Functional Interaction of Ligands and Receptors of the Hematopoietic Superfamily in Yeast

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Circulating peptide hormones and growth factors interact with cell surface receptors to initiate specific cellular responses. These complexes can consist of a simple association between two proteins or a more elaborate association of multiple proteins. We describe the functional expression of ligands and corresponding receptors in a microbial system useful for the rapid dissection of these important protein interactions. GH or PRL and extracellular domains of their respective receptors were functionally expressed as fusion proteins in an extended two-hybrid protein-protein interaction system. Reversible and specific ligand-receptor interactions were demonstrated by concurrent expression of free ligand peptides (GH or PRL) as binding competitors. The versatility established by expressing three heterologous proteins allowed for the investigation of higher order structures. Ligand-dependent GH receptor dimerization was demonstrated but PRL receptor dimerization was not observed in an analogous assay, suggesting that these related growth factors may not engage receptors in a similar manner. Additionally, significant association of GH receptors was observed in the absence of ligand, suggesting that there may be substantial avidity between these receptor proteins before ligand binding. Ligand-dependent and ligand-independent receptor dimerization was demonstrated by vascular endothelial growth factor and receptor proteins in similar assays. These findings indicate that extracellular protein interactions such as ligand-receptor association, as well as the formation of higher order protein structures important for the activation of hematopoietic receptors, can be rapidly investigated in this microbial expression system. (Molecular Endocrinology 9: 1321–1329, 1995

INTRODUCTION

Mammalian cells respond to a wide variety of circulating peptide hormones, often by engaging single transmembrane domain receptors, such as those of the cytokine/hematopoietic receptor family. Recent examination of this receptor family reveals differing ligand-receptor stoichiometries that include two-protein ligand-receptor interactions (1–3) and three-protein ligand-receptor-receptor or ligand-receptor-transducer interactions (4–8). Human GH binds to recombinant GH-binding protein in a 1:2 stoichiometry (9, 10). PRL is related to GH and shares amino acid homology at both the ligand and receptor level. Given these similarities, PRL has been thought to associate with the PRL receptor (PRLR) in a manner similar to GH association with the GH receptor (GHR). PRLR antibodies have been shown to stimulate cell proliferation, although to a reduced maximal response when compared with PRL, suggesting that PRLR dimerization may play a role in receptor function (11). However, direct demonstration of PRL-induced receptor dimerization is still needed.

An alternate ligand-receptor stoichiometry (2:2) (12, 13) is observed for vascular endothelial growth factor (VEGF) (14, 15) and its receptor flk1/KDR (16–18). VEGF induces angiogenesis and is an important factor in tumorigenesis (19), via association with its receptor on vascular endothelial cells. Homodimerization of the ligand is essential for receptor dimerization in a manner analogous to platelet-derived growth factor (13, 20).

Protein-protein interactions occurring at the mammalian cell surface, such as the binding of peptide ligand to receptor, are of paramount importance to cellular functions and biological responses. The two-hybrid expression system (21, 22) to investigate protein-protein interactions has been used extensively in studies of intracellular protein interactions, such as those occurring among signal pathway components and transcription complex proteins (23–26), but has not been applied to protein-protein interactions that normally occur extracellularly. The basis of the two-hybrid system centers on differential interactions between target proteins fused to the separable DNA binding and activation domains of the yeast transcriptional activator Gal4 (21, 22). Interaction of the heterologous proteins brings the two domains of the Gal4 protein in close proximity, activating expression of a reporter gene (UAS_{GAL}-*HIS3*) permitting consequent growth of yeast cells on selective medium. The two-

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hybrid system is considered to be exclusionary of protein-protein interactions that occur at the mammalian cell surface (27) since the specific interaction of fusion proteins must occur in the cytosol and/or nucleus of a yeast cell. However, we demonstrate that this technology is applicable to extracellular protein-protein interactions, focusing on peptide ligand-receptor associations.

We describe the functional expression in yeast of circulating mammalian peptide hormones and cognate extracellular ligand-binding proteins. Specific and reversible ligand-receptor interactions between GH/GHR, PRL/PRLR, and VEGF/KDR were coupled to an easily scorable change in yeast cell phenotype (growth on selective medium). Novel permutations devised to express a third protein enabled examination of reversible and specific ligand-receptor interactions, thereby establishing pharmacology in yeast. Ligand-dependent receptor dimerization was also characterized, demonstrating the utility of these methods for investigating higher order protein complexes. This easily manipulable system creates a unique biological forum for the rapid investigation of hormone-receptor interactions.

RESULTS

Investigation of Peptide Ligand-Receptor Interaction in Yeast

Complementary DNA sequences encoding porcine GH or the extracellular region of the rat GHR were cloned into Gal4 fusion protein expression plasmids. The yeast strain Y190 (24), containing a UAS_{GAL}-*HIS3* reporter gene, was transformed with both fusion plasmids or with a single fusion construct plus the opposing vector containing no heterologous DNA. All strains grew vigorously on nonselective medium (Fig. 1A). These strains were then tested for histidine prototrophy. Only the strain (CY722) containing both ligand and receptor hybrid proteins grew while strains containing either the ligand or receptor fusion alone did not grow (Fig. 1B). These results suggest that GH and GHR can mediate the Gal4-dependent activation of the reporter gene in an interaction suggestive of ligand-receptor binding.

To extend these findings, a similar system was developed using the peptide hormone PRL and its receptor. PRL is structurally related to GH. The PRLR, like the GHR, is a member of the cytokine receptor family. Porcine PRL and the extracellular domain of the porcine PRLR were expressed as protein fusions with Gal4. As in the GH/GHR experiment, the strain expressing both the PRL and PRLR fusions was able to grow on selective medium while strains containing either the ligand or receptor fusion alone did not (Table 1). These results mirror those observed in the GH/GHR expression strains and suggest that this methodology has general utility for examination of ligand binding to members of this receptor superfamily.

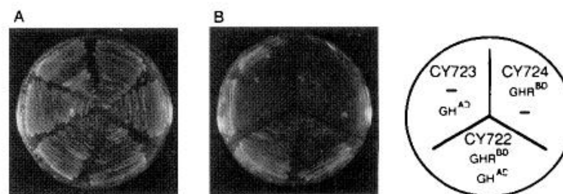

Fig. 1. Growth of Strains Expressing GHR and GH Fusion Proteins

Strains expressing the indicated proteins are described in the text. The position and the expressed heterologous proteins of each strain are indicated in the diagram. *Superscripts* indicate that a protein was expressed as a fusion to the transcriptional activation domain (AD) or DNA-binding domain (BD) of Gal4. A *dash* indicates that the strain contained an unmodified vector (pAS2 or pACT2). Two independent isolates of each strain were streaked on synthetic medium (57) deficient in leucine, tryptophan, and uracil (nonselective; plate A) or on the same medium lacking histidine (selective; plate B). Both plates were supplemented with 60 mM 3-amino-triazole. Plates were incubated for 4 days at 30 C.

Establishment of Pharmacology in Yeast: Reversible and Specific Interaction of Ligand and Receptor

To substantiate the apparent binding of GH to its receptor in the foreign environment of a yeast nucleus, the system was modified to allow the expression of "free" nonfusion ligand from a third plasmid (pCUP) carrying a *URA3* selectable marker. The GH peptide should compete with the GH fusion protein, reversing the interaction of the fusion proteins (Fig. 2A). Strain CY770, a Ura^- derivative of Y190, was transformed with the GH- and GHR-fusion expression plasmids plus a plasmid expressing nonfusion GH. This strain, designated CY781, grew vigorously on nonselective medium (Fig. 3A). Concurrent expression of GH with the GH- and GHR-fusion proteins in strain CY781 substantially attenuated cell proliferation on selective medium in comparison to strain CY722 (Fig. 3B). This experiment typifies an *in vivo* competition assay and illustrates the reversibility of the ligand-receptor interaction. We refer to yeast expression systems employing three heterologous proteins as PRIMES (peptide/receptor interactions in microbial expression systems).

Additional strains were developed to assess ligand-receptor specificity. Strains expressing GH- and GHR-fusion proteins or PRL- and PRLR-fusion proteins were transformed with pCUP, pCUP-GH, or pCUP-PRL. As demonstrated previously, a strain expressing the GH and GHR fusions in the absence of competitor grew on selective medium, and this growth was abrogated with coexpression of free GH (Fig. 3B, Table 1). The strain containing the GH and GHR fusions and also expressing PRL exhibited a strong growth phenotype (CY785; Table 1). These data suggest that GH binding to its receptor in this system retains the pharmacological specificity of the native interaction since the association of fusion proteins can be efficiently competed by GH but not by the related

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Table 1. Summary of Growth and Competition Assays

Strain	pACT2	pAS2	pCUP	Growth
Y190	–	–	–	0
CY722	GH	GHR	–	+++
CY723	Vector	GHR	–	0
CY724	GH	Vector	–	0
CY726	PRL	PRLR	–	++
CY770	–	–	–	0
CY781	GH	GHR	GH	+
CY784	GH	GHR	Vector	+++
CY785	GH	GHR	PRL	+++
CY786	PRL	PRLR	PRL	+
CY787	PRL	PRLR	Vector	++
CY788	PRL	PRLR	GH	++

All yeast strains were derived from strain Y190 (24). Strains with number designations greater than 770 do not contain the *URA3:UAS*$_{GAL}$*-lacZ* gene to allow for selection of the pCUP plasmid. pACT2 carries the *LEU2* selectable marker and expresses the Gal4 transcriptional activation domain as a protein fusion with the indicated peptide. pAS2 carries the *TRP1* selectable marker and expresses the Gal4 DNA-binding domain as a protein fusion with the indicated receptor. pCUP carries the *URA3* selectable marker and is utilized to express nonfusion peptide. A *dash* indicates that a strain does not contain the denoted plasmid. Strains were incubated on selective medium for 4 days at 30 C and scored for growth. A zero indicates that no growth was observed. The most robust growth was observed with the GH/GHR fusion protein interaction (strains CY722 and CY784), indicated by +++. Growth of strains (CY726 and CY787) expressing PRL/PRLR fusion proteins was less robust as indicated by ++. Specific competition of GH/GHR or PRL/PRLR interactions with nonfusion protein (CY781 or CY786, respectively) significantly reduced, but did not eliminate, the growth response as indicated by +.

PRL peptide. The converse experiment produced similar results. Strain CY787, containing PRL and PRLR fusion proteins, grew on selective medium, and this growth was abrogated by expression of free PRL but not GH (CY786 and CY788; Table 1), again illustrating the fidelity of the ligand-receptor interaction in this system.

Investigation of Ligand-Induced Receptor Dimerization in Yeast

The demonstration of a specific ligand-receptor interaction in yeast by using three expression plasmids presented an opportunity to also examine more complex associations of proteins. Differential receptor protein interactions dependent on ligand binding could be observed by concomitantly expressing receptor extracellular domains as fusion proteins and the ligand as a nonfusion protein (Fig. 2B). Experiments were conducted using GH/GHR to investigate ligand-dependent receptor dimer formation. This required the construction of an additional plasmid to express the extracellular domain of GHR as a fusion with the Gal4 activation domain. Yeast strains (CY899, CY901) that expressed any single receptor fusion plasmid with GH failed to grow on selective medium (Fig. 4). The strain that expressed both receptor fusions in the absence of GH (CY887) grew poorly on selective medium as did a strain that expressed both GHR fusions and PRL (data not shown). Only the strain (CY886) that expressed both receptor fusions plus GH exhibited growth substantially greater than control strains (Fig. 4).

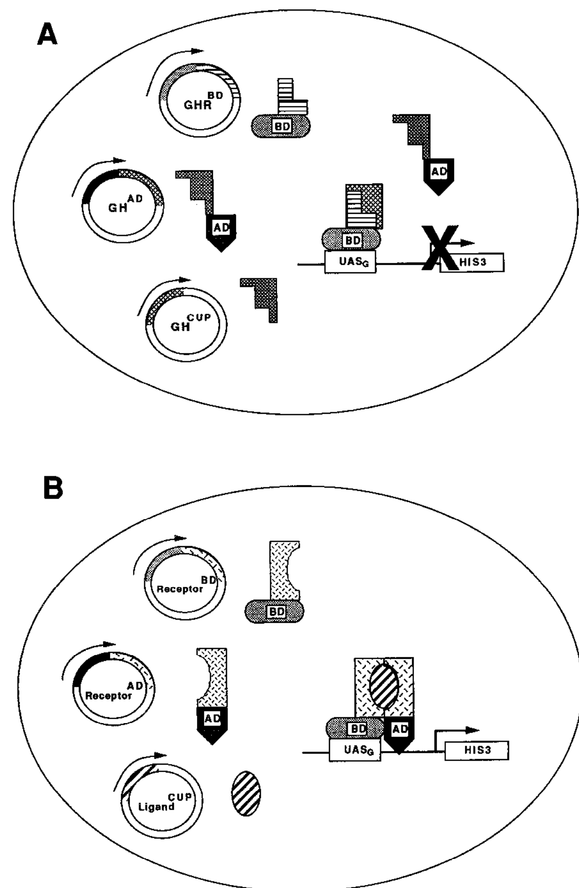

Fig. 2. Diagrams of Peptide/Receptor Interactions in Microbial Expression Systems (PRIMES)

A, Reversible ligand-receptor interaction: The extracellular domain of the rat GHR is expressed as a fusion to the DNA-binding domain (BD) of the Gal4 transcriptional activator protein; mature porcine GH is expressed as a fusion to the Gal4 activation domain (AD). Additionally, GH is expressed from pCUP as a nonfusion protein. In this strain, GH is free to associate with the GHR-fusion protein, thereby blocking the GH fusion from interacting with the GHR fusion. This competitive interaction abrogates the functional reconstitution of DNA binding and activation domains of the Gal4 protein, limiting induction of the UAS$_{GAL}$-*HIS3* reporter gene. B, Ligand-stimulated receptor dimerization: The extracellular domain of a receptor is fused to both the DNA binding (BD) and activation (AD) domains of the Gal4 protein. The mature protein for the cognate ligand is expressed from pCUP as a nonfusion protein. Maximal induction of reporter gene transcription is dependent on ligand-promoted dimerization of receptor fusion proteins.

The growth of strains expressing both GHR fusion proteins was clearly stimulated by coexpression of GH, but there was some growth of the strain (CY887)

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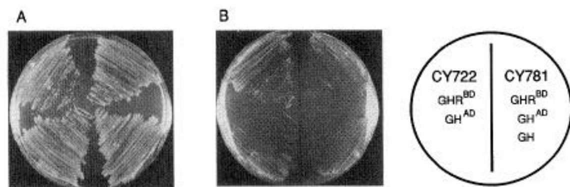

Fig. 3. Growth Inhibition by Coexpression of Nonfusion GH

Strains expressing the indicated proteins are described in the text. The position and expressed heterologous proteins of each strain are indicated in the diagram. *Superscripts* indicate that a protein was expressed as a fusion to the transcriptional activation domain (AD) or DNA-binding domain (BD) of Gal4. Ligand *without designating superscript* denotes expression as a nonfusion protein from pCUP. Two independent isolates of each strain were assayed on nonselective (plate A) or selective medium (plate B) as described in Fig. 1.

Table 2. Growth Rates in Response to Receptor Dimerization

Strain	pACT2	pAS2	pCUP	Growth rate (h^{-1})
CY770	–	–	–	0.022 ± 0.007
CY953	Snf4	Snf1	Vector	0.250 ± 0.001
CY886	GHR	GHR	GH	0.145 ± 0.004[c]
CY887	GHR	GHR	Vector	0.083 ± 0.003[b]
CY899	Vector	GHR	GH	0.027 ± 0.006[a]
CY910	PRLR	PRLR	PRL	0.043 ± 0.007[a]
CY911	PRLR	PRLR	Vector	0.042 ± 0.002[a]
CY931	Vector	PRLR	PRL	0.032 ± 0.003[a]
CY846	KDR	KDR	VEGF	0.086 ± 0.014[c]
CY847	KDR	KDR	Vector	0.061 ± 0.013[b]
CY867	Vector	KDR	VEGF	0.019 ± 0.005[a]

Maximal growth rates were determined and calculated as described in *Materials and Methods*. Plasmid vectors are as described in Table 1. Rates are given in generations per hour and are representative of two or more independent experiments. Values are presented as averages with SD derived from triplicate samples. Within a hormone-receptor group, values with different *superscript letters* are statistically different ($P < 0.05$). Strain CY953, expressing the yeast proteins Snf1 and Snf4 as Gal4 fusion proteins (22), was tested to provide a reference for a well characterized two-hybrid interaction.

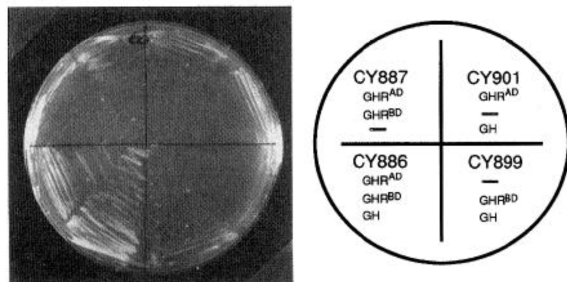

Fig. 4. GH-Induced GHR Dimerization

Strains expressing the indicated GHR fusion proteins and GH are described in the text. The position and the expressed heterologous proteins of each strain are shown in the diagram. *Superscripts* indicate that a protein was expressed as a fusion to the transcriptional activation domain (AD) or DNA-binding domain (BD) of Gal4. A *dash* indicates that the strain contained an unmodified vector (pAS2, pACT2, or pCUP). Two independent isolates of each strain were assayed on selective medium as described in Fig. 1.

that did not express ligand (Fig. 4). To quantitate these effects, the maximal growth rates of strains were determined in selective liquid medium. The baseline level of reporter gene induction in a two-hybrid system generally originates from the DNA-binding fusion protein (expressed from pAS2 in these studies). The control strain CY899 expresses GHR fusion protein from pAS2 and free GH from pCUP and carries unmodified pACT2. The growth rate of this strain was similar to the parental strain CY770 grown under the same conditions (Table 2). Strain CY887, which expresses both GHR-fusion proteins but no GH, grew at a rate significantly greater ($P < 0.05$) than the control, suggesting that some avidity between GHR extracellular domains may exist in the absence of hormone. Expression of ligand further enhanced growth. The growth rate of strain CY886 was 75% greater ($P < 0.05$) than the rate of CY887 (Table 2), demonstrating ligand-dependent GHR dimerization in PRIMES.

With the demonstration of ligand-induced receptor dimer formation for GH/GHR, strains were developed to investigate PRLR dimerization. A plasmid was generated to express the extracellular domain of PRLR as a fusion protein to the Gal4 activation domain. Yeast strains were developed that express both PRLR fusion proteins with nonfusion PRL (CY910) or without ligand (CY911). No induction of growth on selective medium was observed with these strains (Fig. 5). This finding was confirmed by growth rate assays. Expression of receptor fusion proteins (CY911) resulted in a marginal, but insignificant ($P > 0.05$), enhancement of

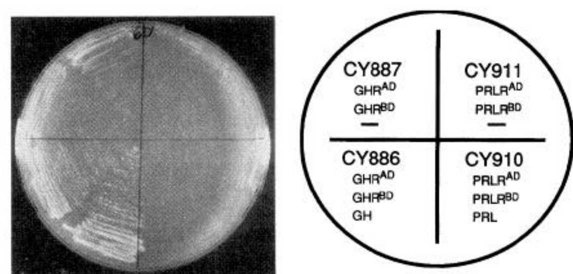

Fig. 5. PRL Does Not Induce PRLR Dimerization

Strains expressing the indicated fusion proteins are described in the text. The position and the expressed heterologous proteins of each strain are shown in the diagram. *Superscripts* indicate that a protein was expressed as a fusion to the transcriptional activation domain (AD) or DNA-binding domain (BD) of Gal4. A *dash* indicates that the strain contained unmodifed pCUP vector. Two independent isolates of each strain were assayed on selective medium as described in Fig. 1.

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growth rate in comparison to the control (CY931) strain. No further enhancement was observed with concomitant expression of PRL (CY910; Table 2). The absence of ligand-stimulated PRLR dimerization was in sharp contrast to the results observed in the GHR dimerization assays (Fig. 5) and suggests that PRL may interact with the PRLR fusion proteins in a manner not conducive to receptor homodimer formation.

The mitogenic peptide VEGF (15) and its receptor, flk1/KDR (16–18), were also investigated in the dimer PRIMES model. Ligand-induced receptor dimer formation is important for receptor activation (12, 17) and occurs with a different (2:2) stoichiometry (13, 20) than that for GH/GHR (1). KDR fusions were generated by cloning sequences encoding the extracellular region of KDR into both fusion protein expression vectors. VEGF was expressed as a nonfusion protein from the pCUP expression vector. Yeast strains (CY867, CY869) that expressed any single KDR fusion protein in the presence of VEGF failed to grow, and the strain (CY847) expressing both KDR-fusion proteins in the absence of VEGF grew poorly on selective medium (Fig. 6). Only the strain (CY846) that expressed both fusion proteins plus VEGF exhibited substantial growth on selective medium (Fig. 6).

Reporter gene activation, observed as growth on selective medium, occurred in the strain (CY847) expressing KDR fusion proteins in the absence of VEGF (Fig. 6), a finding similar to that observed in GHR dimerization assays (Fig. 4). Measurement of maximal growth rates in selective liquid medium produced data consistent with the results shown in Fig. 6. The control strain CY867 grew at a rate equivalent to the parental strain (Table 2). Strain CY847, which expresses both KDR fusion proteins but not VEGF, exhibited a growth rate significantly greater ($P < 0.05$) than that of strain CY867. Coexpression of VEGF in strain CY846 significantly ($P < 0.05$) stimulated cell proliferation to a rate 41% greater than strain CY847 (Table 2). This enhanced growth rate suggests VEGF-dependent formation and/or stabilization of KDR dimers. These findings suggest that some level of receptor association may occur before ligand binding and that this receptor dimerization is enhanced by interaction with the ligand.

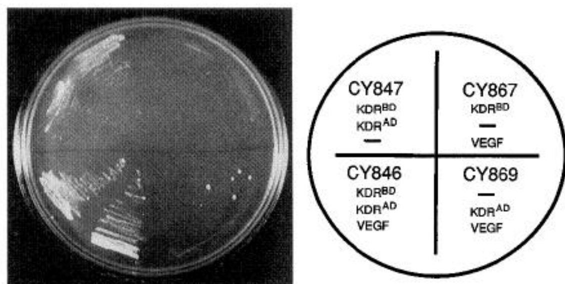

Fig. 6. VEGF Interaction with KDR Fusion Proteins

Strains expressing KDR fusion proteins and VEGF are described in the text. The position and expressed heterologous proteins of each strain are shown in the diagram. *Superscripts* indicate that a protein was expressed as a fusion to the transcriptional activation domain (AD) or DNA-binding domain (BD) of Gal4. A *dash* indicates that the strain contained an unmodified vector (pAS2, pACT2, or pCUP). Two independent isolates of each strain were assayed on selective medium as described in Fig. 1.

DISCUSSION

The two-hybrid protein-protein interaction technique has been successfully employed to study numerous intracellular protein associations (22–26). The novel findings described in this report demonstrate that this method, which depends on gene activation within the yeast nucleus, also can be used to examine protein associations that normally occur at the mammalian cell surface. When expressed in yeast, the peptide hormones GH and PRL were found to interact with their respective receptors. Importantly, ligand-receptor binding was reversible and specific as demonstrated by coexpressing a third protein as an interaction competitor. Utilization of a third heterologous component also enabled the examination of ligand-induced receptor dimerization by expressing two receptor fusion proteins plus free peptide ligand. The functional expression of the peptide ligand with the cognate extracellular ligand-binding domain for GH and PRL, as well as VEGF, suggests broad utility of the PRIMES technology. Additionally, the diversity of the proteins demonstrates that complex structural motifs such as four helix bundles (28), cysteine knots (29), and immunoglobulin (Ig)-like domains (30) are not restrictive for protein-protein interactions in this system.

The peptide ligands and their receptors have structural motifs that utilize disulfide bonds. The extracellular domain of the human GHR contains seven cysteines, configured in a simple disulfide-bonding pattern wherein cysteines in close proximity along the primary structure are linked, defining three short disulfide loops (31). The PRLR contains five cysteines, suggesting the formation of two disulfide loops (32), while the flk1/KDR receptor sequence suggests seven Ig-like domains (12). Mutagenesis of the cysteines in the PRLR extracellular domain abolish binding of PRL to Chinese hamster ovary (CHO) cells expressing this mutant PRLR (33). Reduced VEGF is unable to bind receptor and is functionally inactive (34), as are cysteine mutants of VEGF (13). These data suggest that structural motifs imparted by disulfide bonds are important for the binding integrity of these ligand and receptor proteins. This was an important consideration in the described experiments because the redox potential within cells is thought to be inconducive to disulfide bond formation.

The observations of various ligand-receptor interactions in yeast suggest that the interior of this organism may be suitable for the formation of protein structural motifs stabilized by disulfide bonds. The acidic environment preferred by yeast may be an important distinction in comparison to other eukaryotes. The growth

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media used in the described research had a pH of 4.0–4.3, standard for optimal yeast growth. Regardless of environmental pH, yeast cytosol is maintained at neutral pH via the action of a membrane proton-ATPase (35). The large outward flux of protons across the yeast cell membrane might result in the capacity for disulfide bond formation through localized variations in redox potential. Alternatively, levels of glutathione, the major thiol-disulfide redox buffer in cytosol, can vary significantly in response to changes in the physiological state of cells (36). Yeast grown under standard laboratory conditions may have lower levels of reduced glutathione resulting in a more oxidizing cell interior. When the pH of assay medium was increased in a stepwise manner, GH/GHR interactions and VEGF/KDR interactions were attenuated (37). In these experiments, the interaction of two yeast intracellular proteins, Snf1 and Snf4 (21), was unaffected by changes in the extracellular pH. These findings were further validated by a cysteine substitution analysis of VEGF demonstrating that cysteines essential for *in vivo* activity in a mammalian expression system (13) are also essential for activity in PRIMES (37). These data suggest that the distinct physiology of yeast enables the examination of peptide ligand-receptor interactions in a two-hybrid system.

The peptide hormones and receptors investigated in this system also contain N-linked glycosylation sites and have associated carbohydrate moieties in their native forms (32, 34, 38). Proteins modified by N-linked glycosylation are unlikely to be found in the yeast nucleus. This was less of a concern in these studies because nonglycosylated receptors bind ligand with little or no change in affinity (1, 3, 31). Additionally, many peptide hormones and receptors have been produced recombinantly in *Escherichia coli* and demonstrate appropriate binding affinities (14, 18, 39). In general, the lack of protein glycosylation did not appear to be limiting in this system, although other ligand-receptor pairs may have unique constraints.

As the multiprotein interactions involved in the binding of cytokines, or various other peptide factors, to surface receptors have been described, no simple paradigm has emerged. Receptor proteins (*e.g.* interleukin-2γ, gp130, KH97) can associate with multiple partners to produce binding sites with different specificities for peptide ligands (4–7, 40, 41). In some cases, it appears that ligand binds to one receptor protein and only then interacts with additional proteins to initiate signal transduction (4–7, 41). In other systems, receptors consisting of homo- or heterodimers form high-affinity binding sites for the peptide ligand (4–7, 40). As most of the proteins in the cytokine receptor family function as multimers, the provocative observation that ligand-dependent receptor association can be examined in yeast should catalyze investigations of higher order protein interactions using PRIMES technology.

Monomeric and dimeric associations of GHR with ligands have been observed (2, 39). Human GH contains two distinct domains that bind in an ordered manner to identical regions of two GHRs (1). We demonstrate that GH functionally interacts with its receptor in both monomer and dimer models of PRIMES. In contrast, VEGF functions as a homodimer (13), suggesting that covalently associated ligand molecules engage receptors through identical sites of the peptides. We observed functional interaction of VEGF and KDR fusion proteins in the dimer model of PRIMES but not in a monomer model in which VEGF was expressed as a fusion protein (data not shown). This may have been due to steric constraints of a VEGF fusion protein to properly associate as a homodimer and engage the KDR fusion protein. Heparin may provide an important function in VEGF binding to its receptor (42), although other data suggest that heparin affects VEGF bioavailability rather than having a direct effect on ligand-receptor association (43). In yeast, VEGF bound KDR, inducing receptor dimerization in the absence of heparin, suggesting that heparin does not directly participate in VEGF binding.

PRL was also investigated in both monomer and dimer receptor models of PRIMES. Association of PRL with the PRLR fusion mimicked the response observed for GH/GHR in the monomer system but clearly contrasted with that observed for GH/GHR in the dimer model. Investigation of PRL/PRLR stoichiometries by Bignon *et al.* (3) demonstrated a 1:1 association of PRL with a recombinantly expressed PRLR extracellular domain protein. Additionally, a 1:1 stoichiometry was observed when PRL associated with PRLR from bovine mammary gland (3). Experiments using antibodies to the PRLR suggest that PRLR dimerization can stimulate cell proliferation, although the response is less than that observed using PRL (3, 11). Our results suggest that PRL does not promote receptor-receptor dimer formation. The level of PRL expression may have been too high, forcing a 1:1 ligand-receptor stoichiometry. This is unlikely, however, since PRL was expressed from a centromeric (single copy per cell) plasmid while the receptor fusion proteins were expressed from multicopy 2μ-based plasmids (44). Additionally, GHR dimerization was not blocked by GH in this system, suggesting that expression from the centromeric pCUP plasmid produced GH at a concentration insufficient to force a 1:1 stoichiometry (45). As an alternative to a PRLR homodimerization model, PRL may induce heterodimerization with an as yet unidentified receptor. Receptor heterodimers, or heterotrimers incorporating transducer molecules such as gp130, are prevalent among cytokine receptors (8). Alternatively, PRL may indeed functionally associate with its receptor through a monomeric structure (3).

The quantitative measures of receptor dimerization provide evidence for different modes of ligand-receptor association. As described above, PRL did not promote receptor homodimerization in these studies, nor was there substantial interaction between PRLR fusion proteins in the absence of ligand. In contrast, expres-

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sion of GHR fusion proteins did produce a significant enhancement of growth above control. This suggests that there is some degree of avidity between these receptors in the absence of ligand, although it is noted that the receptor proteins were overexpressed. Interaction between GHRs may occur via the receptor stem region near ser^{201} (9, 45) or via inter-receptor disulfide bond formation at the unpaired membrane-proximal cysteine. The frequency of ligand-independent GHR dimerization in natural systems is unknown. The expression of GH in strain CY886 resulted in a significant stimulation of growth above that observed for CY887, consistent with known structural data for this protein complex (1). The VEGF/KDR assays present a similar paradigm. Strain CY847, expressing only the two KDR fusion proteins, exhibited significant growth above controls. The affinity between KDRs in the absence of VEGF may be indicative of receptor-receptor association via an Ig-loop, as has been observed for the related platelet-derived growth factor receptor (46) and c-*kit* (47, 48). VEGF expression significantly enhanced KDR dimer formation or stabilization. In contrast to the apparent sequential binding of GH to one receptor protein followed by interaction with a second receptor (39), VEGF may simultaneously bind paired receptor proteins, thereby stabilizing dimer formation and consequent receptor activation. Such a model for the formation of GH/GHR complexes is also consistent with the data presented here.

We have described novel methods with which to examine reversible and specific binding of peptide ligands and receptors and the formation of specific multiprotein complexes. Ligand-induced receptor dimerization was observed for GH/GHR and VEGF/KDR but, interestingly, not for PRL/PRLR. These findings suggest that PRL/PRLR may not form a simple 1:2 homodimer complex as has been demonstrated for the related GH/GHR complex. This robust and easily manipulated microbial expression system can be used to rapidly dissect peptide ligand-receptor interactions. Expansion of these findings will likely include the application of various genetic tools available to yeast expression systems to identify new ligands and receptors via cDNA library screening, to perform rapid structure/function analyses, and to characterize receptor protein partners. These applications should contribute to the elucidation of the complex molecular associations involved in ligand-receptor multimerization and potentially lead to the identification of novel therapeutics acting on this important family of molecules.

MATERIALS AND METHODS

Plasmids

Molecular engineering methods were performed by standard protocols (49). Polymerase chain reactions (PCR) (50) used reagents supplied by Perkin Elmer Cetus (Norwalk, CT). Oligonucleotide synthesis and nucleotide sequencing were performed with reagents and instruments supplied by Applied Biosystems (Foster City, CA). Gal4 activation domain fusions were constructed in pACT2, which is identical to pACT (23) with modification of the polylinker region (S. Elledge, personal communication). Gal4 DNA-binding domain fusions were constructed in the vector pAS2 (24).

The cDNA encoding the extracellular domain of the rat GHR (amino acids 1–247) was generated by PCR with oligonucleotide primers incorporating *Nco*I restriction sites and a translational stop codon and using cloned rat GHR sequences (51) as template. The GHR cDNA was cloned into the pAS2 vector as an *Nco*I restriction fragment fusing the coding region for the Gal4 DNA-binding domain to GHR. The cDNA encoding the mature peptide for porcine GH was generated by PCR with oligonucleotides incorporating *Nco*I or *Bam*HI restriction sites and using a pituitary cDNA library as template source. The GH protein-coding region was cloned into pACT2 as an *Nco*I-*Bam*HI restriction fragment fusing the coding region for the Gal4 activation domain to GH. The GH-coding region was amplified by PCR with a sense oligonucleotide incorporating translation start signals and an *Eco*RI restriction site plus an antisense oligonucleotide containing a *Hin*dIII restriction site. This DNA fragment was isolated and cloned into pCUP. pCUP is a yeast expression vector derived from pRS316 (52). Briefly, this vector was constructed by inserting the CUP1 promoter region (53) plus the 3′-end of the yeast PGK gene (54) into the cloning region of the centromeric (low-copy number) vector pRS316 to provide transcriptional initiation and termination signals. In strains described in this report, constitutive expression from the CUP1 promoter in the absence of inducer (Cu^{2+}) was sufficient to attain desired levels of protein. The transcriptional control regions flank unique *Eco*RI and *Hin*dIII restriction sites that were used to clone the GH-coding region. All constructs were confirmed by nucleotide sequence analysis.

Plasmids expressing PRL or PRLR sequences were constructed as follows. DNA encoding porcine PRL (55) was amplified from a pig pituitary cDNA library by PCR utilizing oligonucleotides with terminal *Eco*RI restriction sites. The 5′-oligonucleotide designed to amplify PRL for cloning into pCUP also incorporated translation initiation sequences. PCR products were isolated as *Eco*RI restriction fragments and cloned into pACT2 or pCUP and confirmed by sequence analysis. Sequences encoding the extracellular domain (amino acids 1–229) of the porcine PRLR were amplified by PCR using oligonucleotides designed from the sequence of the mouse PRLR (56). The sense oligonucleotide contained a *Sma*I site, and the antisense oligonucleotide contained a termination codon followed by a *Sal*I site. A pig pituitary cDNA library was used as template source. The PRLR cDNA fragment was cloned into the pAS2 vector via *Sma*I and *Sal*I restriction sites and into the pACT2 vector via *Sma*I and *Xho*I sites. All constructs were confirmed by nucleotide sequence analysis.

VEGF and KDR expression plasmids were constructed by the following methods. The cDNA encoding the ligand-binding domain of KDR (amino acids 1–245) (17, 18) was isolated as an *Nco*I-*Bam*HI fragment and cloned into both the pACT2 and pAS2 vectors. Fusion junctions were confirmed by sequence analysis. The cDNA encoding the mature protein for VEGF (121 residues) (14) was amplified by PCR with a sense oligonucleotide incorporating an *Eco*RI restriction site plus translation start signals and an antisense oligonucleotide containing an *Eco*RI restriction site, cloned into pCUP and confirmed by nucleotide sequence analysis.

Yeast Strains

All yeast strains were derived from strain Y190 (24). Yeast were grown in YPD medium or, if maintaining plasmids, in synthetic drop-out media as described by Rose *et al.* (57). To incorporate a third plasmid, a uracil auxotroph of strain Y190 was identified by passage on 5-fluoro-orotic acid (57). This Ura^- derivative was designated CY770. This modification

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also resulted in the deletion of a UAS_{GAL}-*lacZ* reporter gene. Although this reporter gene is useful for the screening of cDNA expression libraries (24), it was not suitable for quantitative assays of fusion protein interactions (data not shown). Strains were generated by transforming expression plasmids into either Y190 or CY770 by the lithium acetate method (57). To counteract background expression of the UAS_{GAL}-*HIS3* reporter gene, the imidazoleglycerol phosphate dehydratase inhibitor 3-amino-triazole was added to 60 mM in bioassay medium.

Measurement of Growth Rates

Strains were grown overnight at 30 C in 2 ml synthetic medium lacking leucine, tryptophan, and uracil to select for the maintenance of plasmids. Samples were washed twice in 2 ml water to remove traces of histidine. Samples were subsequently diluted 100-fold in 2 ml synthetic medium lacking leucine, tryptophan, uracil, and histidine and supplemented with 3-amino-triazole to 60 mM. The cell density was 2–5 $\times 10^5$ cells/ml. Triplicate test samples were placed on a rotator at 30 C. The optical absorbance at 620 nm (A_{620}) of each sample was measured after 20-h and 44-h incubation times. Cultures exhibited maximal growth rates at these time points (data not shown). In preliminary experiments, cells being grown in assay medium were serially diluted and examined spectrophotometrically and counted in a hemacytometer to determine the mathematical relationship between A_{620} and cell density. These data fit a quadratic equation (58); cells($\times 10^6$)/ml $= 18.75(A_{620}) + 156.25(A_{620})^2$. Cell densities were converted to growth rates using the equation $(\log_2 X - \log_2 X_0)/\Delta time$ (58) where X = cell density at 44 h, X_0 = cell density at 20 h, and $\Delta time = 24$ h. All experiments were performed in triplicate, and results were reported as means with [sd]s. Data were analyzed by a one-way analysis of variance using the General Linear Models procedure of the Statistical Analysis System (59) to detect the main effect of strain within a hormone-receptor group. Fischer's protected least significant difference (LSD) was used to compare means when the main effect of strain was highly significant ($P < 0.001$). Statistical significance between means within a hormone-receptor group was determined by multiple comparison of the least squares means. Significance was declared at $P < 0.05$.

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