0888-8809/08/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 22(9):2190–2202

doi: 10.1210/me.2008-0128

The Extracellular Domain of the Growth Hormone Receptor Interacts with Coactivator Activator to Promote Cell Proliferation

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The presence of GH receptor (GHR) in the cell nucleus correlates with cell division, and targeting the GHR to the nucleus results in constitutive proliferation and transformation because of increased sensitivity to autocrine GH. Here we have sought additional mechanisms that might account for the enhanced proliferation seen with nuclear GHR, commencing with a yeast two-hybrid (Y2H) screen for interactors with the extracellular domain of the GHR [GH-binding protein (GHBP)]. We find that the GHBP is a transcriptional activator in yeast and mammalian cells, and this activity resides in the lower cytokine receptor module. Activity is dependent on S226, the conserved serine of the cytokine receptor consensus WSXWS box. By using parallel GHBP affinity columns and tandem mass spectrometry of tryptic digests of proteins bound to wild-type GHBP and S226A columns, we identified proteins that bind to the transcriptionally active GHBP. These include a nucleoporin and two transcriptional regulators, notably the coactivator activator (CoAA), which is also an RNA binding splicing protein. Binding of CoAA to the GHBP was confirmed by glutathione *S*-transferase pulldown and coimmunoprecipitation, and shown to be GH dependent in pro-B Ba/F3 cells. Importantly, stable expression of CoAA in Ba/F3 cells resulted in an increased maximum proliferation in response to GH, but not IL-3. Because CoAA overexpression has been identified in many cancers and its stable expression promotes cell proliferation and cell transformation in NIH-3T3 cells, we suggest CoAA contributes to the proliferative actions of nuclear GHR by the hormone-dependent recruitment of this powerful coactivator to the GHR. (*Molecular Endocrinology* 22: 2190–2202, 2008)

A NUMBER OF growth factor receptors or their cleaved fragments [epidermal growth factor (EGF) receptor, ErbB-2, fibroblast growth factor receptor (FGFR)1, nerve growth factor receptor, interferon-γR1, prolactin receptor] have been shown to translocate to the cell nucleus (1, 2), and the extent of nuclear localization correlates with proliferation for the FGFR1 (3) and stage of progression of breast cancer in the cases of the EGF receptor and ErbB-2 (1, 4). Nuclear translocation of FGFR1 and ErbB-2 requires importin-β (3, 5), but the precise mechanism of the retrotranslocation from endocytotic vesicle to cytoplasm is not known. Retrotranslocation is thought to involve the endoplasmic reticulum-associated degradation pathway and the sec 61b complex (2), analogous to the entry of ricin and certain other toxins (6). Within the nucleus, the carboxy terminus of EGF receptor has been shown to bind and directly transactivate the cyclin D promoter, whereas ErbB2 is able to transactivate the COX-2 promoter (7, 8).

We have reported the presence of both the full-length GH receptor (GHR) and the alternatively spliced rodent GHR extracellular domain [GH-binding protein (GHBP)] in the cell nucleus (9, 10) and nuclear localization of GHR is seen in hepatocytes during liver regeneration, in a number of solid tumors and in chronic liver disease (9, 11, 12). Moreover, forced nuclear localization of the full-length GHR, but not the GHBP, with a nuclear targeting sequence (NLS) confers strikingly increased sensitivity to GH, such that the low levels of endogenous GH in Ba/F3 proB cells are able to drive autonomous proliferation (9). Nuclear localization of the extracellular domain was shown to be dependent on Importin-β, although a canonical nuclear localization sequence was not identified, raising the possibility that the GHR or GHBP is carried to the

First Published Online July 17, 2008

Abbreviations: CHO, Chinese hamster ovary; CoAA, coactivator activator; CPG, controlled pore glass; DBD, DNA-binding domain; DTT, dithiothreitol; EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FGFR, fibroblast growth factor receptor; FOR, forward; GHBP, GH-binding protein; GHR, GH receptor; HA, hemagglutinin; IPTG, isopropyl-β-D-thiogalactopyranoside; JAK, Janus family of tyrosine kinases; mab, monoclonal antibody; NLS, nuclear localization sequence; rb, rabbit; REV, reverse; SDS, sodium dodecyl sulfate; SIP, SYT-interacting protein; STAT, signal transducer and activator of transcription; SYT, synovial sarcoma translocation; TR, thyroid receptor; WT, wild type; Y2H, yeast two-hybrid; YPD, yeast protein database.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

nucleus in association with a NLS-containing carrier protein, or that it uses a noncanonical localization sequence such as the scramblase1 protein (13). Nuclear localization of the GHR has previously been shown to be dependent on GH binding, as well as on the appropriate extent of serum starvation (9, 14).

Although our previous study (9) indicated a key requirement for constitutive signal transducer and activator of transcription (STAT)5 activation in the autonomous proliferation of nuclear targeted GHR, it did not exclude the possibility of a direct interaction between the native GHR/GHBP and nuclear localizing proteins other than STAT5. Accordingly, we used the yeast two-hybrid (Y2H) system with the GHBP as bait to identify proteins that interact with the extracellular domain of the GHR and that might play a role in its actions within the nucleus.

RESULTS

A Functional Transactivation Domain in GHR Identified by Y2H

Figure 1 provides a schematic for the GHR and GHBP structure. We were unable to identify interactors with the GHBP in the Y2H assay because the GHBP bait was itself strongly transcriptionally active. Thus, the GHBP was able to activate transcription of the β-galactosidase reporter gene from the GAL4 upstream activation sequence when expressed as an N-terminal fusion with the GAL4 DNA-binding domain (DBD) in yeast (result not shown). This transactivation activity was not restricted to the GAL fusion proteins described above, because the same result was reproduced with the LexA-GHBP fusion protein, which was able to induce transcription of the β-galactosidase reporter from the LexA operator (Fig. 2). Therefore, the GHBP activation domain was functional in different contexts, recapitulating the findings of Ma and Ptashne (15) where the specificity is determined by the appropriate DBD. To identify the active sequence within the GHBP, deletions constructs were created that ensured the integrity of the structure of each of the two cytokine receptor subdomains, which are separated by a four-residue linker region. Therefore, the linker region was chosen to separate the domains so that the β-sheet structure of each domain would remain intact. The domain fusion proteins expressed well in yeast with levels of protein shown by Western blot (Fig. 1B). The transactivation domain of GHBP was thus mapped to the 100-residue domain 2, comprising the lower cytokine homology domain of the GHBP. Domain 2 contained full transactivation activity, whereas domain 1 was without activity. The serine at position 226 of domain 2 appears to be essential for the transactivation ability because the mutation S226A is transcriptionally inactive, as shown in Fig. 2. This serine is conserved across all cytokine receptors and is part of the WSXWS consensus motif that is replaced

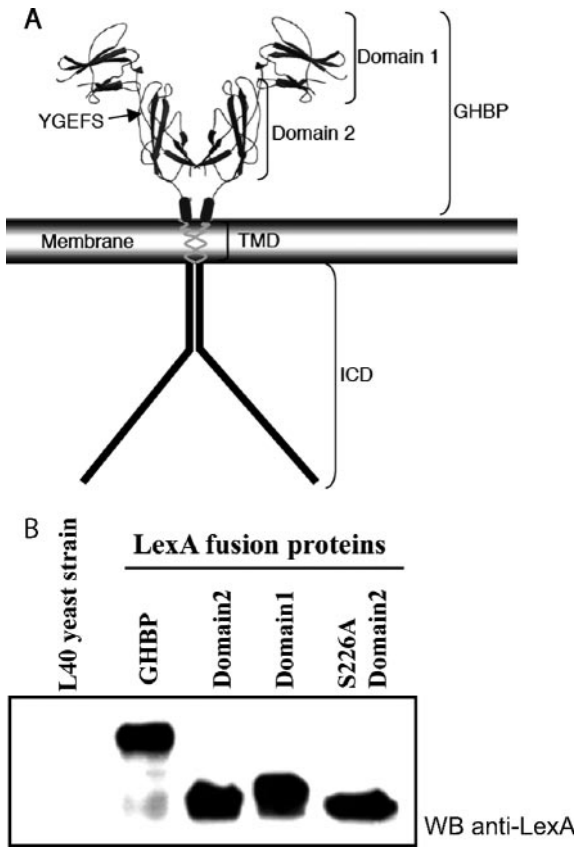

Fig. 1. Schematic of GHBP Domains, Mutations, and Expression in Yeast

A, Schematic of GHR extracellular domain. B, Western blot of yeast lysates from L40 yeast strain transformed with the various constructs as indicated, showing that all constructs are expressed to similar levels. The blot was probed with anti-LexA antibody at 1:1000. TMD, Transmembrane domain; WB, Western blot; ICD, intracellular domain.

by the conservative substitutions YGEFS in the GHR. Two other residues in this consensus motif appear to be unnecessary for transcriptional activity because the Y222A and the E224A mutants retain full transcriptional activity.

To determine whether these findings applied to mammalian cells, the ability of GHBP-GAL4 fusions to transactivate a GAL4 upstream activation sequence-luciferase reporter was tested in Chinese hamster ovary (CHO)-K1 cells. As shown in Fig. 3, the 246-residue GHBP was able to induce a 40-fold induction of a luciferase activity. This ability was restricted to the 100-residue domain 2 of the GHBP and was not seen with the S226A mutant, as had been observed in yeast.

Affinity Chromatography and Identification of GHBP Interactors

In light of the potent transactivation of the reporter genes by GHBP in yeast, affinity chromatography

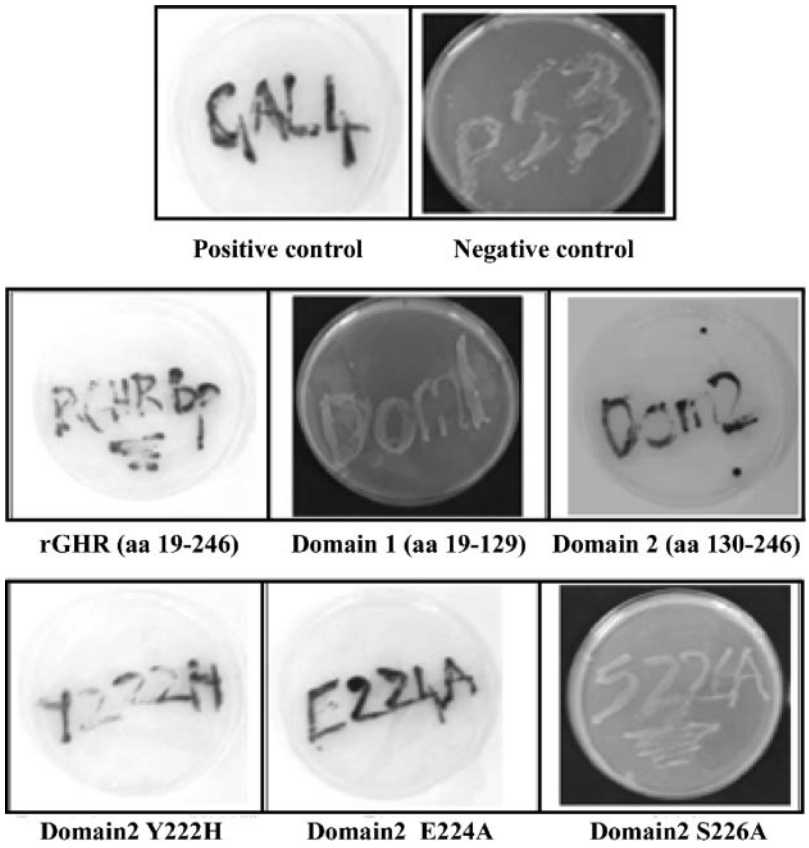

Fig. 2. Identification of a Transactivation Domain in GHR Using the Y2H System

All constructs shown are fusion proteins with GAL DBD or LexA, and the reporter gene is β-galactosidase with IPTG substrate, yielding blue when there is a transcriptional activation domain present. Transactivation ability is evident in GHBP and mapped to the 100-amino acid region of domain 2. The S226A domain 2 mutant had no activity, but the Y222A and E224A mutants retained full activity. aa, Amino acids.

with nuclear extracts was undertaken to find interacting proteins. The mechanism whereby transactivators initiate the process of transcription is thought to involve their recruitment of key components of the transcription machinery to the transcription start site. Therefore, if GHBP possesses transcriptional activity in yeast as well as in mammalian cells, it would be expected to interact with a conserved complex of nuclear proteins in both contexts. The approach here was to identify yeast nuclear proteins

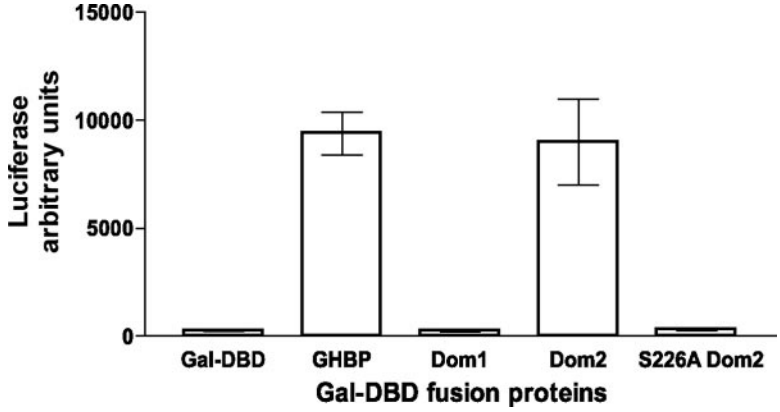

Fig. 3. Transactivation by GHBP in CHO-K1 Cells

Mammalian β-galactosidase reporter assay as described in *Materials and Methods*. The mean ± SEM from three individual experiments is shown here. Within each experiment, each GAL fusion construct was transfected into six replicate wells.

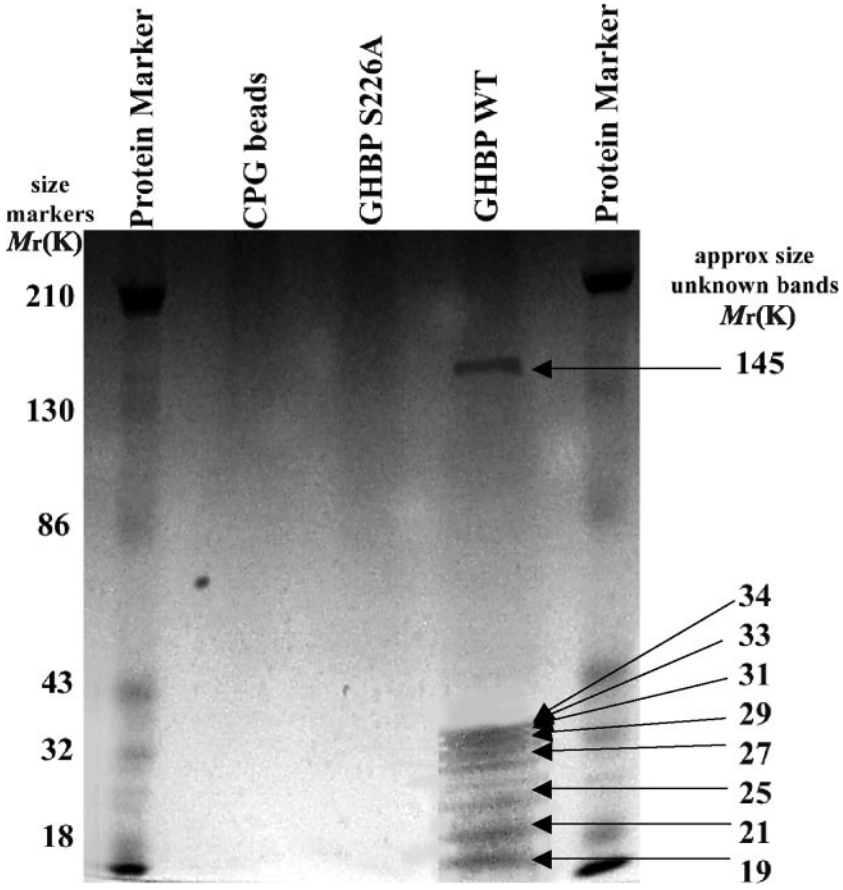

Fig. 4. GHR-Interacting Proteins in Nuclear Extracts Using Affinity Chromatography

Affinity chromatography was carried out with yeast nuclear extracts using three parallel glass bead columns (CPG beads alone, coupled to GHBP or to S226A GHBP) as described in *Materials and Methods*. The $MgCl_2$ eluates from washed columns were dialysed and concentrated, and a sample was run on a linear gradient SDS-PAGE gel (4–20%) and then visualized with Coomassie blue. The eluant from each column was loaded onto individual lanes as marked. Standard protein markers were run in flanking lanes to derive a standard curve (*left lane*), from which the unknown protein sizes were calculated. Individual bands were excised and tryptic digested before mass spectrophometric analysis and protein sequencing.

that interact with the GHBP WT (wild type) but not the transcriptionally inactive S226A, and then find the mammalian homolog to verify the interactions in both models. The result from the parallel affinity column purifications showed that the GHBP WT was able to recruit a large multiprotein complex from yeast nuclear extracts, whereas the transcriptionally inactive S226A was not (Fig. 4).

Identification of all bands was not possible; however, some interesting identifications were made (Table 1). The P160 band was identified as nucleoporin 145 (Nup145), an integral nuclear pore protein that contains FG repeats that are docking sites for the importin-α/β/cargo complex through the nuclear pore (16). This result suggests that the nuclear import of GHBP mediated by the importin-α/β complex in mam-

Table 1. Identification of GHR Binding Proteins by Affinity Chromatography and Tandem Mass Spectroscopy

Unknown	ID	Accession No.	Mammalian Homolog	Function
P31	RPL4	AAA20990.1 GI:172444	L7A/TRUP	Ribosomal protein transcriptional regulation
P33	Sbp1	NP_011829.1 GI:6321753	RNA binding motif protein 14 (SIP); CoAA	RNA binding splicing factor transcriptional regulation
P50	EF1 α	NP_015405.1 GI:6325337	EF1 α	Translational regulation
P160	Nucleoporin	CAA83584.1 GI:496731	Nup145	Nuclear import

Homologous mammalian proteins to the identified yeast proteins were found using the YPD data base http://www.biobase-international.com/pages/index.php?id = 139. TRUP, TR-uncoupling protein.

malian cells (9) may also be conserved in the yeast system.

For the p33 band, two tryptic peptides were sequenced, yielding SKDTLYINNVPFK and IDSDNIK.

Based on these sequences, P33 was identified as single-stranded binding protein 1 (sbp1), an RNA binding protein. The closest mammalian homolog was identified by the yeast protein database (YPD) as RNA-binding protein 14. Identical sequences entered into National Center for Biotechnology Information have also been named SIP [synovial sarcoma translocation (SYT)-interacting protein] or CoAA (coactivator activator) (17). This protein is now commonly referred to as CoAA.

The p31 band was identified as the yeast ribosomal protein L4, and its mammalian homolog was identified as L7a. L7a was renamed as thyroid receptor (TR)-uncoupling protein, when it was characterized as involved in transcriptional regulation with the TR (18). The identification of two yeast homologs of mammalian proteins involved in transcriptional regulation appears to validate the original hypothesis that GHBP is

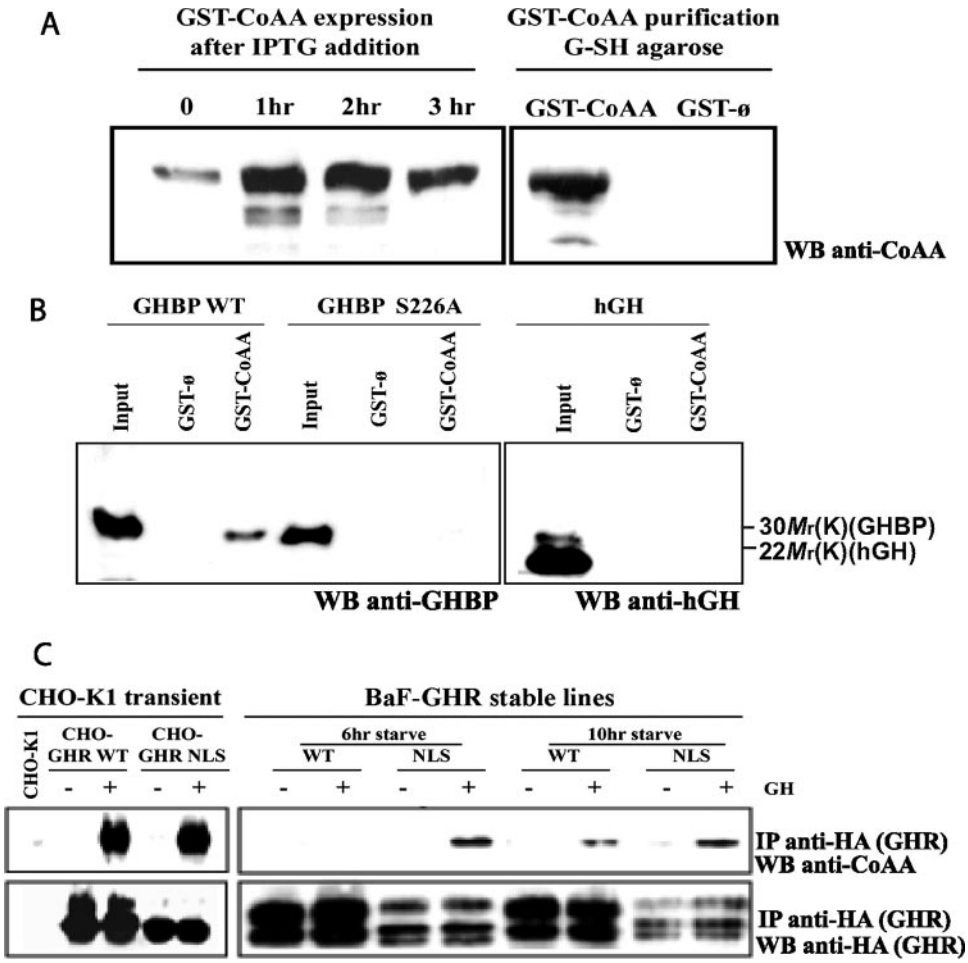

Fig. 5. CoAA Associates with GHBP But Not 226A GHBP in a GH-Dependent Manner

A, Expression of GST-CoAA fusion. The *left panel* shows GST fusion protein expressed in BL21 *E. coli*, with induction by IPTG addition shown at 0, 1, 2, and 3 h after IPTG. The *right panel* shows the pure protein CoAA-GST, which was purified on a glutathione (G-SH) agarose resin. CoAA was detected in these Western blots with a rabbit antibody to CoAA (see *Materials and Methods*). GST-0 is Glutathione *S*-transferase alone. B, GST pull-down assay verifies interaction between CoAA and GHBP but not S226A mutant. Equivalent amounts of lysates expressing GHBP WT or S226A were added to either the GST-CoAA resin or GST resin alone, and the beads were washed thoroughly, then boiled in Laemelli sample buffer, and run on an SDS gel as described in *Materials and Methods*. The GST pull-down assay was performed with hGH also, and hGH did not interact with GST-CoAA beads. GHBP was detected in Western blot with a guinea pig polyclonal antibody to recombinant GHBP, whereas hGH was detected with Mab20. C, Endogenous CoAA associates with GHR in a GH-dependent manner in CHO-K1 cells and Ba/F3 stable lines. HA-GHR (N-terminal HA tag) was transiently expressed in CHO-K1 cells (*left panel*), or stably in Ba/F3 stable lines (*right panel*). The interaction occurs in a GH-dependent manner for both GHR WT and nuclear localized GHR [amino-terminal SV-40 NLS, NLS-GHR (9)], although interaction is stronger for nuclear localized GHR, and independent of serum starvation. In the Ba/F3 stable lines, a time course of serum starvation followed by GH treatment showed that CoAA only associates with WT GHR after a 10-h serum starvation, but not a 6-h serum starvation. In all cases, exposure to hGH was for 10 min at 5 nM. IP, Immunoprecipitation; WB, Western blot.

involved in a transcriptional mechanism conserved from yeast to mammalian cells.

Coassociation of CoAA and GHR

The best candidate from Table 1 appeared to be the transcriptional activator CoAA, because this interacts with the transcriptional coactivators p300 and cAMP response element-binding protein-binding protein (17) and with the protooncogene SYT (19), which is an intrinsic component of the two SWI/SNF complexes (29). Therefore, interaction of CoAA with GHBP was first verified by GST pulldown assay using a specific CoAA antibody (19). The specificity of this antibody is evident in Fig. 4A, which shows detection of GST-CoAA in bacterial lysates before and after the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression, and then after purification on the glutathione agarose beads.

As can be seen in Fig. 5, the affinity chromatography interaction was reproduced in the GST pulldown assay. The GST-CoAA fusion protein purified on glutathione agarose beads was able to pull down GHBP WT but not S226A from whole-cell lysates. Pure recombinant hGH was not pulled down in the assay, which provides another negative control for the specificity of the interaction between CoAA and GHBP WT. Therefore, the association between CoAA and GHBP is shown both by affinity chromatography and GST fusion pulldown; each immobilized protein is able to capture the other from solution.

Further evidence for coassociation between full-length GHR and CoAA in whole cells was then shown by immunoprecipitation. CoAA was expressed in all the GH-responsive cell lines tested including CHO-K1, 3T3 F442A, Cos 1, human embryonic kidney 293, and Ba/F3 (immunoblot result not shown). In CHO-K1 cells transiently transfected with the HA-tagged GHR WT and GHR NLS (9) constructs, it was possible to immunoprecipitate with anti-hemagglutinin (HA) antibody and detect coassociation with endogenous CoAA by Western blot with the anti-CoAA antibody (Fig. 5C). The association occurred only after GH addition, in both WT and NLS-expressing cells. This implies that a GH-induced signal is necessary for the interaction to occur. In the BaF-GHR WT lines, there appeared to be the strict requirement regarding serum starvation time before GH treatment for coassociation to occur. Thus, GH was not able to induce association between GHR and CoAA after a 6-h starvation, but could do so after a 10-h starvation. In contrast, the NLS lines were not restricted in the same way, because coassociation occurred at either time point.

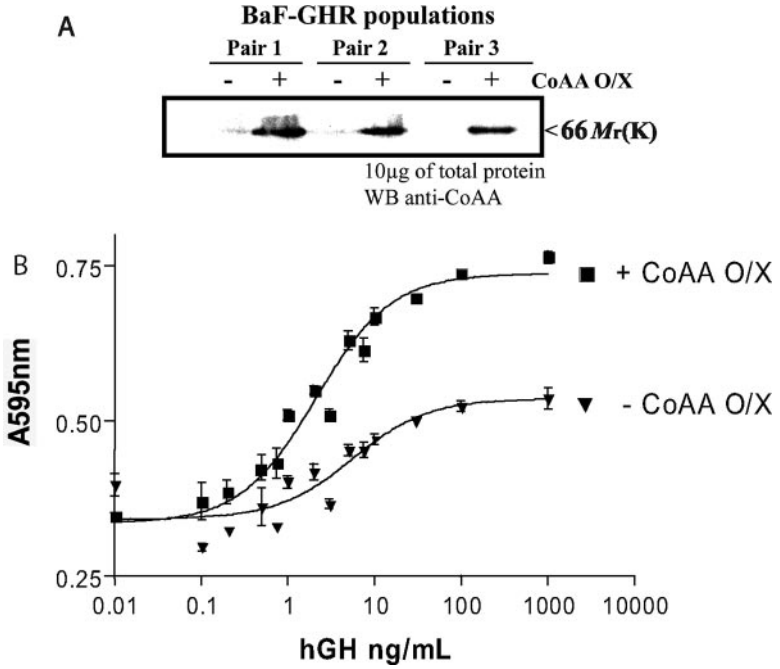

Fig. 6. CoAA Overexpression in BaF-GHR Stable Populations Increases Maximum Proliferative Response to GH But Not IL-3

A, Western blot of total cell lysates (10 μg of total protein loaded per lane) from three stable populations of BaF-GHR WT, and three stable populations of BaF-GHR WT with CoAA overexpression (O/X). The three pairs of populations are matched based on GHR expression. CoAA expression was detected by Western blot with anti-CoAA antibody as described in *Materials and Methods*. B, GH dose response for proliferation in BaF-GHR stable populations overexpressing CoAA compared with BaF-GHR populations alone using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye assay as described in *Materials and Methods*. When IL-3 was used to drive proliferation of these cells, the maximum response was not different between BaF-GHR and BaF-GHR expressing CoAA (0.89 $\pm$ 0.01 *vs.* 0.89 $\pm$ 0.01 absorbance units; mean $\pm$ SEM, n = 3). WB, Western blot.

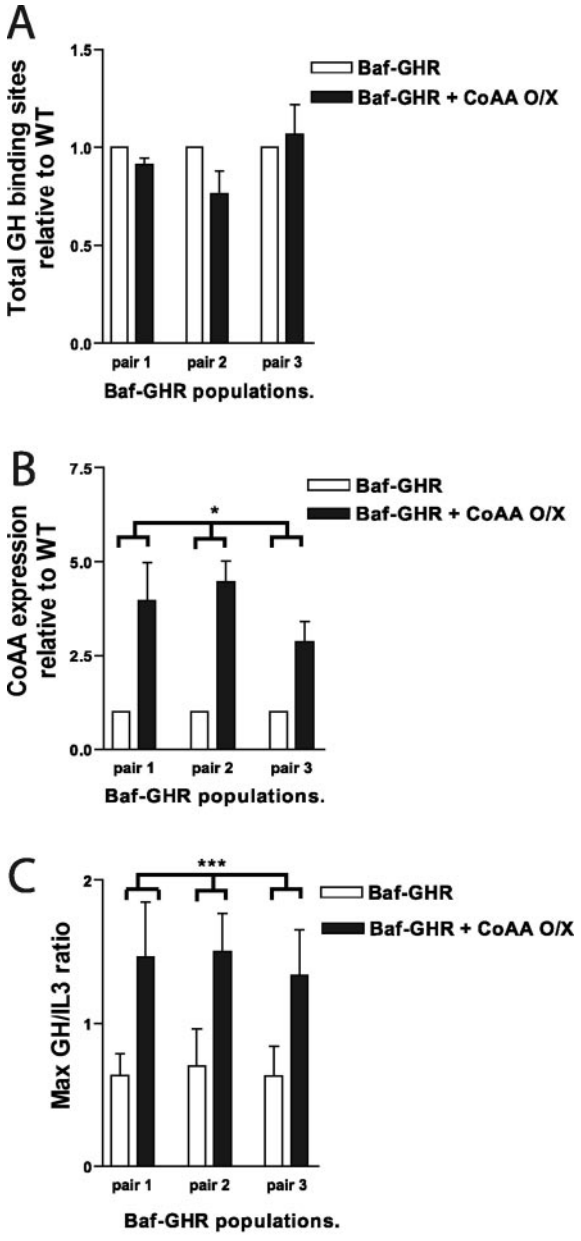

Fig. 7. Characterization of BaF-GHR Populations Shown in Fig. 6 in Relation to CoAA Overexpression

A, Relative specific GH binding in GHR populations. There was no statistically significant difference between the BaF-GHR populations in their cell surface GHR expression, based on Scatchard analysis using [^{125}I]hGH binding as described in *Materials and Methods* ($P = 0.43$). B, Relative CoAA expression in GHR populations. There was a significant difference in the levels of CoAA protein detected by Western blot based on densitometry (endogenous level *vs.* overexpression level, $P = 0.028$). C, Maximum proliferation rate in GHR populations. There was an highly significant difference in maximum proliferation rate between BaF-GHR populations expressing endogenous levels and BaF-GHR populations with overexpression of CoAA ($P = 0.0002$). BaF-GHR and BaF-GHR/CoAA populations were initially matched for GHR expression by FACS (data not shown), and this was verified with [^{125}I]GH binding assays. Mean and SEM data shown, derived from at least three separate experiments by densitometry. Within each experiment, each data point was the derived from at least three replicates. Paired *t* tests were used to analyze the results. O/X, Overexpression.

The Effect of Overexpression of CoAA in Ba/F3 Cells

As a low-abundance GHR-binding protein, CoAA may be a limiting factor in GHR function. Therefore, a CoAA-pCDNA3.1+ expression construct (17) was used for overexpression studies in the Ba/F3 model, with proliferation as the endpoint. The results described below are based on stable populations either transfected with rabbit (rb)GHR-pCDNA3.1+ alone, or cotransfected with rbGHR-pCDNA3.1+ and CoAA-pcDNA3.1+, as described in *Materials and Methods*. The stable populations were sorted by fluorescence-activated cell sorting (FACS) after immunostaining with monoclonal antibody (Mab)263-fluorescein isothiocyanate to yield populations with similar GHR cell surface expression levels. Equivalent GHR expression was verified by [^{125}I]GH binding studies. Subsequently, each matched pair of populations, with or without CoAA overexpression (Fig. 6), was tested for proliferative response to GH with a full dose-response curve. The CoAA-overexpressing populations were found to be proliferating at a faster rate than non-CoAA-transfected cells. In the GH dose-response curve, this is seen as an increase in maximum proliferation (Fig. 6B). However, there was no effect on the maximum proliferative response to IL-3 of the CoAA-overexpressing populations. Because IL-3 is the internal control for proliferation of the Ba/F3 cells, the CoAA effect is specific to GHR, and not a selection artifact. Figure 7 gives the summary data for three sets of populations overexpressing CoAA compared with endogenous expression of CoAA, with quantification of CoAA expression levels shown in Fig. 6. There was a highly significant difference in maximum proliferative response to GH between CoAA-overexpressing and control cells for each of the three populations ($P < 0.01$). This was despite similar extents of total GHR binding. No significant difference in proliferation was observed between the lines at zero GH or low GH concentrations, nor was the ED50 changed significantly ($P = 0.069$; data not shown), implying no change in the sensitivity to GH. However, the increase in the maximum rate of proliferation suggests that CoAA is an important, but limiting, protein in the GH proliferative pathway.

DISCUSSION

The function of the nuclear GHR appears to be different from its function at the plasma membrane (9). Therefore, it seems reasonable to propose the hypothesis that the GHR may be in the category of proteins found to exhibit dual function, termed "moonlighting" proteins (20). In the case of GHR, this dual function appears to be subcellular location dependent. GHR has long been known to copurify with chromatin in nuclear preparations (14); therefore, the discovery of a functional transactivation domain in the GHR has led to our hypothesis that the moonlighting function of the nuclear GHR involves transcriptional regulation, either directly as a transactivator or as a coactivator. Taken together, the data reported here indicate that the nuclear GHR exhibits many of the properties normally associated with transcription factors. It harbors a potent transactivation domain that is functional in both yeast and mammalian systems and requires the conserved serine at position 226, which is a part of the WSXWS equivalent motif. This serine has not been described as a serine phosphorylation site, although this has not been ruled out. A clinical study of a Laron GH-insensitive dwarf with the mutation S226A has been reported (21). Whether this is due to impaired signaling of the GHR at the plasma membrane of target cells, related to the impaired expression and, or processing of the GHR, as described in early work on the WSXWS motif by Baumgartner *et al.* (22) or as a result of the novel function described here, is certainly worthy of investigation.

Graichen *et al.* (23) have previously reported that transiently overexpressed GHBP enhances the GH response of a STAT5-driven reporter gene, and that the enhancement is greater when the GHBP is fused to a NLS. These findings differ from ours in that domain 1 of the GHBP is responsible, and transactivation by the GHBP is seen with other class 1 cytokines receptors responding to their ligands (prolactin, erythropoietin). There was no apparent association of STAT5 with the GHBP. These workers, however, did report GH-dependent nuclear translocation of a GHBP-GFP fusion protein after serum starvation.

The results from the GHBP affinity column further validate the view that protein complexes that form in yeast are recapitulated in mammalian cells. The theme of nuclear import and gene transactivation are again intrinsically linked, by the result that yeast Nucleoporin145 was found in a complex with GHBP on the affinity column. The nuclear import mechanism is known to be highly conserved between yeast and higher eukaryotes (24). The interaction between the yeast nucleoporin, Nup145, and GHBP on the affinity column suggests that the GHBP mechanism of nuclear import (described in Ref. 9) is also conserved in the yeast system.

An additional piece of evidence supports a conserved mechanism for nuclear import of GHBP in yeast, in that GHBP was able to nuclear localize in the Y2H system. Although the GAL4 DBD has an NLS that will target the fusion protein to the yeast nucleus, the LexA protein is a bacterial protein and therefore has no NLS. This fact is the basis for its reported ability to decrease false positives in the lexA derivative of the Y2H system, because the bait should not access the reporter genes inside the nucleus unless an interaction is made with the target, thus causing nuclear import (25). In the case of GHBP-LexA, transactivation again occurred in the absence of an AD-fused target, implying that GHBP was able to nuclear localize in this context.

The discovery that the nuclear transcriptional regulators CoAA and TR-uncoupling protein are interacting partners for the GHBP is novel and, in the case of CoAA, provides a putative mechanism for the transcriptional activity of the nuclear GHR. CoAA was originally identified by its interaction with coactivator thyroid receptor binding protein and the histone acetyltransferases cAMP response element-binding protein-binding protein and p300 (17). It has been shown to function as a general coactivator through thyroid hormone, glucocorticoid, estrogen, progesterone, and activator protein 1 and nuclear factor-κB elements (17, 26). It is widely expressed in human tissues, including major GH targets such as liver, kidney, and heart (17). Importantly, CoAA is a heterogenous nuclear ribonucleoprotein, possessing two N-terminal RNA recognition motifs, which allow it to also function as a regulator of mRNA splicing, dependent on the nature of the promoter and of the mRNA product (27, 28). Splicing and transactivation functions can be independently regulated although the RNA recognition motifs are needed for transcriptional activation (27). The central transactivation domain possesses 27 tyrosine/glutamine-rich hexapeptide repeats that are also present in the oncoproteins EWS (Ewings sarcoma), TLS/FUS (translocation/fusion in liposarcoma), and SYT (19). CoAA was independently identified as interacting with SYT via its QPGY motif, and SYT is known to bind to adenosine triphosphatases hBRM (human homolog of *Drosophila* brahma)/BRG1 within the SWI/SNF chromatin-remodeling complex (29). Importantly, CoAA is overexpressed in a variety of cancers as a result of a chromosomal breakage at 11q13, which removes an upstream Alu-rich silencer sequence (30). In particular, elevated CoAA transcript expression is present in 60–80% of lung, skin, stomach, and testicular cancer, and CoAA protein overexpression is evident in lung, squamous cell, pancreas, lymphoma, and gastric carcinoma (30). CoAA amplification is evident in cells with undifferentiated morphology and is frequently located in stromal locations where progenitor cells may reside. Interestingly, it is widely expressed in the mouse embryo and in embryonic stem cells, yet its expression decreases during differentiation (31). This correlates with similar observations on the nuclear localization of the GHR (9, 32). Importantly, 3- to 5-fold overexpression of CoAA in NIH-3T3 cells very significantly

increased cell proliferation (30). Conversely, the alternatively spliced form of CoAA, the coactivator modulator CoAM, acts as a dominant negative for CoAA because it lacks the central transactivation domain, and becomes the predominant form during embryonic stem cell differentiation (31).

The finding that CoAA appears to play an important role in GH-dependent (but not IL-3-dependent) proliferation in Ba/F3 cells provides support for, but does not prove its involvement in, direct transcriptional regulation by the GHR in progenitor myeloid proliferation. The interaction of GHR with CoAA is mediated by GH addition, which implies the signaling domain of GHR is responsible. This has likewise been shown to be the case for the GHR nuclear translocation process, which is ablated when the cytoplasmic domain is truncated (14). Association between the GHR and CoAA also coincides with GHR nuclear translocation kinetics, in that it is dependent on the length of serum starvation before GH treatment. The refractory period for nuclear translocation, exemplified by the need for serum starvation (9, 14), suggests a highly regulated cell cycle dependence. The current results suggest this regulation also exists for association with CoAA. It is therefore possible that CoAA, possessing several putative NLSs similar to those of Gal4 and SW16 (33), and displaying a nucleocytoplasmic distribution (19) could act as a piggyback protein in conjunction with importin-β (9) to facilitate transport of the GHBP/GHR to the nucleus.

In the case of the GHR NLS, it was perplexing to find that association with CoAA was GH dependent and not constitutive, as was the case for proliferation, survival, and a subset of genes associated with these endpoints. Therefore, CoAA association appears to be regulated in a manner more like activation of Janus family of tyrosine kinases, GHR phosphorylation, and STAT5 activation by NLS (as discussed in Ref. 9). Thus, GHR-CoAA association may be regulated by conventional cytoplasmic signaling pathways, most likely phosphorylation. The NLS, however, is free of the refractory period associated with CoAA binding, because GH treatment results in association after both 6-h and 10-h fasting periods. This lack of a refractory period may contribute to the increased sensitivity observed in the NLS lines. Of course, because the limiting amount of CoAA is a factor in both BaF-GHR WT and NLS

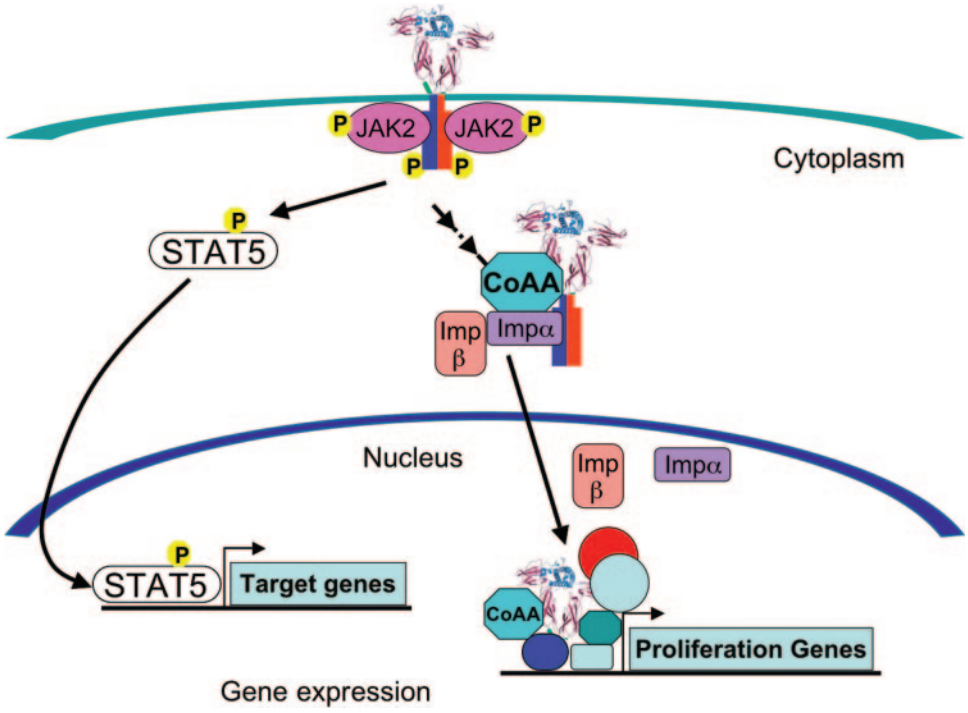

Fig. 8. Model of Proposed Mechanism for Nuclear GHR Action

Plasma membrane localized GHR induces STAT5 activation, and other pathways, by the classical Janus family of tyrosine kinases-STAT pathway. STAT5 translocates to the nucleus and binds to its DNA recognition sequence to initiate target gene transcription. In certain cellular conditions, such as during the proliferative phase in liver regeneration and at defined cell cycle-regulated times in progenitor proliferative cells, the GHR escapes its degradative pathway after GH addition and is translocated to the cytoplasm (possibly via the sec61 translocon) and then to the nucleus by the importin-α/β mediated classical import pathway. This process may involve its interaction with the NLS-containing protein CoAA, which mediates the interaction between GHR and nuclear import machinery. Once in the nucleus, the GHR can act as a transcriptional activator in conjunction with CoAA to initiate transcription of a subset of target genes to regulate cell cycle progression. Imp, Importin; JAK, Janus family of tyrosine kinases.

lines, maximum proliferation would be limited also, as observed.

To conclude, we have shown a novel association between the extracellular domain of the GHR and a powerful coactivator protein that is hormone regulated and results in increased cell proliferation in a receptor-specific manner (Fig. 8). CoAA overexpression has been implicated in a variety of carcinomas, and its association with the nuclear GHR may contribute to the oncogenic actions of autocrine GH (9, 34).

MATERIALS AND METHODS

Constructs

GHBP-DBD GAL. Pfu (Stratagene, La Jolla, CA) was used to PCR amplify the 246 residues corresponding to rbGHBP from a rbGHR-pCDNA3.1+ construct. The primers were designed to omit the secretory sequence and to introduce an *Eco*RI site at the 5′-end, and to introduce a STOP codon (TAG) and a *Xho*I site at the 3′-end of the sequence, so that the PCR product was cloned into the polylinker of pBD-GAL4 Cam (Stratagene) at the *Eco*RI and *Sal*I sites, and in frame with the DBD of GAL4. Restriction sites are *underlined*, and start and stop codons, are in *italics*.

rbGHBP forward (FOR) 5′-TCAGAATTC*ATG* TTTTCTGGGAGTGAGG-3′

rbGHBP reverse (REV) 5′-TCCTCTCGAG*CTA* ATCTTCTTCACATGTG-3′

GHBP-pLexA. The same GHBP PCR fragment was inserted into pBTM116 (pLexA) using *Eco*RI and *Sal*I sites in the polylinker. pBTM116 and the yeast strain L40 were both generous gifts from Susan Nixon (Institute for Molecular Bioscience, University of Queensland, St Lucia, Australia) and have been described previously (35).

The subdomains of GHBP were made as follows: domain 1 (Dom1) was designed to include Phe 1, which is the first residue of the mature protein (after the secretory signal sequence), extending to Val 129. The forward PCR primers included an *Eco*RI site, and the reverse primer included a STOP codon and no restriction site, so that the PCR product was inserted into the *Eco*RI site and blunt *Sma*I site of the polylinker of pBTM116. Domain 2 (Dom2) was designed to include glutamine 130 extending to aspartic acid 246. The forward PCR primer contained no restriction site, and the reverse primer included a stop codon and a *Sal*I site, so that the PCR product was inserted into the blunt *Sma*I site and *Sa*I site of the polylinker of pBTM116. Restriction sites are *underlined*, and start and stop codons are in *italics*.

Dom 1 FOR 5′-TCAGAATTC*ATG* TTTTCTGGGAGTGAGG-3′

Dom 1 REV 5′-*CTA* CACTATTTCCTCAACAGAGAAAC-3′

Dom 2 FOR 5′-*ATG* CAACCAGATCCACCCATTGGCCTC-3′

Dom 2 REV 5′-CTGTCGAC*CTA* ATCTTCTTCACATGTGAATG-3′

Mutations of Domain 2 GHBP

Site-directed mutagenesis was carried out using primers for each mutation as indicated (mutated bases corresponding to amino acid substitution are *underlined*):

Y222H-pLexA (TAT to CAT)

FOR 5′-CGAAGCTCTGAAAAACATGGCGAGTTCAGTGAGG-3′

REV 5′-CCTCACTGAACTCGCCATGTTTTTCAGAGCTTCG-3′

E224A-pLexA (GAG to GCG)

FOR 5′-CTCTGAAAAATATGGCGCGTTCAGTGAGGTGCTC-3′

REV 5′-GAGCACCTCACTGAACGCGCCATATTTTTCAGAG-3′

S226A-pLexA (AGT to GCT)

FOR 5′-CTGAAAAATATGGCGAGTTCGCTGAGGTGCTCTATGTAACC-3′

REV, 5′-GGTTACATAGAGCACCTCAGCGAACTCGCCATATTTTTCAG-3′

Mammalian GAL Constructs

The plasmids G5E1b-LUC, pGALO were a generous gift from George Muscat (Institute for Molecular Bioscience, University of Queensland, Saint Lucia, Australia), and have been previously described (36). Inserts for GHBP, domain 1, domain 2, and S226A domain 2 were subcloned out of the pLexA yeast expression vectors and placed into the pGALO vector using the *Eco*RI and *Pst*I sites in the polylinker of both vectors, such that the inserts were in frame with GAL DBD.

GHBP-PET20b+. The PCR primers described above were used to amplify GHBP to insert onto the bacterial expression vector pET20b+ (Novagen Corp., Madison, WI) using *Eco*RI and *Sal*I sites in the polylinker. S226A GHBP-pET20b+ was generated using the same site-directed mutagenesis primers as described above.

CoAA. CoAA-pCDNA3.1 was a generous gift from Lan Ko, and has been described previously (17). CoAA-pGSTag and anti-SIP 907 (rabbit polyclonal to CoAA) are described in Ref. 19.

Yeast Transactivation Assays

Yeast-competent cells (YRG2 and L40 strains; Stratagene) were prepared by the LiAc/SS-DNA/PEG procedure and then transformed as described (www.stratagene.com/manuals/235611.pdf). Dropout media omitted *l*-tryptophan for the GAL DBD plasmid and histidine for activation of the histidine auxotrophy reporter. GHBP-GAL was transformed into the YRG2 yeast strain, which contains the histidine auxotrophy and β-galactosidase reporter genes upstream of a concatamer of four GAL binding sites. The LexA fusion proteins of GHBP, domain 1, domain 2, and the domain 2 mutants Y222H, E224A, and S226A were transformed into the L40 yeast strain containing reporter genes for histidine auxotrophy and β-galactosidase reporter genes upstream of the LexA operator (37). Transcriptional activation of the reporter genes was measured initially by growth on histidine dropout plates, and then quantitatively by β-galactosidase activity measured by colorimetric assay of yellow product formed from the ortho-nitrophenyl-β-galactoside substrate and blue product formed from X-gal substrate.

CHO-K1(Mammalian) Transactivation Assays

CHO-K1 cells were cultured in Hams-F12 supplemented with 10% Serum Supreme. Cells for transfection were grown in 12-well dishes to 60–70% confluence and transiently transfected with 1 μg of the G5E1B-LUC reporter plasmid and 0.33μg of the GAL fusion GHBP constructs and 0.33μg β-galactosidase-pCDNA3.1 in 1ml of Hams-F12 media. The medium was replaced 24 h after transfection after which the cells were grown an additional 24–48 h before harvesting for luciferase assay. Each experiment represented three sets of independent triplicates.

GHBP Expression and Purification and Affinity Column Preparation

GHBP-PET20b+ or GHBP S226A-PET20b+ was transformed into BL21 DE3 *Escherichia coli* (Stratagene) and in-

duced with 1 mM IPTG for 3 h. The cell pellets were freeze thawed, and mechanically ruptured with a French pressure cell, and then washed inclusion bodies were prepared, solubilized in urea, refolded and purified by ion exchange as previously described (38). To prepare affinity columns, controlled pore glass (CPG) beads (1 g/affinity column) were activated with cyanogen bromide and coupled to 10 mg of either GHBP WT or GHBP S226A (or no protein), and then blocked and as described previously (39).

Yeast Nuclear Extract Preparation

This was a modification of the method of Ponticelli and Struhl (40), as follows. Yeast culture (2 liters) from L40 yeast strain was grown to an OD_{600} of 1.2, and then harvested by centrifugation at 100 × *g* for 10 min. The cell pellet (~20 g) was resuspended in 35 ml of 50 mM Tris (pH 7.5), 30 mM dithiothreitol (DTT) for 15 min at 30 C, then recentrifuged and resuspended in 20 ml YPD with 18 mg Zymolyase 100T (ICN Biomedicals, Cleveland, OH). This was incubated at 30 C for 2.5 h, 100 ml of yeast peptone adenine dextrose media was added, and the spheroplasts were pelleted by centrifugation at 1000 × *g* for 12 min, resuspended in 250 ml YPD media, and then incubated at 30 C for 30 min to allow cells to recover. Cells were subsequently pelleted at 1000 × *g* for 12 min and resuspended in 100 ml of buffer A [18% Ficoll 400, 10 mM Tris (pH 7.5), 20 mM K acetate, 5 mM Mg acetate, 1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, supplemented with 3 mM DTT, and complete protease inhibitor (Boehringer)] immediately before use. Nuclei were released by three passes through a Dounce homogenizer. The resulting supernatant was transferred to 50-ml centrifuge tubes and centrifuged at 10,000 × *g* for 30 min in a Sorvall SS34 rotor to pellet crude nuclei. The nuclei were then resuspended in buffer C (20 mM HEPES, pH 7.6; 10 mM $MgSO_4$; 1 mM EGTA; 20% glycerol; supplemented with 3 mM DTT and protease inhibitors immediately before use), and homogenized with a microtip homogenizer for 3 × 30 sec on ice. Debris was pelleted by centrifugation at 10,000 × *g* for 20 min, and the resulting nuclear supernatant was used for affinity chromatography.

Affinity Chromatography Isolation and Identification of Interacting Proteins

Nuclear extracts from yeast were divided into three equal aliquots and applied to three affinity columns in parallel (GHBP WT, S226A GHBP, and CPG beads alone). The columns were washed with 150 ml of buffer C, after which the interacting proteins were eluted with 4.5 M $MgCl_2$. The eluants were dialysed against buffer C, then concentrated in Centricon spin columns to 50 μl, denatured with sodium dodecyl sulfate (SDS) loading buffer, and boiled for 5 min before loading onto a Laemmli 4–20% linear gradient gel. The bands were excised, tryptic digested. and sequenced by tandem mass spectroscopy using a Finnegan Triple-Quad mass spectrometer.

GST Pull-Down Interaction Assays

The GST-CoAA fusion construct (and GST) was transformed into BL21 DE3, and protein was expressed for 3 h, the pellet sonicated, and GST fusion protein purified with glutathione agarose by standard methods. Aliquots (200 μl each) of glutathione agarose beads with GST-CoAA or GST alone, corresponding to each GST pull-down assay, were stored at −20 C until the assays were performed. Equivalent amounts of protein lysate from BL21 DE3 *E. coli* expressing either GHBP WT or GHBP S226A were incubated with either GST-CoAA or GST beads for 30 min at 4 C. Each GST pull-down tube was washed four times in buffer C to remove noninteracting proteins, after which the glutathione resin and any interacting proteins were denatured by boiling for 5 min in the presence of 10 μl of SDS loading buffer with DTT. The supernatants were then run on 10% PAGE, and Western blot was performed to detect the presence of GHBP WT or S226A associated with the GST fusions.

Coimmunoprecipitation Assays

CHO-K1 cells transiently transfected with GHR WT or NLS were serum starved in 0.5% Serum Supreme/Hams F12 medium (−GH), and then treated with 100 ng/ml hGH for 10 min (+GH), and harvested. BaF-GHR WT or NLS stable lines (1×10^8 cells) were starved for either 6 or 10 h in 0.5% Serum Supreme/RPMI 1640, after which they were treated with 100 ng/ml hGH and then harvested.

Immunoprecipitation and immunoblotting were performed exactly as described previously (9), with 2 μg of anti-HA antibody used to immunoprecipitate the GHR with an N-terminal HA tag. Immunoblotting to detect coassociation of endogenously expressed CoAA was performed using the rabbit polyclonal antibody to CoAA (anti-SIP 907) diluted 1:10000 in 0.5% skim milk powder. For loading normalization, membranes were stripped after immunoblotting with anti-SIP 907 and reprobed with the immunoprecipitating antibody at 1:1000 dilution.

Coexpression of CoAA in Stable Ba/F3 Lines

Ba/F3 cells were transfected by electroporation as described previously (41), except 10 μg of GHR-pCDNA3.1+ and 10 μg of CoAA-pCDNA3.1+ was used for coexpression. Cells were selected on G418 and GH as described previously (41).

Ba/F3 stable populations coexpressing either GHR WT or GHR WT and CoAA were then matched for surface receptor expression by FACS Mab263-antimouse fluorescein isothiocyanate-immunostained populations with the FACS Vantage (Becton Dickinson, Franklin Lakes, NJ). Protein levels of CoAA in the stable GHR-matched populations, expressing either endogenous levels (−) or overexpression levels (+) of CoAA, were determined by Western blot of BaF-GHR cell lysates (10 μg/lane) probed with the anti-CoAA antibody (anti-SIP 907). Proliferation assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye incorporation were undertaken as previously described (41). This required a parallel assay with a maximum dose of IL-3 in the absence of hGH to determine maximum intrinsic .proliferation rate of the particular population in response to the endogenous ligand, IL-3. This rate was used to normalize the GH response to account for population variation.

Acknowledgements

We thank Dr. Valerie Wasinger and Val Valova (MA Spec and Protein Analysis Lab, Garvan Institute of Medical Research, Australia) for assistance with the mass spectrograph.

Received April 18, 2008. Accepted July 7, 2008.

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This work was supported by National Health and Medical Research Council Grants (Australia) (to M.J.W. and P.J.R.).

Disclosure Statement: The authors have nothing to disclose.

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