Available online at www.sciencedirect.com
SCIENCE DIRECT
Gene 360 (2005) 35 – 44
GENE
SECTION
FUNCTIONAL GENOMICS
www.elsevier.com/locate/gene

Utilization of mammalian cells for efficient and reliable evaluation of specificity of antibodies to unravel the cellular function of mKIAA proteins

Akiyuki Ozaki [a,1], Takahiro Nagase [b], Ayako Watanabe [a], Daisuke Nakajima [b], Kiyo Shimada [c], Mihoko Nagano [c], Osamu Ohara [b,d], Hisashi Koga [b,c], Susumu Inamoto [a,c,*]

[a] *Department of Biotechnology, Institute of Research and Innovation, 1201 Takada, Kashiwa, Chiba 277-0861, Japan*
[b] *Kazusa DNA Research Institute, 2-6-7 Kazusa-Kamatari, Kisarazu, Chiba 292-0818, Japan*
[c] *Chiba Industry Advancement Center, 2-6 Nakase, Mihama-ku, Chiba 261-7126, Japan*
[d] *RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama City, Kanagawa, 230-0045, Japan*

Received 2 February 2005; received in revised form 2 June 2005; accepted 16 June 2005

Received by K. Gardiner

Abstract

Complementary DNA (cDNA) clones for human KIAA genes have been isolated as long cDNAs (>4 kb) with unknown functions. To facilitate the functional analysis of these human clones, we have isolated and determined the structures of their respective mouse homologues (mKIAA genes). Furthermore, we have comprehensively raised antibodies against the translated mKIAA proteins in order to establish a platform for their functional analysis. Since the specificity of these antibodies is critical for subsequent analyses of protein function, here we introduce two assays utilizing mammalian cells to improve their evaluation. First, we have established a semi-high-throughput production of C-terminally FLAG epitope-tagged proteins for Western blotting using specially designed mammalian expression vectors. Secondly, we have utilized immunofluorescence staining of mouse cells to analyze the subcellular localization of endogenous mKIAA proteins. Importantly, these methods allow us to detect potential posttranslational modification of the mKIAA/KIAA proteins and to predict their biological function based on their subcellular localization.

Keywords: Western blotting; Expression vector; Immunofluorescence staining

1. Introduction

The sequencing of human genome has been completed (International Human Genome Sequencing Consortium, 2004) and draft sequences of other animals are also available (http://www.genome.gov/). Given that the most important molecules for biological processes are the proteins made from mRNA, the sequence information of cloned copies of mRNA (i.e. cDNA) is indispensable in predicting protein structure and ultimately understanding biological function. Since 1994, we have accumulated sequence information from long human cDNAs (>4 kb) encoding proteins of unknown function at the time of sequencing (Nomura et al., 1994). The function of many of these proteins can be

Abbreviations: KIAA, KI is Kazusa DNA Research Institute and AA are reference characters; mKIAA, mouse counterparts of human KIAA; cDNA, DNA complementary to RNA; mRNA, messenger RNA; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); kDa, kilodaltons; MW, molecular weight; siRNA, small interfering RNA; PBS, phosphate-buffered saline; GST, glutathione-*S*-transferase; ORF, open reading frame; CMV, cytomegalovirus; GFP, green fluorescent protein; APC2, anaphase promoting complex subunit 2; BLAST, Basic Local Alignment Search Tool; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride; ELISA, enzyme-linked immunosorbent assay; Tet, tetracycline.

* Corresponding author. Department of Biotechnology, Institute of Research and Innovation, 1201 Takada, Kashiwa, Chiba 277-0861, Japan. Tel.: +81 4 7146 0053; fax: +81 4 7144 9007.
E-mail address: inamots@iri.or.jp (S. Inamoto).
[1] Present address: Genetic Breeding Group, Farming Biology Division, National Research Institute of Aquaculture, Fisheries Research Agency (NRIA-FRA), Nansei, Watarai, Mie 516-0193, Japan.

0378-1119/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2005.06.033

speculated (and in some cases identified) as being related to higher-order cell function and/or diseases (Mushegian et al., 1997; Ohara et al., 1997). Until now, we have isolated more than 2000 genes (KIAA genes) (Nagase et al., 2003; http://www.kazusa.or.jp/huge/).

As a next step in understanding the function of the KIAA genes, we have begun to isolate their respective mouse homologues (mKIAA genes) (Okazaki et al., 2002, 2003a,b, 2004; http://www.kazusa.or.jp/rouge/). Since approximately 99% of mouse genes have human counterparts (Mouse Genome Sequencing Consortium, 2002), we can bypass the legal and ethical restrictions by using mouse counterparts instead of using human materials to analyze the function of KIAA genes. To reveal the function of the corresponding proteins, it is important to prepare a set of probes that can specifically recognize and distinguish each protein. To this end, we have begun to comprehensively produce antibodies against mKIAA proteins (Hara et al., 2003; Koga et al., 2004a). Using these antibodies, we have begun to examine protein expression by Western blot analyses using samples from adult mouse tissues and mouse tissue culture cells as well as by immunohistochemical analyses of mouse tissues. These data, together with the gene expression profiles of 127 mKIAA genes/proteins, were integrated and released to public as the InGaP database (Koga et al., 2004b; http://www.kazusa.or.jp/ingap/).

In this work, we introduce two assays using mammalian tissue culture cells as a means of developing a reliable set of reagents for the functional analyses of mKIAA/KIAA proteins. First, we have established semi-high-throughput production of mammalian expression vectors in order to express C-terminally FLAG epitope-tagged proteins for Western blotting. Secondly, we have utilized immunofluorescence staining of mouse cells to analyze the subcellular localization of endogenous mKIAA proteins. We show that these assays are effective in evaluating antibody specificity to be used for further functional studies of mKIAA/KIAA proteins. We also present data indicating that these assays are useful in detecting potential posttranslational modification and in predicting possible protein function based on specific intracellular localization.

2. Materials and methods

2.1. Plasmids

ORFs (from the initiation codon to the termination codon) of mKIAA/KIAA were cloned between in vitro recombination sites (*att*L1 and *att*L2 sites) into a plasmid by using *E. colli* homologous recombination. In these plasmids, ORFs of mKIAA/KIAA were preceded by the Kozak consensus sequence for efficient translation in mammalian cells and termination codons were destroyed by one base substitution (Nakajima et al., in press). These ORFs were cloned into pTREx FLAGFRT-DEST vector (Nakajima et al., unpublished data) to construct mammalian expression vectors by using the Gateway recombination cloning system (Invitrogen Corp., Carlsbad, CA) (Fig. 1) so that mKIAA/KIAA proteins were expressed as fusions with 3xFLAG (DYKDHDGDYKDHDIDYKDDDDK) at their carboxyl terminus. Since there is also the linker sequence containing the *att*B2 site in between ORFs and 3xFLAG, expressed proteins are 47 amino acids longer than native ones. Identity of each construct was checked either by digestion with appropriate restriction enzymes or by sequencing 3′-ends of mKIAA/KIAA cDNAs.

2.2. Cell culture and nucleic acid transfection

The human cell line, HEK293, was cultured in Dulbeco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Inc., St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA) in a 5% CO_2 incubator at 37 °C, while mouse cell lines, P388D1, L929 and Swiss3T3 were cultured in DMEM with 5% fetal bovine serum in a 5% CO_2 incubator at 37 °C.

One day before transfection, 3×10^5 of HEK293 cells were plated in 35-mm plates and 500 ng of expression vectors were transfected by using FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN). Cells were harvested 36–45 h after transfection for Western blotting analysis.

2.3. Anti-mKIAA antibodies and Western blotting

Rabbit polyclonal antibodies were prepared by immunization with recombinant antigens as described previously (Hara et al., 2003; Koga et al., 2004a). All immune sera were preabsorbed by a large amount of GST immobilized on the resin to remove contaminating anti-GST antibodies.

Western blotting was performed basically as described previously (Magae et al., 1997; Koga et al., 2004b). ECL Western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) were used for detection. As an

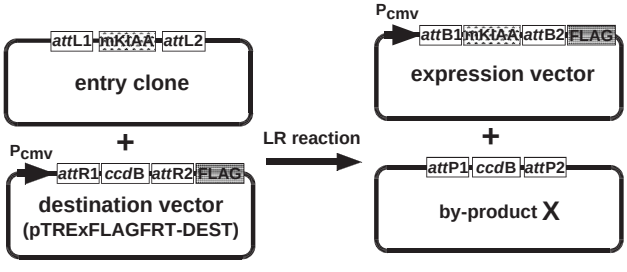

Fig. 1. Outline of the strategy to construct mammalian mKIAA/KIAA-3xFLAG protein expression vectors by in vitro recombination-assisted method. After LR reaction, ORFs of mKIAA/KIAA genes (mKIAA in the figure) in entry clones were transferred between the CMV promoter (P_{CMV}) and the 3xFLAG epitope (FLAG). The *ccd*B gene encoding a toxin targeting the essential DNA gyrase of *E. coli* and the phage λ recombination sites (*att*B, *att*P, *att*L, and *att*R) are also indicated.