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Isolation of serpin-interacting proteins in *C. elegans* using protein affinity purification

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ABSTRACT

Caenorhabditis elegans is a useful model organism for combining multiple imaging, genetic, and biochemical methodologies to gain more insight into the biological function of specific proteins. Combining both biochemical and genetic analyses can lead to a better understanding of how a given protein may function within the context of a network of other proteins or specific pathway. Here, we describe a protocol for the biochemical isolation of serpin-interacting proteins using affinity purification and proteomic analysis. As the knowledge of *in vivo* serpin interacting partners in *C. elegans* has largely been obtained using genetic and *in vitro* recombinant protein studies, this protocol serves as a complementary approach to provide insight into the biological function and regulation of serpins.

1. Introduction

Proteases are core components of many biological pathways where they cleave peptide bonds to either activate or degrade other proteins. Thus, regulation of proteolytic activity is essential [1]. Serine and cysteine protease inhibitors (serpins) are the largest family of active site inhibitors, where the majority of members function to prevent the negative effects of excessive peptide bond hydrolysis *via* a unique suicide substrate-like mechanism where a serpin inhibits a target protease with a 1:1 stoichiometry [2–6].

Members of the serpin family are also conserved within the *Caenorhabditis elegans* genome, including 5 members that have *bona fide* inhibitory function [6,7]. Using both genetic and RNA interference (RNAi)-based studies, we have previously demonstrated that one of these serpins, SRP-6, exhibits a pro-survival function by blocking intestinal cell necrotic cell death in response to several stress-inducing stimuli [8]. The *C. elegans* model system has proven ideal for providing information regarding the identification of SRP-6 regulatory targets from a genetic standpoint. Although informative, these genetic approaches do not demonstrate that SRP-6 can be found in complex with a particular protease or non-protease regulatory target *in vivo*. Overall, there is little direct biochemical evidence regarding the identity of the specific proteins that interact with SRP-6 in *C. elegans*. Recently, proteomics-based approaches have been used more frequently to complement the genetic evidence obtained through chemical mutagenesis and RNAi studies. To date, multiple strategies have been developed and employed to isolate protein complexes from *C. elegans* [9–13]. In this report, we describe a modified version of our biochemical approach to isolate SRP-6 interacting proteins using affinity purification steps in combination with nano-scale liquid chromatography online coupled with tandem mass spectrometry (nanoLC-MS/MS) [10]. This methodology is advantageous because it is an unbiased approach and provides the specificity needed to isolate and identify *in vivo* SRP-6 containing protein complexes from lysates prepared from animals that express the affinity tagged version of SRP-6. This approach serves as an important way to combine both biochemical and genetic information in combination to further define the biological function of serpins. We describe methods for large-scale worm growth, whole worm lysate preparation, affinity purification procedures, proteomic analysis, and bioinformatic analysis of identified proteins.

2. Materials and methods

2.1. TAP tag design

There are several considerations when designing a construct for affinity purification: (1) promoter choice, (2) epitope tag (3)

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enzymatic cleavage site choice, and (4) position of the affinity tag relative to the protein of interest. Using a strategy previously described by our lab [10], we have generated a triple affinity purification (TrAP) SRP-6 expression construct that is expressed *via* the nhx-2 promoter to drive strong intestinal expression (Fig. 1). The expressed SRP-6 protein is fused N-terminally to the TrAP tag, consisting of *GFP::TEV(tobacco etch virus peptidase cleavage site)::myc epitope::Pre(PreScission peptidase cleavage site)::Flag epitope* (P_{nhx-2}-TrAP::SRP-6). The use of the GFP tag is advantageous because it allows for the visual detection of SRP-6 expression patterns, to determine relative expression levels, and to assess the amount of chimerism that is sometimes problematic when expressing extrachromosomal transgenes in the *C. elegans* system. Previous work from our lab has shown that insertion of N-terminal fusions (GFP or GST tags) does not alter serpin inhibitory function [7,8,14].

2.2. Generation of transgenic strains that express TrAP tagged SRP-6

Transgenic animals are created by microinjection of plasmid DNA directly into the distal arm of the gonad and are subsequently expressed as an extrachromosomal array [15,16]. We generated transgenic animals in the *srp-6(ok319)* background by injecting P_{nhx-2}*TrAP::SRP-6* with the co-injection marker P_{myo-2}*mCherry*, which drives mCherry expression in the pharynx. Additionally, TrAP::SRP6 is expressed in *srp-6* null animals rather than in N2 control [*srp-6(+)*] animals to eliminate competition for target interacting protein binding to endogenous SRP-6. The extrachromosomal array was integrated into the genome by gamma irradiation [17]. Integration allows for the uniform expression of TrAP::SRP-6 and 100% transmission of the transgene among progeny. The integrated lines were then outcrossed six times to remove background mutations that were produced as a result of the irradiation procedure. The resulting TrAP::SRP-6 strains were examined using fluorescence microscopy and immunoblot to verify subcellular localization and protein expression patterns in these animals. As predicted, strong intestinal GFP expression is observed in animals expressing TrAP::SRP-6 (Fig. 2A), and the transgene is detected at varying levels at the predicted molecular mass of 72 kDa upon immunoblot analysis of lysates prepared from three independent transgenic lines (Fig. 2B). The following sections will describe in detail the methodology employed to purify SRP-6::interacting protein complexes from animals expressing this transgene.

2.3. Large scale growth of C. elegans

A critical aspect to the identification of serpin interacting proteins is to generate an adequate amount TrAP::SRP-6 in complex with an interacting partner so that it is suitable for detection using mass spectrometry. Once the integrated strain of choice has been generated, we employ two common methods for generating large populations of TrAP::SRP-6 expressing animals.

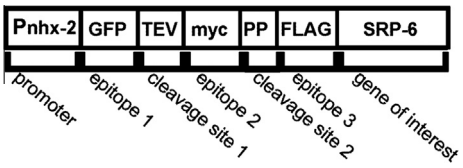

Fig. 1. Schematic representation of the triple affinity purification (TrAP) SRP-6 expression construct. Expression of SRP-6 is driven by the intestinal-specific promoter P_{nhx-2}. This construct contains 3 epitope/fluorescent tags (GFP, c-myc, and FLAG) as well as 2 protease cleavage sites for purification steps (tobacco etch virus; TEV and PreScission Protease; PP). The TrAP tag is fused to the *srp-6* gene.

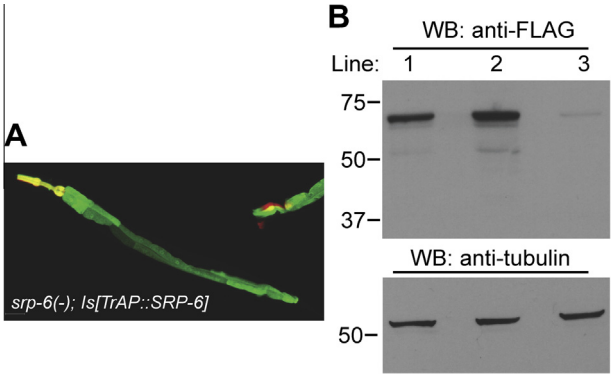

Fig. 2. Analysis of TrAP::SRP-6 expression. (A) Widefield image of an integrated *srp-6(−)* animal expressing the TrAP::SRP-6 construct (green) and the mCherry::myo-2 pharyngeal marker (red). Note the strong intestinal expression pattern. (B) Lysates prepared from 3 independent TrAP::SRP-6 transgenic lines were used to demonstrate that the TrAP::SRP-6 fusion protein can be detected at the correct molecular mass (72 kDa) by Western blot of whole animal lysate using the FLAG epitope. 75 μg total protein was run in each lane. The membrane was stripped and re-probed with anti-tubulin (52 kDa) to demonstrate equal loading in each lane. Note the varying level of expression in each independent line.

2.3.1. Growth of C. elegans on large nematode growth medium (NGM) plates

Unless otherwise noted, we use the reagents for growth and maintenance of *C. elegans* populations as previously described, and the laboratory food source for *C. elegans* is the *Escherichia coli* strain OP50.

1. ~100 gravid young adults are transferred to a 15 cm seeded NGM plates. To generate a final worm pellet size of ~0.5 mL, 20 15 cm NGM plates are used.
2. Animals are allowed to lay eggs for 8 h.
3. Egg-laying adults are removed by gently washing plates with 7 mL of phosphate buffered saline (PBS). This will allow the eggs to adhere to the OP50 and remain on the plate.
4. Animals are incubated for 36–48 h until most of the animals on the plates have reached the L4-young adult stages.
5. Animals are allowed to grow for an additional 8–12 h before being harvested. If necessary, more OP50 may be added to plates to prevent starvation.
6. Animals are transferred to a 50 mL conical tubes stored on ice by washing each plate with PBS containing 0.01% Triton X-100. This ensures that animals do not adhere to the plastic of the pipet wall during transfer.
7. Animals are allowed to gravity pellet for 5–10 min and the supernantant is removed.
8. Animals are subsequently washed 2X more with PBS and allowed to gravity settle to remove remaining OP50.
9. Once the supernatant from the final wash has been removed, the worm pellet is immediately snap frozen with liquid nitrogen and can be stored at −80 °C until use.

2.3.2. Growth of C. elegans on egg plates

1. The yolks from 10 large chicken eggs are placed into a 2L sterile bottle and mixed with 250 mL Luria broth (LB).
2. The bottle is then incubated at 60 °C for 1 h and subsequently cooled rapidly to room temperature on ice.
3. 30 mL of OP50 prepared from an overnight culture is added to the LB solution and 10 mL is added to each 15 cm NGM plate.
4. The seeded egg plates are allowed to incubate overnight at room temperature. After this incubation any excess liquid is either pipetted off or incubated in a laminar flow hood until dry. Egg plates are then stored at 4 °C until use.