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David Dunn

Major professor

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# USE OF THE YEAST TWO-HYBRID SYSTEM TO IDENTIFY PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE ASSOCIATED PROTEINS

By

Liqun Gu

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## ABSTRACT

USE OF THE YEAST TWO-HYBRID SYSTEM TO IDENTIFY PROSTAGLANDIN ENDOPEROXIDE H SYNTHASE ASSOCIATED PROTEINS

By

Liqun Gu

PGHS-1 and PGHS-2, the two central isozymes in the pathway for conversion of arachidonic acid to the biologically active prostaglandins and thromboxanes, are very similar in structure and show nearly identical catalytic properties toward arachidonate metabolism. However the two isozymes are not redundant, they each play separate and specialized roles in cellular signaling and developmental processes.

To test whether PGHS-1 and PGHS-2 form complexes with other proteins, thereby conferring isozyme differences in apparent substrate affinity, signal transduction, or catalytic regulation, we used the yeast two-hybrid system to identify candidate genes of proteins that interacted with PGHS. Two candidate proteins AT15 and AT18 were shown to interact with PGHS-2 carboxyl terminus in the yeast two-hybrid system. However after serial testing both *in vitro* and *in vivo*, neither AT15 nor AT18 could be demonstrated to associated with PGHS-2.

To

my family

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| PGHS | Prostaglandin endoperoxide H synthases |
| COX | Cyclooxygenase |
| SP | Signal peptide |
| EGF | Epidermal growth factor |
| MBD | Membrane binding domain |
| NSAID | Nonsteroidal anti-inflammatory drug |
| EPA | 5,8,11,14,17-eicosapentaenoic acid |
| FLAP | 5-lipoxygenase-activating protein |
| DNA-BD | DNA binding domain |
| AD | Activation domain |
| 3-AT | 1,2,4-3-aminotriazole |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactoside |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| HRP | Horseradish peroxidase |
| mAb | monoclonal antibody |
| ER | endoplasmic reticulum |

# INTRODUCTION

## Literature Review

The prostaglandin endoperoxide H synthases (PGHS) are the central enzymes in the pathway for conversion of arachidonic acid to the biologically active prostaglandins and thromboxanes. There are two PGHS isozymes, PGHS-1 and PGHS-2, also known as cyclooxygenase-1 and -2 (COX-1 and COX-2). PGHS-1 is constitutively expressed in most tissues, and is thought to mediate "housekeeping" functions, including cytoprotection of the gastric mucosa, regulation of renal blood flow, and platelet aggregation. In contrast, PGHS-2 is usually undetectable in most tissues, but it can be induced to express at high levels in migratory and other responding cells by pro-inflammatory and mitogenic stimuli. PGHS-2 is generally considered to be a mediator of inflammation (1-4).

PGHS-1 and PGHS-2 are very similar in structure and show nearly identical catalytic properties toward arachidonate metabolism (5). The two enzymes are about 60% identical in amino acid sequence and both have four main functional domains (Figure 1): a) a signal peptide (SP); b) a short amino-terminal epidermal growth factor homology domain (EGF); c) a putative membrane binding domain (MBD); and d) a large catalytic domain. The catalytic domains are highly conserved. However, the amino terminus of PGHS-2 is slightly truncated compared with that of PGHS-1, and the carboxyl terminus of PGHS-2 possesses an 18–residue cassette that is absent in PGHS-1. The biological significance of these subtle differences in the primary amino acid sequence still needs to be determined.

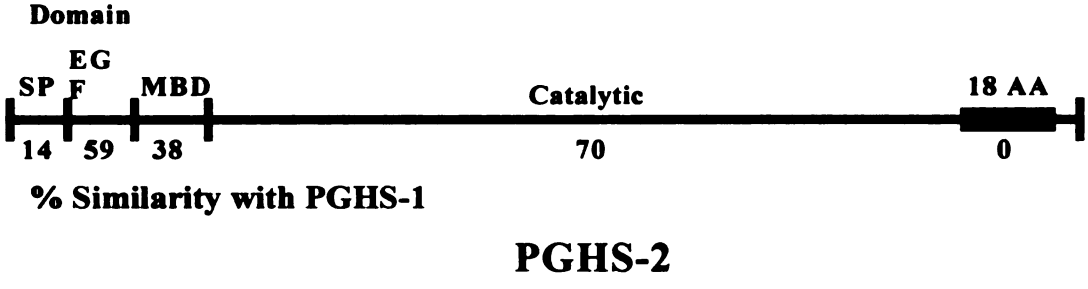



**Figure 1. Comparison of the primary structure of PGHS-2 and PGHS-1.**

Knockout mice for PGHS-1 and PGHS-2 display distinct phenotypes suggesting that these two enzymes are not redundant and that each play separate and specialized roles in cellular signaling and developmental processes (6,7). The inducible PGHS-2 functions more than simply to augment constitutive prostaglandin synthesis by PGHS-1, because induction of PGHS-2 often results in only a nominal increase in cellular $PGH_2$ biosynthetic activity (8). In addition, prostanoid synthesis catalyzed by PGHS-1 or PGHS-2 is coupled to different extracellular stimuli and derives from different arachidonate substrate pools, possibly via coupling to different phospholipase systems. In some cells, prostaglandin synthesis proceeds via PGHS-2, even though significant PGHS-1 enzyme is present (9). Taken together, these observations imply the existence of two partially independent PGHS-1 and PGHS-2 prostanoid biosynthetic pathways (10), which may result from different microenvironments in which the enzymes are located, or due to specific interactions of each enzyme with other proteins.

Besides the cellular traits that distinguish the two isozymes from one another, PGHS isozymes differ subtly in their substrate specificity and nonsteroidal anti-inflammatory drug (NSAID) affinities (11,12). While both enzymes have similar Km's and catalytic efficiencies for arachidonic acid, PGHS-2 has been shown to use alternate fatty acid substrates, such as α-linolenic acid and 5,8,11,14,17-eicosapentaenoic acid (EPA), more efficiently than PGHS-1. NSAIDs have been identified that selectively inhibit either PGHS-1 or PGHS-2. The larger and more accommodating active site of PGHS-2 compared with that of PGHS-1 might be responsible for some of these differences. However, other factors might account for their differential binding activities. Another important enzyme for eicosanoid metabolism, 5-lipoxygenase, depends on FLAP

(an 18kDa subsidiary protein known as 5-lipoxygenase-activating protein) for arachidonate delivery (13,14). This precedent opens the possibility that regulation of prostaglandin synthesis by PGHS-1 or PGHS-2 could also depend on protein-protein interactions, thus modifying the activity or subcelllular location of PGHS-1 or PGHS-2.

Another argument supporting separate biological roles for PGHS-1 and PGHS-2 has come from recent research on the mechanisms whereby chronic aspirin users have reduced incidence of colon cancer (15). Normal colon epithelial cells are found to constitutively express PGHS-1, but not PGHS-2, while most colon carcinoma cells express normal levels of PGHS-1 and high levels of PGHS-2 (16-18). Furthermore, NSAIDs which specifically inhibit PGHS-2, but not PGHS-1, reduce intestinal polyp formation in normal mice. Genetic ablation of PGHS-2 in knockout mice also reduces polyp formation (19). These results suggest that PGHS-2 plays a specific signaling role in colon epithelial cells.

All the above data raise the question: Do PGHS-1 and PGHS-2 form complexes with other proteins, thereby conferring isozyme differences in apparent substrate affinity, signal transduction, or catalytic regulation?

**Research Project Goals**

The primary hypothesis that we would like to test is that the unique biological properties of PGHS-1 and PGHS-2 result from specific interaction of the enzyme with other proteins that may modify the activity and/or subcellular location of PGHS. Since there are obvious structural differences between PGHS-1 and PGHS-1 that could possibly mediate differential protein-protein interactions (i.e. the 18–residue cassette), we plan:

Aim 1: To identify candidate genes of proteins that interact with PGHS, using the yeast

two-hybrid system.

Aim 2: To confirm the specific association of the candidate protein and PGHS *in vitro*.

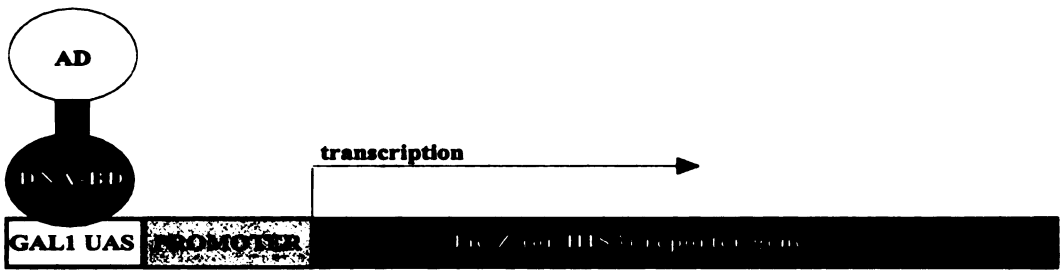
Aim 3: To confirm the association of PGHS and the candidate protein *in vivo*.

**Background**

The yeast two-hybrid assay is based on the fact that many eukaryotic transcriptional activators are composed of two physically separable, functionally independent domains. The yeast GAL4 transcriptional activator, for example, contains a DNA binding domain (DNA-BD) and a transcriptional activation domain (AD). The DNA-BD recognizes and binds to a sequence (UAS) in the upstream regions of GAL4-responsive genes, while the AD interacts with other components of the transcription machinery needed to initiate transcription. When both domains are part of the same protein, they are able to activate gene transcription. If physically separated by recombinant DNA technology and expressed in the same host cell, however, the GAL4 DNA-BD and AD peptides do not directly interact with each other and cannot activate the responsive genes.

In the yeast two-hybrid systems, two different cloning vectors are used to generate separate fusion proteins of the two GAL4 domains. The recombinant hybrid proteins are co-expressed in yeast where they are targeted to the yeast nucleus. If the non-GAL4 portions of the two hybrid proteins interact with each other, the DNA-BD and transcriptional AD are re-united and can again activate transcription (Figure 2). Thus, as a result of a two-hybrid interaction, the GAL4 transcriptional activator will be functionally reconstituted and will activate transcription of reporter genes (*lacZ* and *HIS3*) having upstream GAL4 binding sites. This makes the protein interaction

1. GAL4 transcriptional activator (wild type)




2. GAL4 DNA-binding domain and transcriptional activation domain (physically separated by recomvbinant DNA technology)


3. In two-hybrid system, two different cloning vectors are used to generate separate fusions of these GAL4 doamins to genes encoding proteins that potentially interact with each other

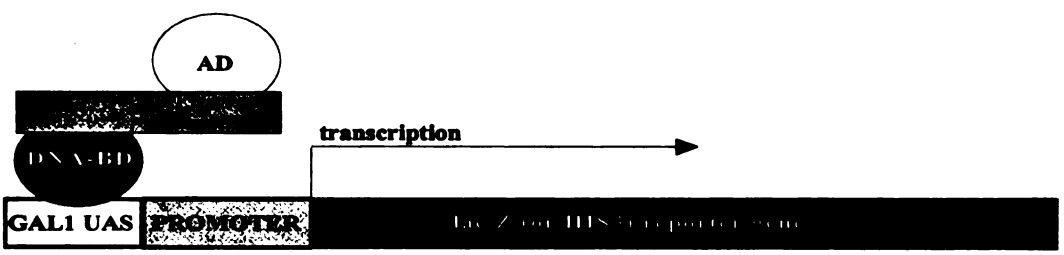



**Figure 2. The principle of yeast two-hybrid system.**

phenotypically detectable.

The same principle applies to screening a yeast two-hybrid library . The gene encoding a target protein is cloned into the DNA-BD vector to generate a fusion protein, referred to as a "bait". Likewise, a cDNA library is constructed in the AD vector to generate chimeras of various proteins encoded by the library cDNAs fused to the GAL4 AD. The two types of hybrid plasmids are then cotransformed into a yeast host strain, which is auxotrophic for Trp and Leu, for library screening. The transformants are plated on minimal medium lacking Leu, Trp, and His to select for those that contain both types of plasmids (i.e., $Leu^+$, $Trp^+$) and that also express interacting hybrid proteins ($His^+$). Primary $His^+$ transformants are tested for expression of a second reporter gene (*lacZ*) using a sensitive and rapid filter assay for β-galactosidase activity (20).

The yeast two-hybrid system has previously been used successfully in identifying one protein that interacts with the catalytic regions of both PGHS-1 and PGHS-2 (residues 381- 498, using the translation start site in human PGHS-1 as residue 1) (21). However, these early experiments determined that, when large regions of PGHS (300-400 residues) or regions of PGHS containing the membrane-binding domains (MBD) were used as baits, no interacting proteins were detected. These results could have been obtained because no other interacting proteins exist, or because these baits were poorly expressed or were not translocated to the nucleus because of the MBD in the bait. Short carboxyl terminal PGHS-1 baits were found to be unsuitable for library screening as well, because they intrinsically transactivated the reporter genes.

To avoid the same problems and to identify proteins that interact specifically with PGHS-1 or PGHS-2, we decided to choose three PGHS short sequences as baits in our

yeast two-hybrid screening. The baits we employed were the human PGHS-1-EGF domain, residues 24-84, the human PGHS-2-EGF domain, residues 18-70, (using the translation start site in human PGHS-2 as residue 1) and the human PGHS-2 carboxyl terminal sequence, residues 555-604 (Figure 3 and 4). We chose the PGHS-2 carboxyl terminal sequence because this sequence contains an 18-amino acid cassette that is absent in PGHS-1, which represents the most pronounced amino acid sequence difference between PGHS-1 and PGHS-2. The PGHS-EGF domains were chosen because the EGF domains in other proteins are often responsible for protein-protein interaction. Although the MBDs represent the second most dissimilar region between PGHS-1 and PGHS-2, these sequences are too hydrophobic to translocate into the nucleus, and cannot be used in yeast two-hybrid system.

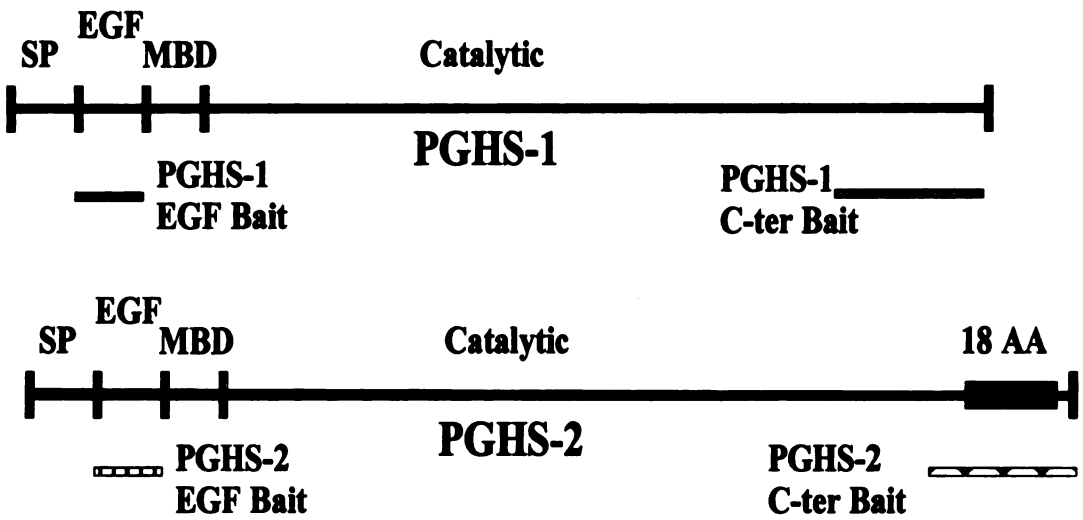



**Figure 3. PGHS protein sequences used for bait to screen the yeast two-hybrid system.** The four PGHS sequences depicted were cloned into pAS2-1 vector. The resulting plasmids expressed PGHS-GAL4-BD fusion proteins.The PGHS-1 constructs contained the following amino acids: PGHS-1-EGF, residues 24-84; and PGHS-1-COOH, residues 556-599. The PGHS-2 constructs contained the following amino acids: PGHS-2-EGF, residues 18-70; and PGHS-2-COOH, residues 555-604 .

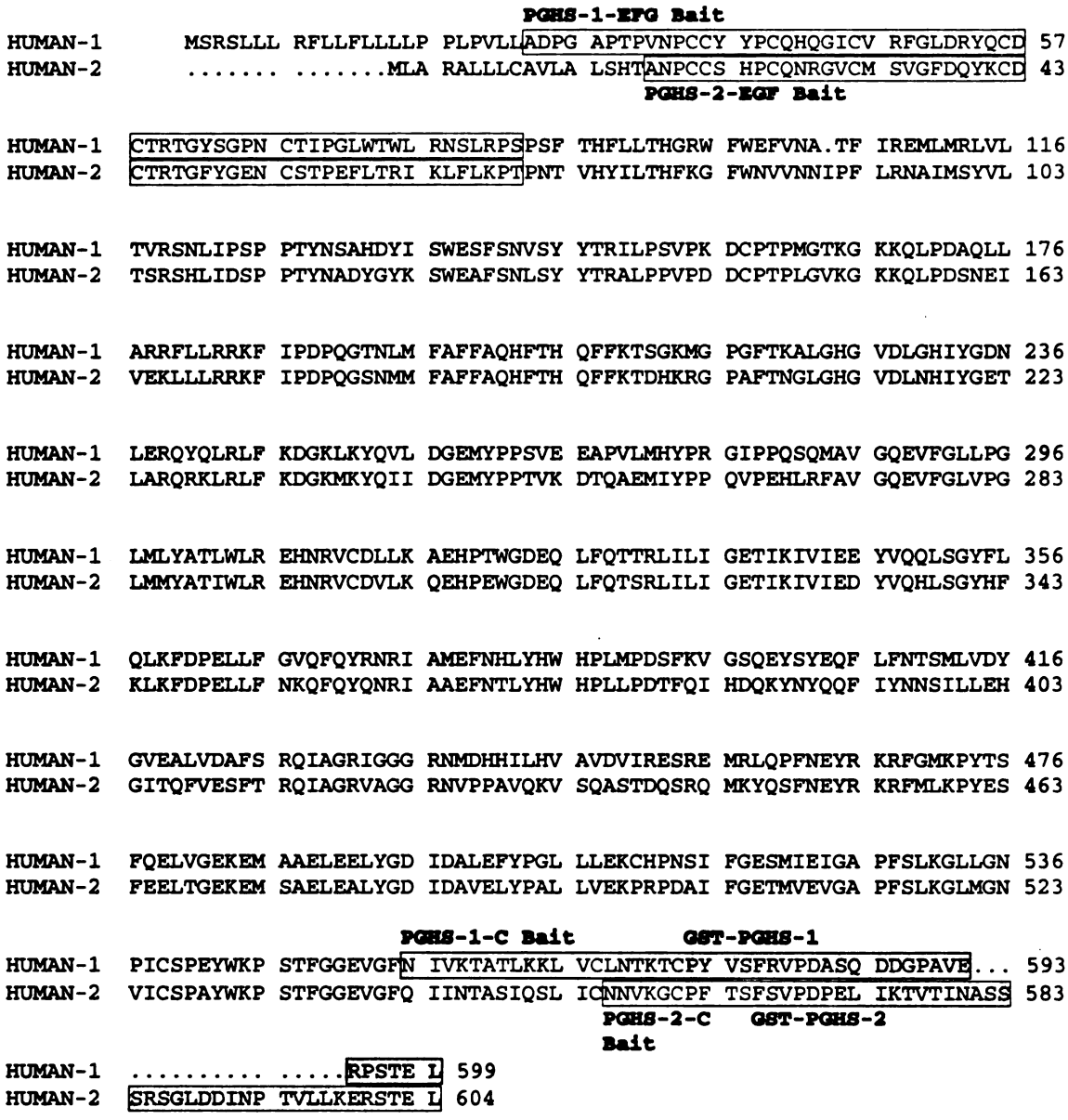



**Figure 4. Location in the PGHS-1 and PGHS-2 proteins of the amino acid sequences used to construct the yeast two-hybrid bait plamids (standard one-letter symbols).** The numbers on the far right refer to the amino acid position. Those sequences used as baits in yeast two-hybrid screening are boxed (residues 24-84 and 556-599 of PGHS-1, residues 18-70 and 555-604 of PGHS-2). Sequences used in GST fusion proteins are shaded (residues 575-599 of PGHS-1, residues 565-604 of PGHS-2).

## MATERIALS AND METHODS

### Construction of bait Plasmids

For yeast two-hybrid screening, four pairs of upper and lower primers (Figure 5) complementary to the human PGHS-1-EGF domain, human PGHS-2-EGF domain, human PGHS-1 carboxyl terminal sequence, and human PGHS-2 carboxyl terminal sequence were synthesized and used in PCR reactions to generate four corresponding PGHS cDNA fragments. The PCR products were first cloned into the plasmid pCR2.1 TA (Invitrogen), then subcloned into GAL4-DNA-binding-domain vector pAS2-1 (which carries a selectable marker *TRP*1) (Table I). Structures of the four pAS2-1 constructs were confirmed by restriction endonuclease analysis and DNA sequencing. The three pAS2-1 constructs, which encoded the human PGHS-1-EGF domain (residues 24-84), the PGHS-2-EGF domain (residues 18-70), or the PGHS-2 carboxyl terminal sequence (residues 555-604) fusion proteins were designated as pAS1EFG, pAS2EGF and pAS2C respectively, and were used as baits in the yeast two-hybrid system (Table I). The fourth pAS2-1 construct, which encoded the human PGHS-1 carboxyl terminal sequence (residues 556-599) fusion protein, was designated as pAS1C and was used as a control for clones that reacted with pAS2C. To ensure that these PGHS baits by themselves did not activate transcription, they were used to transform the yeast strain Y190 (Table II) separately. The Y190 transformants were selected on minimal media lacking tryptophan and were tested for autonomous reporter gene (*lacZ*) activation by the β-galactosidase assay. The transformants expressing the GAL4 fusion proteins were confirmed by western blot using a monoclonal antibody (mAb) against the GAL4-DNA-binding-

**Human PGHS-1-EGF Bait**
5' Primer, 29mer, nucleotides coding for the peptide sequence
Ala-Asp-Pro-Gly-Ala-Pro-Thr (residues 24-30):
5' CCC ATA TGG CGG ACC CAG GGG CGC CCA CG 3'
NdeI

3' Primer, 31mer, complementary to nucleotides coding for the peptide sequence
Arg-Asn-Ser-Leu-Arg-Pro-Ser-AMB (residues 78-84):
5' CCG ACG TCC TAG CTG GGC CGC AGT GAA TTC C 3'

**Human PGHS-1 C' Bait**
5' Primer, 28mer, nucleotides coding for the peptide sequence
Asn-Ile-Val-Lys-Thr-Ala-Thr (residues 556-562):
5' TCC ATA TGA ACA TTG TCA AGA CGG CCA C 3'
NdeI

3' Primer, 29mer, complementary to nucleotides coding for the peptide sequence
Arg-Pro-Ser-Thr-Glu-Leu-OPA (residues 594-599):
5' CGG AAT TCT CAG AGC TCT GTG GAT GGT CG 3'
EcoRI

**Human PGHS-2-EGF Bait**
5' Primer, 29mer, nucleotides coding for the peptide sequence
Ala-Asn-Pro-Cys-Cys-Ser-His (residues 18-25):
5' CCC ATA TGG CAA ATC CTC CTT GCT GTT CC 3'
NdeI

3' Primer, 33mer, complementary to nucleotides coding for the peptide sequence
Lys-Leu-Phe-Leu-Lys-Pro-Thr-AMB (residues 64-70):
5' GGC CTA GGC TAA GTG GGT TTC AGA AAT AAT TTT 3'

**Human PGHS-2 C' Bait**
5' Primer, 28mer, nucleotides coding for the peptide sequence
Asn-Asn-Val-Lys-Gly-Cys-Pro (residues 555-560):
5' CCC ATA TGA ATA ACG TGA AGG GCT GTC C 3'
NdeI

3' Primer, 29mer, complementary to nucleotides coding for the peptide sequence
Glu-Arg-Ser-Thr-Glu-Leu-AMB (residues 598-604):
5' GGC TTA AGC TAC AGT TCA GTC GAA CGT TC 3'

**Figure 5. Specific primers used in PCR reactions to generate PGHS-1-EGF, PGHS-1 carboxyl terminal, PGHS-2-EGF and PGHS-2 carboxyl terminal cDNA sequences for construction of the yeast two-hybrid bait plasmid. (OPA: opal stop codon; AMB: amber stop codon)**

## TABLE I DESCRIPTION OF THE PLASMIDS

| Vector | Description | Size |
|---|---|---|
| **Clonming vectors** | | |
| pAS2-1 | *GAL4(1-147) DNA-BD, TRP1, amp$^r$,CYH$^s$2,* | 8.4kb |
| pACT2 | *GAL4(768-881) AD, LEU2,, amp$^r$, HA epitope tag* | 8.1kb |
| **Recombinant plasmids** | | |
| pAS1EGF | PGHS-1-EGF (24-84) in pAS2-1 *TRP1, amp$^r$* | 8.5kb |
| pAS2EGF | PGHS-2-EGF (18-70) in pAS2-1 *TRP1, amp$^r$* | 8.5kb |
| pAS1C | PGHS-1-COOH (556-599) in pAS2-1 *TRP1, amp$^r$* | 8.5kb |
| pAS2C | PGHS-2-COOH (555-604) in pAS2-1 *TRP1, amp$^r$* | 8.5kb |
| pASn15 | AT15 in pAS2-1 *TRP1, amp$^r$* | 9.1kb |
| pASn18 | AT18 in pAS2-1 *TRP1, amp$^r$* | 9.6kb |
| pACT2C | PGHS-2-COOH (555-604) in pACT2 *LEU2,, amp$^r$* | 8.3kb |
| pAT15 | AT15 in pACT2 *LEU2,, amp$^r$* | 8.8kb |
| pAT18 | AT18 in pACT2 *LEU2,, amp$^r$* | 9.3kb |

## TABLE I DESCRIPTION OF THE PLASMIDS
(continued)

| Vector | Description | Size |
|---|---|---|
| **Clonming vectors** | | |
| pCR2.1 | *kan$^r$, amp$^r$* | 3.9kb |
| pET-28a | *kan$^r$, 6 X Histidine-tag* | 5.4kb |
| pGEX-4T | *amp$^r$, GST-tag* | 5.0kb |
| **Recombinant plasmids** | | |
| pET-AT15 | AT15 in pET-28a<br>*kan$^r$, 6 X Histidine-tag* | 6.2kb |
| pET-AT18 | AT18 in pET-28a<br>*kan$^r$, 6 X Histidine-tag* | 6.7kb |

**TABLE II GENOTYPES OF THE YEAST HOST STRAINS**

| Strain | Genotype | Reporters | Auxotrophy |
|---|---|---|---|
| Y190 | *MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal4-del, gal80-del, cyhr2, LYS2::GAL1$_{UAS}$-HIS3$_{TATA}$-HIS3, URS3::GAL1$_{UAS}$-GAL1$_{TATA}$-lacZ* | *HIS3, lacZ* | *trp1,leu2,cyh$^r$2* |
| CG1945 | *MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal4-del, gal4-542, gal80-538,cyhr2, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URS3::GAL4 17-mers(x3)-CyC1$_{TATA}$-lacZ* | *HIS3, lacZ* | *trp1,leu2,cyh$^r$2* |

domain (Clontech).

**Library Plasmids**

The human brain cDNA library was constructed in the GAL4-activation-domain vector pACT2 (Table I), which carries a selectable marker *LEU2*. It was purchased from Clontech.

**Yeast Two-Hybrid Screening and Testing of Positive Clones**

The yeast two-hybrid library was screened following transformation into the Y190 yeast strain, which harbors the reporter genes *HIS3* and *lacZ* under the control of upstream GAL4 transcription regulatory element (Clontech, Matchmaker Two-Hybrid System 2). Briefly, Y190 was co-transformed with a PGHS bait plasmid and cDNA library constructed in the GAL4-transcriptional-activation-domain vector pACT2. An estimated 3,500,000 recombinants from the human brain cDNA library were screened. Yeast co-transformation was done by a procedure using lithium acetate, single stranded DNA and polyethylene glycol (22). Double-transformants containing plasmids encoding PGHS bait and interacting proteins were selected for on minimal media deficient in tryptophan, histidine and leucine, and containing 25 mM 1,2,4-3-aminotriazole (3-AT) (Sigma). Yeast colonies surviving the medium selection were subsequently assayed for β-galactosidase activity in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) by filter lift assays (Figure 6).

Plasmids containing the library genes that interacted with PGHS baits were isolated from β-galactosidase-positive colonies and transformed into *E. coli* strain *DH5α* for amplification. These plasmids were then co-transformed into yeast strain CD1945 (Table II) along with their PGHS bait partners. The double transformants were subjected

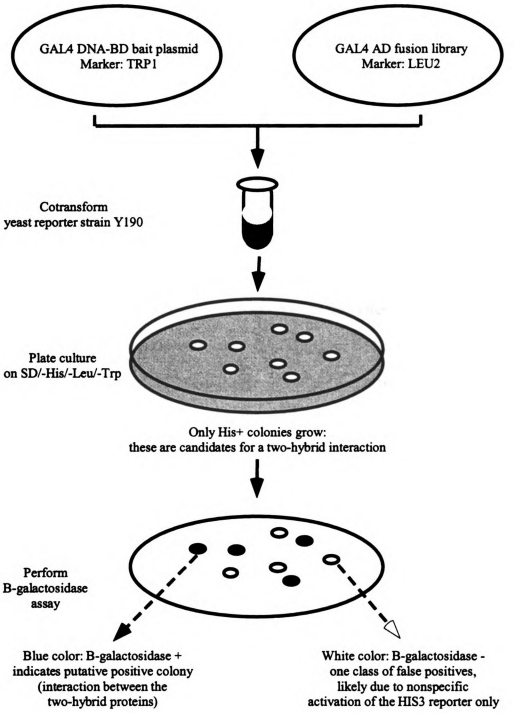



**Figure 6. Screening an GAL4-Activation Domain (AD) fusion library for proteins that interact with a bait protein.** Double-transformant colonies, which survive the $His^-$ selective medium and have β-galactosidase activity, contain potential-bait-interacting hybrid proteins.

to a second round of testing to verify the ability of the library plasmids to induce transactivation of the *HIS3* and *lacZ* reporter genes. All library plasmids that passed the above tests were transformed alone into CG1945 yeast. If their encoded proteins possessed intrinsic transactivating activities toward the reporter gene, they were discarded. The plasmids that passed those verification procedures were again transformed into CG1945 with a plasmid encoding lamin C fused to GAL4-BD or the fourth pAS2-1 bait construct, which encoded the human PGHS-1 carboxyl terminal sequence (residues 556-599) fused to GAL4-BD (23). These pairings tested for non-specific activation by the pACT2 encoded proteins via a mechanism other than interacting with GAL4-BD. The clones encoding proteins that interacted with lamin C or PGHS-1 carboxyl terminal sequence were discarded. Plasmids that passed all the above tests, were demonstrated to encode proteins that interacted specifically only with the PGHS baits (Figure 7). Sequences of the candidate library cDNA clones were compared to GenBank database with the Blasta and Tfasta sequence comparison programs.

**Expression of AT15 and AT18 proteins in Bacteria**

To express the two proteins AT15 and AT18, found to interact with pAS2C, their respective cDNA insert was digested out of the pACT2 library plasmid, and subcloned, in-frame, into the prokaryotic expression vector pET-28a (Novagen), giving rise to pET-AT15 and pET-AT18. The identity and orientation of the constructs were confirmed by DNA sequencing. The pET-AT15 or pET-AT18 vector expressed the AT15 or AT18 protein as a fusion protein containing 6 consecutive histidine residues (His-tag) at the $NH_2$ terminus. The His-tag was used for purification of the recombinant AT15 or AT18 protein. The plasmids were transformed into *E. coli* strain BL21(DE3), a *lon* mutant

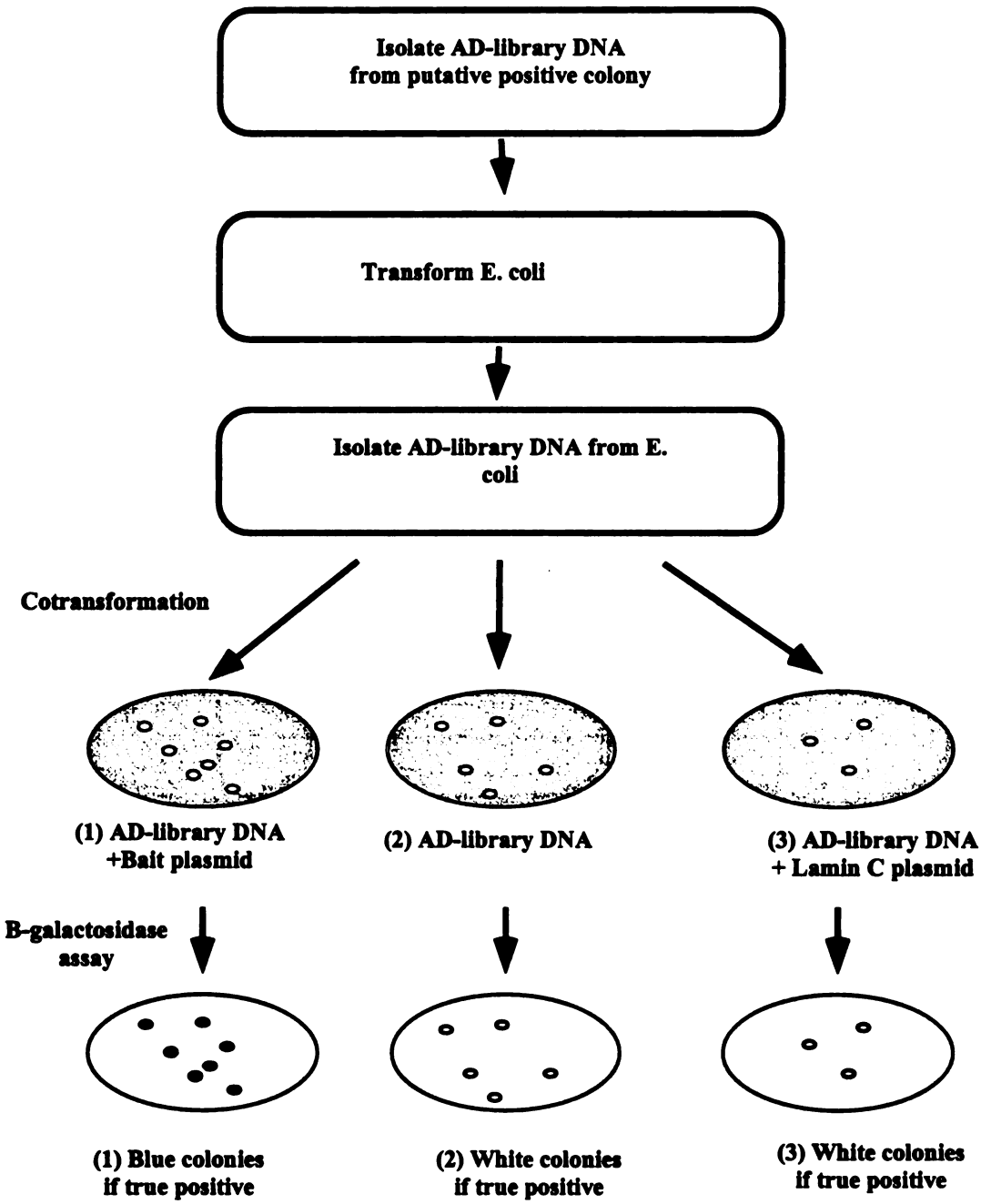



**Figure 7. Protocol used to eliminate false positives that arise in a two-hybrid library screening.** Plasmids, which didn't intrinsically activate transcription, which encoded proteins interacting specifically only with the PGHS baits, not with nonspecific protein, e.g. Lamin C, were selected as true postives.

strain containing the T7 polymerase under the control of *lac UV5* promoter. Addition of isopropyl-β-D-thiogalactopyranoside (IPTG) induces the expression of the fusion proteins in this system. The His-tag-AT15 and His-tag-AT18 were purified from crude bacterial lysates by Ni-NTA Resin (Qiagen) according to the manufacturer's instruction.

***In vitro* Elution Assay**

50 μg of purified ovine PGHS-1 or mouse PGHS-2 were incubated together with 50 μg of either His-tag-AT15 or His-tag-AT18 in 1 ml PBS (50 mM sodium phosphate, 300 mM NaCl, pH8.0) at 4 °C for 1 h to allow dimerization. Ovine PGHS-1 and mouse PGHS-2 were provided by Mike Malkowski. 200 μl of 50% slurry of Ni-NTA agarose was then added to each mixture, and the mixture was incubated at 4 °C for 1 h, followed by centrifugation at 1000 rpm for 5 min at 4 °C. The supernatants were aspirated, and the pellets were washed 3 times with 1 ml PBS and twice with PBS containing 10% glycerol (pH6.0) by repeated centrifugation. Bound proteins were eluted with 1 ml PBS containing 10% glycerol and 300mM imidazole. Aliquots (20 μl) of the eluted proteins were separated by electrophoresis on a 15% polyacrylamide gel using Tris glycine/SDS buffer (Qiagen).

***In vitro* Slot Blotting**

The carboxyl terminal sequences of PGHS-1 (residues 575-599) and PGHS-2 (residues 566-604) were generated by PCR, subcloned in-frame into pGEX-4T (Pharmacia) and transformed into *E. coli* to generate GST-PGHS-1-COOH and GST-PGHS-2-COOH fusions (Figure 4). The fusion proteins were purified from crude bacterial lysates by Mike Malkowski under non-denaturing conditions by chromatography on glutathione-Sepharose (Pharmacia).

Purified GST-PGHS-1-COOH, GST-PGHS-2-COOH, ovine PGHS-1, and mouse PGHS-2 were blotted onto nitrocellulose, and incubated with 0.5 μg/ml His-tag-AT15 or His-tag-AT18 in PBS at 4 °C for 1 h. After washing the nitrocellulose filters with PBS (3 times), bound His-tag-AT15 and His-tag-AT18 were visualized on X-ray film using Ni-NTA conjugated horseradish peroxidase (HRP) and a chemiluminescence reagent. BSA and His-tag-AT15, His-tag-AT18 were also blotted onto the nitrocellulose as controls.

***In vitro* Affigel Assay**

AT15 and AT18 affinity agarose were prepared by irreversibly cross-linking His-tag-AT15 or AT18 protein to Affigel agarose beads (1 mg/ml bed volume, Bio-Rad Laboratories). 40 μl of beads were then incubated in PBS with 4 μg purified human PGHS-2 for 1 h at 4 °C, and washed three times with PBS. Bound PGHS-2 was eluted by boiling in 1 x SDS-PAGE loading buffer and the samples were analyzed by western blotting using polyclonal antibody against PGHS-2 (24).

**Additional Two-Hybrid Testing in Yeast to Verify Interaction**

AT15 and AT18 cDNA inserts were released by digestion with restriction enzymes from the yeast library plasmids, GAL4-activation-domain vector pACT2 (Table I), and subcloned in-frame into a GAL4-DNA-binding-domain vector, pAS2-1, to construct pASn15 and pASn18. The two pAS2-1 constructs were confirmed by restriction endonuclease analysis and DNA sequencing. These plasmids were transformed into CG1945 (Table II) separately. The CG1945 transformants were selected on minimal media lacking tryptophan and were tested for autonomous reporter gene (*lacZ*) activation by a β-galactosidase assay. The transformants were also analyzed for expression of the fusion proteins by western blot using the monoclonal antibody (mAb) against the GAL4-

DNA-binding-domain (Clontech).

The PGHS-2 carboxyl terminal sequence (residues 555-604) was excised from the GAL4-DNA-binding-domain vector pAS2-1, and subcloned in-frame into the GAL4-activation-domain vector pACT2 to construct pACT2C. The structure of the pACT2C construct was confirmed by restriction endonuclease analysis and DNA sequencing. CG1945 transformant of pACT2C was selected on minimal media lacking leucine and was tested for autonomous reporter gene (*lacZ*) activation by a β-galactosidase assay. The transformant was also analyzed by western blot using the monoclonal antibody (mAb) against the GAL4-activation-domain (Clontech).

The pASn15 and pASn18 plasmids were then transformed into CG1945 either alone or with pACT2C or with the empty GAL4-activation-domain vector pACT2. Single and double transformants were tested for autonomous reporter gene *lacZ* activation by a β-galactosidase assay and autonomous reporter gene *HIS* activation by re-streaking on minimal media lacking histidine.

# RESULTS

## Cloning of AT15 and AT18 by Yeast Two-Hybrid Screening

Four cDNA sequences, corresponding to the PGHS-1-EGF domain (residues 24-84), the PGHS-2-EGF domain (residues 18-70), PGHS-2 carboxyl terminal sequence (residues 555-604), and PGHS-1 carboxyl terminal sequence (residues 556-599) (Figure 3 and 4), were cloned into the GAL4-DNA-binding-domain vector pAS2-1, to generate four PGHS-bait plasmids: pAS1EGF, pAS2EGF, pAS2C and pAS1C. Three of them: pAS1EGF, pAS2EGF, pAS2C were used as baits for yeast two-hybrid screening to identify proteins that might interact with the PGHS isozymes. All four of them were used separately to transform the yeast strain Y190 to check for intrinsic transactivation activity toward the host reporter genes. The transformants were subjected to a β-galactosidase assay and were found to express no *lacZ* activity, showing that the bait plasmids were suitable for library screening. Western blot analysis, using the transformant lysates and monoclonal antibody (mAb) against the GAL4-DNA-binding-domain, showed that all four baits were properly expressed and fused with the GAL4-DNA-binding-domain in the correct orientation and reading frame.

An estimated 3,500,000 human brain library transformants were screened using pAS1EGF, pAS2EGF and pAS2C. We used this library, because PGHS-2 is constitutively expressed in the brain. Among these, approximately 940,000 were screened with pAS2C, 300,000 with pAS2EGF, and 2,200,000 with pAS1EGF (Table III). From these combined screens, a total of 58 colonies survived selection on minimal media lacking Trp, His and Leu, but only 21 of these contained detectable β-galactosidase

## TABLE III YEAST TWO-HYBRID SCREEN RESULT

| Library | Bait | Transformants Screened | Surviving Colonies | B-gal positive colonies | Positive clones after cotransformation | Clones | Homology | Outcome |
|---|---|---|---|---|---|---|---|---|
| Human Brain | PGHS-2 C-ter | 940000 | 27 | 9 | 3 | AT14 | PDZ | Tested *in vitro & in vivo* |
| | | | | | | AT15 | PDZ | Tested *in vitro & in vivo* |
| | | | | | | AT18 | Cortactin Binding Protein | Tested *in vitro & in vivo* |
| | PGHS-2 EGF | 300000 | 2 | 0 | 0 | | | |
| | PGHS-1 EGF | 2200000 | 29 | 12 | 5 | AT29 | No-homology | Unknown protein Not tested |
| | | | | | | AT32 | ATP synthase | Non-specific Not tested |
| | | | | | | AT31 | No-homology | Unknown protein Not tested |
| | | | | | | AT37 | No-homology | Unknown protein Not tested |
| | | | | | | AT6-1-1 | ATP synthase | Non-specific Not tested |

activity and were studied further. When transformed alone, 4 of the 21 clones were eliminated because they intrinsically transactivated the reporter genes. Another 9 were eliminated because they showed nonspecific binding activity to heterologous lamin C protein. Eight clones survived all tests. These clones were sequenced.

It was determined that two clones which interacted with the carboxyl terminal PGHS-2 bait (AT14 and AT15), contained identical cDNA fragments. The open reading frame of this cDNA encoded a 260 amino acid fragment showing 70% amino acid identity to Lin-7 (*C. elegans*), 41% identity to PSD-95 and 38% identity to hDlg-1/-2 over its length. An alignment of these cDNAs revealed the homology of AT14 with AT15 corresponded to the shared PDZ repeat domains in these proteins (Figure 8), which are protein modules that bind to the carboxyl terminal ends of target proteins (25-27). Another clone AT18 that also interacted with the carboxyl terminal PGHS-2 bait, encoded for a fragment of 566 amino acids with 69% amino acid identity to a cortactin-binding protein. Two of the clones that interacted with the PGHS-1-EGF domain, were ATP synthase: a protein commonly obtained as an artifact in the yeast two-hybrid screening. They were not examined further. Three other clones identified by the interaction with the PGHS-1-EGF domain, were novel proteins not present in the genetic databases, and were also not examined further (Table III).

To verify that the proteins encoded by the pAT14/15 and pAT18 interacted specifically with PGHS-2, these clones were transformed into the CG1945 yeast host strain in the presence or absence of plasmid pAS2-1 which encoded the GAL4 DNA-binding-domain, or pAS2C which encoded the PGHS-2 carboxyl terminal sequence, or pAS1C which encoded the PGHS-1 carboxyl terminal sequence. Both pAT14/15 and

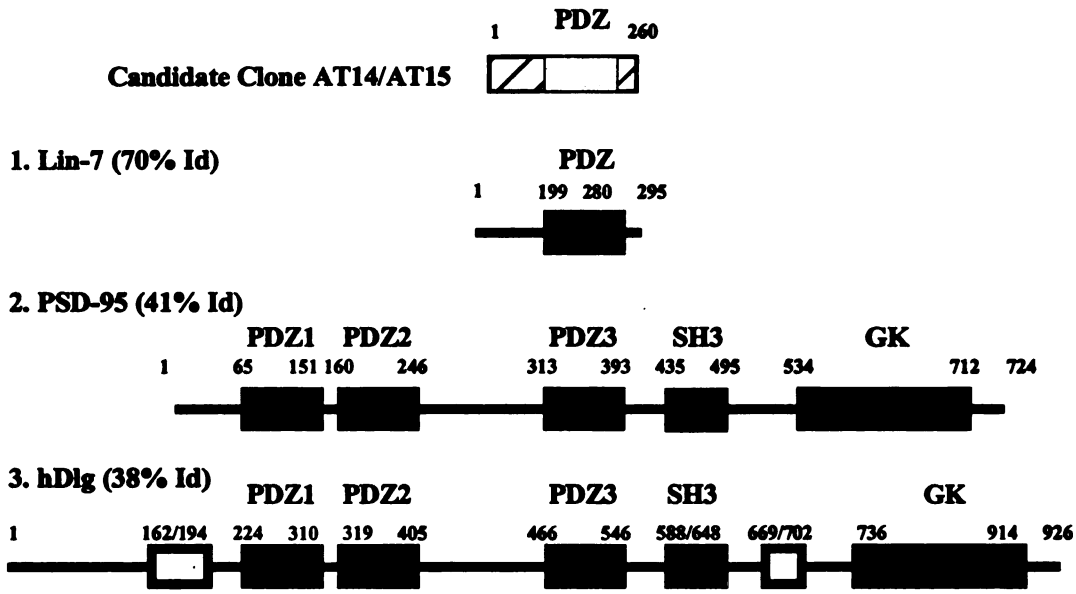



**Figure 8. Two of the candidate clones isolated by the yeast two-hybrid screening: AT14 and AT15.** Showing are the alignment between the candidate clone and the PDZ domains of Lin-7, PSD-95 repeat 3 and hDlg repeat 3. Percent identidies for the PDZ domains are shown (percent Id.). (Lin-7: C. elegans protein invoved in vulval development, PSD-95: post-synaptic density protein, hDlg: human DlgA homologous protein, DlgA: Drosophila discs-large tumor suppressor protein.)

pAT18 clones were positive in the β-galactosidase activity assay only when co-transformed with the plasmid encoding the PGHS-2 carboxyl terminus (pAS2C). Therefore, we concluded that the isolated clones AT14/15 and AT18 represent proteins that interact specifically only with the carboxyl terminus of PGHS-2, and not with the carboxyl terminus of PGHS-1, or the GAL4-DNA-binding-domain alone.

***In vitro* Testing of PGHS-2-AT15 and PGHS-2-AT18 Interaction**

To test the *in vitro* binding of AT15 and AT18 to PGHS-2, AT15 and AT18 were expressed as His-tag fusion proteins in bacteria and were purified by chromatography using Ni-NTA resin (Figure 9). Purified His-tag-AT15 or His-tag-AT18 was incubated with purified ovine PGHS-1 or mouse PGHS-2 to allow the formation of AT15-PGHS-2 or AT18-PGHS-2 complexes, which were then precipitated by nickel chelated Ni-NTA agarose. As shown in Figure 10, His-tag fusion proteins AT15 and AT18 were precipitated with Ni-NTA agarose, but PGHS-2 was not co-precipitated with either AT15 or AT18 in this assay.

A second approach to demonstrate an interaction with AT15 and AT18 was then tried in which purified GST-fusion PGHS-1 carboxyl terminus, GST-fusion PGHS-2 carboxyl terminus, ovine PGHS-1, and mouse PGHS-2 were applied by slot-blotting to nitrocellulose filters. These filters were then incubated with His-tag-AT15 or His-tag-AT18, and bound His-tag proteins were detected with Ni-NTA conjugated HRP. Neither His-tag-AT15 nor His-tag-AT18 could be detected bound to either GST fusion PGHS-2 carboxyl terminus or the holo-PGHS-2 protein (Figure 11).

Purified His-tag-AT15 or AT18 protein was next cross-linked to Affigel agarose beads (1 mg/ml) and incubated with 1/10 the amount of the purified human PGHS-2.

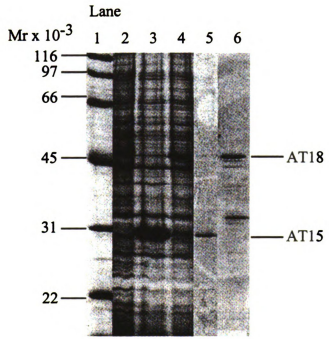



**Figure 9. SDS-PAGE analysis of the expression of His-tag-AT18 and AT15 protein.** AT18 and AT15 were subcloned into pET-28a to generate His-tag fusion proteins. *E. coli* strain BL21(DE3) was transformed with the indicated recombinant pET-28a plasmid, and protein expression was induced by addition of IPTG. The His-tag fusion proteins were purified by Ni-NTA resin. Homogenates (20 μg) of BL21(DE3) bacteria transformed with no plasmid (lane 2), pET-AT15 (lane 3), or pET-AT18 (lane 4) and purified His-tag-AT15 (lane 5) and His-tag-AT18 (lane 6). Molecular weight marker is in lane 1.

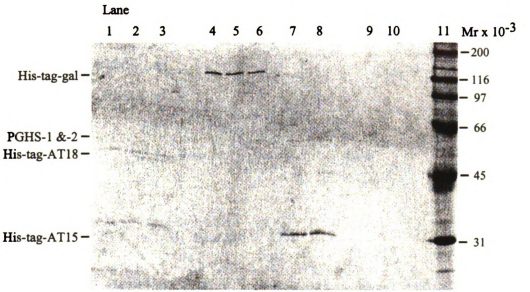



**Figure 10.** ***In vitro*** **Binding Assay.** 50 μg purified His-tag-AT15 or -AT18 were incubated with 50 μg PGHS to allow heterodimerization. The His-tag-AT15 and His-tag-AT18 were then precipitated with Ni-NTA agarose. Following washing of the agarose beads, bound proteins were eluted with 0.3M imidizole, separated by 15% SDS-PAGE, and stained with Coumassi Blue.
Lane 1. His-tag-AT18. Lane 2. His-tag-AT18 + ovine PGHS-1. Lane 3. His-tag-AT18 + mouse PGHS-2. Lane 4. His-tag-galactosidase (control). Lane 5. His-tag-galactosidase + ovine PGHS-1 (control). Lane 6. His-tag-galactosidase + mouse PGHS-2 (control). Lane 7. His-tag-AT15. Lane 8. His-tag-AT15 + mouse PGHS-2. Lane 9. ovine PGHS-1. Lane 10. mouse PGHS-2. Lane 11. Bio-Rad SDS-PAGE MW standard.

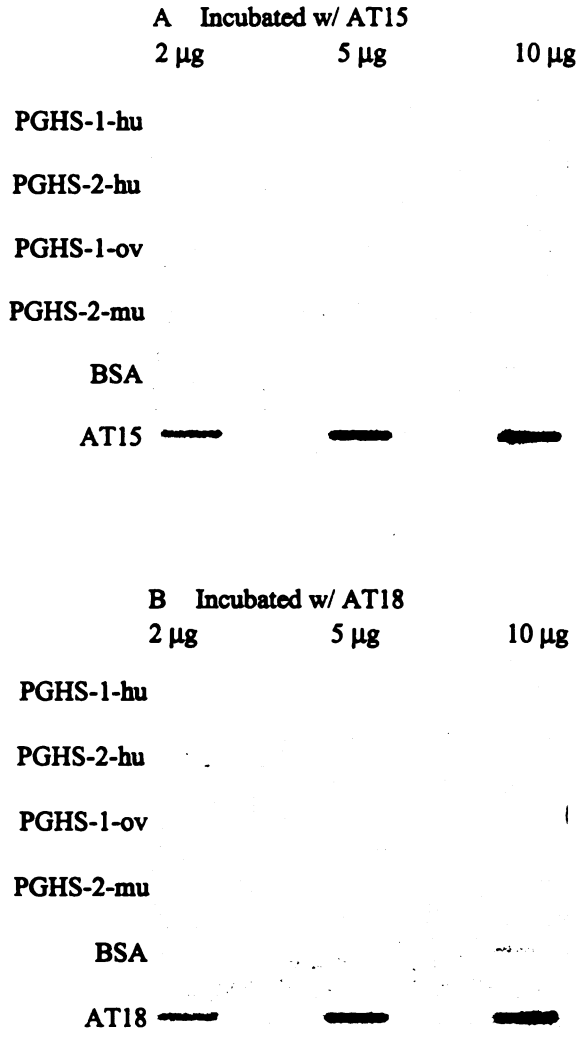



**Figure 11. Slot Blotting.** Three aliquots (2 µg, 5 µg, or 10 µg) of GST fusion human PGHS-1 carboxyl terminus, GST fusion human PGHS-2 carboxyl terminus, ovine PGHS-1, mouse PGHS-2, BSA, His-tag-AT15 or His-tag-AT18 were blotted onto nitrocellulose. The filters were probed with either 0.5 mg/ml His-tag-AT15 (Panel A) or His-tag-AT18 (Panel B), and bound His-tag-AT15 and His-tag-AT18 were detected with Ni-NTA conjugated HRP.

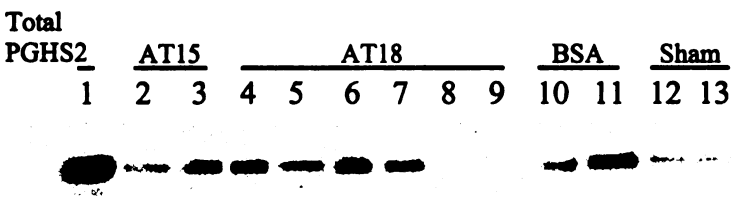



**Figure 12. Immunoblot of PGHS-2 retained on Affigel.** Equal amounts of purified human PGHS-2 (lane 1) were incubated with Affigel beads (at a ratio of 1 μg PGHS-2/10 μl beads) containing either His-tag AT15 (lane 2, 3) or His-tag AT18 (lane 4-9) or BSA (lane 10, 11) or sham (lane 12, 13). The retained PGHS-2 were eluted with 1x SDS loading buffer and detected by Western blotting. No consistent reproducible or specific binding of PGHS-2 to either Affigel-His-tag-AT15 or -AT18 was observed.

Following washing of the Affigel, the Affigel was treated with 1 x SDS sample buffer and the sample buffer was analyzed. As shown in Figure 12, no consistent reproducible or specific binding of PGHS-2 to either Affigel-His-tag-AT15 or -AT18 could be detected by western blot analysis using a PGHS-2 polyclonal antibody (24).

***In vivo* Testing in Yeast to Verify Interaction**

Since the above *in vitro* binding assays could not demonstrate that AT15 and AT18 bind PGHS-2 *in vitro*, additional experiments were designed to detect the existence of AT15-PGHS-2 and AT18-PGHS-2 complexes in yeast. Cloning vectors were switched by moving the AT15 and AT18 insert from the GAL4-activation-domain to the GAL4-DNA-binding-domain vector, and PGHS-2 carboxyl cDNA sequence from the DNA-BD to AD vector. Six constructs pASn15, pASn18, pAS2C (containing AT15, AT18 or PGHS-2 carboxyl terminal cDNA in the pAS2-1 vector, respectively), pAT15, pAT18 and pACT2C (containing AT15, AT18 or PGHS-2 carboxyl terminal cDNA in the pACT2 vector, respectively) were used to transform CG1945 separately. The transformants were selected on minimal media lacking Trp or Leu. Western blotting, using the transformant lysates and monoclonal antibody (mAb) against the GAL4-DNA-binding-domain or GAL4-activation-domain, showed that all six insert proteins were properly expressed and fused with GAL4-DNA-binding-domain or GAL4-activation-domain in the correct orientation and reading frame (Figure 13).

When the two-hybrid assay was repeated, yeast co-transformed with pASn15 (containing the AT15 cDNA in pAS2-1 vector) and pACT2C (containing the PGHS-2 carboxyl terminal cDNA in pACT2 vector) had no detectable β-galactosidase activity. These co-transformant colonies also couldn't grow on histidine selected media when re-

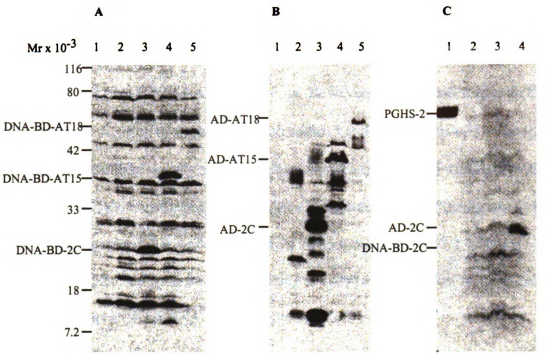



**Figure 13. Western blots of candidate proteins and bait protein using the GAL4 DNA-BD, AD mAbs and Dinah (Ab against PGHS2 carboxy terminus).** Soluble protein extracts were prepared from yeast strain CG1945 transformed (separately) with the indicated vector. Samples equivalent to ~1-1.5 OD600 units of cells were resolved on a 15% polyacrylamide/SDS gel and electroblotted to a PVDF filter. The blots were probed with either the GAL4 DNA-BD mAb (0.5 μg/ml; Panel A) or the GAL4 AD mAb (0.4 μg/ml; Panel B) or Dinah (0.1 μg/ml; Panel C), followed by HRP-conjugated polyclonal Goat Anti-Mouse IgG or HRP-conjugated polyclonal Goat Anti-Rabbit IgG. Signals were detected using a ECL detection assay and a 1-5min exposure of X-ray film.
Panel A. Lane 1: untransformed CG1945 control. Lane 2: pAS2 (a GAL4 DNA-BD vector). Lane 3: pAS2C. Lane 4: pASn15. Lane 5: pASn18.
Panel B. Lane 1: untransformed CG1945 control. Lane 2: pACT2 (a GAL4 AD vector). Lane 3: pACT2C. Lane 4: pAT15. Lane 5: pAT18.
Panel C. Lane 1. Human PGHS-2 standard. Lane 2. untransformed CG1945 control. Lane 3. pAS2C. Lane 4: pACT2C.

streaked (Figure 14). The fact that no interaction of these hybrid proteins could be observed after switching the vectors suggests that AT15 does not interact with the PGHS-2 carboxyl terminus in a biologically relevant manner.

Yeast co-transformed with pASn18 (containing AT18 cDNA in pAS2-1 vector) and pACT2C had strong β-galactosidase activity. However, yeast transformed with the pASn18 plasmid alone or co-transformed with pASn18 and the empty pACT2 vector, also had β-galactosidase activity, albeit less than when co-transformed with both pASn18 and pACT2C. All the transformant colonies grew on histidine selected media when re-streaked. This suggested that while AT18 might interact with the PGHS-2 carboxyl terminus, AT18 also had intrinsic transactivating activity toward the host reporter genes when expressed as a GAL4 DNA-BD chimeric protein.

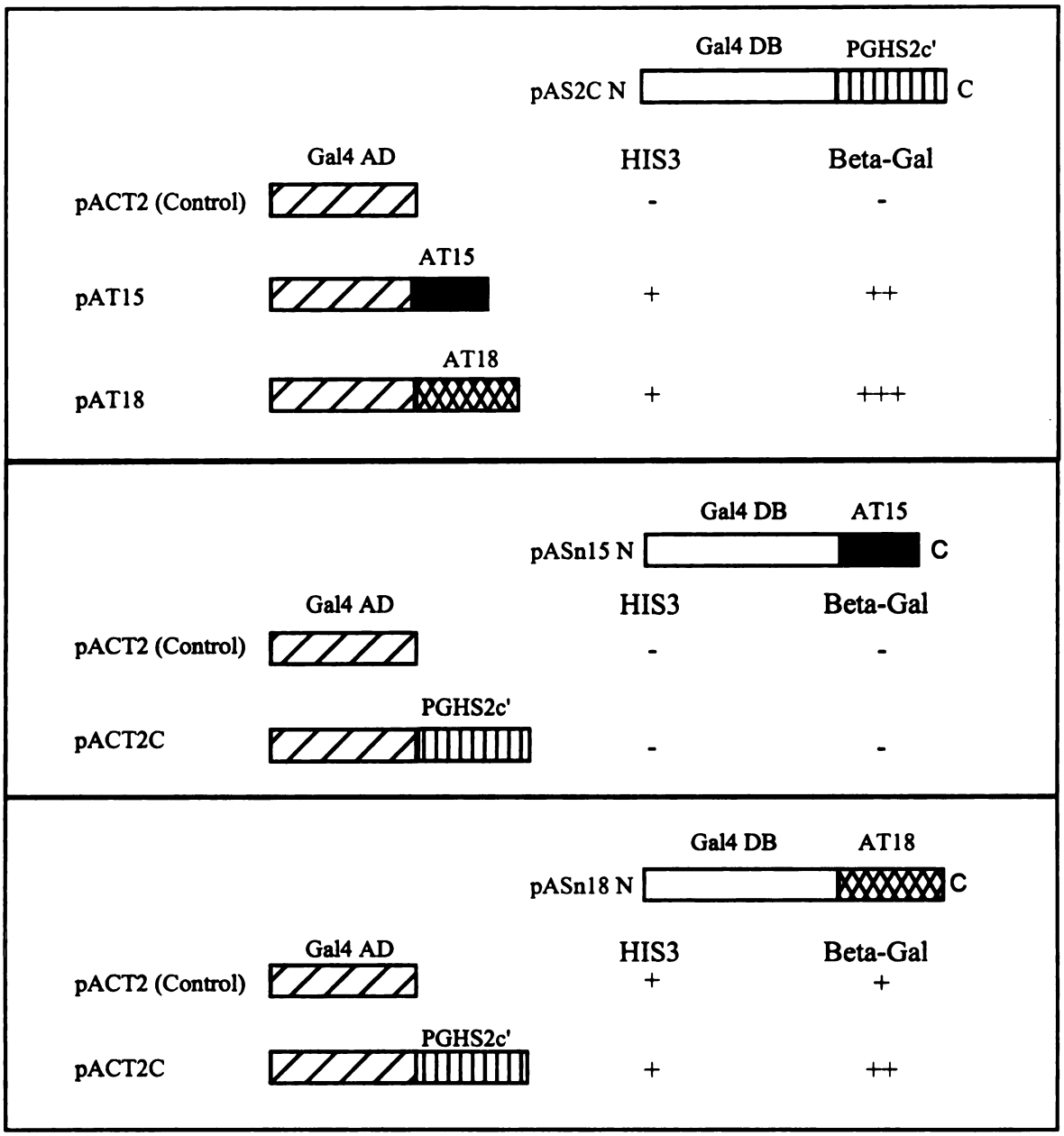



**Figure 14. Interaction between AT15, AT18 protein with PGHS-2 C-terminus as determined by semiquantitative yeast two-hybrid assays based on the degree of induction of the two reporter genes HIS3 and β-gal.** Yeast strain CG1945 was co-transformed with the indicated plasmids. HIS3 activity was determined by the yeast colonies grown on medium lacking histidine: +, growth; -, no significant growth. β-gal activity was estimated from the time taken for colonies to turn blue in 5-bromo -4-chloro-3-indolyl b-D-galactoside filter lift assays at room temperature: +++, <30 min; ++, 45-90 min; +, 90-480 min; -, no significant activity.

## DISCUSSION

The use of the yeast two-hybrid system to identify PGHS associated proteins in the endoplasmic reticulum (ER) has been found to be more complicated than searching for cytosolic proteins (21). The main difficulties result because PGHS contains a membrane-binding domain, and requires disulfide-bond formation and N-glycosylation for proper folding. Since the yeast system lacks the proper protein modification system, it should be advantageous to use small PGHS domains as baits in the yeast two-hybrid screening. This logic formed the partial basis for our choosing of the carboxyl terminal sequence of PGHS-2 as bait for these studies. Another reason that this sequence was chosen is that the carboxyl terminus of PGHS-2 contains sequences unique to this isozyme that we thought likely to have a specific function. Our choosing of the EGF domains of PGHS-1 and PGHS-2 as baits was based on the fact that EGF domains commonly function as protein-protein interaction domains, and they have already been identified as dimerization domains in the PGHS homodimer (28).

Five candidate clones were obtained with the PGHS-1-EGF bait, three of which are novel genes not known in the database, and were not characterized. Three candidate clones were obtained with the PGHS-2 carboxyl terminal bait, among which was a novel protein, AT15, possessing a PDZ domain, and AT18 a novel protein similar to cortactin binding protein. Since these clones interacted only with the PGHS-2 carboxyl terminus, which contains the 18 amino acid insert, and not with the PGHS-1 carboxyl terminus, we focused our characterization on these two candidate proteins: AT15 and AT18.

The PDZ domains are protein modules that bind to the carboxyl terminal ends of

target proteins. They were originally identified in the post-synaptic density protein PSD-95 as three repeats of about 90 residues containing the conserved motif Gly-Leu-Gly-Phe (GLGF) (26). PDZ domains have since been found in a diverse and growing set of proteins with different functions, e.g. p55, hDlg, Dsh, etc (29). Since PDZ domains appear to mediate direct protein-protein interactions by interacting with the carboxyl termini of the target proteins, it was particularly interesting for us to further characterize AT15's binding specificity to PGHS-2.

We expressed and purified His-tag fusion proteins of AT15 and AT18 in *E. coli*, and tried different approaches to detect the interaction between AT15 and PGHS-2, or AT18 and PGHS-2. However, none of the *in vitro* assays could prove that AT15 or AT18 bound PGHS-2.

The lack of protein modification in *E. coli* suggested that perhaps only in yeast were AT15 and AT18 properly modified, or that there might be a third protein in yeast required for the interaction of AT15 and AT18 with the PGHS-2 carboxyl terminus. Therefore we re-tested the binding capacity of AT15 and AT18 to PGHS-2 carboxyl terminus in yeast by switching vectors, moving the AT15 and AT18 insert from the activation-domain (AD) vector to the DNA-binding-domain (DNA-BD) vector, and PGHS-2 carboxyl cDNA sequence from the DNA-BD vector to the AD vector. When the two-hybrid assay was repeated for AT15 and PGHS-2 carboxyl terminus after switching vector, neither the β-galactosidase nor the histidine reporter gene's transcription was transactivated. The lack of reporter gene expression suggests that AT15 does not interact with the PGHS-2 carboxyl terminus after the vector switching.

On the other hand, transcription of both β-galactosidase and histidine genes were

activated in yeast either transformed with AT18 in the GAL4-DNA-binding-domain (DNA-BD) vector alone, or co-transformed with AT18 and the empty activation-domain (AD) vector, or with AT18 and PGHS-2 carboxyl terminus in the AD vector. The intensity of β-galactosidase activity in yeast co-transformed with both AT18 and PGHS-2 carboxyl terminus was stronger than that with single AT18 or with AT18 and the empty AD vector. The expression of β-galactosidase and histidine might suggest that AT18 interacted with the PGHS-2 carboxyl terminus after the vector switching. However, AT18 also had intrinsic transactivating activity toward the host reporter genes when it was fused with GAL4-DNA-binding-domain, suggests that AT18 may be a transcriptional activator by itself. The cumulative evidence led us to conclude that neither AT15 nor AT18 interacts with PGHS-2 in any biologically relevant manner.

In general, the yeast two-hybrid system has been used extensively to detect protein-protein interactions (20, 30). However, detection of a specific interaction between the bait protein and the library-encoded proteins in this heterologous assay system does not necessarily indicate that there is a corresponding interaction in the proteins' native environment. For example, two members of a protein family (i.e., leucine zipper protein family) may produce a signal in the yeast two-hybrid assay, but they may never normally be present in the same cell type, cellular compartment, or during the same stage of the cell cycle. Similarly, the two-hybrid system may detect interactions between domains of proteins that are not present in the same subcellular location in a cell. Alternatively, if an interaction is mediated via a short sequence, that sequence may not be exposed on the native protein. Therefore, it is very important to verify the protein-protein interactions detected by the two-hybrid system by other biological or biochemical experiments.

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