Determining Zebrafish Epitope Reactivity to Commercially Available Antibodies

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FIG. 1. Identification of immunoreactive MTNR1 peptides in zebrafish retina. (A) The SUMO-based recombinant protein platform for screening putative peptide epitopes. Between the SUMO protein sequence and the epitope of interest, there is a short linker of Gly-Gly-Ser (GGS). (B) Z-projection of confocal images taken of zebrafish retinas, labeled using anti-HsMTNR1 antibody, indicating the antibody recognizes a zebrafish protein. Brackets indicate the photoreceptor layer, where the antibody strongly reacts with a protein. Scale bar, 50 μ m. (C) Multiple alignment of the human immunogenic peptide and respective regions of the six Mtnr1 proteins in zebrafish. Sequence divergence between the zebrafish sequences and the human sequence is noted in **bolded gray**; chemically nonconservative differences are additionally underlined. (D) Western blots of titrations of the five recombinant SUMO fusion proteins, each containing one of the unique putative epitopes in zebrafish. Identical blots were probed with either anti-His or anti-HsMTNR1 antibody, as indicated. HsMTNR1, human melatonin receptor 1 protein.

Abstract

Antibodies raised against mammalian proteins may exhibit cross-reactivity with zebrafish proteins, making these antibodies useful for fish studies. However, zebrafish may express multiple paralogues of similar sequence and size, making them difficult to distinguish by traditional Western blot analysis. To identify the zebrafish proteins that are recognized by an anti-mammalian antibody, we developed a system to screen putative epitopes by cloning the sequences between the yeast SUMO protein and a C-terminal 6xHis tag. The recombinant fusion protein was expressed in *Escherichia coli* and analyzed by Western blot to conclusively identify epitopes that exhibit cross-reactivity with the antibodies of interest. This approach can be used to determine the species cross-reactivity and epitope specificity of a wide variety of peptide antigen-derived antibodies.

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Keywords: immunohistochemistry, antibody specificity, antigen specificity, recombinant protein, epitope mapping

HISTORICALLY, COMMERCIAL ANTIBODIES raised against zebrafish (*Danio rerio*) proteins have not been readily available. Although this circumstance is rapidly changing as aquatic model systems are becoming more widely used, a considerable amount of work has been performed using zebrafish tissue and antibodies raised against proteins from other vertebrates (primarily mammalian). BLAST searches can provide reasonable confidence in species cross-reactivity. However, conservative sequence differences exist between species, and even between paralogues within zebrafish. For example, the effect of a conservative amino acid change (e.g., isoleucine to valine) on the binding affinity between antibody and antigen cannot be predicted by sequence analysis alone.^{1–3} Moreover, similar sizes of paralogous proteins make it difficult, if not impossible, to determine cross-reactivity of individual paralogues using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of tissue lysates (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/zeb).

We have used the recombinant SUMO solubility tag expression system to experimentally determine (1) whether a commercially available anti-human antibody recognizes zebrafish homologues of a human protein and (2) whether the antibody discriminates among zebrafish paralogues. To accomplish this, we first modified the pET28-SUMO vector, by deleting the N-terminal 10xHis tag, creating the vector pET28-SUMOΔNHis (Supplementary Fig. S1). This modification makes only the C-terminal 6xHis tag available for purification and detection. The putative zebrafish epitope was then inserted between the N-terminal SUMO tag and the C-terminal 6xHis tag (Fig. 1A).⁴ Fusion proteins were recombinantly expressed in and purified from *Escherichia coli* (Supplementary Materials and Methods section and Supplementary Figs. S1 and S2) before analysis by western blotting with both an anti-His probe and the antibody of interest to test cross-reactivity.

The C-terminal location of the 6xHis tag affords multiple advantages beyond facilitating rapid purification. A control western blot that probes the 6xHis tag serves as a direct loading control for the test antibody western blot. In addition, it provides a mechanism for monitoring the integrity of the putative epitope peptide. If the recombinant protein is not able to bind to either the affinity resin or the anti-His antibody, then there is reason to suspect that both the C-terminal 6xHis and the putative epitope have been degraded during cell lysis and/or purification. In that case, a new protein preparation should be made before performing the test antibody western blot.

We have demonstrated the facility of this approach using a commercial antibody (ab87639; Abcam) raised against a 13-residue peptide within the human melatonin receptor 1 protein (*HsMTNR1*). Immunohistochemistry experiments clearly show antibody reactivity with a protein in zebrafish retinas (Fig. 1B)⁵; however, the zebrafish genome contains six different *mntnr1* paralogues (Fig. 1C). Of the proteins encoded by these genes, two (*DrMtnr1Aa* and *DrMtnr1Ab*) share the exact same putative epitope sequence with one another. We have recombinantly expressed and purified SUMO fusion peptides of the five unique putative epitopes from the zebrafish *Mtnr1* proteins (*DrMtnr1Aa/b*, *DrMtnr1A-like*, *DrMtnr1Ba*, *DrMtnr1Bb*, and *DrMtnr1C*). Titrations of these proteins were analyzed by SDS-PAGE and two western blots (anti-His and anti-*HsMTNR1*) (Fig. 1D and Supplementary Materials and Methods section and Supplementary Fig. S3). These data clearly show that the anti-*HsMTNR1* antibody reacts with both the *DrMtnr1Aa/b* epitope and the *DrMtnr1A-like* epitope. The antibody does not recognize the putative epitope within the *DrMtnr1Ba*, *DrMtnr1Bb*, or *DrMtnr1C* paralogues. It is notable that this approach allows us to probe how precisely the antibody recognizes the chemical features of the epitope. *DrMtnr1C* contains only one nonconservative chemical difference between the two sequences (Ser10→Arg10 within the epitope), whereas the other four differences with the antigenic sequence are very chemically conservative differences (Lys→Arg and Ser→Thr). Even so, the antibody does not recognize the *DrMtnr1C* sequence at all, demonstrating that the size, shape, and charge of these amino acid side chains matter greatly. This approach can, therefore, also be used to probe the features of a putative epitope that are necessary for antibody binding.

Acknowledgments

We thank Benjamin Euhus, Katie Shaw, and Leticia Gonzalez for assistance with protein expression and purification; the Texas State University Student Undergraduate Research Fund (to M.A.V. and B.J.R.); and Chris Lima (Rockefeller University) and Cornell University for the pET28-SUMO expression plasmid. Confocal microscopy was performed on an instrument supported by NSF grant DBI-0821252 (to J. Koke and D.M.G.).

Disclosure Statement

No competing financial interests exist.

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