1 domain mutation of only 2 amino acids, Arg-14 and Phe-64 to alanine, produced a decrease in affinity that was too great to be determined (13). Mutation of Asn-15 to alanine produced a 250-fold decrease in affinity, and mutation of Phe-39 to alanine caused a 35-fold decrease (13). Mutation of the remaining amino acids implicated in insulin binding only produced decreases in affinity ranging from 3- to 15-fold (13). In contrast, in the scan of amino acids 704–717, mutation of 4 amino acids (Thr-704, Phe-705, Glu-706, and His-710) produced decreases in affinity that were too great to be accurately quantitated, and mutation of 3 amino acids (Tyr-708, Leu-709, and Phe-714) caused a greater than 120-fold decrease in affinity. Certainly the magnitude of these free energy contributions is sufficient to account for the finding that secreted recombinant proteins generated from receptor cDNAs encoding truncations N-terminal to this region fail to bind insulin, although they are secreted from transfected cells as stable proteins (6). Also they are of sufficient magnitude to account for the low affinity of the uncleaved precursor of the alternatively spliced isoform of the receptor lacking the amino acids encoded by exon 11 (15). The proteolytic cleavage of this form of the receptor may, therefore, be a prerequisite for this region of the receptor to adopt its normal conformation. Also their role in insulin binding may account for the variation in the affinity for insulin of the two alternatively spliced isoforms of the receptor (33).

DISCUSSION

Our results demonstrate that the region between amino acids 704 and 717 of the insulin receptor α subunit appears to play a major role in high affinity insulin binding by the secreted recombinant receptor. Of the 14 residues in this region only 4 amino acids, Asp-707, Val-712, Pro-716, and Arg-717, can be mutated to alanine without severely compromising insulin binding. The reduction in affinity resulting from the individual mutation of the remaining amino acids varied from an increase in Kd of 3.69 × 10−9 M (Asn-711) to greater than 10−6 M (Thr-704, Phe-705, Glu-706, and His-710). As with our previous findings for the L1 domain (13), it appears that the aromatic amino acids of this region of the receptor (Phe-705, Tyr-708, and Phe-715) play a prominent role in insulin binding.

The equivalent region of the insulin-like growth factor-1 receptor (amino acids 690–704) is highly conserved (34) and thus may play a similar role in ligand binding. There are only two nonconservative substitutions, Val-690 for Thr-704 and Ser-699 for Val-712, and of these substituted residues only Thr-704 appears to be critical for high affinity insulin binding.
However, its role may be indirect. Chimeric receptors produced by replacement of the N terminus of the insulin-like growth factor-1 receptor by the corresponding region of the insulin receptor bind insulin with relatively high affinity (8, 10). This observation effectively excludes the possibility of the participation of other side chains critical for insulin binding. It has also been suggested that the C-terminal region of the α subunit is a determinant of ligand specificity of the insulin and insulin-like growth factor-1 receptors (35). The conservatively substituted residues, Asp-707, Tyr-708, and Val-713, could, therefore, play a role in mediating specificity for insulin.

In two recently proposed models of insulin-receptor interactions (28, 36), it has been suggested that insulin has two receptor binding sites and that the receptor heterodimer in turn has two ligand binding sites. High affinity insulin binding is generated by the asymmetrical binding of one insulin molecule to the two receptor heterodimers constituting the holoreceptor, i.e. Site 1 of the insulin molecule binds to Site 1 on the first heterodimer and Site 2 of the insulin molecule binds to Site 2 on the second heterodimer according to Schaffer’s nomenclature (28). In the isolated heterodimer and the secreted receptor extracellular domain, only one receptor insulin binding site on the insulin molecule (Site 1) binds to its corresponding site on the receptor heterodimer (Site 1), producing low affinity interactions (28). Since in both this and our previous study (13) we have examined the effects of alanine mutations on the affinity of the secreted recombinant insulin receptor, the ligand binding determinants we have identified are those of binding Site 1 according to this model of insulin-receptor interactions. More quantitative analyses will be necessary to determine whether the free energy contributions of the amino acids, which we have identified in the N and C terminus of the α subunit as being essential for high affinity insulin binding, are sufficient to account for the free energy of insulin binding of this form of the receptor. In addition, high resolution structural analysis will be essential to fully characterize the role of these amino acids in insulin binding.

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