Interactions between Neurogranin and Calmodulin in Vivo*

(Received for publication, June 25, 1998, and in revised form, December 3, 1998)

Lisa Prichard‡§, Jean Christophe Deloulme¶, and Daniel R. Storm‡¶

From the §Department of Pharmacology, University of Washington, Seattle, Washington 98195 and ¶INSERM U 244, DBMS/BRCE, CENG, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France

Neurogranin is a neural-specific, calmodulin (CaM)-binding protein that is phosphorylated by protein kinase C (PKC) within its IQ domain at serine 36. Since CaM binds to neurogranin through the IQ domain, PKC phosphorylation and CaM binding are mutually exclusive. Consequently, we hypothesize that neurogranin may function to concentrate CaM at specific sites in neurons and release free CaM in response to increased Ca²⁺ and PKC activation. However, it has not been established that neurogranin interacts with CaM in vivo. In this study, we examined this question using yeast two-hybrid methodology. We also searched for additional proteins that might interact with neurogranin by screening brain cDNA libraries. Our data illustrate that CaM binds to neurogranin in vivo and that CaM is the only neurogranin-interacting protein isolated from brain cDNA libraries. Single amino acid mutagenesis indicated that residues within the IQ domain are important for CaM binding to neurogranin in vivo. The Ile-33 Gln point mutant completely inhibited and Arg-38 Asp-36 Arg-38 mutants reduced neurogranin/CaM interactions. These data demonstrate that CaM is the major protein that interacts with neurogranin in vivo and support the hypothesis that phosphorylation of neurogranin at Ser-36 regulates its binding to CaM.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, T4 DNA ligase, and β-agarase were purchased from New England Biolabs. The TA cloning kit was from Stratagene. Yeast media was purchased from Difco. Sheared DNA and Lex A antibody were from CLONTECH. X-Gal was purchased from 5 Prime-3 Prime, Inc., Boulder, CO. Amino acids and other chemicals were from Sigma.

Cell Culture—HEK-293 cells were maintained in Heps-buffered Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and 1% penicillin and streptomycin in a humidified 95% O₂/5% CO₂ incubator.

Yeast Media—Yeast were grown on YPAD or YC solid or liquid media. The coding sequence of a synthetic mammalian CaM (34) was polymerase chain reaction-amplified with the addition of new BamHI and EcoRI restriction sites using the neurogranin primers 5′-CCCCGAAATTCTAGGGACTGTCG-3′ and 5′-CAATGGATCCCTTAATCTCCGCTG-3′. The polymerase chain reaction product was cloned into a TA vector and then digested with BamHI and EcoRI. The gel-isolated insert was ligated into the two-hybrid BTM116 yeast expression vector.

Preparation of Neurogranin and Calmodulin Constructs—The coding sequence of rat neurogranin was polymerase chain reaction-amplified with the addition of new BamHI and EcoRI restriction sites using the neurogranin primers 5′-CCCCGAAATTCTAGGGACTGTCG-3′ and 5′-CAATGGATCCCTTAATCTCCGCTG-3′. The polymerase chain reaction product was cloned into a TA vector and then digested with BamHI and EcoRI. The gel-isolated insert was ligated into the two-hybrid BTM116 yeast expression vector.

Preparation of Neurogranin and Calmodulin Constructs—The coding sequence of rat neurogranin was polymerase chain reaction-amplified with the addition of new BamHI and EcoRI restriction sites using the neurogranin primers 5′-CCCCGAAATTCTAGGGACTGTCG-3′ and 5′-CAATGGATCCCTTAATCTCCGCTG-3′. The polymerase chain reaction product was cloned into a TA vector and then digested with BamHI and EcoRI. The gel-isolated insert was ligated into the two-hybrid BTM116 yeast expression vector.

This paper is available on line at http://www.jbc.org

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

‡ Both authors contributed equally to this paper.

§ To whom correspondence should be addressed: Dept. of Pharmacology, University of Washington, Box 357280, Seattle, WA 98195-7280. Tel.: 206-543-7028; Fax: 206-616-8621.

¶ The abbreviations used are: PKC, protein kinase C; CaM, calmodulin; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; LTP, long term potentiation.
ligated into the VP16 activation domain vector (35). Each construct was sequenced to ensure accuracy during cloning.

Site-directed Mutagenesis—Specific amino acids were mutated within the IQ domain of the BTM116-neurogranin wild-type construct. Oligonucleotides were designed to introduce the following changes: Ile-33→Gln, 5′-CCCTGCGACGCAAAAACACAGCGGAGTTTTCGGGCCCATATGGCCCAGG-3′ and 5′-CTCTGCGATAGGGCTTGGCCCAGG-3′; Arg-38→Gln, 5′-GCCAAATTCAGGGCGAGTTTTCGGGCCCATATGGCCCAGG-3′; and Ser-36→Asp, 5′-GCCAAATTCAGGGCGAGTTTTCGGGCCCATATGGCCCAGG-3′. Filter lifts and β-galactosidase assays were performed on the yeast re-streaked to the −Leu/−Trp media. For large scale transformations and two-hybrid screens, BTM116-neurogranin wild-type and mutant constructs were transformed singly into the L40 strain of yeast. The constructs were transformed into the VP16 activation domain construct as described under "Experimental Procedures." Positive transformants were streaked to media lacking histidine and leucine/tryptophan (−Leu/−Trp). Growth was assayed on the YC media lacking histidine (−His) or again to −Leu/−Trp. Growth was assayed on the −His plates after 2 days. Filter lifts and β-galactosidase assays were performed on the yeast re-streaked to the −Leu/−Trp media. Large scale transformations and two-hybrid screens, BTM116-neurogranin wild-type and mutant constructs were transformed singly into L40 and were grown on YC media lacking tryptophan (−Trp) for 2 days. A single positive transformant colony was grown in 10 ml of liquid −Trp YC media overnight. The culture was diluted into 100 ml of −Trp YC media for another 24 h and then harvested and resuspended to an OD of 0.1 in YPAD media. Large scale transformations were performed using either a rat brain library (CLONTECH) or a mouse brain library (36). Library cDNAs were isolated from colonies that grew in the absence of histidine and had β-galactosidase activity. To check for specificity, each cDNA was re-tested in a mating assay against BTM116 lamin (37) and against the original bait construct. cDNAs that retested as positive were sequenced and identified.

Western Blotting—Western blotting was utilized to demonstrate appropriate expression of wild-type and mutant neurogranin constructs in yeast. The constructs were transformed singly into the L40 strain of yeast, and positive transformants were grown for 2 days on YC media lacking leucine and tryptophan (−Leu/−Trp). Briefly, for small scale transformations, BTM116-neurogranin wild-type and two-hybrid screens were performed essentially as described (32). Yeast Transformations—

Output text:

**RESULTS**

**Interaction between Neurogranin and CaM in Vivo**—To determine if neurogranin interacts with CaM in vivo, we utilized yeast two-hybrid technology. In this system, the interaction of two proteins, expressed as fusion constructs, is monitored within the confines of a yeast cellular environment (42). Interactions are measured by read-out from two transcripational reporters. Activation of one reporter results in the production of histidine, allowing yeast to grow in the absence of this amino acid. Activation of the other reporter construct results in β-galactosidase activity which is measured by hydrolisis of an X-gal substrate that leads yeast to turn blue.

An L40 strain of yeast was transformed with a wild-type neurogranin DNA-binding domain construct and a CaM activation domain construct as described under "Experimental Procedures." Positive transformants were streaked to media lacking histidine and were assayed for growth (Fig. 1). This assay showed that neurogranin interacts strongly with CaM in...
Protein lysates were prepared as described under “Experimental Procedures,” and 15 μg of protein was loaded per lane. Molecular mass markers are in kDa on the left. Lex A protein is expressed from yeast transformed with the BTM116 vector. Expression of each of the neurogranin (NG) constructs results in a fusion protein of the appropriate size, approximately 30.5 kDa. From left to right the constructs are: BTM116, wild-type BTM116 neurogranin; Ile-33 → Gln, Arg-38 → Gln, Ser-36 → Asp, and Ser-36 → Ala.

**Fig. 2. Expression of wild-type neurogranin and neurogranin IQ domain mutants.** L40 was transformed singly with each BTM116-neurogranin construct or with BTM116 alone. Positive transformants were grown at 30 °C for 36 h in liquid media lacking tryptophan. Protein lysates were prepared as described under “Experimental Procedures,” and 15 μg of protein was loaded per lane. Molecular mass markers are in kDa on the left. Lex A protein is expressed from yeast transformed with the BTM116 vector. Expression of each of the neurogranin (NG) constructs results in a fusion protein of the appropriate size, approximately 30.5 kDa. From left to right the constructs are: BTM116, wild-type BTM116 neurogranin; Ile-33 → Gln, Arg-38 → Gln, Ser-36 → Asp, and Ser-36 → Ala.

**Fig. 3. Interaction of neurogranin IQ domain mutants with CaM.** L40 was transformed with each neurogranin mutant and VP16 CaM, and positive transformants were streaked to media lacking histidine. Plates were incubated for 2 days at 30 °C. Each plate is separated into three regions. Two separate colonies are shown for each condition. A, the BTM116-wild-type neurogranin and VP16-CaM interaction is shown as a control. Neurogranin (NG) Ile-33 → Gln is on the left, and Arg-38 → Gln is shown on the right. B, the BTM116-wild-type neurogranin and VP16-CaM interaction is shown as a control. The neurogranin Ser-36 → Asp mutant is on the left, and neurogranin Ser-36 → Ala is on the right. Results shown are representative of five separate experiments.

**Interaction between CaM and Neurogranin IQ Domain Mutants**—Site-specific point mutants were created to determine if amino acids within the IQ domain of neurogranin are important for its interaction with CaM in vivo. These point mutants included Ile-33 → Gln, Arg-38 → Gln, Ser-36 → Ala, and Ser-36 → Asp. To determine whether the mutants were adequately expressed in yeast, Western analysis was performed on yeast lysates as described under “Experimental Procedures.” An antibody that recognizes the Lex A DNA-binding domain was used to detect the fusion proteins. All of the neurogranin mutants were expressed and electrophoresed with an apparent mass of 30 kDa, the expected size for the fusion proteins (Fig. 2).

Each of the neurogranin mutants was co-expressed with CaM, and the interaction between the expressed proteins was examined. A range of interaction strengths was observed by growth on media lacking histidine (Fig. 3, A and B). Each mutant was compared with wild-type neurogranin on the same plate. The best indication of strong interactions is the ability of the yeast to form single colonies at the interior of each yeast streak. The Ile-33 → Gln fusion protein did not interact with CaM, indicating that the isoleucine within the IQ domain is particularly important for neurogranin/CaM interactions. The Arg-38 → Gln mutant interacted with CaM, but to a lesser degree than wild-type neurogranin (Fig. 3A).

In vitro data indicate that the serine within the IQ domain is a PKC phosphorylation site in neurogranin and other IQ domain proteins (8, 15, 19, 22). When phosphorylated at this site, neurogranin no longer interacts with CaM in vitro. The two-hybrid point mutants at Ser-36 were examined to define the importance of this amino acid for CaM binding and to determine if introduction of negative charge at this site inhibits CaM binding in vivo. The Ser-36 → Asp mutant was created to mimic neurogranin phosphorylated at this site by introduction of a negative charge. Mutation of the serine to an alanine (Ser-36 → Ala) removes the putative PKC phosphorylation site. The change from serine to alanine did not affect the neurogranin/CaM interaction; yeast growth was comparable to that seen with wild-type neurogranin and CaM (Fig. 3B). Conversion of serine to an aspartic acid reduced but did not completely inhibit the neurogranin/CaM interaction. This is consistent with the hypothesis that introduction of negative charge by phosphorylation at Ser-36 may reduce CaM binding.

Since the results described above using growth on media lacking histidine are qualitative, the β-galactosidase reporter was used to provide a more quantitative evaluation of these interactions (Fig. 4). The data obtained with β-galactosidase expression are consistent with those reported in Fig. 3. The Ile-33 → Gln, Arg-38 → Gln, and Ser-36 → Asp mutants did not show any interaction with CaM over background (BTM116 alone). Both wild-type neurogranin and Ser-36 → Ala constructs interacted strongly with CaM. Because of variation between individual colonies and the sensitivity of the assay, it cannot be concluded that the Ser-36 → Ala mutation has a higher affinity for CaM than native neurogranin. However, the slightly stronger interaction seen with the Ser-36 → Ala mutant may reflect basal phosphorylation of native neurogranin that lowers the signal somewhat compared with the alanine mutant.

**Does Neurogranin Interact with Other Proteins in Vivo?**—Although the data described above indicate that neurogranin interacts with CaM in vivo, this does not preclude interactions between neurogranin and other proteins. Therefore, it was of
IQ Domain of Neurogranin Is Needed for Calmodulin Interactions

Following the mating assays, we isolated 98 positive cDNAs from the rat brain transformation and 90 from the mouse brain transformation. Each of these DNAs was digested with Sau3A1, and the restriction patterns were compared. All of the digested DNAs had the same restriction pattern, suggesting that they were identical. Twenty cDNAs from each transformation were sequenced. Each of these cDNAs were identified as either rat or mouse CaM, indicating that CaM is the only neurogranin-binding protein detectable in yeast two-hybrid screens from two different rodent cDNA libraries.

Although the data described above suggests that CaM may be the only protein that interacts with neurogranin in vivo, there may be other neurogranin-binding proteins whose interactions are inhibited by CaM. The high levels of CaM in brain (10–20 μM) may have obscured the detection of other neurogranin-binding proteins. Furthermore, phosphorylated neurogranin may bind to other proteins, which would not have been detected in the original yeast two-hybrid screen. For these reasons, we re-screened the brain cDNA libraries using the Ile-33 → Gln and Ser-36 → Asp neurogranin mutants, neither of which interact strongly with CaM in vivo.

The Ile-33 → Gln mutant was screened against both libraries. Results are shown in Table I. In both screens, none of the isolated cDNAs retested as positive in the mating assay. In addition, each of the positive cDNAs were tested for interactions against wild-type neurogranin and the other neurogranin mutants in mating assays. None of the cDNAs showed positive interactions with any of the baits tested. A subset of these cDNAs were sequenced, and none of the sequences correlated to “in frame” sequences. Similarly, in the Ser-36 → Asp neurogranin mouse brain screen, none of the isolated cDNAs retested as positive in mating assays or when retransformed in yeast with each of the neurogranin constructs. Sequencing of 12 of the putative positives did not reveal in frame sequences.

Obvious differences were seen between the wild-type and mutant neurogranin two-hybrid screens. All transformations were equally efficient, but many fewer positives were found in the mutant screens. The β-galactosidase activity in the wild-type screens appeared within minutes, whereas positives detected in the mutant screens appeared in hours. At each step in the screening process, the number of mutant positives decreased substantially, while very few of the positives in the wild-type screens were lost during successive screens. We were unable to detect any neurogranin-binding proteins by screening the mutant neurogranins suggesting that CaM is the major protein that interacts with neurogranin in vivo.

Neurogranin Regulates Calmodulin-sensitive Targets in Vivo—Previous studies have shown that neurogranin and neuromodulin can regulate calcium/CaM-sensitive enzymes in vitro (24, 25). For example, nitric oxide synthase activity decreases in the presence of increasing concentrations of neuromodulin and neurogranin. We used the CaM-stimulated adenyl cyclases, AC1 and AC8, to determine whether neurogranin could regulate CaM-sensitive targets in vivo. AC1 and AC8 are both stimulated by Ca2+ and CaM, in vivo. In these experiments, 293 cells were transiently co-transfected with neurogranin and either AC1 or AC8. Enzyme activity was measured in response to the calcium ionophore, A23187. Ca2+ stimulation of AC1 or AC8 in vivo was markedly reduced when neurogranin was co-expressed (Fig. 5). These data indicate that neurogranin has the potential to regulate the activity of CaM-regulated enzymes in vivo by complexing and lowering the effective concentration of free CaM.
Histidine-positive transformants grew on media lacking histidine 48 h following transformation. These colonies were retested for activities were measured in triplicate and were normalized to as the ratio of cAMP to a total pool of adenine nucleotides. Acti-

enzyme expression. Error shown is three separate experiments.

either AC1 (293 cells were transiently transfected with pcDNA3-neurogranin and

bait plasmids were segregated. L40 containing the library plasmid was then mated to the AMR70 strain transformed with the bait plasmid, but not lamin, were identified as positive after mating. The percent of colonies that retested as positive is shown in the mating column. Proteins found to interact with the neurogranin baits are indicated as isolated clones.

Ca2+ response to PKC phosphorylation and increases in intracellular

43, 44), and recently it was shown that neuromodulin binds CaM in vivo (45, 46). The objectives of this study were to determine if neurogranin binds CaM through its IQ domain and to determine if other neurogranin-binding proteins can be detected by yeast two-hybrid technology. The discovery of other neurogranin-binding proteins would provide mechanistic insights concerning the role of neurogranin in signal transduction and would suggest alternative biochemical mechanisms for neurogranin in neurons.

Our data indicate that neurogranin and CaM are also in vivo binding partners and that the interaction occurs primarily through the IQ domain of neurogranin. The Ile-33 → Gln mutation completely inhibited the CaM/neurogranin interaction, illustrating the importance of hydrophobic interactions for CaM binding to neurogranin. Reduced binding of CaM to the Ser-36 → Asp mutant of neurogranin is consistent with the hypothesis that introduction of negative charge at Ser-36 by PKC phosphorylation lowers CaM binding affinity.

CaM was the only neurogranin-interacting protein isolated from the rat and mouse brain two-hybrid screens. In addition, neurogranin-binding proteins were not detected using neurogranin and neuromodulin are PKC substrates, yeast two-hybrid screens did not identify PKC as an interacting protein with either neuromodulin or neurogranin. Nevertheless, our data indicate that CaM is the only neurogranin-binding protein detectable by an extensive yeast two-hybrid screen; no other neurogranin-interacting proteins other than PKC have been identified (48). Yeast two-hybrid screens do not necessarily detect all significant interactions. For example, although neurogranin and neuromodulin have been shown to regulate the activity of CaM-stimulated enzymes (24, 25). Addition of neurogranin or neuromodulin to Ca2+/CaM-activated nitric oxide synthase re-

whether these proteins bind CaM and are phosphorylated by PKC in vivo. Both neurogranin and neuromodulin are phosphorylated by PKC in vivo (8, 43, 44), and recently it was shown that neuromodulin binds CaM in vivo (45, 46). The objectives of this study were to determine if neurogranin binds CaM in vivo through its IQ domain and to determine if other neurogranin-binding proteins can be detected by yeast two-hybrid technology. The discovery of other neurogranin-binding proteins would provide mechanistic insights concerning the role of neurogranin in signal transduction and would suggest alternative biochemical mechanisms for neurogranin in neurons.

Our data indicate that neurogranin and CaM are also in vivo binding partners and that the interaction occurs primarily through the IQ domain of neurogranin. The Ile-33 → Gln mutation completely inhibited the CaM/neurogranin interaction, illustrating the importance of hydrophobic interactions for CaM binding to neurogranin. Reduced binding of CaM to the Ser-36 → Asp mutant of neurogranin is consistent with the hypothesis that introduction of negative charge at Ser-36 by PKC phosphorylation lowers CaM binding affinity.

CaM was the only neurogranin-interacting protein isolated from the rat and mouse brain two-hybrid screens. In addition, neurogranin-binding proteins were not detected using neurogranin mutant proteins that do not bind CaM. It is interesting that other two-hybrid screens performed on IQ domain proteins have yielded similar results. After extensive testing, CaM was the only protein found to interact with neuromodulin (45), and it was also the only protein isolated using Igloo, a human putative Ras GTPase-activating protein that contains four IQ domains, CaM was the most frequent interacting protein identified (48). Yeast two-hybrid screens do not necessarily detect all significant interactions. For example, although neurogranin and neuromodulin are PKC substrates, yeast two-hybrid screens did not identify PKC as an interacting protein with either neuromodulin or neurogranin. Nevertheless, our data indicate that CaM is the only neurogranin-binding protein detectable by an extensive yeast two-hybrid screen; no other neurogranin-interacting proteins other than PKC have been identified in vitro or in vivo.

If neuromodulin and neurogranin regulate free CaM levels, these proteins should regulate the activity of CaM-stimulated enzymes. In support of this hypothesis, both neurogranin and neuromodulin have been shown to regulate the activity of CaM-dependent enzymes (24, 25). Addition of neurogranin or neuromodulin to Ca2+/CaM-activated nitric oxide synthase re-

FIG. 5. Neurogranin inhibits adenylyl cyclase activity in vivo. 293 cells were transiently transfected with pcDNA3-neurogranin and either AC1 (A) or AC8 (B). As a control, when not co-expressed with neurogranin, adenylyl cyclases were expressed with equivalent amounts of a green fluorescent protein. Adenylyl cyclase activity was measured as the change in intracellular cAMP levels ± 5 mM A23187 as described under "Experimental Procedures.” Enzyme activity is shown as the ratio of cAMP to a total pool of adenine nucleotides. Activities were measured in triplicate and were normalized to β-galactosidase expression. Error shown is ± S.D. Data shown are representative of three separate experiments.

Table I

Summary of wild type and mutant neurogranin two-hybrid screens

Steps of each two-hybrid screen are outlined to demonstrate screen efficiency. Each bait was screened against a rat and/or mouse brain library. The transformation efficiency is shown as number of transformants. Each library expresses between 1 × 106 and 3 × 106 independent clones. Histidine-positive transformants grew on media lacking histidine 48 h following transformation. These colonies were retested for β-galactosidase (β-Gal) activity. Strong activity indicates conversion of X-Gal, demonstrated by the color blue, in 30–60 min. Any activity seen from 4–24 h was identified as weak. Library and bait plasmids were segregated. L40 containing the library plasmid was then mated to the AMR70 strain

DISCUSSION

Neurogranin and the related IQ domain protein, neuromodulin, are hypothesized to regulate neuronal CaM levels in response to PKC phosphorylation and increases in intracellular Ca2+ (3, 16, 23). Therefore, it has been important to determine...
duces NO synthase activity. This inhibition is not seen when neurogranin or neuromodulin are phosphorylated, or at high Ca$^{2+}$ concentrations, indicating that sequestration of CaM by neurogranin or neuromodulin regulates the activity of CaM-stimulated enzymes. Our results show that neurogranin may also regulate calmodulin-stimulated targets in vitro. When co-expressed with neurogranin, the calcium/calmodulin-stimulated activities of adenyl cyclases AC1 and AC8 were inhibited. As both AC1 and AC8 are expressed in brain, they may represent physiological targets for neurogranin.

The existence of presynaptic and postsynaptic CaM-binding proteins that may regulate free CaM in neurons has led to the interesting hypothesis that these proteins may play a pivotal role during synaptic plasticity, e.g. long term potentiation (LTP) (30, 31). For example, neurogranin and neuromodulin are both phosphorylated by PKC during LTP (49–53). There are several forms of mechanistically distinct LTP expressed in the hippocampus and other areas of brain, including forms that are presynaptically and others that are hypothesized to be postsynaptically (54). In either case, initial increases in intracellular Ca$^{2+}$ arising because of activation of voltage-sensitive Ca$^{2+}$ channels or glutamate receptors are thought to initiate signal transduction cascades leading to enhanced synaptic efficacy (55–57). CaM-stimulated enzymes including CaM kinases, NO synthase (58), and adenyl cyclases (59, 60) are thought to play major roles in the initiation, maintenance, and propagation of LTP. Consequently, neuromodulin and neurogranin may be critical components of the molecular machinery used for modulation of synaptic plasticity and the development of learning and memory in vertebrates. Our demonstration that neurogranin can regulate brain adenyl cyclases through its interactions with calmodulin is consistent with this theory.

Acknowledgments—We thank Dr. J. Camonis for use of the mouse brain two-hybrid library. We thank Dr. A. Waskiewicz, Dr. A. Vojtek, and Dr. J. A. Cooper for reagents and assistance in performing the two-hybrid screens. We also thank Dr. Guy Chan for adenylyl cyclase two-hybrid screens. We also thank Dr. A. Waskiewicz, Dr. A. Vojtek, and Dr. J. A. Cooper for reagents and assistance in performing the two-hybrid screens. We also thank Dr. Guy Chan for adenylyl cyclase two-hybrid screens. We also thank Dr. A. Waskiewicz, Dr. A. Vojtek, and Dr. J. A. Cooper for reagents and assistance in performing the two-hybrid screens.