A novel mechanism-based mammalian cell assay for the identification of SH2-domain-specific protein–protein inhibitors

Richard J Rickles, Pamela A Henry, Wei Guan, Mihai Azimioara, William C Shakespeare, Shelia Violette and Mark J Zoller

Background: Many intracellular signal-transduction pathways are regulated by specific protein–protein interactions. These interactions are mediated by structural domains within signaling proteins that modulate a protein's cellular location, stability or activity. For example, Src-homology 2 (SH2) domains mediate protein–protein interactions through short contiguous amino acid motifs containing phosphotyrosine. As SH2 domains have been recognized as key regulatory molecules in a variety of cellular processes, they have become attractive drug targets.

Results: We have developed a novel mechanism-based cellular assay to monitor specific SH2-domain-dependent protein–protein interactions. The assay is based on a two-hybrid system adapted to function in mammalian cells where the SH2 domain ligand is phosphorylated, and binding to a specific SH2 domain can be induced and easily monitored. As examples, we have generated a series of mammalian cell lines that can be used to monitor SH2-domain-dependent activity of the signaling proteins ZAP-70 and Src. We are utilizing these cell lines to screen for immunosuppressive and anti-osteoclastic compounds, respectively, and demonstrate here the utility of this system for the identification of small-molecule, cell-permeant SH2 domain inhibitors.

Conclusions: A mechanism-based mammalian cell assay has been developed to identify inhibitors of SH2-domain-dependent protein–protein interactions. Mechanism-based assays similar to that described here might have general use as screens for cell-permeant, nontoxic inhibitors of protein–protein interactions.

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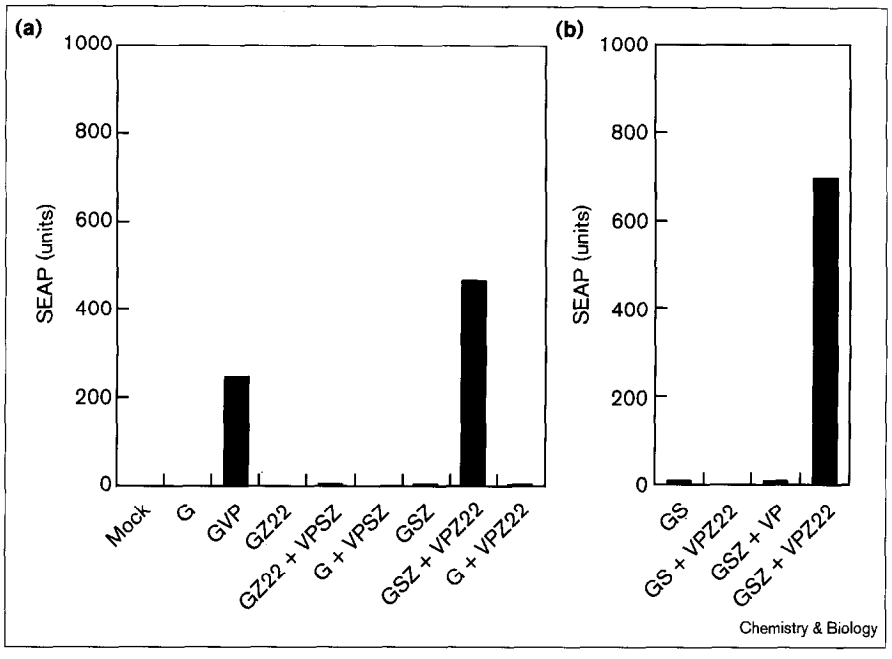
Introduction

Changes in cellular activity are triggered as a physiological response to environmental and developmental conditions. The mediators of these responses include small molecules, ions, peptides and cell surfaces. The relay of signals within the cell occurs via a cascade of specific molecular interactions, involving proteins that have either enzymatic or binding activity [1,2]. During the past 15 years, many components of pathways regulated by a variety of mediators have been elucidated. This knowledge is being applied to the development of pharmaceutical agents that interfere with signal transduction for the treatment or prevention of disease [3,4]. Many of the signaling pathways have redundancies, where multiple proteins of a gene family will simultaneously perform overlapping functions or parallel distinct signaling events take place to yield the same net result. The ability to target unique and essential steps of a given signal cascade provides a means to discover new therapeutic agents that are effective and safe.

Targeted disruption of genes in mice, as well as clinical, genetic and biochemical studies of human diseases, has provided important information about signaling proteins essential for basic biological processes. For example, studies on mice that are deficient in Src have revealed that the Src tyrosine kinase is essential for osteoclast-mediated bone resorption [5]. As the Src SH2 and SH3 domains mediate important protein–protein interactions [6], molecules that antagonize Src SH2 or SH3 domain function might inhibit bone resorption and therefore could be useful for the treatment of osteoporosis. ZAP-70 is another essential signaling protein. Humans lacking ZAP-70, a T-cell-receptor (TCR)-associated protein tyrosine kinase, have a form of severe combined immunodeficiency syndrome [7,8]. Individuals lacking ZAP-70 exhibit defective TCR-dependent signal transduction and abnormal T-cell development. ZAP-70-deficient mice have a similar phenotype [9]. ZAP-70 contains two SH2 domains that mediate the association of ZAP-70 with immunoregulatory tyrosine-activation motifs (ITAMs) present in the cytoplasmic domains of the TCR [10]. TCR ligation induces ITAM-bound ZAP-70 tyrosine phosphorylation and kinase activity. Molecules that block the binding of ZAP-70 SH2 domains to ITAMs inhibit T-cell activation [11]. As defects in ZAP-70 function

Figure 1



Conditions for tandem ZAP-70 SH2 domain-ζ two-hybrid formation in mammalian cells. 5 × GAL4 IL-2 SEAP HT1080 cells were transfected with the effector plasmids described in the text. Following transfection, cells were cultured for 24 h and the media were then assayed for SEAP. Mock transfected cells received carrier DNA and no effector plasmids. The results from two transfections are shown **(a,b)**. The SEAP assay was performed for 1 h at 37°C. SEAP activity is given as relative arbitrary units. G, GAL4 (DNA-binding domain, residues 1–147); VP, VP16 transcriptional activation domain; GVP, GAL4–VP16; GZ22, GAL4–ZAP SH2 domains; VPSZ, VP16–Src-kinase-ζ ITAM; GS, GAL4–Src-kinase; GSZ, GAL4–Src-kinase-ζ ITAM.

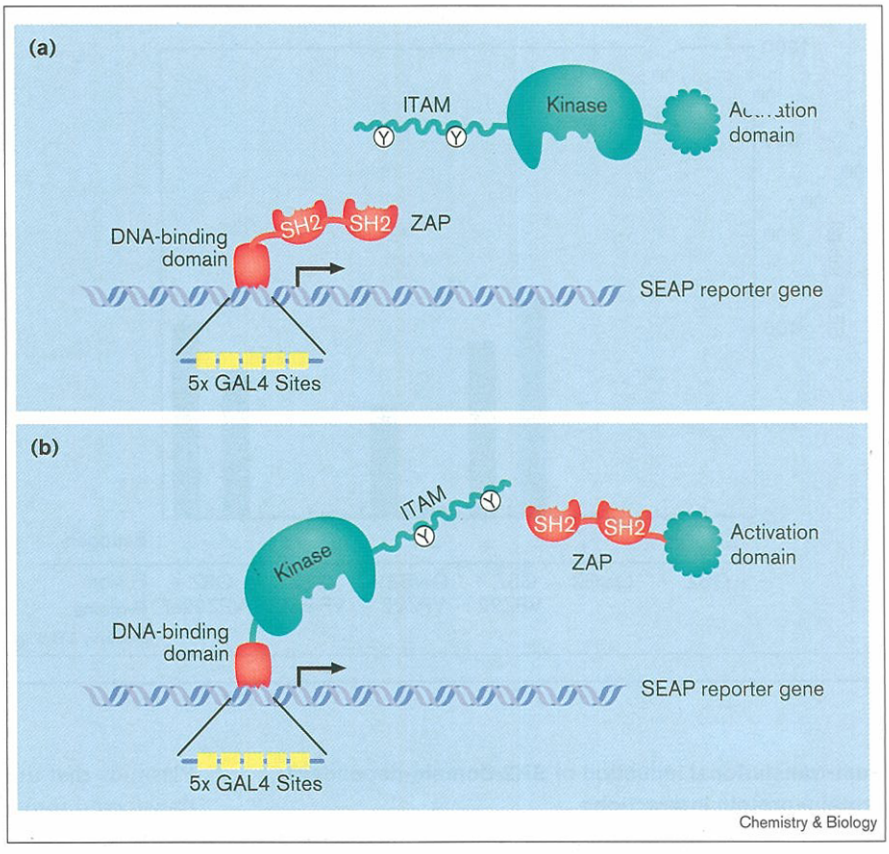
compromise T-cell function, pharmaceutical agents that selectively block ZAP-70 SH2 domain binding would be immunosuppressive agents.

Our goal has been to identify inhibitors of specific SH2-domain-dependent protein–protein interactions using an *in vivo* screening assay. Such molecules would be ideal pharmacological agents. A cellular assay offers the advantage over an *in vitro* assay of identifying cell-permeant compounds and allowing an assessment of non-specific effects on cellular functions. Mammalian cells are preferable for our purposes because the permeability of compounds into yeast cells is often not the same as that into mammalian cells [12–14]. Given the plethora of SH2-domain-containing proteins in a mammalian cell, the identification of specific inhibitors requires the use of a mechanism-based screen. One technique, the two-hybrid interaction assay, is a widely used method for identifying interacting proteins [15,16]. The two-hybrid method is typically performed using yeast cells to identify the association of known proteins with unidentified cellular proteins and to map determinants within proteins important for protein–protein interactions [15,16]. This assay is based on the interaction of hybrid transcription factors. One protein (the target protein) is fused to a DNA-binding domain and a second, interacting protein (or cDNA if it is a library screen) is fused to a transcriptional activation domain. The two proteins are coexpressed in cells that contain a reporter gene with upstream cognate binding sites for the DNA-binding domain. If the two chimeric proteins interact, the reporter gene will be transcriptionally activated, allowing detection of the interaction.

Here, we have modified the two-hybrid assay to develop a mechanism-based cellular assay for inhibitors of SH2-dependent interactions. The SH2 domain and ligand were expressed in a mammalian cell line as components of molecules used for a two-hybrid interaction screen. The application of the two-hybrid assay to SH2-domain–peptide-ligand interactions is complicated by the need to phosphorylate the ligand on tyrosine. The hybrid molecules have therefore been engineered such that the binding partner is tyrosine phosphorylated, so that it serves as a good binding site for the SH2 domain. Both the effector and reporter gene plasmids have been stably integrated into mammalian cells. Furthermore, the two-hybrid system has been designed so that the interaction of the SH2 domain with ligand can be post-translationally induced using estrogen and transcription can be easily monitored. We have developed two different two-hybrid systems. One is a ZAP-70 SH2 domain/TCR ζ chain two-hybrid assay, a system designed to mimic the interaction of ZAP-70 with the TCR in activated T cells. We also designed a Src SH2-domain-dependent two-hybrid assay. Here, we demonstrate that compounds that selectively bind to the Src SH2 domain selectively inhibit Src, but not ZAP-70, SH2-domain-dependent two-hybrid activity. The two-hybrid assay we describe can be used to monitor other SH2-dependent interactions and hence could be used to identify inhibitors of other SH2-domain-dependent processes.

Figure 2

Schematic diagram of conditions tested for SH2-dependent two-hybrid formation in mammalian cells. HT1080 fibrosarcoma cells containing an integrated SEAP reporter gene were used for all transfections. SEAP gene transcription was GAL4-dependent because of the placement of five GAL4-binding sites and the IL-2 basal promoter upstream of SEAP coding sequences. Two different conditions were examined where the ZAP-70 SH2-ζ ITAM interaction might occur. Transfections were carried using plasmids designed to produce the following proteins in cells: in **(a)** the ZAP-70 SH2 domains were fused to the GAL4 DNA-binding domain and the vSrc kinase-ζ was linked to the VP16 transcriptional activation domain. In **(b)** vSrc kinase-ζ residues were fused to the GAL4 DNA-binding domain and the tandem ZAP SH2 domains were linked to the VP16 transcriptional activation domain. The production of vSrc kinase-ζ fusion protein in one of these configurations was reasoned to lead to appropriately tyrosine phosphorylated ζ chain residues. Only when fusion proteins were expressed as shown in (b) was there an induction of SEAP gene transcription.



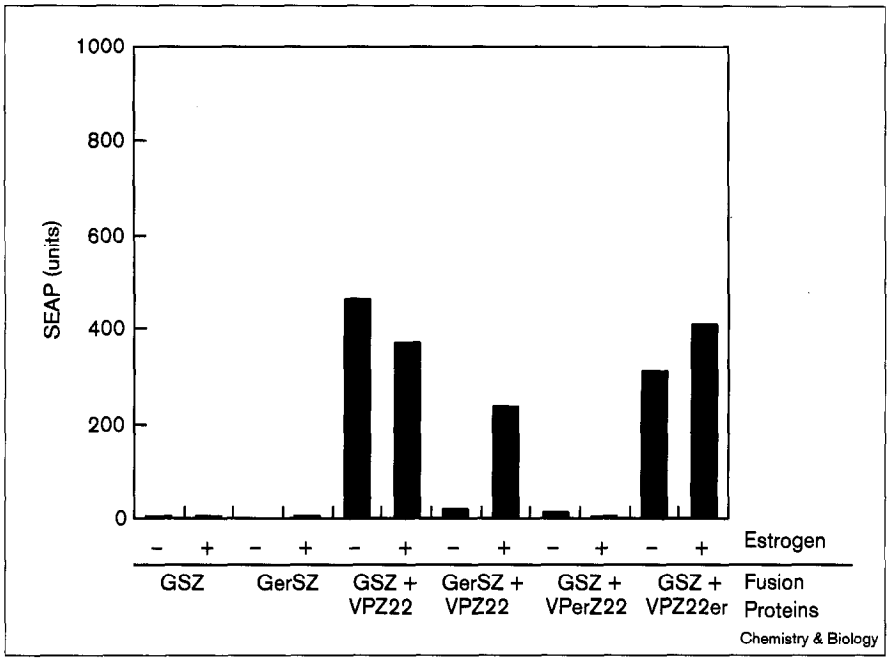
Results and discussion

We have employed a human HT1080 fibrosarcoma cell line (HT1080B), containing an integrated secreted alkaline phosphatase (SEAP) reporter gene under the control of a GAL4-responsive promoter to define conditions for monitoring two-hybrid interactions. HT1080B does not normally produce SEAP (Figure 1a, mock). Transfection with an effector plasmid directing the expression of GAL4-DNA-binding-domain (GAL4DBD)–VP16-transcriptional-activation-domain fusion protein (GAL4–VP16) induces SEAP production (Figure 1a, GVP), demonstrating the potential utility of this cell line for detecting two-hybrid interactions.

ZAP-70 SH2-domain-dependent two-hybrid assay

The tyrosine residues within the ζ ITAM ligand must be phosphorylated for the ZAP-70 SH2 domains to bind with high affinity [17]. To facilitate phosphorylation of ITAM residues, the ζ chain was fused to the vSrc (viral Src) tyrosine kinase domain (vSrc-ζ). We reasoned that the vSrc kinase domain, in this configuration, would appropriately phosphorylate, via an intramolecular or intermolecular interaction, the ζ chain. Two different orientations were tested for the ability to allow ZAP-70 SH2-ζ two-hybrid formation. In one orientation, effector plasmids were constructed such that one plasmid contained the ZAP-70 SH2 domains fused to the GAL4DBD with the other plasmid containing vSrc kinase-ζ fused to the VP16 activation domain (Figure 2a). In the other orientation, the vSrc kinase-ζ was fused to the GAL4DBD and the tandem ZAP SH2 domains were fused to VP16 (Figure 2b). Neither GAL4–ZAP-SH2 (Figure 1a, GZ22) nor the GAL4–vSrc-kinase-ζ (Figure 1a, GSZ) fusion proteins, when expressed alone in cells, was capable of acting as a transcriptional activator. In addition, the orientation shown in Figure 2a did not lead to transcriptional activation of the reporter gene (Figure 1a, GZ22/VPSZ). In contrast, the orientation shown in Figure 2b triggered a robust stimulation of SEAP gene transcription (Figure 1a, GSZ/VPZ22). Transcriptional activation did not occur if the vSrc kinase-ζ residues were omitted (Figure 1a, G/VPZ22). Furthermore, GAL4DBD fusion proteins that contain the vSrc kinase domain but lack the ζ residues (Figure 1b, GS/VPZ22) or contain the ζ ITAMs but lack the vSrc kinase domain (GAL4DBD-ζ, data not shown) failed to induce transcriptional activation. Thus, the ζ chain, kinase and SH2 domains are required and must be positioned appropriately for two-hybrid formation to occur. The results using HT1080B mammalian cell line were reproduced in yeast when using the appropriate analogous effector plasmids (data not shown).

Figure 3



Estrogen regulation of tandem ZAP-70 SH2 domain-ζ two-hybrid formation in mammalian cells. Cells were transfected with effector plasmids and then cultured in media alone or supplemented with 10 nM estrogen for 18 h and the media were assayed for SEAP. The results shown are from a 1 h SEAP assay. The fusion proteins examined include GSZ (GAL4–Src-kinase-ζ ITAM) and VPZ22 (VP16–ZAP SH2 domains) and derivatives containing estrogen-receptor ligand-binding-domain residues, denoted GerSZ, VPerZ22 and VPZ22er.

Post-translational induction of SH2-domain-dependent protein–protein interactions

When assaying for inhibitors of specific protein–protein interactions, under certain conditions, it might be easier to block protein–protein interactions than disrupt a pre-formed complex [18]. This would be particularly relevant when dealing with high-affinity interactions or complexes with slow dissociation rates. Hence, the ability to induce a protein–protein interaction would be a useful feature of a cell-based screen for inhibitors of molecular interactions. Cells containing the components necessary for two-hybrid formation could first be exposed to potential inhibitory compounds and then protein–protein (two-hybrid) interaction could be induced. To generate such a system, we fused the ligand-binding domain of a steroid hormone receptor to one of the binding partners.

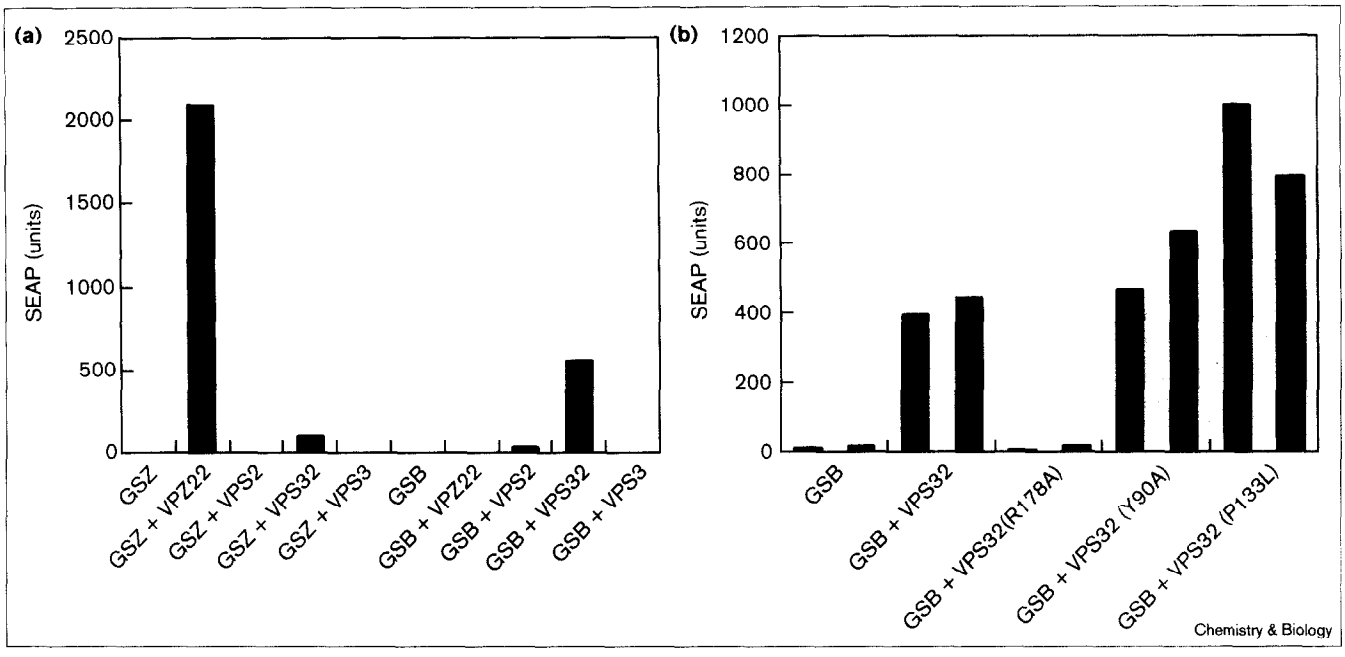
The modular ligand-binding domains of steroid hormone receptors have been widely used to generate protein chimeras that are ligand-dependent for activity [19,20]. The steroid hormone ligand-binding domain can act as a regulatory domain and subject heterologous activities on the same polypeptide to hormonal control. To examine whether SH2-domain-dependent two-hybrid formation can be hormonally controlled, we fused the ligand-binding domain of the estrogen receptor (ER-LBD) to either the GAL4DBD–vSrc-kinase-ζ or the VP16–tandem ZAP SH2 chimeric protein, and assayed for estrogen-inducible reporter-gene expression.

We first examined the properties of a GAL4DBD–vSrc-kinase-ζ fusion protein containing ER-LBD (GerSZ). Plasmids that direct the expression of GSZ or GerSZ were transfected into HT1080B reporter cells and then the cells were treated with 10 nM estrogen for 18 h. As shown in Figure 3, GerSZ is not, by itself, a transcriptional activator. Furthermore, neither GSZ or GerSZ fusion protein expressed alone activates SEAP production with estrogen treatment. Estrogen had no effect on SEAP production in cells with constitutive SH2-domain-dependent two-hybrid formation (GSZ + VPZ22). When cells expressing GerSZ and VPZ22 were cultured for 18 h in media containing 10 nM estrogen, two-hybrid formation was induced, however, with a tenfold increase in SEAP production observed. In contrast, chimeric VP16–tandem-ZAP-SH2 fusion proteins containing the ER-LBD at either of two positions were expressed in cells along with the GSZ fusion protein, estrogen regulation was not observed. Placement of the ER-LBD residues between the VP16 transcriptional activation domain and tandem ZAP SH2 abolished two-hybrid formation (Figure 3, GSZ + VPerZ22). When placed at carboxyl terminus of the protein, two-hybrid formation still occurred but estrogen regulation was not conferred (Figure 3, GSZ + VPZ22er).

Src SH2-domain-dependent two-hybrid assay

Having defined conditions for the detection of tandem ZAP-70 SH2 binding in a mammalian cell-based two-hybrid assay format, we next investigated whether these conditions could be used to monitor other SH2-domain–ligand interactions. Initially, we analyzed whether the Src SH2 domain can bind to the ligand recognized by the ZAP SH2 domains (the phosphorylated ITAM tyrosine residues

Figure 4



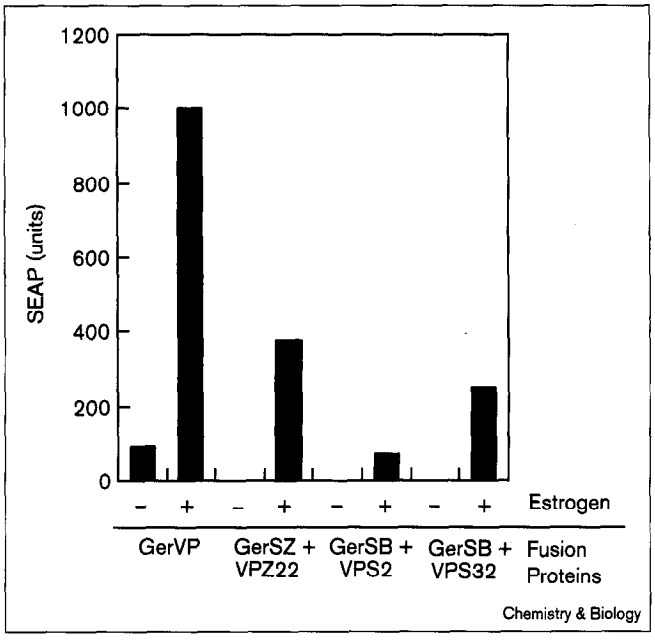
Conditions for Src SH2 domain-dependent two-hybrid formation in mammalian cells. Cells were transfected with the plasmids described in the text. Following transfection, cells were cultured for 24 h and the media were assayed for SEAP. Two separate transfections are shown **(a,b)**, with the transfections shown in (b) performed in duplicate. The results shown are from a 3 h SEAP assay. GSZ, GAL4–Src-kinase-ζ ITAM; GSB, GAL4–Src-kinase-β ITAM; VPZ22, VP16–ZAP-SH2 domains; VPS2, VP16–Src-SH2; VPS32, VP16–Src-SH3-SH2. VPS32(R178A) contains a mutation in the Src SH2 domain and VPS32(Y90A) and VPS32(P133L) each have a mutation in the Src SH3 domain predicted to dramatically reduce domain function.

of the GAL4DBD–vSrc kinase-ζ fusion protein). Three VP16–Src fusion proteins were prepared for this study: VP16–Src-SH2-domain (VPS2), VP16–Src-SH3/SH2-domains (VPS32) and VP16–Src-SH3-domain (VPS3). VPS2 and VPS32 both contain the Src SH2 domain but in different contexts (alone or with the Src SH3 domain in its natural orientation). The VPS3 fusion protein should not bind to SH2 ligands and served as a control for the detection of any SH3-domain-dependent interactions. Expression plasmids for each of these proteins, as well as for VPZ22, were co-transfected into HT1080B reporter cells with a plasmid directing the expression of GAL4DBD–vSrc kinase-ζ ITAMs fusion protein (GSZ) and SEAP production was assessed. As shown in Figure 4a, VPS32 induced moderate SEAP production, whereas VPS2 and VPS3 failed to induce SEAP.

Although the phosphorylated ζ subunit of the TCR is a high-affinity ligand for the ZAP-70 SH2 domains, a high-affinity consensus binding site for the Src SH2 domain (YEEI) is not present [21], hence phosphorylated ζ might not efficiently recruit VP16–Src-SH2-domain fusion proteins to the SEAP gene promoter. The chain of the IgE receptor encodes a high-affinity Src SH2-domain-binding site, YEEL [22]. A plasmid directing the expression of GAL4DBD–vSrc-kinase-β fusion protein (GSB) was made and tested in the two-hybrid assay. GSB expressed alone in HT1080B reporter cells did not activate SEAP reporter gene transcription (Figure 4a, GSB). The coexpression of GSB with either VPZ22 or VPS3 also failed to induce SEAP production. In contrast, coexpression of GSB with VPS32 or VPS2 induced SEAP production, although, the combination of GSB with VPS2 was approximately 13-fold less efficient than VPS32 in the assay.

The finding that VP16 fusion proteins containing both the Src SH2 and SH3 domains induce SEAP production more efficiently than VP16–SH2 suggested that there might be weak binding sites for the Src SH3 domain in the GSB fusion protein. To delineate the contributions that the Src SH2 and Src SH3 domains make to GSB-dependent two-hybrid formation, amino-acid substitutions were made in the VPS32 fusion protein that should dramatically affect SH2- or SH3-domain binding. As shown in Figure 4b, a mutation in the phosphotyrosine-binding pocket of the Src SH2 domain (Arg178→Ala, R178A [23]) abolished two-hybrid formation/SEAP production. In contrast, mutations in the SH3 domain that have been shown previously to affect ligand binding (Tyr90→Ala, Y90A, and Pro133→Leu, P133L [24]), did not reduce SEAP production. These experiments support the hypothesis that GSB-dependent two-hybrid formation is Src SH2-dependent.

Figure 5



Estrogen-regulated two-hybrid stable cell lines. Cells were co-transfected with a plasmid conferring neomycin resistance and with plasmids directing the expression of either GerSB + VPZ22, GerSB + VPS2 or GerSB + VPS32. Colonies were selected, expanded and analyzed for estrogen-dependent SEAP production. Of the 12 ZAP-70 SH2 cell lines analyzed, two were estrogen-dependent for SEAP induction. Of the 24 Src SH2 cell lines screened, two were positive for estrogen-dependent SEAP activity and of the 12 Src SH3/SH2 cell lines screened, three scored positive. Representative results obtained examining estrogen-dependent SEAP production using ZAP-70 SH2 (GerSG + VPZ22), Src SH2 (GerSB + VPS2) and Src SH3/SH2 (GerSB + VPS32) cell lines are shown. The results shown are from a 1 h SEAP assay.

The addition of the SH3 domain to the fusion protein (VPS32) could lead to more efficient SEAP production because the protein might be more stable in cells than the VP16–Src SH2 fusion protein or the presence of the SH3 domain in the VP16–SH3/SH2 fusion protein might contribute to SH2-domain binding or folding.

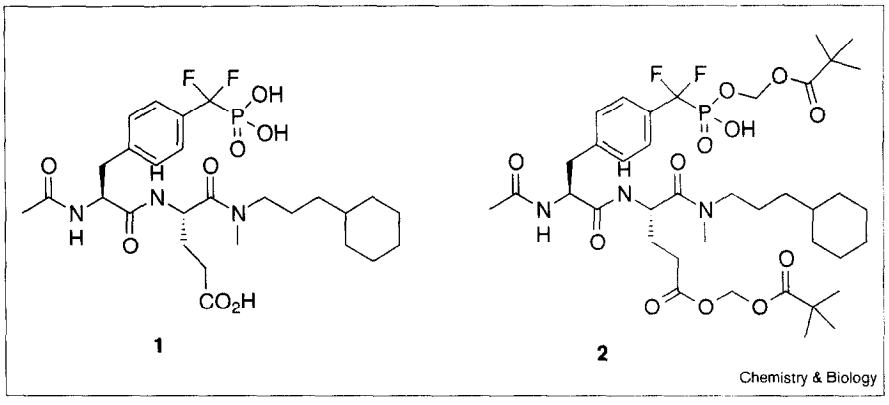
Construction and use of mammalian cell lines for the identification of cell-permeant SH2 domain inhibitors

We have described conditions for regulated SH2-domain-dependent two-hybrid formation in mammalian cells, which should be useful for the identification of compounds that inhibit SH2-domain–ligand interactions. One technical problem with the assay described above, however, is that the effector plasmids have been introduced into cells by transient transfection. This would not be practical for a routine screening assay, as large-scale transfections are cumbersome and too variable. Because a reproducible, robust, high-throughput assay requires stable cell lines, the effector plasmids were stably integrated into HT1080B reporter cells. To first test if it would be possible to generate a stable estrogen-inducible SEAP-producing cell line, HT1080B cells were co-transfected with a plasmid that directs the constitutive expression of GAL4DBD–ER-LBD–VP16 activation-domain fusion protein (GerVP) and a second plasmid that confers neomycin resistance. Stable neomycin-resistant transfected cells were expanded and tested for estrogen-dependent SEAP production. The results for one cell line are shown in Figure 5 (GerVP). This cell line produced high levels of SEAP in an estrogen-dependent manner. Having demonstrated that the stable selection of estrogen-inducible SEAP cell lines was possible using HT1080B reporter cells, transfections were then carried out to select cells that would have estrogen-regulated ZAP-70 SH2- and Src SH2-dependent two-hybrid formation. Representative results obtained examining estrogen-dependent SEAP production using ZAP-70 SH2 (GerSZ + VPZ22), Src SH2 (GerSB + VPS2) and Src SH3/SH2 (GerSB + VPS32) cell lines are shown in Figure 5. Both ZAP SH2- and Src SH2-dependent estrogen-regulated two-hybrid stable cell lines were obtained.

To determine whether the two-hybrid cell lines would be useful for the identification of SH2-domain inhibitors, we synthesized a high affinity Src SH2 ligand and the corresponding ester prodrug [25], a derivative shown to have increased cell permeability (Figure 6). When tested *in vitro* using fluorescence polarization [26], the parent compound had an IC_{50} of 4–9 μM for the Src SH2 domain and exhibited an IC_{50} of >1000 μM for ZAP-70 SH2 domains. As expected, the prodrug did not exhibit any binding activity in the *in vitro* assays. Both compounds were tested for their effect on inhibiting the production of SEAP from the GerVP, ZAP SH2-ζ ITAM and Src SH2-β ITAM cell lines (Figure 7). Alamar blue reactivity or ^{3}H-uridine incorporation were monitored to determine if the compounds non-specifically inhibited transcription or were toxic to cells. Both the parent compound (Figure 7a,b) and prodrug (Figure 7c,d) significantly inhibited SEAP production from the Src SH2-β ITAM cell line. The parent compound had an IC_{50} of 150 μM in the Src SH2 two hybrid cell line with little or no inhibition of the ZAP or GerVP cells at 200 μM. The prodrug was a more effective inhibitor with an IC_{50} of 18 μM in the Src SH2 two-hybrid cells. In contrast, this compound had an IC_{50} of 60–70 μM in the ZAP and GerVP two-hybrid cells, which overlapped in a concentration-dependent manner with inhibition of ^{3}H-uridine incorporation and alamar reactivity. Thus, the concentrations of prodrug that inhibit SEAP production from the ZAP and GVP cells correlate with nonspecific inhibition of transcription or toxicity. The higher potency exhibited by the prodrug compared to the parent compound in the Src SH2 cell line is probably due to increased cellular permeability. It is noted that the parent compound contains three negative charges and still demonstrates inhibitory activity in the two-hybrid cells. We believe that even small amounts of

Figure 6

Compounds that bind to the Src SH2 domain. Two Src SH2 domain binding compounds, Ac-(4-(difluorophosphonomethyl))-L-Phe–L-Glu–*N*(methyl)(cyclohexylpropyl) (**1**) and the bis(pivaloxymethyl) prodrug (**2**).



highly charged compounds can enter the cells via pinocytosis, particularly as the cells are incubated with compound for a relatively long duration (20 h). It is also of note that this compound is relatively lipophilic, potentially compensating for poor membrane permeability exerted by the high overall charge. We have compared the effects of several structurally similar compounds containing an equivalent negative charge and with different IC_{50} values in the *in vitro* binding assay. We have found a direct relationship between the extent of inhibition of SH2 binding *in vitro* and the inhibition measured in the two-hybrid system.

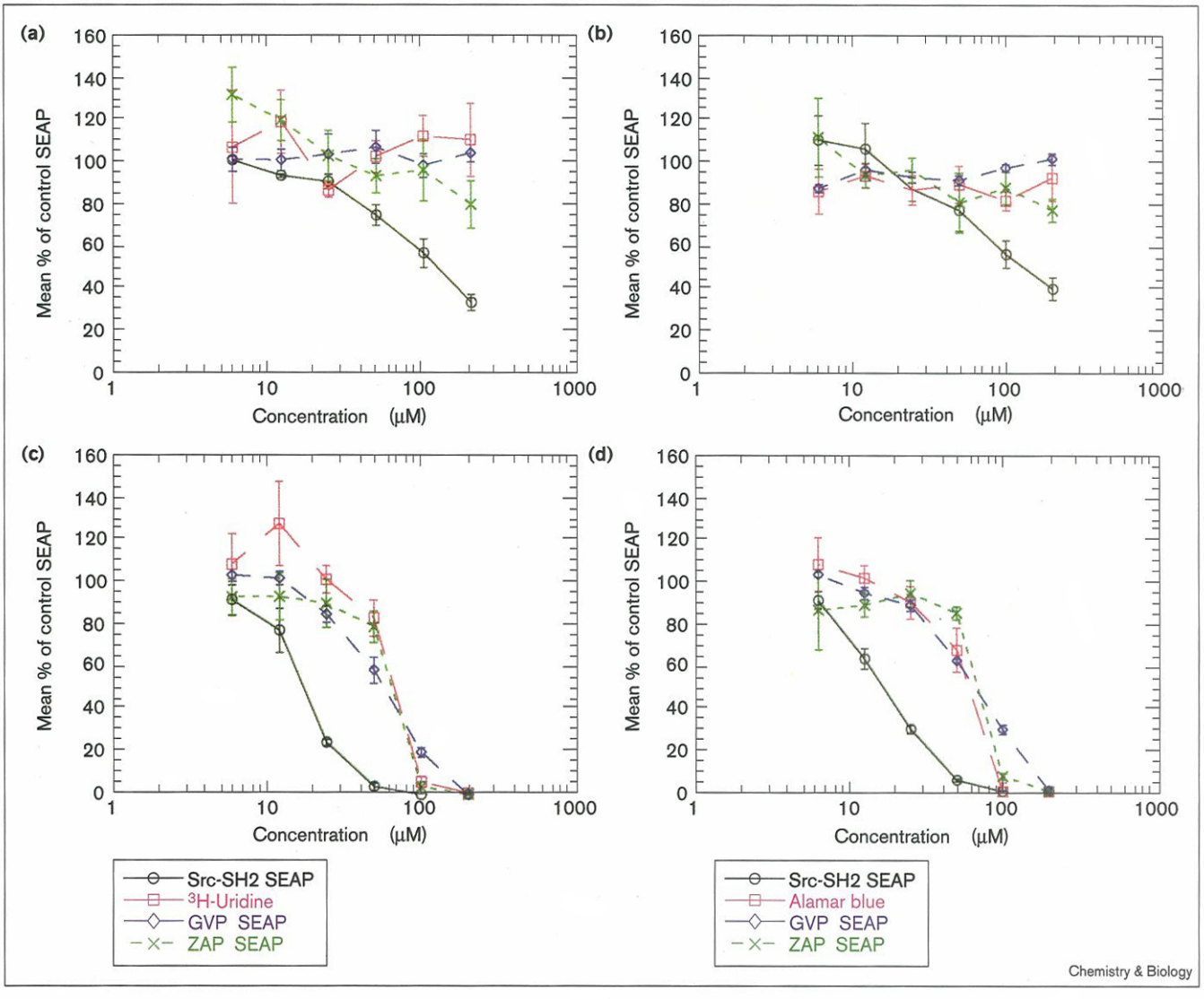
Therapeutic agents that specifically target proteins whose functions are unique and critical for particular disease states offer the potential of reduced side effects. New cellular assays with target-dependent readouts would facilitate the identification of such agents. In this report, we have described a novel two-hybrid assay using human components in mammalian cells, where specific SH2-domain–ligand interactions control the expression of a reporter gene. Stable cell lines have been constructed in which the association of the human ZAP-70 or Src SH2 domains with a phosphorylated ligand can be induced and readily monitored. These cells form the basis of an assay to identify cell-permeant inhibitors of SH2-mediated interactions. In contrast to target cell assays (e.g., using T cells or osteoclasts), in which an inhibitor can interfere with the assay readout at many points in a pathway, these assays are dependent on the target interaction of interest. Compounds that selectively inhibit the Src SH2 domain might be useful for the treatment of osteoporosis. ZAP SH2 domain inhibitors would be expected to be selective T-cell inhibitors.

Configuring the mechanism-based two-hybrid assay in a mammalian cell offers a number of advantages over a yeast-based system. First, as the cellular permeability of compounds into human and yeast cells does not always correlate, the mammalian cell assay might identify active compounds that would not be detected in a yeast cell assay. Second, the mammalian cell could contain auxiliary factors that stabilize or activate the protein of interest. In addition, the mammalian cell might tolerate certain aspects of the assay, such as the requirement for a protein that is difficult to express or toxic in a yeast cell.

The development of the SH2-domain-based two-hybrid assay was complicated by the need to phosphorylate the ligand. In contrast to the 'tri-hybrid' system reported in yeast [27], we solved the problem by linking the ligand and kinase within the DNA-binding-domain fusion. This orientation appears to be critical, because the reverse orientation, with the kinase and ligand linked to the activation domain, failed to express SEAP. Similarly, the ability to hormonally regulate the system was context dependent. The estrogen receptor ligand-binding domain conferred regulation when linked to the DNA-binding-domain fusion but not when part of the activation-domain fusion protein. The mechanism of this context dependence is not understood and our results suggest that, in general, multiple experimental designs must be explored to define conditions for two-hybrid formation. The ability to post-translationally regulate fusion proteins could find general application to evaluate protein–protein interactions in mammalian cells dependent on serine/threonine phosphorylation, acetylation, or perhaps proteolysis.

We have shown that the SH2 mechanism-based assay can be used to detect Src SH2 domain inhibitors. The specificity of action of these inhibitors has been assessed by testing for nonspecific effects on transcription (^{3}H-uridine incorporation) and cell viability (alamar blue reactivity) and by using other appropriately engineered cell lines as counterscreens. For example, general inhibitory effects on translation, secretion, GAL4 DNA binding or ER-LBD function would be identified when using cells that produce SEAP under the control of a GAL4–ER-LBD–VP16 transcription factor. Src-kinase inhibitors

Figure 7



In vivo assay of compounds that bind to the Src SH2 domain. Compounds **1 (a,b)** and **2 (c,d)** were assayed as described in the Materials and methods section. Data are shown as mean % of control SEAP production relative to vehicle alone treated cells. In one set of experiments, ^{3}H-uridine incorporation was monitored to measure nonspecific effects on transcription (a,c). In a separate experiment, alamar blue reactivity was monitored to measure effects on cell viability (b,d). Each data point represents the results of triplicate samples ± the standard deviation.

should similarly affect both the Src and ZAP SH2 two-hybrid cells. Cell lines in which two-hybrid formation is dependent on the SH2 domains from Syk and Fyn have been constructed (data not shown). These and additional cell lines could be used to assess the specificity of a compound for a particular SH2 domain.

In a manner similar to that described here, we have designed a Src SH3 mechanism-based mammalian cell assay to identify compounds that block Src SH3 binding. The affinity of SH3 domain interactions is 10–100-fold lower than that observed with SH2 domains [28]. To detect lower affinity interactions, we expressed the SH3 domain in a novel manner, linked to bundled transcriptional activation domains (R.J.R. and S. Natesan, unpublished observations). By increasing the number of cognate DNA-binding sites in the reporter-gene promoter [29] or by increasing the strength of the transcriptional-activation domain fusion protein (data not shown), the sensitivity of detection of two-hybrid protein–protein interactions could also be improved, allowing the detection of weak protein–protein interactions.

Significance

We have described an SH2 mechanism-based two-hybrid assay using mammalian cells. This assay will be useful as a screen for antagonists of SH2-mediated protein–protein

interactions that mediate disease states in humans. Cells can be cultured and all assay steps performed using 96-well dishes, convenient for high-throughput screening purposes. Using the appropriate cell lines, counter-screens can be designed to address the specificity of inhibitor action. The assay can provide rapid structure–activity relationship information, taking into account not just binding, but also cell membrane permeability. Mechanism-based assays might have general utility as a screen for inhibitors of specific protein–protein interactions and could be particularly useful when performing cellular screens for inhibitory compounds using protein domains where binding partners have been identified but cellular functional assays are not yet available.

Materials and methods

Plasmid constructions

The retroviral vector pLH, which contains the Hygromycin B resistance gene (hph) driven by the Moloney murine leukemia virus LTR and a unique downstream *Cla*I site, was constructed as follows: pWZL-Bleo (a gift of J. Morganstern) was cut with *Bam*HI and *Cla*I to release the bleo gene and a *Hin*dIII-*Cla*I fragment, from pBabe-Hygro [30], containing the hph gene was inserted. pLH-5×GAL4-IL2-SEAP was created by cloning a *Cla*I-BstBI fragment from 5×GAL4-IL2-SEAP, which contains five GAL4 sites upstream of a minimal IL2 promoter driving expression of the SEAP gene (a gift of J. Morganstern and S. Ho) into the *Cla*I site of pLH. It was oriented such that the directions of transcription from the viral LTR and the internal GAL4-IL2 promoters are the same. The properties of SH2-domain– and vSrc-kinase–ITAM fusion proteins were first examined by expression in yeast (R.J.R., unpublished observations). Sequences encoding SH2 domains and vSrc kinase-ITAM residues were then shuttled from the yeast expression plasmids into mammalian cell expression vectors for transfection into the HT1080B reporter cell line. Two mammalian cell expression plasmids were use in this study for the production of novel fusion proteins. pBXG1 directs the expression of GAL4 DNA-binding domain fusion proteins in mammalian cells. pBXG1 is a pECE72-based vector [31]. pECE72 has the SV40 virus origin of replication, SV40 early promoter and SV40 polyadenylation regulatory sequences. GAL4 (1–147) and multiple cloning sites C-terminal to GAL4 coding sequences were obtained from pSKGAL147 [32] as a *Hin*dIII/*Xba*1 fragment and inserted into *Hin*dIII/*Xba*1 cut pECE72 to generate pBXG1. pMVN1 (kindly provided by I. Sadowski) was used for the production of transcriptional activation domain fusion proteins in mammalian cells. pMVN1 contains the SV40 early promoter, HSV TK translational leader, SV40 nuclear localization sequences and VP16 activation domain residues followed by multiple cloning sites for the construction of novel fusion proteins. Residues 248–526 of the vSrc kinase (Genbank Accession #J02342) domain was placed downstream of GAL4 DNA binding domain or transcriptional activation domain sequences. Sequences encoding residues 52–164 of the human Zeta chain (Genbank Accession # J04132) or sequences encoding residues 203–244 of the human Beta ITAM were then placed 3′ to the vSrc kinase DNA. Sequences encoding residues 1–259 of human ZAP-70 (a gift of M. Botfield, Genebank Accession #L05148), 144–249 of Src (SH2) or 84–249 of Src (SH3/SH2) were used for the construction of SH2 domain fusion proteins [33]. VPS3 contains Src residues 84–143. The ER-LBD consists of residues 282–595 of the human estrogen receptor (Genbank Accession #M12674). Additional details about the constructs described in this report are available by request.

Cell culture

HT1080 cells (ATCC CCL-121), derived from a human fibrosarcoma, were grown in MEM supplemented with nonessential amino acids and 10% Fetal Bovine Serum. Helper-free retroviruses containing the 5×GAL4-IL2-SEAP reporter gene were generated by transient co-transfection of 293T cells [34] with a Psi(–) amphotropic packaging vector (a gift of D. Afar and O. Witte) and the retroviral vector pLH-5×GAL4-IL2-SEAP. To generate a clonal cell line containing the SEAP reporter gene stably integrated, HT1080B cells infected with retroviral stock were diluted and selected in the presence of 300 μg/ml Hygromycin B. Individual clones were screened for the presence of integrated reporter gene by transient transfection of a plasmid encoding a chimeric transcription factor containing a GAL4-DNA-binding domain. The most responsive clone was used for subsequent analysis. Cells were plated in six-well dishes (1×10^6 cells/well) and transfected with Lipofectamine (GIBCO/BRL) using the manufacturers recommended conditions. A total of 2.5 μg of DNA was transfected into each well: 1 μg GAL4 DNA-binding domain fusion protein, 1 μg VP16 activation domain fusion protein and 0.5 μg pUC19. Following lipofection (16 h), 1 ml of fresh media was added to each well. After 18–24 h, media was removed and assayed for SEAP as described [35] using a Luminesence Spectrometer (Perkin Elmer) at 350 nm excitation and 450 nm emission. If estrogen inducible SEAP production was to be examined, after removal of media containing lipofectamine, cells received 1 ml of fresh media containing 10 nM β-estradiol. Each transient transfection result has been reproduced at least twice and the results of a representative experiment are shown. The β-estradiol (Sigma Chemicals) was stored as a 10 mM stock solution in 100% ethanol and diluted just before use. Stable cell lines were generated by co-transfection of reporter cells with effector plasmids and pBabeNeo [30] and selected in medium containing 500 μg/ml Geneticin (GIBCO BRL).

Two-hybrid assay protocol

Stable transfected two-hybrid cells were plated into 96-well plates at 20,000 cells/well in 100 μl culture medium and then incubated for 4 h at 37°C and 5% CO_2. Test compounds were then added at the appropriate concentration with each well containing a final concentration of dimethyl sulfoxide (DMSO) equivalent to 0.1%. Untreated control samples contained culture medium supplemented with 0.1% DMSO. Compounds were tested in triplicate wells. Cells were incubated with compound for 3 h at 37°C and 5% CO_2 followed by the addition of estrogen at a final concentration of 1 μM. Cells were then incubated for an additional 16 h at 37°C and 5% CO_2. Cell-culture supernatant was collected and heat inactivated at 65°C for 60 min to inactivate background phosphatase activity. The remaining monolayer of cells was monitored for alamar reactivity or ^{3}H-uridine incorporation. Heat-inactivated supernatants from the various two-hybrid cells lines were transferred into a 96-well fluorimeter plate (Perkin Elmer) and monitored for SEAP activity by incubating with 0.66 mM 4-methylumbelliferylphosphate and 1 M diethanolamine at 37°C. Samples were analyzed on a Perkin-Elmer LS50B fluorimeter using an excitation of 350 nM and an emission of 445 nM. Cells were monitored for alamar reactivity by incubating cells with 100 μl/well of alamar blue (Alamar Biosciences, Sacramento, CA) diluted 1:10 in culture medium for 2–4 h at 37°C. Alternatively, cells were monitored for ^{3}H-uridine incorporation by incubating cells with 4 μCi/ml ^{3}H-5,6-uridine (NEN Dupont) for 90 min, washing cells three times with ice-cold phosphate -buffered saline (PBS), lysing cells with 0.2 N sodium hydroxide and then immediately adding ice cold trichloracetic acid (TCA) to a final concentration of 20%. Lysed and TCA precipitated cells were harvested onto filter mats using a TomTec (Wallac, Gaithersburg, MD) cell harvester and incorporated ^{3}H-5,6-uridine was quantitated using a Betaplate reader (Wallac, Gaithersburg, MD).

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