Protein Purification and Western Blot Detection from Single Zebrafish Embryo

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Abstract

Characterization of a protein of interest during development is essential for functional studies. A general strategy for understanding the function of a particular protein involves the generation of null mutations, or treatment with drugs, that interfere with its activity. To demonstrate that the synthesis, stability, or activity of a protein has been affected, accurate and efficient detection of low amounts of protein is essential. This can be achieved by immunohistochemistry or by western blot. Here we describe a method for the detection of proteins from single de-yolked zebrafish embryos. This procedure includes a fixation step and the concomitant elimination of lipids from the yolk cell. We show that this approach allows the rapid analysis of proteins in embryos without having to manually remove the yolk. This method provides a convenient alternative for genotyping of mutant embryos as early as the 128 cell stage. In addition, in drug- or morpholino-treated embryos, the correlation between the penetrance of a phenotype and the concentration of a protein present may be established.

Keywords: protein purification, western blot, zebrafish embryo

Introduction

ZEBRAFISH HAS BEEN used for decades as an excellent model organism. Its use has increased exponentially with the characterization of novel genes involved in vertebrate development and disease. Every functional study eventually involves the demonstration of differential expression or activity of a protein of interest. The presence or absence of the protein can be determined either by immunohistochemistry or gel electrophoresis followed by a western blot. Successful detection of a target protein depends on the affinity and specificity of the corresponding antibody and on the abundance of the protein of interest. Standard western blots in zebrafish are achieved by pooling tens to thousands of embryos.¹ Owing to natural variability between individuals with a mutation or after treatment with an inhibitor, a gradient of phenotypes can be observed; when embryos with variable phenotypes are pooled and further analyzed by western blot, the specific amount of a protein in an embryo cannot be correlated with the particularities of its phenotype. Another common problem inherent to zebrafish embryos is the abundance of yolk proteins that interfere with the detection of the protein of interest; when the yolk is not completely removed the sensitivity for detecting other cellular proteins is limited.² Although other methods of protein extraction of single embryos or larvae have been described,

they either manually remove the yolk,³ which is time consuming, or the yolk is not removed.^{4,5} We have adapted a method whereby the whole protein extracts can be obtained from a single embryo with the concomitant easy removal of yolk. This procedure eliminates the lipids from the sample by using a mix of heptane and methanol, which forms a double layer where the lipids rest, whereas the yolk-free embryos remain at the bottom from where an efficient extraction of proteins is achieved. By using this procedure, we obtained protein from single embryos at different embryonic stages from 128-cells to 5 days postfertilization (dpf) being able to identify proteins present in the nuclei, cytoplasm, and membrane. Moreover, we showed that this protocol is reproducible and it is useful for associating a distinctive phenotype to the absence of protein produced in embryos in CRISPR-Cas9 mutants

Materials and Methods

Fish maintenance and strains

AB wild-type zebrafish (*Danio rerio*) embryos and wild-type embryos from an undefined strain sourced from a local pet store and inbred for several generations were obtained from natural crosses and raised at 28°C. *smarcel1* mutant crosses were obtained as described previously.⁶

Zebrafish embryos were incubated at 28°C in embryo water until reaching the desired developmental stage. Embryo stages were determined by morphological criteria according to Kimmel *et al.*⁷ Zebrafish were handled in compliance with local animal welfare regulations and approved by the Institute's Ethics Committee (Instituto de Biotecnología, UNAM).

smarce1 mutants and genotyping

smarce1 mutants were previously generated and described in Castillo-Robles *et al.*⁶ Embryos were obtained by crossing *smarce1*^{+/-} adults; embryos were incubated at 28°C in embryo water until 5 dpf. Larvae were anesthetized with tricaine, immobilized with methylcellulose on agar plates and visualized with a stereomicroscope (Leica MZ 12.5), and photographed using a CCD camera (AxioCam MRc 5; Zeiss) and the AxioVision Rel. 48 software.

The posterior part of the larvae was used for genotyping; a sharp cut with an insulin needle was performed at the posterior end of the yolk extension, whereas the anterior part was used for protein purification. The primers 5'-TCTGGCCTACAA CAACTACA-3' and 5'-TCCTCTCGGCTGGGCTTTTA-3' were used to amplify a 121 bp fragment, and polymerase chain reaction products were separated on 14% polyacrylamide gels where an 8-bp deletion can be distinguished.⁸

Total protein preparation and western blotting

Total protein was obtained from single zebrafish embryos with the protocol modified from Sue.⁹ In brief, individual embryos or larvae are gently pipetted in a 1.5 mL eppendorf tube. The embryo water is discarded completely and 500 μ L of heptane is added, immediately after 500 μ L of cold methanol is added, and samples are fixed for 5 min with gentle shaking and rinsed twice with 500 μ L of cold methanol. Embryos or larvae were washed twice with 100 μ L of cold embryo buffer (Tris 10 mM pH 7.5, sodium β -glycerophosphate 80 mM pH 7.5, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 20 mM, MgCl₂ 15 mM, Na₃VO₄ 2 mM, benzamidine 1 mM, sodium metabisulfite 1 mM, phenylmethylsulfonyl fluoride (PMSF) 2 mM, and Tween-20 0.05%), each sample was suspended in 20 μ L of cold embryo buffer (EB). Tris 10 mM pH 7.5, 5 dpf embryos were homogenized with a small plastic pestle in 30 μ L of cold EB. An aliquot was obtained to quantify total protein with the Bradford assay. 5 $\times$ sodium dodecyl sulfate (SDS) sample buffer was added and boiled for 5 min. The western blotting was performed as previously described¹⁰ with some modifications. The antibodies used were anti-E-cadherin (610182, AB_397581; BD Biosciences), anti-Erk-1 (sc153; Santa Cruz Biotechnology), anti-SMARCE1 antibody (Abcam ab131328), and the secondary antibody used was anti-rabbit IgG-HRP (sc2004; Santa Cruz Biotechnology).

Results

Total proteins extracts were obtained at several distinctive embryonic stages, from 128-cell stage to 5 dpf (Fig. 1A). To demonstrate that this method is suitable to detect proteins with different cellular localization from single embryos, we used antibodies for the identification of E-cadherin, a cell adhesion protein located in the membrane; Erk1 a serine/threonine kinase present in the cytoplasm and Smarce1, a

FIG. 1. Single embryo protein purification. (A) Western blot of single proteins purified from embryos at different stages 128, 512, sphere, 30% epiboly, shield, 70% epiboly, and 18 somites. The use of antibodies for E-cadherin (E-cad), Erk1, and Smarce1 proteins is indicated (representative blots are depicted here). (B) Wild-type (+/+), heterozygous (+/-), and homozygous embryos. The characteristic phenotype of the *smarce1* mutants is observed with a cardiac edema, and the body curvature. (C) Genotypes of polymerase chain reaction *smarce1* embryos. A 121 bp amplicon, including the mutated region, is obtained from the wild-type embryos while a 113 fragment is amplified from the mutated allele. (D) Western blot with the Smarce1 antibody from 4 dpf wild-type single embryos (+/+) and normal looking heterozygous embryos (+/-) derived from *smarce1* (+/-) in crosses and their edematous curved siblings (-/-). Erk1 protein was used as a control to confirm that the same amount of protein was loaded in each well (representative blots are depicted here).

subunit of the BAF chromatin remodeling complex, that acts in the nuclei. These three proteins were successfully detected in all the embryonic stages tested (Fig. 1A).

Next, we evaluated this technique for the characterization of the phenotypes produced in embryos with mutations. For this purpose, we isolated protein from single embryos of *smarce1* mutants previously described.⁶ *smarce1* mutants exhibit very distinctive defects at 72 hours postfertilization, including smaller eyes, abnormal body curvature, and heart abnormalities. We previously showed that mutant embryos have heart defects, including dysmorphic chambers, absence of looping, slower beating, and absence of circulation; these defects were accompanied with an abnormal morphology of the endocardium.⁶ Since *smarce1* mutants are easily distinguished by their phenotype, larvae can be separated into groups, defective and normal phenotype (Fig. 1B). After

taking photographs of each larva, the posterior part of the larva was used to genotype and larvae were classified as wild-type, heterozygous, and homozygous larvae (Fig. 1C). The anterior part of each larva was used to obtain total protein. The cell lysates were loaded in an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and blotted onto a nitrocellulose membrane to be analyzed by western blot against Smarce1 and as a loading control we included Erk1 (Fig. 1D). We were able to demonstrate that larvae with a distinctive phenotype were those in which Smarce1 could not be detected, whereas wild type and heterozygotes presented the expected levels of Smarce1 protein (Fig. 1D), with heterozygous embryos showing a lower concentration of protein compared with the homozygous wild-type embryo.

In conclusion, we have adapted a protocol to identify proteins from yolk-free single embryos at different stages of development, which is less time consuming, reproducible, and permits many biological and experimental replicates to be performed.

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Disclosure Statement

No competing financial interests exist.

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