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Subtractive phage display technology identifies zebrafish *marcksb* that is required for gastrulation

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ABSTRACT

In the present study, we used a phage display technique to screen differentially expressed proteins from zebrafish post-gastrula embryos. With a subtractive screening approach, 6 types of single-chain Fv fragments (scFvs) were screened out from an scFv antibody phage display library by biopanning against zebrafish embryonic homogenate. Four scFv fragments (scFv1, scFv3, scFv4 and scFv6) showed significantly stronger binding to the tailbud embryos than to the 30%-epiboly embryos. A T7 phage display cDNA library was constructed from zebrafish tailbud embryos and used to identify the antigens potentially recognized by scFv1, which showed the highest frequency and strongest binding against the tailbud embryos. We acquired 4 candidate epitopes using scFv1 and the corresponding genes showed significantly higher expression levels at tailbud stage than at 30%-epiboly. The most potent epitope of scFv1 was the clone scFv1-2, which showed strong homology to zebrafish myristoylated alanine-rich C-kinase substrate b (Marcksb). Western blot analysis confirmed the high expression of *marcksb* in the post-gastrula embryos, and the endogenous expression of Marcksb was interfered by injection of scFv1. Zebrafish *marcksb* showed dynamic expression patterns during embryonic development. Knockdown of *marcksb* strongly affected gastrulation movements. Moreover, we revealed that zebrafish *marcksb* is required for cell membrane protrusion and F-actin alignment. Thus, our study uncovered 4 types of scFvs binding to zebrafish post-gastrula embryos, and the epitope of scFv1 was found to be required for normal gastrulation of zebrafish. To our knowledge, this was the first attempt to combine phage display technique with the embryonic and developmental study of vertebrates, and we were able to identify zebrafish *marcksb* that was required for gastrulation.

1. Introduction

Zebrafish have proven to be an excellent animal model of vertebrate developmental studies (Key and Devine, 2003). The gastrulation of vertebrates involves both massive cell differentiation and dynamic cell movements (Solnica-Krezel, 2006). Previous reports have shown that the ability to undergo developmental programmed cell death and induced apoptosis is acquired no earlier than the gastrula stage (Greenwood and Gautier, 2005). The early embryo is sensitive to the toxicity and subject to phenotypic abnormalities (Gurdon and Byrne, 2004). In addition, our previous studies demonstrated that the developmental failure of nuclear transferred embryos often occurs at the transition between the blastula and post-gastrula stages (Luo et al., 2009; Pei et al., 2007; Sun et al., 2005). All these facts indicate that various developmentally critical genes are expressed during the gastrulation of zebrafish. For this reason, we decided to isolate gastrulation-related proteins using a high-throughput screening technique.

High-throughput screening techniques, such as subtractive hybridization, differential display, and microarray technology, can produce results at the level of DNA or mRNA sequence (Donson et al., 2002; Linney et al., 2004; Sargent and Dawid, 1983; Stanton, 2001). The so-called post-genomic era calls for further approaches at the protein level (Ko, 2001). Phage display techniques can be used to express peptides, proteins, and antibody fragments at the surfaces of phage particles. This system can be used for large-scale studies and the selection of proteins based

Abbreviations: ABTS, 2, 2′-amino-di (2-ethyl-benzothiazoline sulphonic acid-6) ammonium salt; BLAST, basic local alignment search tool; BSA, albumin from bovine serum; cDNA, DNA complementary to RNA; CE, convergence and extension; DIG, digoxigenin; *E. coli*, *Escherichia coli*; ECL, enhanced chemiluminescence; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; F-actin, filamentous actin; GFP, green fluorescent protein; hpf, hours post fertilization; HRP, horseradish peroxidase; Marcks, myristoylated alanine-rich C-kinase substrate; MO, morpholino; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PKC, protein kinase C; rpm, revolutions per minute; scFv, single-chain Fv fragment; SDS, sodium dodecyl sulfate; TBST, tris-buffered saline; WISH, whole mount *in situ* hybridization; μl, microliter.

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on their binding and specificity. These techniques have been used in many different fields because of their speed and sensitivity (Burton, 1995; Clackson and Wells, 1994; Koohapitagtam et al., 2010; Scott and Smith, 1990; Sidhu, 2000; Smith, 1985; Winter et al., 1994; Zou et al., 2004). An advantage of phage display is the enormous diversity of variant proteins that can be represented. For instance, phage display antibody libraries with levels of diversity as high as 10^{10} are routinely constructed (Clackson et al., 1991). In addition, a phage display library is usually constructed by inserting DNA fragments into phage or phagemid genomes and the corresponding proteins are expressed on the phage coat. This creates a direct physical link between the DNA sequences and their encoding proteins (Paschke, 2006). In previous reports, phage display technology has been used to produce specific antibodies capable of recognizing different cells, tissues, and organs (Deflorian et al., 2009; Kemp et al., 2002; Sche et al., 1999), or utilized for fragment-based ligand discovery (Erlanson, 2007). In the present study, by using a subtractive biopanning method, we tried to produce specific phage-displayed scFv antibodies capable of distinguishing the homogenate of zebrafish embryos at tailbud stage from the embryos at 30%-epiboly stage. We then attempted to use one scFv antibody showing higher binding to tailbud embryos, to isolate certain interacting epitopes using a T7 cDNA phage display library from zebrafish tailbud embryos. Fortunately, we have characterized zebrafish *marcksb* by this method and showed *marcksb* is necessary for normal gastrulation of zebrafish.

2. Material and methods

2.1. Zebrafish

Mature zebrafish (from the China Zebrafish Resource Center) were maintained at 28 °C with a photoperiod of 14 h light and 10 h dark in an aquarium supplied with fresh water and aeration. Embryos were produced using standard procedures and staged according to standard criteria (hours post fertilization, hpf) (Kimmel et al., 1995). All the animals used in the study were cared for in accordance with the Institutional Animal Care and Use Committee of Institute of Hydrobiology, Chinese Academy of Sciences.

2.2. Biopanning and production of soluble scFv antibodies against zebrafish homogenates

A single-chain Fv (scFv) antibody phage display library was constructed and isolated as described in our previous study (Dai et al., 2003), except the mice were immunized with combined homogenate of zebrafish embryos at tailbud and 30%-epiboly stages. The scFv antibody phage display library was incubated with the homogenate of zebrafish 30%-epiboly embryos, to absorb the antibodies which could bind to the proteins present in the embryos at 30%-epiboly. They were then added to the Nunc immuno test tube, which had been coated with zebrafish homogenate at tailbud stage. After three rounds of biopanning, we examined whether certain populations of phages might be enriched during these biopanning procedures by percentage recovery. The colonies from the 3rd round were inoculated and amplified. The nucleotide sequences of the scFvs were detected using the primers of pCANTAB-S6 (5′-GTA AAT GAA TTT TCT GTA TGA GG-3′) and pCANTAB-R1 (5′-CCA TAG TTA CGC CAA GCT TTG GAG CC-3′). They were inoculated into a 96 well plate with 400 μl of 2 × YT-AG overnight at 37 °C with shaking at 150 rpm. Four hundred microliters of 2 × YT-AG was pipetted into each well in a fresh 96 well plate. Then 40 μl of overnight culture was transferred to corresponding well in new plate and incubated at 30 °C for 2 h. The plate was centrifuged at 1500 ×*g* for 20 min at room temperature (Eppendoff centrifuge 5801R) and the supernatant was carefully removed. Then 400 μl of 2 × YT-AI was added to each well and the plate was incubated 3 h at 30 °C in a humidified incubator. The plate was centrifuged as described above. Then 320 μl of each supernatant (containing scFv antibodies) was carefully transferred to a corresponding well in a new 96 well plate.

2.3. Binding assay of scFv antibodies with enzyme-linked immunosorbent assay (ELISA)

A 96-well ELISA plate was coated alternately with the homogenate of zebrafish tailbud embryos and 30%-epiboly embryos at 4 °C. The following day, the coated wells were blocked with 4% skim milk in PBS and added with the supernatant containing scFvs. The binding of the scFv antibodies was detected using the mouse monoclonal antibody of HRP/anti-E Tag conjugate (Amersham Pharmacia Biotech Ltd., U.K.). Finally, an ABTS substrate solution (Sigma, Taufenstein, Germany) was used for the color reaction with peroxidase and the absorbance was determined at 405 nm. One-way ANOVA was used to analyze the absorbance values of two stages. P value < 0.05 was regarded as statistically significant.

2.4. Periplasmic expression of soluble antibody fragments

The periplasmic extract soluble scFvs were produced and isolated as previously reported (Xiao et al., 2006). The interested colony, scFv1, was inoculated into 50 ml of 2 × YT-AG overnight at 30 °C with shaking at 200 rpm. The mixture was allowed to culture overnight and then transferred to 500 ml of 2 × YT-AG. The culture was centrifuged at 1500 ×*g* for 20 min at room temperature. The supernatant was discarded and the sediment cells were resuspended in 500 ml 2 × YT-AI. They were incubated overnight at 30 °C with shaking at 200 rpm. After centrifugation at 1500 ×*g* for 20 min, cell pellets obtained from 500 ml overnight culture volumes were resuspended in 10 ml ice-cold TES (0.2 M Tris–HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0), followed by addition of 16.5 ml ice-cold 1:4 water-diluted TES. The suspension was incubated for 30 min on ice and then centrifuged at 1500 ×*g* for 10 min. The supernatant that contained periplasmic soluble antibody fragments was collected and transferred to a clear container and stored at −70 °C. The scFv2, which was used as a control in this study, was produced using the same methods.

2.5. Construction and biopanning of T7 cDNA phage display library

Total RNA was extracted from zebrafish at the tailbud stage using SV total RNA kits (Promega, U.S.), and poly A^+ mRNA was isolated by polyATract mRNA Isolation System III and IV (Promega, U.S.). A directional cDNA library was established using a Directional RH Random Primer cDNA Synthesis Kit and T7Select 10 Cloning Kit (Novagen, WI, U.S.). Ligation efficiencies were evaluated using a small aliquot of the packaged phage and a total of 1.72×10^7 pfu/ml (plaque forming units per milliliter). These initial transformants were subjected to one amplification step by infecting log phase BLT5403 cells, providing a zebrafish T7 cDNA phage display library with an overall titer of 5.16×10^{11} pfu/ml. The library was stored in aliquots (100 μl) at −70 °C as 8% glycerol stocks. The T7 cDNA phage display library was biopanned with periplasmic soluble scFvs captured by anti Etag antibody. A 96-well ELISA plate (Nunc, Rochester, NY) was coated with anti Etag antibody, and added with periplasmic extract soluble scFvs. The plate was blocked with 3% PBS-BSA and washed several times with PBS. The wells were coated with anti Etag-scFv complex and incubated with 10^8 pfu/ml phages in Tris-buffered saline (TBST). The bound phages were amplified directly *in situ* by exposure mid-log phase to *E. coli* BLT5403 for 1 h at room temperature. The contents were transferred into L-broth supplemented with 50 mg/ml carbenicillin for further culture, and the phages were grown until lysis occurred. Phage lysate was then diluted for subsequent biopanning. The entire procedure was repeated 6 times. To determine the enrichment effect of biopanning, phage lysate containing selected populations of phages from each round of biopanning were used as templates for