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

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Abstract

C. 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.

Keywords

Caenorhabditis elegans; affinity purification; proteomics; serpin

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].

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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) 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.

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 supernatant 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.
5. The steps outlined in 2.4.1 are now followed to generate animal pellets. Typically, a worm pellet of 0.5 mL can be obtained by using 7–10 15 cm egg plates.

2.4 Preparation of whole worm extracts

Once the desired amount of transgenic animal pellets has been obtained, the following protocol is used to prepare whole worm extracts that will be used for subsequent immunoprecipitation (IP) experiments. As a general reference, batches of lysate are prepared when the total pellet size is ~ 3 mL (six sets of twenty 15 cm plates).

1. Worm pellets are thawed on ice and resuspended in an equal volume of worm lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM CaCl_2 and 1 mM MgCl_2) that is supplemented with Complete EDTA-free protease inhibitor cocktail (Roche).
2. Resuspended animals are then homogenized by passing them five times through a French press (Thermo Fisher) at 2500 PSI (40,000 cell PSI). Alternatively, homogenization can be achieved by sonication in short 10 s bursts on ice. The total number of sonication bursts required to completely homogenize animals is variable and small amounts of lysate should be frequently visualized using a light microscope to verify that animals have been sufficiently homogenized.
3. The resulting lysate is centrifuged for 5 min at 500 Xg at 4°C to pellet animal cuticles. The remaining supernatant is transferred to 1.7 mL microcentrifuge tubes.
4. Lysate is then centrifuged at 16,000 Xg for 15 min to remove nuclei and mitochondria. The clarified supernatant is removed and individual 100 μL aliquots are snap frozen in liquid nitrogen for storage at -80°C until use.

5. Total lysate protein concentration is determined by performing a Bradford Assay using the Coomassie Plus protein assay kit (Pierce). Total protein concentration of a lysate prep is ~ 4–6 mg/mL.

2.5 Immunoprecipitation of TrAP::SRP-6

To purify protein complexes from TrAP::SRP-6 lysate, we take advantage of specific design features of the TrAP tag (Fig 1) to perform up to two successive rounds of protein purification. First round purification is accomplished by using the GFP and c-myc epitopes, separated by a TEV™ cleavage site. To further purify isolated complexes a second round of purification using the c-myc and FLAG epitopes in conjunction with a PreScission™ protease cleavage site is used. In the section described below, all antibodies and enzymes used in the purification procedure are commercially available.

2.5.1 First and second round purifications

1. TrAP::SRP-6 lysate is prepared to a final protein concentration of 4–6 mg/mL. The lysate is then diluted 1:1 in worm lysis buffer containing protease inhibitor cocktail to a final volume of 1.0 mL (2–3 mg/mL total protein concentration).
2. 50 µl (5 µg IgG) of polyclonal anti-GFP coupled to sepharose beads (Abcam) is added to each IP. As controls for specificity, we also include samples derived from *srp-6(ok319)* lysate (untagged) incubated with GFP-sepharose beads as well as TrAP::SRP-6 lysate incubated with uncoupled sepharose beads (no antibody).
3. IPs are rotated for 8–12 h at 4°C.
4. Beads (~ 25 µl pellet) are centrifuged at 500 Xg and the supernatant is removed. A small amount of supernatant may be saved to check for immunodepletion of TrAP::SRP-6 from the original lysate. The beads are washed a total of 3X with worm lysis buffer containing protease inhibitors.
5. To liberate the TrAP::SRP-6 protein complexes from the GFP beads, the pelleted beads are resuspended in a volume of 50 µl worm lysis buffer without protease inhibitor. To this slurry, 1 µl (50 Units) of 2Ez TEV™ (Genlantis) is added and gently rotated 8–12 h at 4°C.
6. The supernatant containing complexed TrAP::SRP-6 is removed and stored on ice. The remaining beads are then washed 2X with 50 µl worm lysis buffer containing protease inhibitors. Each wash is added to the original supernatant creating a final volume of 150 µl. At this point the remaining beads can be saved for later analysis of TEV efficiency.
7. For the second round of purification, the 150 µl TEV cleavage supernatant is diluted 1:1 with worm lysis buffer containing protease inhibitors (300 µl final volume). 100 µl (5 µg IgG) of polyclonal anti-c-myc coupled to agarose beads (Sigma) is added to each IP.
8. IPs are then rotated at 4°C for 4 h. Following this incubation, the beads (50 µl pellet) are washed under the same conditions as described in step 4.

9. To liberate TrAP::SRP-6 protein complexes from the c-myc beads, the pelleted beads are resuspended in a volume of 30 μ l worm lysis buffer without protease inhibitor. To this slurry, 2.0 μ l (50 Units) of PreScission™ Protease (GE Healthcare) is added and samples are gently rotated for 4 h at 4°C.
10. For the removal of the PreScission™ Protease, the supernatant is passed one time through a prepacked GST SpinTrap™ microspin column (GE Healthcare).
11. Following removal of the PreScission™ Protease, a Bradford Assay is performed on the supernatant to determine the total protein concentration of the sample. A typical sample will have a protein concentration of 10–50 ng/ μ l. The sample is digested with trypsin and the resultant tryptic peptides are analyzed using LC-MS/MS for peptide/protein identification. If the overall protein concentration is too low for MS/MS, multiple IPs may be combined and concentrated to a suitable volume to provide an increased protein yield using Amicon® UltraCel (10 K membrane) centrifugal filters (Thermo Fisher).

2.5.2 In solution trypsin digestion of purified protein

1. Adjust the samples to final volume of 90 μ l with 50 mM ammonium bicarbonate (NH_4HCO_3 , pH 8) buffer.
2. Add 1 μ l 1M dithiothreitol (DTT; Sigma-Aldrich) to each sample and place at 99°C for 10 min.
3. After samples cool down, add 4.5 μ l 450 mM iodoacetamide (IAA; Sigma-Aldrich) to each sample and incubate at room temperature in the dark for 1 hour.
4. Add trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega) to a final protease:protein ratio of 1:20.
5. Incubate at 37°C overnight.
6. Terminate trypsin activity by adding 100 μ l 0.1% trifluoroacetic acid (TFA).
7. Prepare C18 spin column (Pierce®) by washing with 200 μ l 70% acetonitrile (ACN) /0.1% TFA twice, then equilibrating with 200 μ l 0.1% TFA twice. Each wash with 1 min centrifugation at 1500 x g. Discard the flow-through.
8. Load the samples into prepared C18 spin column. Centrifuge at 1500 x g for 1 min. Re-load the flow-through once to ensure complete binding of tryptic peptides to C18 resin.
9. Wash the spin column with 200 μ l 0.1% TFA three times. Each with 1 min centrifugation at 1500 x g.
10. Elute with 40 μ l 70% ACN/0.1% TFA twice, each with 1 min centrifugation at 1500 x g. Combine both flow-through.
11. Lyophilize the samples and resuspend in 10 μ l 0.1% formic acid (FA).

2.5.3. Tandem mass spectrometry—Desalted tryptic peptides were analyzed with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Detailed setup for LC-

MS/MS will vary according to different systems used. The results showed in this work were analyzed with a Waters nanoACQUITY UPLC system coupled online to LTQ/Orbitrap Velos hybrid mass spectrometer (ThermoFisher) in the following manner:

- 1 Tryptic peptides were separated using a C18 reverse-phase column using a 90 min gradient running at 300nl/min (5% B for 3 min, 5–30% B in 60 min, 30–95% B in 1 min, 95% B for 5 min, 95%–5% B in 1min, 5% B for 20 min).
- 2 The LTQ/Orbitrap instrument was operated in a data-dependent MS/MS mode in which each high resolution broad-band full MS spectra ($R = 60,000$ at mass to charge (m/z) 400, precursor ion selection range of m/z 300 to 2000) was followed by 9 MS/MS scans in the linear ion trap where the 9 most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 35%. Dynamic exclusion was enabled to minimize redundant selection of peptides previously selected for CID.
- 1 MS/MS spectra were searched with the MASCOT search engine against a UniProt human proteome database (February 2013 release) from the European Bioinformatics Institute (<http://www.ebi.ac.uk/integr8>) merged with a contaminant database from AB Sciex (Framingham, MA) with the following modifications: static modification of cysteine (carboxyamidomethylation, +57.0214 Da) and variable modification of methionine (oxidation, +15.9949 Da). The mass tolerance was set at 10 ppm for the precursor ions and 0.5 Da for the fragment ions. Peptide/protein identifications were filtered using Scaffold (Proteome Software, Inc) based on peptide threshold of 5% false discovery rate (FDR) and protein threshold of 1% FDR according to PeptideProphet and ProteinProphet algorithms, respectively, and with a minimum identification of 2 different peptide sequences.

3. Results

3.1 Overview

In the following section, we present data demonstrating the effectiveness of the individual steps of the two-round purification process. In addition, we describe the workflow, quality control steps, and data analysis of a typical one-round purification IP experiment using TrAP::SRP-6.

3.2 Validation of TrAP::SRP-6 purification procedure

As shown in Fig 3, using immunoblot analysis TrAP::SRP-6 is immunoprecipitated from whole animal lysates using a polyclonal GFP antibody coupled to sepharose beads (lane 4). Using the FLAG epitope to detect TrAP::SRP-6, a band with a molecular mass of 72 kDa is detected from un-immunoprecipitated TrAP::SRP-6 lysate (lane 1), corresponding to the predicted molecular mass of full-length TrAP::SRP-6. A band of similar mass is detected when TrAP::SRP-6 lysate is incubated with anti-GFP sepharose beads (lane 4), demonstrating that TrAP::SRP-6 is effectively immunisolated from whole animal lysates. However, no FLAG-reactive bands were detected in IPs performed with GFP coupled beads

incubated with untagged lysate (lane 2) or IPs in which uncoupled beads were incubated with TrAP::SRP-6 lysate (lane 3), indicating the specificity of the first round IP. Incubation of the GFP beads with TEV results in the detection of a truncated TrAP::SRP-6 product at the predicted mass of 42 kDa (lane 5), consistent with efficient removal of the GFP epitope and completion of the first round purification step. To begin the second purification round, TEV-cleaved TrAP::SRP-6 eluate is efficiently recovered by incubation with anti-c-myc agarose beads, as demonstrated by the presence of a 42 kDa band (lane 6). Finally, incubation of the c-myc beads with PreScission protease results in the detection of a 40 kDa product, consistent with the removal of the c-myc epitope (lane 7). This experiment highlights the effectiveness of the individual purification steps, demonstrating the overall functionality of TrAP tag system. Purified samples are routinely analyzed after one round of purification, following TEV cleavage. However, the second round purification step using PreScission cleavage may also be employed to refine interacting protein specificity. The following section describes the assay quality control steps and classification of SRP-6 interacting proteins identified in a single purification experiment.

3.3 Identification of distinct groups of SRP-6 interacting proteins using TrAP::SRP-6 purification

3.3.1 Assay quality control—Following incubation of lysate with anti-GFP beads a band of 72 kDa representing TrAP::SRP-6 is detected by Coomassie staining (Fig 4A). Following TEV cleavage to remove the isolated TrAP::SRP-6 from the beads, a band of ~42 kDa is observed (Fig 4B) indicating that TEV cleavage is efficient. Additionally, as no FLAG-positive band was detected when GFP beads were re-eluted with sample buffer after TEV cleavage (Fig 4B, lane 1). This demonstrates that minimal TrAP::SRP-6 remains associated with the GFP beads after TEV cleavage. In contrast, a 42 kDa band is detected in the TEV eluate (lane 2), demonstrating that the GFP epitope has been removed from the isolated TrAP::SRP-6. To further enhance the total amount of TrAP::SRP-6 and any potential interacting proteins present in the TEV eluate for downstream proteomic analysis, three individual TEV eluates were combined and concentrated (lane 3). As a result of this step, TEV eluate concentrations were increased from 0.02 µg/mL to 0.07 µg/mL total protein.

3.3.2 Proteomic analysis of IP samples and description of identified proteins

—Concentrated IP samples were prepared for proteomic analysis by digestion of the eluates with trypsin. Digested samples were analyzed by nano-LC-MS/MS and individual peptides were identified from the collision-induced dissociation (CID) spectra and are then queried against a *C. elegans* proteome database. The obtained list of identified peptides was correlated to identify specific proteins using MASCOT search engine. In this experiment, six unique peptide sequences corresponding to SRP-6 were identified, representing 23% total coverage of the protein (Table 1). The list of 181 identified proteins from this experiment was analyzed using the database for annotation, visualization, and integrated discovery (DAVID) bioinformatics resources. DAVID is an open-access resource for the analysis of large data sets generated from high-throughput genetic and proteomic experiments that uses various data-mining parameters to assign biological relevance and functional groupings to a given data set [18]. The list of putative SRP-6 interacting proteins

was uploaded to DAVID using the UniProt accession number as the primary gene identification feature. Once uploaded, lists of individual proteins are analyzed using a specific DAVID analytic tool. The data set from this experiment was analyzed using the 'gene functional classification' tool that separates a specific list of proteins into distinct protein groups using various text and biological pathway-mining applications. The advantage of this analytic tool is that it groups individual proteins, allowing for the identification of potential protein networks with biological significance. The main disadvantage to this analytic method is that specific proteins from a data set without strong associations to other proteins from within the set are left out from the overall grouping analysis. For our set of identified proteins, 159 out of 181 total proteins were analyzed in this manner (88% of the total list). As shown in Fig 5, the list of putative SRP-6 interacting proteins from this IP experiment can be grouped into seven functional categories. The proteins identified contain a broad list both protease and non-protease putative serpin regulatory targets.

4. Discussion

The overall goal of these studies was to determine whether the TrAP::SRP-6 transgene was detectable using our protocol. Taken together, our data demonstrate that this affinity purification procedure yields a SRP-6 species that is detectable by nanoLC-MS/MS after a single round of purification. We are working to verify which of the associated proteins are legitimate SRP-6 interacting partners by addressing several issues. It is a possibility that the overexpression of SRP-6 in our system may lead to the identification of false positive interacting proteins. However, serpin complexes are degraded *via* the proteasome, resulting in a short predicted half-life [19]. Thus, an overexpression system is used to increase overall SRP-6 abundance to maximize the probability of detecting *in vivo* SRP-6 complexes. To alleviate the concern of increased false positive detection, we are carrying out our procedure in several different ways. First, we are comparing mass spectrometry data obtained from multiple different IP reactions from a single round of purification and identifying those proteins that arise in all data sets. Second, we are performing a second round of purification. Ideally, if the interacting protein is in a true complex with SRP-6, it should remain throughout the purification. However, we expect that with increased purification steps the sensitivity for detecting SRP-6 complexes will decrease, but the specificity of proteins identified will increase. Third, we can also eliminate potential contaminating proteins by increasing the stringency of the IP wash conditions. Our current protocol uses 150 mM NaCl, but other reports have used up to 300 mM NaCl [13]. Fourth, Quantitative mass spectrometry can also be applied to compare the relative abundance of pull-down proteins in the presence of SRP-6 *vs.* in the absence of SRP-6 to overcome the problem of false positive identification [20]. Finally, once these steps are applied, to confirm that the identified proteins are "true" interactors of SRP-6 and not false positives, it is important to perform secondary screening using genetics and cell biological techniques to confirm the interaction.

In the purification procedure described here, the addition of protease inhibitors is added to animal lysates post-lysis to (1) prevent spurious interactions between SRP-6 and proteases released from non-cytosolic compartments during the lysis process, and (2) to prevent the degradation of SRP-6:protease complexes that are present in the prepared lysate. The

addition of protease inhibitors does not occur while SRP-6-expressing animals are intact, eliminating the concern that their addition could interfere with *in vivo* SRP-6:protease complex formation. As described above, serpin:protease complexes are short-lived and could potentially be degraded by the proteasome [19], thus one way in which to increase the detection of the complex may be to perform immunoprecipitations under conditions of proteasomal inhibition. We have demonstrated that treatment of *C. elegans* with bortezomib efficiently inhibits proteasomal function [21], suggesting that this treatment may increase the concentration of SRP-6 containing complexes in our experimental system. In addition, while serpin:protease complexes are held together *via* a covalent bond, non-protease SRP-6 interacting partners are most likely held via ionic interactions, creating the potential risk for loss of these components throughout the purification process. To alleviate this issue we are developing the use of chemical cross-linking reagents to stabilize these non-covalent interactions in either animal lysates or live animals.

To confirm that any potential SRP-6:protease interaction is specific, we can perform pulldown experiments using transgenic animals expressing a *srp-6* gene with either a wild-type or mutated reactive site loop (RSL). The classical P14 mutation prevents covalent serpin:protease complex formation with any target protease [22]. We would predict that a putative protease target identified from pulldown performed from wild-type SRP-6 lysates would not be present in lysates prepared from animals harboring the P14 mutation. This mutant strain will provide specificity to any proteases isolated as SRP-6 interacting partners however, will not prevent non-protease regulatory proteins that bind to SRP-6 in other locations other than the RSL. To validate the specificity of non-protease SRP-6 interacting proteins, we can perform pulldown experiments using control transgenic animals expressing a non-serpin *C. elegans* protein, such as glutathione S-transferase 44 (GST-44). This 29 KDa protein will be expressed under the control of the *nhx-2* promoter and contain an N-terminal TrAP tag, providing us with a cytosolic, non-serpin control to identify the identity of non-specific protein interactions that are present within our purification setup. Once the identities of non-specific interacting proteins have been identified, these specific proteins can be eliminated as potential SRP-6 interactors.

Using the controls described above will provide increased specificity to the process of identifying specific SRP-6 interacting proteins. Once a specific candidate protein has been identified through MS analysis, the interaction will be verified in multiple ways. First, we will verify the interaction by performing reciprocal immunoprecipitations between SRP-6 and the candidate protein. Second, we can verify the biological relevance of this interaction by using RNAi to rescue known phenotypes associated with *srp-6* deletion in *C. elegans* [8]. Thirdly we can use visualize the interaction *in vivo* by microscopy using FRET based reporter constructs.

5. Conclusion

C. elegans is a useful model organism for combining multiple imaging, genetic, and biochemical methodologies to gain more insight into the biological function of serpins. We have described a method to biochemically isolate *in vivo* serpin-interacting proteins using an affinity tagged version of SRP-6. As the knowledge of *in vivo* SRP-6 interacting partners

using *C. elegans* has largely been obtained using genetic and *in vitro* recombinant protein studies, we sought to complement these findings using an affinity protein purification setup to isolate interacting proteins from animals that express TrAP::SRP-6. Towards this end, we have demonstrated that our overall affinity purification procedure is effective in recovering TrAP::SRP-6, and we have used this protocol to identify candidate SRP-6 interacting proteins with a variety of biological functions that have been isolated after one round of affinity purification. Collectively, our findings using this experimental approach will introduce insight into the biological function of SRP-6 in the *C. elegans* model system and provide the basis for overlapping studies in mammalian systems.

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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 μ g 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.

Fig 3.

Validation of TrAP::SRP-6 purification procedure. Western blot analysis using the FLAG epitope can be used to demonstrate the effectiveness of each step in the TrAP::SRP-6 purification procedure. (Lane 1) TrAP::SRP-6 is detected in whole animal lysate prepared from transgenic animals. (Lane 2) No FLAG-positive band is detected in IP samples where anti-GFP beads are incubated with lysate prepared from animals that do not express TrAP::SRP-6. (Lane 3) No FLAG-positive band is detected in IP samples where uncoupled sepharose beads are incubated with lysate prepared from TrAP::SRP-6 expressing animals. (Lane 4) A FLAG-positive band is detected at 72 kDa in IP samples where TrAP::SRP-6 lysate is incubated with anti-GFP beads. (Lane 5) GFP beads incubated with TEV display a FLAG-positive band at 42 kDa demonstrating effective recovery after TEV cleavage. (Lane 6) TrAP::SRP-6 is recovered post-TEV by incubation with anti-c-myc beads. (Lane 7) c-myc beads incubated with PreScission Protease display a FLAG-positive band is detected at 40 kDa.

Fig 4.

Isolation of TrAP::SRP-6 protein complexes for proteomic analysis. (A) Coomassie stained SDS-PAGE identifying TrAP::SRP-6 following first round IP with anti-GFP. A distinct band is detected at 72 kDa (arrowhead), indicating TrAP::SRP isolation following first round IP with anti-GFP. (B) Western blot following TEV cleavage and subsequent concentration of three post-TEV supernatants. Following IP with anti-GFP, the beads are processed for TEV cleavage to liberate TrAP::SRP-6. A 42 kDa FLAG-positive band is detected in the TEV sup fraction (lane 2), but no band is detected in the GFP beads fraction (lane 1), demonstrating efficient TEV cleavage. A more intense FLAG-positive signal was observed (lane 3) by combining and concentrating the supernatants from 3 individual TEV reactions to generate a larger amount of TrAP::SRP-6 for MS/MS analysis

Fig 5.

Classification of putative TrAP:SRP-6 interacting proteins. This chart summarizes the major functional category of putative SRP-6 interacting proteins obtained from proteomic analysis. Individual proteins identified *via* MS/MS were grouped into biologically relevant categories using DAVID software, a large database of bioinformatics resources used to assign biological relevance to large gene or protein data sets. These results were obtained from one representative TrAP::SRP-6 IP experiment. Using DAVID, 159 of 181 proteins identified from the IP were grouped into specific categories. The data for each category is expressed as an overall percent of the proteins identified.

Table 1

List of TrAP::SRP-6 peptides identified by MS/MS.

Identified Peptide Sequence	Amino Acid Position
QQNLTESFVFSPLSIALALSLVHVAAK	27-53
IINPDSISEELVAVLTNAFYFK	153-174
EFFSSNSKR	190-199
RETEFLHSR	199-207
ALMAVGIEK	286-294
DNHPLFIGIHN	365-375