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Analysis of a peptide hormone–receptor interaction in the yeast two-hybrid system

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ABSTRACT **Interaction between a peptide hormone and extracellular domains of its receptor is a crucial step for initiation of hormone action. We have developed a modification of the yeast two-hybrid system to study this interaction and have used it to characterize the interaction of insulin-like growth factor 1 (IGF-1) with its receptor by using GAL4 transcriptional regulation with a β-galactosidase assay as readout. In this system, IGF-1 and proIGF-1 bound to the cysteine-rich domain, extracellular domain, or entire IGF-1 proreceptor. This interaction was specific. Thus, proinsulin showed no significant interaction with the IGF-1 receptor, while a chimeric proinsulin containing the C-peptide of IGF-1 had an intermediate interaction, consistent with its affinity for the IGF-1 receptor. Over 2000 IGF-1 mutants were generated by PCR and screened for interaction with the color assay. About 40% showed a strong interaction, 20% showed an intermediate interaction, and 40% give little or no signal. Of 50 mutants that were sequenced, several (Leu-5 → His, Glu-9 → Val, Arg-37 → Gly, and Met-59 → Leu) appeared to enhance receptor association, others resulted in weaker receptor interaction (Tyr-31 → Phe and Ile-43 → Phe), and two gave no detectable signal (Leu-14 → Arg and Glu-46 → Ala). Using PCR-based mutagenesis with proinsulin, we also identified a gain of function mutant (proinsulin Leu-17 → Pro) that allowed for a strong IGF-1–receptor interaction. These data demonstrate that the specificity of the interaction between a hormone and its receptor can be characterized with high efficiency in the two-hybrid system and that novel hormone analogues may be found by this method.**

The interaction between peptide hormones or growth factors and the extracellular domains of their cell surface receptors is the initial step in regulation of cellular metabolism, growth, and differentiation. Methods for characterizing this interaction generally rely on having cells expressing sufficient quantities of receptor and biologically active labeled ligands (1), or physical methods such as affinity chromatography, affinity labeling, immunoprecipitation, or the use of the Biacore surface plasmon resonance detector (2–4). Defining the specificity and structural requirements of the hormone–receptor interaction is extremely tedious, requiring synthesis of numerous hormone analogues or *in vitro* mutants of the receptor and analysis of the interaction of these proteins in the binding assay (5).

The yeast two-hybrid system has provided a powerful method for analysis of protein–protein interactions, especially when the domains of the protein involved in the interaction can be defined (6, 7). This method has been widely used to study the interaction between enzymes and their substrates (8–10), to study interactions between intracellular signaling molecules, and to screen for unknown molecules that interact with various cellular proteins (11–13). This method has generally not been considered for analysis of the interaction between the extracellular domain of a receptor and its ligand (7, 14), although recently a three-hybrid system has been used to detect such interactions between small ligands and their protein receptors (15).

In this study, we demonstrate that the yeast two-hybrid system can be used to analyze the interaction between a hormone and its receptor, using insulin-like growth factor 1 (IGF-1) as a model system. We have demonstrated that the assay has specificity similar to that of conventional ligand binding and have applied random mutagenesis to the ligand to study the structural requirements of the IGF-1 ligand required for receptor interaction, as well as to identify variants of proinsulin with high affinity for the IGF-1 receptor.

MATERIALS AND METHODS

Yeast Strain. The yeast strain *Saccharomyces cerevisiae* Y153 (*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2–3,112 +URA3*::*GAL→lacZ, LYS2*::*GAL→HIS3*) was provided by Steve Elledge (Baylor College of Medicine, Houston, TX). Yeast were grown in YPD (yeast extract/peptone/dextrose) or appropriate medium to maintain plasmids (16).

Plasmid and Construction. Fusions with the GAL4 DNA-binding domain (GAL4DB) were constructed in pGBT9, and those with the GAL4 activation domain (GAL4AD) were constructed in pGAD424 (17). The cDNA fusions were performed by ligation of cDNA that had been amplified with the polymerase chain reaction (PCR) (18) using oligonucleotide linkers to allow the in-frame insertion into the yeast expression vectors. Oligonucleotide synthesis and nucleotide sequencing were performed with reagents and instruments supplied by Applied Biosystems in the Molecular Biology Core Facility at Joslin Diabetes Center. All PCR products and all junctions of the constructs were sequenced to confirm correct insertion. All mutants created by PCR were manually sequenced using Sequenase 2.0 protocols (United States Biochemical).

The full-length IGF-1 receptor fusion was constructed by inserting two fragments, the PCR product amplified by oligonucleotides5′-CGGAATTCGAAATCTGCGGGCCAGGCAT-3′ and 5′-GGCTCTCGAGGCCAGCCACTCG-3′ and the *Xho*I–*Bam*HI fragment of the IGF-1 receptor cDNA, into *Eco*RI–*Bam*HI site of the vectors pGBT9 and pGAD424. The fusion protein containing the extracellular domain of the receptor was constructed by inserting two fragments, the PCR product amplified by oligonucleotides 5′-CGGAATTCGAAATCT-GCGGGCCAGGCAT-3′ and 5′-GGCTCTCGAGGCCAGC-CACTCG-3′ and the *Xho*I–*Sma*I fragment of the IGF-1 receptor, into the *Eco*RI–*Sma*I site of the vectors pGBT9 and pGAD424. The cysteine-rich domain of the insulin receptor-related receptor (IRR) was generated by PCR with 5′- CGGAATTCGAAATC-

Abbreviations: IGF-1, insulin-like growth factor 1; GAL4DB, GAL4 DNA-binding domain; GAL4AD, GAL4 activation domain; IRR, insulin receptor-related receptor; InIGF, chimeric molecule with the C-peptide of IGF-1 inserted into human insulin; IGF1R, full-length IGF-1 proreceptor.

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TGCGGGCCAGGCAT-3′ and 5′-AGAGTCGACAGAATCAATGGTCTTT-3′ using a full-length cDNA (kindly provided by S. Taylor, National Institutes of Health) as a template. The proIGF-1 fragment was generated by inserting a 28-bp *Bam*HI–*Hga*I adaptor and cutting with *Bam*HI. The fragment was then inserted into the vectors pGBT9 and pGAD424 at the *Bam*HI site. The proinsulin and IGF-1 constructs were created by PCR with oligonucleotides to generate *Eco*RI and *Bam*HI sites at the end of fragments to allow insertion into pGBT9 or pGAD424.

β-Galactosidase Assay. The colony β-galactosidase color assay and quantitative β-galactosidase assay were performed as described by Vojtek *et al.* (19). Activity is expressed in standard units (20) multiplied by 1,000. All results were reproducible in at least two independent assays.

PCR Random Mutagenesis. Two pairs of primers were designed for amplifying IGF-1 and proinsulin. 5′-ATCGAATTCCCGGGAATCTTAGGT-3′ and 5′-CGCTGGGCACGGATCCATAAGCTGA-3′ were used to amplify IGF-1 with proIGF-1/pGBT9 as a template, and 5′-GCCGAATTCTTTGTGAACCAACAC-3′ and 5′-GGCGGATCCTAGTTGCAGTTC-3′were used to amplify proinsulin with proinsulin/pGBT9 as a template. The PCR conditions were as previously described (21). Several modifications relative to standard PCR were used to increase the rate of mutagenesis, including increased concentration (25 units/100 μl) of *Taq* DNA polymerase, increased concentration of $MgCl_2$ (7 mM), and increased concentrations of dCTP and dTTP (1 μM) with standard concentrations of dGTP and dATP (0.2 μM). All reaction mixtures contained 20 fmol of cDNA, 30 pmol (each) PCR primers, 50 mM KCl, and 10 mM Tris·HCl (pH 8.3), and were amplified for 40 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min. The PCR products were digested with *Eco*RI and *Bam*HI at 37°C for 6 h and inserted into the *Eco*RI–*Bam*HI sites of pGBT9. The mixture then was amplified in *Escherichia coli* and transformed into yeast cells for further analysis.

Competitive Ligand-Binding Assay. The ligand-binding assay was performed according to a published method (22). Chinese hamster ovary (CHO) cells overexpressing the IGF-1 receptor (23) were grown to confluence in 24-well cluster trays and incubated with F12 medium without serum for 15 h. Cells then were washed twice with PBS at 4°C and incubated with ^{125}I-labeled IGF-1 (^{125}I-IGF-1) and unlabeled IGF-1, proinsulin (kindly provided by R. Chance, Eli Lilly, Indianapolis, IN), insulin, and a chimeric molecule in which the C-peptide of IGF-1 was inserted into human insulin termed InIGF (kindly provided by Claus Kristensen, Novo Nordisk, Bagsvaerd, Denmark) at the indicated concentrations for 15 h at 4°C in Hepes binding buffer: 100 mM Hepes, pH 7.4)/118 mM NaCl/50 mM KCl/1.2 mM $MgSO_4$/8.8 mM dextrose/1% BSA. Unbound ligand was removed by washing the cells once with PBS containing 1% BSA and twice with PBS. The cells then solubilized in 1 ml of 0.1 M NaOH containing 0.1% SDS at 22°C, and bound radioactivity was determined by γ counting.

RESULTS

The Interaction of proIGF-1 and IGF-1 Receptor. To test the interaction of IGF-1 and its receptor, we fused the GAL4DB with proIGF-1 and the GAL4AD with a series of constructs prepared from the IGF-1 receptor, including the N-terminal cysteine-rich region (amino acids 1–315), the entire extracellular domain (1–868), or the full-length IGF-1 proreceptor (IGF1R). These were then co-expressed in *S. cerevisiae* Y153, and the interaction was detected by the β-galactosidase color assay (Fig. 1*A*). Interaction between proIGF-1 and the N-terminal receptor fragment, IGF1R(1–315), was the strongest, with a blue color being detectable within 2 h by colony filter color assay. Constructs for the entire extracellular domain or the full-length receptor also interacted with proIGF-1, but the blue color appeared only after 4 and 6 h, respectively (Fig. 1*A*). The strength of the interaction correlated with the level of expression of the IGF-1 receptor construct as judged by immunoblotting of yeast extracts with a polyclonal antibody (Upstate Biotechnology, Lake Placid, New York) to GAL4 (data not shown). Controls in which either the ligand or the receptor was omitted showed no blue color for up to 12 h (Fig. 1*B*). Likewise, there was no interaction when proIGF-1 was paired with constructs containing the intracellular domain of the IGF-1 receptor.

Interaction between IGF-1 and its receptor was orientation dependent. When the receptor was fused with GAL4DB and the ligand was fused with GAL4AD, there was an almost 10-fold decrease in the strength of the association. However, the rank order of interaction was similar and was above the levels observed with the negative controls (Fig. 1*A*).

The Binding Specificity of IGF-1 Receptor and Different Ligands. To further explore the specificity of the interaction among the different ligands and different receptors in the IGF-1 family, GAL4DB hybrids containing IGF-1, proIGF-1, or proinsulin and GAL4AD hybrids containing IGF1R(1–315) or the corresponding cysteine-rich region of IRR [IRR(1–302)] were constructed. As shown in Table 1, both IGF-1 and proIGF-1 interacted strongly with IGF1R(1–315), whereas the activity of proinsulin with the IGF-1 receptor was marginally above background. The N-terminal cysteine-rich region of IRR(1–302) did not show any interaction with IGF-1 (Table 1). Thus, IGF-1 and proIGF-1 bind specifically to IGF-1 receptor but not to IRR, and proinsulin, which has many features similar to those of IGF-1, binds to the IGF-1 receptor with much lower affinity, consistent with other *in vitro* studies (24).

The Interaction of Insulin/IGF-1 Single Chain Hybrid and IGF-1 Receptor. To further validate the system for detecting ligand–receptor interactions, a single-chain insulin/IGF-1 chimeric peptide (InIGF) (25) was tested both in the yeast two-hybrid system and in the cell surface ligand receptor binding assay system. As previously described (25), the InIGF chimeric molecule has an affinity for the IGF-1 receptor intermediate to the affinities of IGF-1 and proinsulin. Thus, when the affinity of these ligands was assessed in a conventional binding assay using CHO cells overexpressing IGF-1 receptors, the IC_{50} values for IGF-1, InIGF, and insulin were 8.0×10^{-10} M, 6.4×10^{-9} M, and 2.5×10^{-7} M, respectively (Fig. 2*B*). Proinsulin showed an affinity intermediate between insulin and the InIGF chimeric molecule (data not shown). In the yeast two-hybrid sytem, the InIGF chimera bound to the cysteine-rich domain of the IGF-1 proreceptor, resulting in β-galactosidase activity intermediate between proinsulin and IGF-1 (Fig. 2*A*). Thus, the interaction of IGF-1 and related peptides in the two-hybrid system paralleled closely the results from assessment of the ligand–receptor interaction in conventional binding assays. Proinsulin showed an affinity inter-

Table 1. Interaction of various ligands with the cysteine-rich domain of the IGF-1 receptor family

GAL4DB hybrid	GAL4AD hybrid	Colony color	β-Galactosidase activity, units
Empty	Empty	−	<1
Empty	IGF1R(1–315)	−	2
IGF-1	Empty	−	
IGF-1	IGF1R(1–315)	+++	320
proIGF-1	Empty	−	11
proIGF-1	IGF1R(1–315)	+++	328
Proinsulin	Empty	−	
Proinsulin	IGF1R(1–315)	±	29
proIGF-1	IRR(1–302)	−	<1

The intensity of the interaction was measured either in the colony color assay or as β-galactosidase activity as described in the legend of Fig. 1. The cysteine-rich domain of the IGF-1 receptor is denoted IGFR(1–315) and that of the IRR as IRR(1–302). "Empty" indicates no insert in the vector.

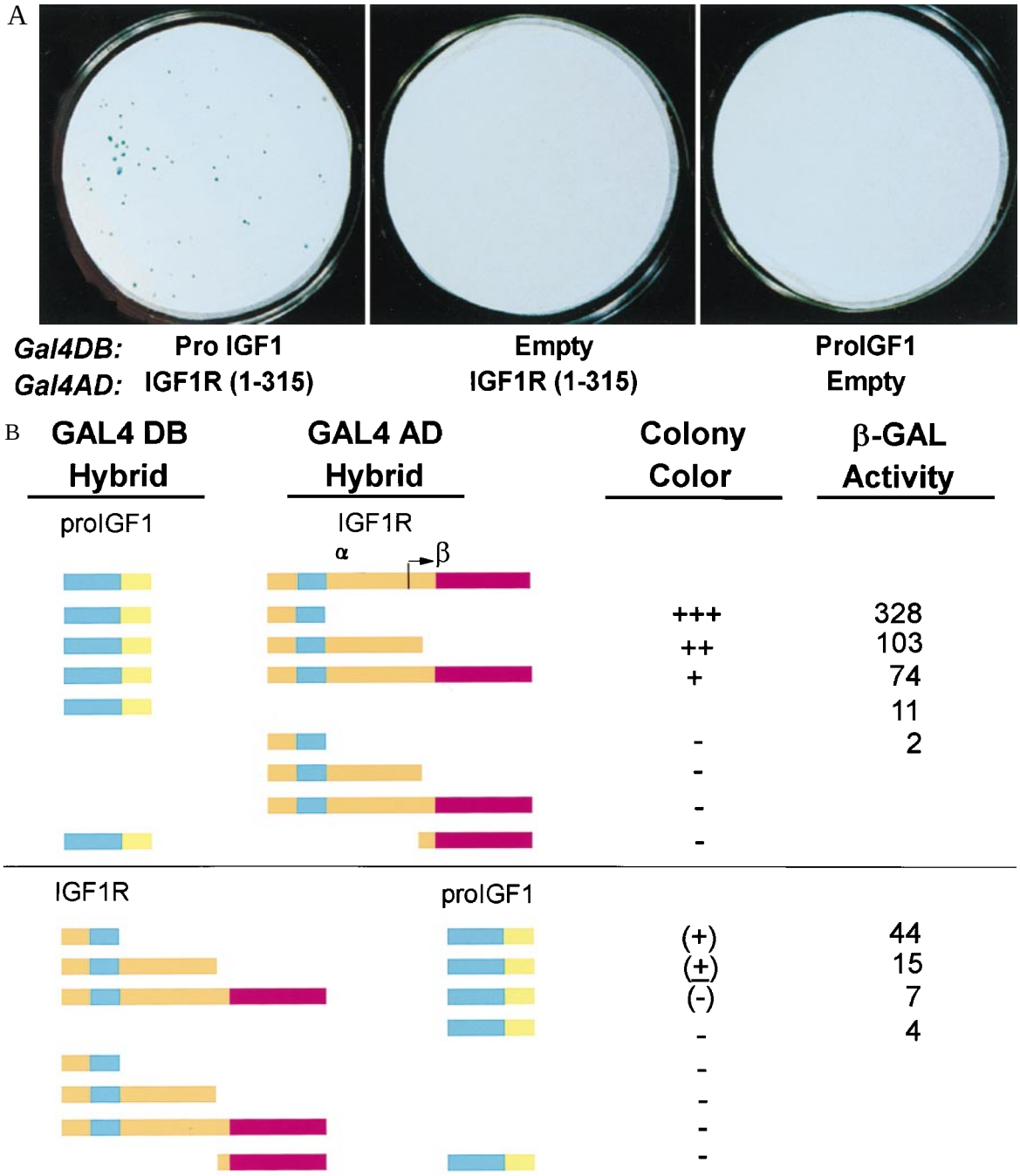

FIG. 1. Interaction of proIGF-1 with an IGF-1 receptor fragment (1–315) in the yeast two-hybrid system. (*A*) Colony color assay. The colonies were grown on a plate containing appropriate medium, transferred onto a nitrocellulose filter, and lysed by rapid freezing in liquid nitrogen. The filters then were placed on a plate containing Z-buffer and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) to analyze the enzyme activity. When proIGF-1 and IGF-1 receptor were coexpressed in yeast, colonies showed a blue color within 2 h, whereas controls in which either the ligand or the receptor were omitted showed no blue color for up to 12 h. (*B*) Quantitation of the interaction between proIGF-1 and the IGF-1 receptor. The structures of the proIGF-1 and IGF-1 receptor (IGF1R) are presented schematically. Filled box in proIGF-1 molecule indicates the IGF-1 region, and the hatched box indicates the E chain of the proIGF-1. The filled box in IGF-1 receptor represents the cysteine-rich domain; the shaded and cross-hatched boxes represent the extracellular and intracellular domains of the receptor, respectively. The colony color assay is graded as white (−), equivocal (±), light blue (+), medium blue (++), or dark blue (+++). β-Galactosidase activity was quantitated and expressed in standard units multiplied by 1,000. The results were reproducible in at least two independent assays.

mediate between insulin and the InIGF chimeric molecule (data not shown).

Random Mutagenesis of IGF-1 by PCR Approach. One advantage of the yeast two-hybrid system is the ability to introduce point mutations randomly and thereby to analyze the residues that might be involved in the protein–protein interaction. A mixture of IGF-1 mutants was created by using the modified PCR protocol described in *Materials and Methods*, and the mutant mixture was inserted into GAL4DB, amplified in *E. coli*., and used to transform yeast containing the IGF1R(1–315) GAL4AD hybrid construct. Interaction between ≈2,000 mutants and IGF1R(1–315) was detected and graded by using the β-galactosidase color assay. After random mutagenesis, the intensity of blue color of the yeast colonies became variable as compared with the relatively homogeneous color before mutation (Fig. 3). Rescreening of 200 colonies of

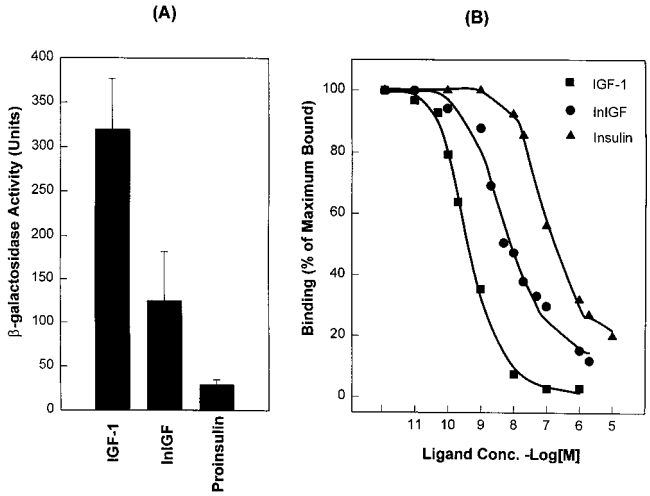

FIG. 2. Assessment of a single-chain insulin/IGF1 chimeric molecule (InIGF) in binding to the IGF-1 receptor. (*A*) β-Galactosidase quantitative assays for yeast containing IGF-1/IGF1R(1–315), InIGF/IGF1R(1–315), and proinsulin/IGF1R(1–315) were performed as described in the legend of Fig. 1. The data are the mean of at least two experiments. (*B*) Competition curves for ^{125}I-IGF-1 binding to IGF1 receptor. CHO cells overexpressing IGF-1 receptor (23) were incubated with labeled ligand for 15 h at 4°C together with increasing concentrations of unlabeled insulin, IGF-1, or InIGF. The amount of labeled hormone bound, as a percentage of labeled hormone bound in the absence of unlabeled hormone, is plotted against the hormone concentration.

the original 2,000 revealed that about 40% of the colonies had a strong association with the receptor, while 20% showed intermediate interaction, and 40% gave little or no signal.

Fifty plasmids containing mutant cDNAs were then isolated from the yeast, used to transform *E. coli*, and sequenced. Fifteen single point mutants showing different binding activity with IGF1R(1–315) are illustrated in Fig. 4. Several mutants (Leu-5 → His, Glu-9 → Val, Arg-37 → Gly, and Met-59 → Leu) showed enhanced intensity of the blue color compared with native IGF-1. For this group of mutants, quantitative β-galactosidase activity increased from ≈350 units for wild-type IGF-1 to 500–2,000 units. Five mutations of IGF-1 did not affect the interaction with IGF1R(1–315). All of these were in the B chain at residues Thr-4, Asp-12, Cys-18, and Tyr-24. By contrast, point mutations in the C chain (Tyr-31 → Phe) and the A chain (Ile-43 → Phe) resulted in a noticeably weaker color reaction and modestly reduced

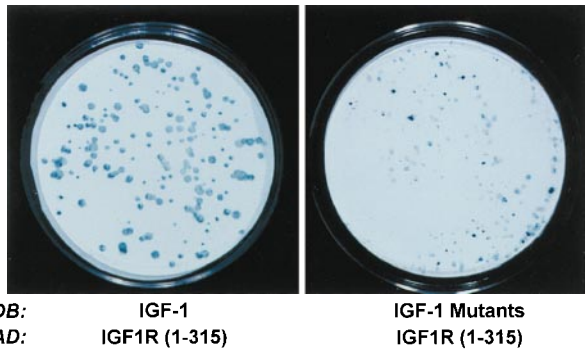

FIG. 3. The colony color assay of the IGF-1 wild-type or mutants. IGF-1 was mutagenized by PCR and co-transformed into yeast with IGF1R(1–315), and the color assay was performed as described in the legend of Fig. 1. Wild-type co-transformants showed relatively homogeneous color (*Left*), whereas the blue color intensity of the yeast colonies became variable (*Right*) after random mutagenesis of IGF-1. Approximately 40% of the colonies showed dark or medium blue (++++ or +++), 20% showed intermediate intensity (+ or ++), and 40% gave little or no signal (± or −) (*Right*).

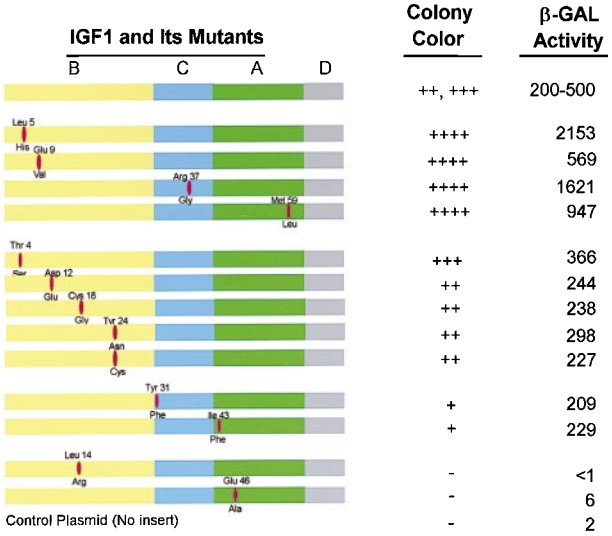

FIG. 4. Quantitation of the interaction of IGF-1 mutants with cysteine-rich domain of IGF-1 receptor. The structure of the IGF-1 is presented schematically with the B, C, A, and D domains. The solid bars indicate the position of the mutation and the number is the amino acid number. The amino acid above the bar is the native sequence; that below the bar is the mutation. Colony color and β-galactosidase activity were measured as described in the legend of Fig. 1.

activity in the quantitative assay. Furthermore, one B-chain mutant (Leu-14 → Arg) and one A-chain mutant (Glu-46 → Ala) had completely lost ability to interact with the IGF-1 receptor in this assay. Fig. 5 shows the position of these various mutations detected in the yeast two-hybrid system superimposed the predicted three-dimensional structure of IGF-1.

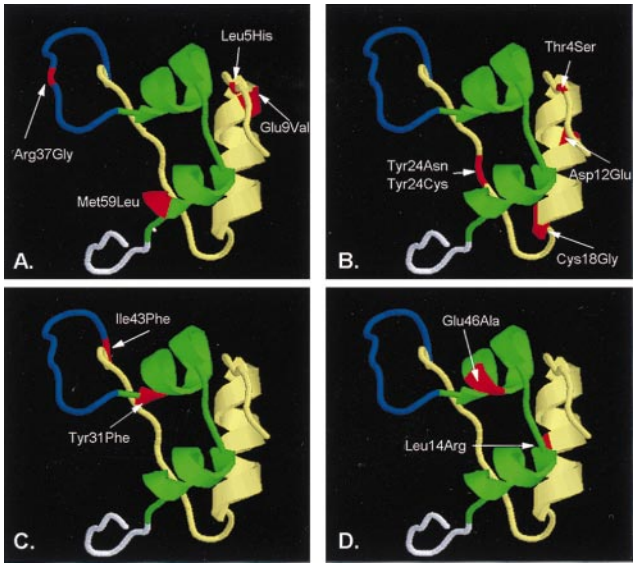

FIG. 5. IGF-1 mutants indicated on the three-dimensional structure of the IGF-1 molecule. The residues marked in red indicate the site of mutation; the domains of the IGF-1 molecule are indicated as follows: A, green; B, yellow; C, blue; and D, white. After random mutagenesis four groups of the mutants could be distinguished by the intensity of the blue color or β-galactosidase activity. (*A*) The strong interaction group. These mutants showed enhanced intensity of β-galactosidase reaction compared with native IGF-1. (*B*) The intermediate interaction group. This group of mutants did not affect the interaction with IGF-1 receptor. (*C*) The reduced interaction group. These mutants had significantly weaker color reaction and modestly reduced activity in the quantitative assay. (*D*) The negative interaction group. These mutants had completely lost ability to interact with the IGF-1 receptor in the yeast two-hybrid assay.

All mutants were also tested with both empty control pGAD424 vector and the pGAD424 vector containing the IGF-1 receptor intracellular domain. In most cases, the background enzyme activity was less than 20 units. About 12% of the mutants, including Tyr-24 → Lys, showed very high background with these controls and thus could not be accurately evaluated in the yeast system (data not shown). About 10% of constructs that had no signal after mutagenesis either had completely lost the IGF-1 insert or had a frameshift mutation, further confirming the specificity of the interaction.

A Mutant of Proinsulin Gaining the Function of Binding to IGF-1 Receptor. Although proinsulin has a three-dimensional structure similar to to that of IGF-1, its affinity for the IGF-1 receptor is low (26). To determine if the random mutation library and yeast two-hybrid system could be used to identify a gain-of-function mutation, we employed the approach described above for proinsulin and screened more than 1,000 colonies. Greater than 99% showed no interaction with IGF1R(1–315), a few showed very weak binding, and one appeared to have a strong association with the receptor (Fig. 6*A*). Sequencing of the very weakly positive clones revealed no change in sequence, whereas the one clone interacting strongly with the receptor was found to have mutations in positions B17 (Leu → Pro) and B30 (Thr → Ala) (Fig. 6*B*). For this mutant the β-galactosidase activity was increased about 30-fold compared with the control and was similar to that for IGF-1. Because the change in the B30 position of Thr to Ala is the same as that which occurs normally in human vs. porcine proinsulin and has no effect on IGF-like activity, the difference in receptor activity is almost certainly due to the change at position B17 from Leu to Pro. B17 occurs in the third loop of the α-helical portion of proinsulin and would be predicted to disrupt the helix and cause a significant change in conformation of this portion of the molecule.

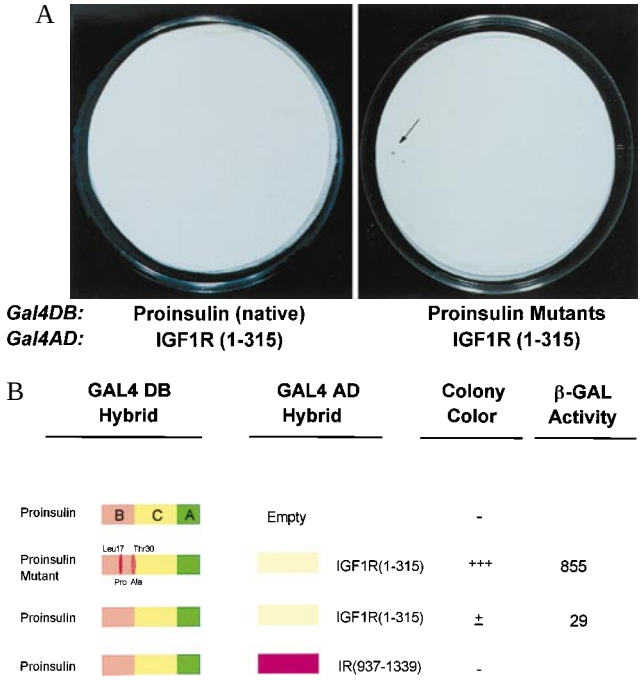

FIG. 6. Interaction of proinsulin with IGF-1 receptor. (*A*) The colony color assay. When co-transformed into yeast, native proinsulin did not significantly interact with the cysteine-rich region of IGF-1 receptor (*Left*). After random mutagenesis, one colony showed strong interaction with the same region of the receptor (*Right*). (*B*) Structural analysis of proinsulin mutants that interact with the IGF-1 receptor. The structure of the proinsulin is represented in schematic form with the domains corresponding to the B, C, and A chains. Solid filled bars indicate the positions of the mutation with the name and number of the amino acids involved as in Fig. 4. The intensity of the interaction was assessed by measuring either color of the colony or β-galactosidase activity.

DISCUSSION

The yeast two-hybrid system has been shown to be a powerful tool for the identification and study of intracellular protein–protein interactions and for mapping the domain requirements for the underlying the association (6, 17). In this paper, we report the successful use of this system to detect and characterize the IGF-1 ligand–receptor interaction, as well as to define structural requirements of this interaction by random *in vitro* mutagenesis. The system was validated not only by testing the interaction of IGF-1 with other receptors and empty vectors, but also by use of a chimeric molecule of insulin and IGF-1 with intermediate affinity in the ligand–receptor binding system. Although the yeast two-hybrid system has been generally thought unsuitable for studies of ligand–receptor interactions, while this work was in progress, Ozenberger *et al.* (27) reported another variant of the two-hybrid system in which they could detect the interaction between the extracellular domains of the growth hormone and prolactin receptors with their ligands, further indicating the feasibility, and perhaps general applicability, of this approach. The use of the β-galactosidase reporter assay in our system, as opposed to simply density of cell growth, facilitates both the qualitative and quantitative (or at least semiquantitative) analysis required for structure–function studies. The current work also demonstrates not only that this approach can be utilized with the entire extracellular domain of a receptor and its ligand but also that it can be used to map specific domain interactions and to scan for structure/function features by random mutagenesis. Thus, the yeast two-hybrid system can be used to examine protein–protein associations that normally occur at the mammalian cell surface, and the information can be accumulated to help understand which are the functionally significant amino acids within a protein involved in the interaction. In theory, the method should also allow one to search cDNA libraries to find ligands for orphan receptors and binding proteins for ligands where these are not known.

The IGF-1 ligand–receptor interaction presents several interesting aspects for use in this system. IGF-1 is one member of a family of structurally and evolutionary related peptides that includes insulin, IGF-1, IGF-2, and relaxin. In mammalian cells, IGF-1 binds to an IGF-1 receptor, which is a covalent tetramer of two α (binding) and two β (kinase) subunits. Although it has been suggested that high-affinity binding depends on the dimeric nature of the subunits, an interaction between IGF-1 and its receptor (or fragments of the receptor) is easily detected by the yeast two-hybrid system, in which the receptor is presumably monomeric. Furthermore, the interaction shows appropriate specificity. Thus proinsulin, which binds to the IGF-1 receptor with 1/100 the affinity of IGF-1, produces a barely detectable interaction in the two-hybrid system, helping to define the limits of sensitivity of the assay. The intermediate affinity of the insulin-IGF chimeric molecule (InIGF) and ability to identify a gain-of-function mutant of proinsulin that binds to the IGF-1 receptor indicates that the low binding of native proinsulin is due not to a defect in processing or nonspecific steric factors but to a true change in affinity. In the latter case the binding activity of proinsulin was dramatically increased by mutations in the molecule at positions B17 and B30. As noted above, however, because the B30 mutation is the same as that which occurs normally between porcine and human proinsulin, the increased affinity of the mutant proinsulin for the IGF-1 receptor must be due to the B17 alteration, which occurs in the third α-helical loop of the B-chain. Although this must be confirmed and validated by *in vitro* studies, it seems likely this proinsulin analogue will bind to and could be an agonist for both the IGF-1 and insulin receptors, similar to the insulin-IGF chimera (25).

Our data also indicate that several factors other than the primary sequence of the proteins is involved in determining

the strength of interactions. For example, different intensities of interaction were observed between proIGF-1 and different receptor fragments. This was due, at least in part, to differences in expression levels among the receptor fragments. Western blotting data revealed that, in this case, the shorter the receptor fragment, the higher the level of expression. This could be one reason why the cysteine-rich domain shows the strongest binding activity, no matter what the orientation. On the other hand, in this system orientation is also important with GAL4DB–proIGF-1 and GAL4AD–IGF1R activating transcription much more efficiently than the reverse orientation. Whether this is due to increased stability between the hybrids containing ligand and receptor in the preferred orientation or is due to differences in the three-dimensional structure after fusion to other proteins is unclear. Previous work using insulin dimers made by covalent cross-linking has suggested that the insulin receptor interaction is orientation dependent (28), and the same is likely true when one has a relatively small hormone molecule fused to a much larger GAL4AD fragment.

To analyze the role of individual residues of IGF-1 in the ligand–receptor interaction, we took advantage of the ability in the two-hybrid system to rapidly screen for changes in binding and transcriptional activity by scoring the intensity of blue color of colonies in the X-Gal filter assay. Using a PCR protocol that would introduce only one or two random mutations into IGF-1, we could clearly distinguish four groups of mutants on the basis of the intensity of the color and the transcriptional activity in the two-hybrid system. Most of these mutants have not been described before and thus present novel IGF-1 analogues.

Several points from this aspect of the study are worth noting. First, one of the mutants (Cys-18 → Gly) that still interacts in the two-hybrid system involves a cysteine residue that participates in disulfide bond formation with Cys-61 (29). Although the role of this disulfide bond in the structure and activity of IGF-1 has not been directly analyzed, our data suggest that in yeast, the IGF-1 molecule adopts a proper conformation in the absence of this disulfide bond or that the proper conformation is induced by receptor binding. It is also possible that the primary amino acid sequence, rather than the three-dimensional conformation of IGF-1, is sufficient for interaction in this system. However, on the basis of the presumed complex three-dimensional structure of IGF-1 and the presumed importance of this folding in creating a proper binding interface, this seems less likely.

In this system, two other mutations (Tyr-24 → Asn and Tyr-24 → Cys) bind to the cysteine-rich domain of the receptor with strength similar to that of native IGF-1. Although these analogues have not been directly tested, these findings are somewhat discordant with a report by Bayne *et al.* (30), who have shown a decrease in receptor binding affinity for IGF-1 with Tyr-24 → Leu when studied in a conventional receptor assay. Whether this reflects the fact that replacement of Tyr-24 by Asn or Cys, but not Leu, is tolerated, that the receptor fragment has a different specificity than the holoreceptor, that the nature of the interaction between the IGF-1 and the receptor is different in the nucleus as compared with in solution, or that the iodinated IGF-1 used in the *in vitro* assays behaves differently from native IGF-1 with regard to tyrosine mutation is not known. Obviously, testing for the association between IGF-1 derivatives and a single domain of its receptor might reflect only a part of the binding affinity of the holoreceptor expressed on the cell surface. Indeed, in ongoing experiments we have identified a second interface of the IGF-1–receptor interaction involving a more C-terminal region of the α-subunit similar to the secondary domain described for insulin (31–34), and we have shown that the specificity of IGF-1 mutants for this domain of the receptor varies independently of the interaction with the cysteine-rich region (unpublished results). Because transcriptional activation usually reflects the affinity of a protein–protein interaction in the yeast system (35), it is important to have the mutants from screening analyzed for secondary interactions to have a more complete picture of the interaction.

In summary, our findings suggest that the specificity of the hormone–receptor interaction can be characterized with high efficiency by using PCR random mutagenesis in the yeast two-hybrid system. Although this approach may not be applicable to all peptide hormone–receptor interactions and the results in yeast may not always reflect exactly the specificity and conditions of interaction observed with cells or cell membranes, when this procedure is operative, it may be used as a primary screen to characterize the structure–function relationship for both the hormone and its receptor partner. In addition, one could also screen random peptide libraries (36) to find peptide agonists and antagonists to receptors and could screen cDNA libraries to find receptors for ligands or ligands for receptors that are unknown.

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