Gateway RFP-Fusion Vectors for High Throughput Functional Analysis of Genes

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There is an increasing demand for high throughput (HTP) methods for gene analysis on a genome-wide scale. However, the current repertoire of HTP detection methodologies allows only a limited range of cellular phenotypes to be studied. We have constructed two HTP-optimized expression vectors generated from the red fluorescent reporter protein (RFP) gene. These vectors produce RFP-tagged target proteins in a multiple expression system using gateway cloning technology (GCT). The RFP tag was fused with the cloned genes, thereby allowing us to localize the expressed proteins in mammalian cells. The effectiveness of the vectors was evaluated using an HTP-screening system. Sixty representative human C2 domains were tagged with RFP and overexpressed in HiB5 neuronal progenitor cells, and we studied in detail two C2 domains that promoted the neuronal differentiation of HiB5 cells. Our results show that the two vectors developed in this study are useful for functional gene analysis using an HTP-screening system on a genome-wide scale.

Keywords: Gateway Cloning System; Red Fluorescent Protein; High Throughput Screening System.

Introduction

Over the last decade rapid progress in genome sequencing has led to the identification of great numbers of genes, and has made it possible to create and apply repositories of defined functional elements to perform high throughput (HTP) genome-wide analyses (Gibbs et al., 2004; Waterston et al., 2002). However, most gene products predicted from the currently available genome sequences remain functionally uncharacterized. One essential step in the development of genome-wide analyses is the systematic mapping of macromolecular interactions and biochemical reactions using reverse proteomics approaches (Walhout and Vidal, 2001). Reverse proteomics projects, in turn, require the cloning and manipulation of large numbers of protein-encoding sequences, or open reading frames (ORFs).

Recently, cloning systems employing in vitro recombination have been developed, and are amenable to HTP cloning (Hartley et al., 2000; Siegel et al., 2004). Gateway cloning technology (GCT) is one such system, which uses in vitro site-specific recombination to clone and subsequently transfer DNA segments between vector backbones (Hartley et al., 2000). In this system, a fragment carrying attB1 and attB2 recombination sites at its ends is prepared by the polymerase chain reaction (PCR), and is introduced into a donor vector carrying attP1 and attP2 recombination sites by an in vitro recombination reaction driven by BP clonase. In turn, master vectors (so-called ‘entry clones’) are produced, which contain the fragment of interest carrying attL1 and attL2 recombination sites.

Abbreviations: GCT, gateway cloning technology; HTP, high throughput; ORF, open reading frames; RFP, red fluorescent protein.

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This system has been used to generate several large clone collections, such as those for Xenopus (http://sgc.nci.nih.gov) and zebrafish (http://zgc.nci.nih.gov), as well as many human, mouse, and rat genes (http://mgc.nci.nih.gov). Some full-length clones have also been used to establish entry clones through GCT. Furthermore, an initial version of the human ORFeome, comprising 8,076 human ORFs, was recently generated using GCT (Rua et al., 2004).

Although the GCT is a powerful tool for multi-gene cloning, few practical expression vectors for tagging with fluorescent proteins are commercially available. In this work we generated novel vectors that express red fluorescent protein (RFP)-tagged proteins using GCT. These vectors can be used with any entry clones and can produce any protein of interest fused to RFP in mammalian cell lines. In addition, we tested whether the RFP-tagging vectors could be used in HTS analysis to obtain cellular images of 60 representative human C2 domains.

Materials and Methods

Construction of gateway destination vectors pDsRed-Monomer-N1 (Clontech) was used as a backbone vector for pDEST-N-RFP (Fig. 1A). The suicide ccdB box, which contains the ccdB gene (Bernard and Couturier 1992), the chloramphenicol (CM) cat gene, and attR sites, was amplified with pDEST 15 (Invitrogen) as template, and with a BamHI site in the upper primer and a BglII site in the lower primer (5′-GGGGATCCGCGGACT-3′ and 5′-GGGTGATACACACTTTTGTTGACAAAAGC-3′, respectively). The PCR product was partially digested with the same enzymes, and cloned into the BamHI site of pDsRed-Monomer N1. For pDEST-RFP-C, we used pEGFP-C1 (Clontech) as a backbone vector (Fig. 1B). TheDsRed-Monomer gene was amplified from pDsRed-Monomer N1 vector, with the upper and lower primers containing AgeI and BamHI sites in the lower primer (5′-GGGACCGGTCGCCACC-3′ and 5′-GGGGATCCGCGGACT-3′) and 5′-GGGGATCCGCGGACT-3′ and 5′-GGGGATCCGCGGACT-3′, respectively). The suicide ccdB box was also amplified from the above-mentioned pDEST15 vector, with upper and lower primers containing BglII and BamHI sites, respectively (5′-GGGGATCCAACAGTTGGTACAAAAGGC-3′ and 5′-GGGGATCTCTAACACACTTTTGTTGACAAAAGGC-3′) and 5′-GGGGATCCGCGGACT-3′ and 5′-GGGGATCCGCGGACT-3′). A stop codon (underlined in bold) was added to the lower primer to prevent tagging unnecessary amino acids. The two products were partially digested with the same enzymes and were cloned into the AgeI and BamHI sites of pEGFP-C1 vector.

Construction of expression vectors for RFP-fused C2 domains Entry clones of human C2 domains were constructed as follows. We initially identified 175 published C2 domains with conserved regions from 250 proteins listed in the Pfam (www.sanger.ac.uk/Software/Pfam) and NCBI (www.ncbi.nlm.nih.gov) databases. In order to obtain human C2 domains, reverse transcription (RT)-PCR was performed using total RNAs isolated from human brain, placental tissues and HeLa cells. Human C2 domains were PCR amplified from the pooled complementary DNAs (cDNAs) using target sequence-specific attB1/attB2 primers. The upper primer (5′-GGGGATCCGCGGACT-3′) and the attB1 sequences (underlined) followed by a Kozak sequence and start codon sequences (bold), were introduced. The lower primer was 5′-GGGGATCCGCGGACT-3′ and the attB2 sequence (underlined) was introduced immediately after the last stop codon (bold). The PCR products were cloned into the pDONR207 donor vector (Invitrogen), and their sequences confirmed (Macrogen Inc.). 150 human C2-domain entry clones were obtained by GCT. From these, we selected 60 entry clones at random and introduced them into the pDEST-RFP-C destination vector via the LR reaction of GCT.

Cell culture, HTP transfection, and adenovirus infection Immortalized HiB5 cells were cultured at 33°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) with 0.11 mg/ml sodium pyruvate, 3.7 mg/ml NaHCO3, 50 μ/l penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (FBS). For differentiation as previously reported (Kwon, 1997; Sung et al., 2001), cells were shifted to the chemically defined N2 medium containing 5 μg/ml insulin, 100 μg/ml transferrin, 20 nM progesterone, 30 nM selenium, 60 μM putrescine, 0.11 mg/ml sodium pyruvate, and 2 mM glutamine at 39°C for 2 d.

The expression-ready subset of human C2 domains was transfected into cultured HiB5 cells at 33°C in 96-well plates. In a semi-automated process, each expression vector (200 ng) was incubated with a liposomal transfection reagent (lipofectamine 2000; Invitrogen) and introduced into a well containing ~10,000 HiB5 hippocampal progenitor cells. After further incubating for 48 h, cell images were acquired using an In Cell Analyzer 1000 automated high-content imaging system (Amersham Biosciences). Finally, we screened for human C2 domains that induced neurite outgrowth of HiB5 cells. We also constructed two replication-defective adenoviruses (rAd5) containing GFP-tagged CPN9-C2 and PKCδ-C2, respectively (Neurogenex Co., Korea). HiB5 cells were infected at the 90% level at a multiplicity of infection (MOI) of 50.

Imaging analysis and immunocytochemistry The screened C2 domains were re-analyzed by the RFP fluorescence-detection method with a confocal microscope (Olympus Fluoview FV 1000) mounted on an inverted microscope (Olympus BX81) and fitted with a 60× objective. The excitation light source was the 543-nm line of a green HeNe laser. The emitted fluorescence was passed through a 580-nm (40-nm bandwidth) primary barrier filter before it reached the photo-multiplier tube. The laser intensity was minimized to prevent dye bleaching. The digital image output was 512 × 512 pixels with a 32-bit resolution. For the imaging analysis the cells were plated onto glass coverslips at 4 × 105 cells/ml. After 2 days they were washed twice with
A new set of RFP-tagging expression vectors for HTP

We decided to base our new RFP-tagging expression vec-
tors on recombination-based cloning using the GCT series
of plasmids (Invitrogen). GCT uses a site-specific recom-
bination reaction whereby a gene of interest is cloned into
a so-called entry vector (pENTR) that is then recombined
with a so-called destination vector (pDEST) to produce
the desired expression vector. This cloning system permits
the easy assembly of a variety of expression vectors in
one step. In our case we designed the destination vectors
to contain RFP flanked by a suicide ccdB box.

The destination vectors we developed are essentially
modifications of the existing pDsRed-Monomer-N1 and
pDsRed-Monomer-C1, which encode the DsRed-Monomer
protein (Fig. 1A). This reporter protein is a monomeric
mutant derived from the tetrameric Discosoma sp. RFP
DsRed (Matz et al., 1999). By performing the gateway re-
combination reaction with pENTR containing a gene of
interest, new destination vectors are produced that express
RFP-tagging proteins under the control of the cytomega-
lovirus immediate-early promoter (CMV).

HTP screening of the putative roles of C2 domains in
neuronal differentiation of HiB5 cells

Our destination vectors were designed for the HTP screening of gene functions on a genome-wide scale and the effectiveness of these destination vectors for HTP screening was evaluated using C2 domains. The C2 domain, a conserved protein module of ~120 amino acids, was originally defined as homologous to the C2 regulatory region of protein kinase C (reviewed in Newton and Johnson, 1998). It is now known to be present in numerous eukaryotic signaling proteins, including kinases, GTPase-activating proteins, ubiquitination enzymes and proteins involved in vesicular trafficking. In addition, Benes et al. (2005) recently showed that the C2 domain of PKCδ can directly bind phosphotyrosine.

To screen for C2 domains involved in neuronal differ-
entiation we selected 60 entry clones of human C2 do-
 mains at random and converted them into pDEST-RFP-C
expression vectors by the LR reaction of GCT (Fig. 2A
and Table 1). The RFP-tagging expression vectors were
transiently transfected into HiB5 hippocampal progenitor
cells in 96-well plates. After 48h incubation, cellular im-
ages were captured, and the effects of the C2 domains on
neurite outgrowth were assessed (Fig. 2B).

HiB5 cells comprise a multipotent hippocampal stem
cell line isolated from the embryonic day 16 rat hippo-
campus, in which precursors of pyramidal cells initiate
proliferation (Renfranz et al., 1991). They are immortal-
ized by the temperature-sensitive SV40 large T antigen,
grow at a permissive temperature of 33°C, and express
the stem-cell marker nestin. When incubated in N2 me-
dium at the non-permissive temperature of 39°C, they
stop growing and many die. However, 30–40% of the
cells survive, and less than 30% of the surviving cells
differentiate into cells with neurite-like structures. We

Results and Discussion

A new set of RFP-tagging expression vectors for HTP

We decided to base our new RFP-tagging expression vec-

Fig. 1. Schematic of the new RFP-tagging expression vectors. The
two destination vectors, pDEST-N-RFP (A) and pDEST-RFP-C
(B) are depicted. These vectors contain the suicide ccdB box
(attB1 site, ccdB gene, chloramphenicol cat gene (CM R), and
attR2 site). The cyan boxes indicate the attR1/attR2 recombi-
ation sites. The red and yellow arrows show the locations and ori-
entation of several ORFs and the cytomegalovirus immediate-
early promoter (CMV), respectively. Restriction enzyme sites are
also shown. Kana/Neo R, Kanamycin/Neomycin resistance gene.

DMEM and monitored with the confocal microscope. The immu-
nocytochemistry protocol was modified from Choi et al. (2005).
Briefly, HiB5 cells grown on coverslips (4 × 10^4 cells) were fixed
in 4% paraformaldehyde and permeabilized in cold methanol for
5 min. They were then incubated with primary pan-neuronal neu-
rofilament marker monoclonal antibody (SMI 311, Covance Re-
search Products) against neurofilament (NF; 1:500) overnight at
4°C. The following day the cells were washed and treated with
fluorescein isothiocyanate (FITC)-conjugated secondary antibody
(HTP transfection) or Cy3-conjugated secondary antibody (rAd
infection) for 1 h at room temperature. All images were cap-
tured on the same confocal microscope.
Table 1. Human genes containing C2 domains used in this study. The listed genes are identified by arrows and numbers matched with the images shown in Fig. 2B.

<table>
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<th>Nucleotide region</th>
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measured the effects of the C2 domains on the formation of these neurite-like structures at 33°C. Based on the results, we screened six C2 domains that appeared to promote the formation of neurite-like structures and settled on the C2 domains of PKCδ (PKCδ-C2) and of copine9 (CPNE9-C2) for further study. The selected C2 domains promote the neuronal differentiation of HiB5 cells In order to confirm that neurite outgrowth was induced by PKCδ-C2 and CPNE9-C2, each RFP-tagged C2 domain was transfected into HiB5 cells and their ability to induce morphological changes was monitored with a confocal microscope. Since PKCδ-C2 and CPNE9-C2 were fused to RFP it was very easy to detect the transfected cells, which were examined for the presence of neurites, defined as processes longer than the length of two cell bodies. As shown in Fig. 3A, the cells expressing PKCδ-C2 and CPNE9-C2 had well-defined neurite-like structures. In addition, neuronal differentiation was checked by immunocytochemistry for neurofilament (NF), a differentiation marker for neuronal cells; overexpression of PKCδ-C2 or CPNE9-C2 led to an increase in the number of transfected cells bearing neurites at the permissive temperature (Fig. 3B).

Since only 30−40% of the HiB5 cells survive in N2 medium at the non-permissive temperature of 39°C, and transfection efficiency is very low in this condition, it was difficult to assess the activity of the transfected genes. Therefore we constructed recombinant adenoviruses containing GFP-tagged CPNE9-C2 and PKCδ-C2, to overcome the low efficiency of transfection and measure the effect of the two selected C2 domains on neuronal differentiation at 39°C. As shown in Fig. 3C, PKCδ-C2 and CPNE9-C2 clearly increased the number of cells bearing neurites at 39°C.

In accord with our result it has been reported that PKCδ is involved in the nerve growth factor signaling that elicits neuronal differentiation of PC12 cells (O'Driscoll et al., 1995) and in neurogenic fibroblast growth factor signaling in both PC12 cells and hippocampal cells (Corbit et al., 1999). Moreover the regulatory domain of PKCδ (RD-PKCδ) enhances neurite outgrowth of HiB5 cells independent of the catalytic activity of the protein (Ling et al., 2004; Trollér and Larsson, 2006). Since the regulatory domain contains C1 and C2 domains (reviewed in Newton and Johnson, 1998), the PKCδ-C2 -induced neurite outgrowth described above implies that the C2 domain of PKCδ plays an important role in RD- PKCδ-induced neu-
Fig. 2. Gene mining of C2 domains using GCT, and their expression images. **A.** C2 domains were amplified by RT-PCR with primers containing attB1/attB2 sites, and cloned into pDONR207 by the BP reaction. The C2 domains in pENTR-C2 were converted to pDEST-RFP-C destination vectors by the LR reaction. **B.** pDEST-RFP-C2 expression vectors were transfected into HiB5 cells in 96-well plates. Images were captured using a HTP imaging system (In Cell Analyzer 1000). The captured images are identified by arrows and numbers. Gen, Gentamycin resistance gene; Kana, Kanamycin resistance gene.

Fig. 3. Images of the screened C2 domains, and C2 domain-induced neuronal differentiation. **A.** Two C2 domains selected from the initial screening were reexamined in HiB5 cells with a confocal microscope, and their ability to induce neurite outgrowth was confirmed by immunoreactivity for neurofilament (NF). **B–C.** Quantification of the number of RFP or GFP-positive HiB5 cells with neuritis (NF-positive) longer than two cell bodies expressing RFP- or GFP-tagged CPNE9-C2 and PKCδ-C2. Data are means ± SEM of three separate experiments (** P < 0.001 or * P < 0.05, by one-way ANOVA).

Copine 9 (CPNE9) belongs to the copine family, a novel family of ubiquitous Ca\(^{2+}\)-dependent phospholipid-binding proteins (reviewed in Tomsig and Creutz, 2002) and was recently cloned (Xie et al., 2004). Its role in neuronal differentiation has not yet been elucidated. Although further studies will be required, our results strongly suggest that the two genes containing these C2 domains play pivotal roles in neuronal differentiation of HiB5 cells.

In conclusion, our novel destination vectors can be successfully applied to HTP screening of cellular image data. In addition, the two vectors allow simultaneous examination of two or more proteins of interest by combining them with the EGFP-tagging system, since the fluorescence of RFP can be readily differentiated from that of EGFP.

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