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A Proteomics Approach To Quantify Protein Levels Following RNA Interference: Case Study with Glutathione Transferase Superfamily from the Model Metazoan *Caenorhabditis elegans*

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Loss-of-function phenotypic analysis via interference RNA (RNAi) technology is a revolutionary approach to assigning gene function. While transcript-based methodologies commonly validate RNAi gene suppression investigations, protein-based validation is less developed. This report illustrates the potential for two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-DE) and gel analysis to quantify protein levels following RNAi. This case study involves three glutathione transferase (GST) genes targeted by RNAi from the model organism *Caenorhabditis elegans*.

Keywords: RNAi • Glutathione transferase • *Caenorhabditis elegans*

Introduction

Loss-of-function phenotypic analysis via interference RNA technology (RNAi) offers a revolutionary approach to assigning gene function.[1] However, genome-wide RNAi screens in model organisms, such as the nematode *Caenorhabditis elegans*, report no obvious phenotypic alteration upon suppression of a small, but significant proportion of genes.[2] Where transcript suppression is confirmed, this problematic RNAi outcome may be due to the biological function of the gene product not being appropriately mimicked *in vitro*, or may be the result of redundancy between similar or interacting genes within the genome.

Additionally, despite significant gene transcript reduction, multiply translated mRNAs or highly active enzymes may be suppressed at insufficient levels to produce a practically measurable phenotype. Transcript-based technologies only infer phenotype-inducing protein levels following RNAi. Therefore, to provide quality assurance that RNAi gene suppression has been successful or reveal evidence of RNAi failure, the gene depression outcome should also be robustly examined at the level of protein. However, proteomic-based verification of RNAi suppression is not usually part of the validation process, and often semiquantitative antibody-based approaches are used despite their known cross-specificity, quantification and resolving power issues.

Two-dimensional sodium dodecyl polyacrylamide gel electrophoresis (2-DE) proteomics has the potential to support RNAi experiments by providing a global and subproteomics search platform to robustly resolve and provide relative quantification of proteins within a complex mixture. Additionally, proteomics may uncover evidence of specific and off-target protein knockdown, protein-interaction networks and putative response cascades following the RNAi procedure. This report highlights the support of 2-DE subproteomics following RNAi suppression of selected members of the glutathione transferase (GST) phase II detoxification superfamily genes in the model organism *C. elegans*.

Materials and Methods

***C. elegans* Culture and RNAi.** *C. elegans* N2 Bristol strain wild-type was provided by Caenorhabditis Genetics Center; RNAi clones in vector pL4440 (ampicillin/carbenicillin resistant) of *C. elegans* GST genes CE00302, CE01613 and CE07055 (WormBase codes www.wormbase.org) and control vector pL4440[3] were obtained from Geneservice Ltd. U.K. (http://www.geneservice.co.uk/) and constructed by Dr. Julie Ahringer's laboratory at The Wellcome CRC Institute, University of Cambridge, Cambridge, England.[4,5] The constructs GST-1 and GST-5 do not have recognized secondary targets of RNAi within the *C. elegans* genome database (www.wormbase.org), and although GST-7 has a reported secondary RNAi target in GST-8, this protein product was not detected at a 2-DE level within this study or in a previous study into the *C. elegans* GST proteome.[6] Laboratory stock cultures were maintained at 20 °C on nematode growth media agar (NGM) as described,[7] aside from the food source being changed from *Escherichia coli* HB101 to the RNAi compatible *E. coli* HT115(DE3) tetracycline-resistant, RNaseIII-deficient strain.[8] Culture in liquid media was as described[6] with adaptations for RNAi suppression carried out according to bacterial feeding method protocols from the Fire laboratory (http://firelab.stanford.edu/ accessed (20.05.07)).

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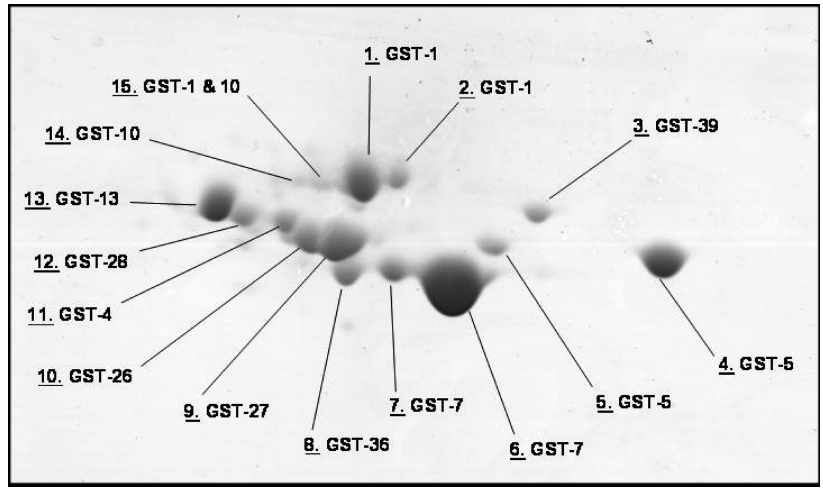

Figure 1. 2-DE profile of GSTs isolated from *C. elegans* N2 under laboratory conditions. The numbered spots were identified via MALDI-ToF, PMF and investigated for expression values in response to RNAi (Tables 1 and 2).

C. elegans eggs were prepared via the hypochlorite method[9] before incubation on a shaker table at 200 rpm, 20 °C overnight (~16 h) to allow hatching to first larval stage (L1). Approximately 200 000 L1 worms were added to each 500 mL of S-Basal liquid culture medium along with 15 mL of *E. coli* HT115(DE3) (including appropriate RNAi vector construct) as food source. A further 25 mL of *E. coli* HT115(DE3) was added after 36 h. Following growth and reproduction in liquid S-Basal for 96 ±5 h, nematodes were harvested as described,[6] cryogenically frozen and stored at −80 °C.

Samples cultured this way were found to contain life-stages from L1 to adult in approximate proportions as follows; 15% adults, 45% L1/L2, 40% L3/L4. Dauer stages were not evident. A typical yield was found to be approximately 4 g of wet-weight worm pellet.

Affinity Isolation and 2-DE Resolution of GSTs. GSTs were purified from *C. elegans* soluble cytosolic supernatants via glutathione (GSH)-affinity chromatography as previously described.[6,10] All purified samples showed significant GST enzyme activity, confirming the presence of GST when assayed for activity with the universal model substrate 1-chloro-2, 4-dinitrobenzene (CDNB) as described previously.[11] 2-DE was performed according to previously described methods.[12,13]

2-DE Image Analysis. 2-DE gels were scanned upon a Bio-Rad GS-800 densitometer for gel image analysis with Progenesis PG220 software, version 2006 (Nonlinear Dynamics Ltd., U.K.). Spots were detected manually and volumes normalized based upon total spot volume multiplied by 100. Background subtraction was via mode of nonspot. Relative quantitation of protein spots between and within 2-DE gels was based on normalized spot volumes. Each experimental analysis was based upon three independent biological replicates for each condition.

Mass Spectrometry and Protein Identification. Proteins spots excised from gels were identified following matrix assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry and peptide mass fingerprinting (PMF) as previously described.[6] Protein Prospector v4.0.8. 'MS-Fit' program (http://prospector.ucsf.edu/prospector/4.0.8/html/msfit.htm accessed May 20, 2007)[14] was used to obtain PMF results.

Results and Discussion

The expression of three major GST proteins in *C. elegans* was suppressed using RNAi-mediated interference. The GST protein complement was then examined following GSH-affinity chromatography and quantitative analysis of the subproteome. Fifteen matching spots from each RNAi suppression experiment were investigated following 2-DE, MALDI-ToF, PMF and gel image analysis, (Figure 1. and Table 1.). Targeted RNAi gene suppression was detectable and relatively quantified on 2-DE gels for the respective protein products (Figures 2 and 3; Table 2.). Proportions of targeted GSTs were significantly different from control experiments, and allow a measure of the specificity of the RNAi constructs to their target with the following Student's *t* test *p*-values for RNAi: GST-1, 1.39×10^{-3}; GST-5, 1.24×10^{-2}; GST-7, 3.57×10^{-6} (Supplementary Table S1). This case study focuses on a large superfamily of proteins, many of which share high sequence identity (>50%), and are consequently candidates for possible off-target effects.[15] However, proportions and suppression levels of the proteins of nontargeted GSTs for each RNAi-mediated GST knockdown were also assessed (Table 2 and Figure 3) but were not significantly different across or between experiments (Supplementary Table S2) except in the case of GST-36 (spot no. 8) which increased

Table 1. PMF Results of Representative Spots Cut From 2-DE Gels and Subjected to MALDI-ToF MS[a]

spot no.	protein ID	acc. no.	MOWSE score	no. of peptides matched (%)	% peptide coverage
1	GST-1	P10299	2.01×10^7	8 (40)	43
2	GST-1	P10299	3.86×10^6	8 (40)	41
3	GST-39	Q9NAB0	8.62×10^4	6 (40)	41
4	GST-5	Q09596	1.52×10^8	13 (86)	70
5	GST-5	Q09596	1.28×10^6	10 (66)	49
6	GST-7	P91253	1.36×10^8	12 (26)	50
7	GST-7	P91253	8.65×10^6	12 (80)	40
8	GST-36	Q09607	8.99×10^4	9 (64)	48
9	GST-27	Q9NAB3	2.09×10^5	9 (60)	47
10	GST-26	Q9NAB4	5.56×10^5	8 (53)	53
11	GST-4	Q21355	1.12×10^5	7 (87)	30
12	GST-28	Q9NAB2	5.27×10^6	9 (60)	49
13	GST-13	Q22814	9.33×10^3	10 (66)	47
14	GST-10	Q9N4X8	1.62×10^6	11 (73)	49
15	GST-1	P10299	7.43×10^4	8 (40)	29
	GST-10	Q9N4X8	1.70×10^7	11 (73)	48

[a] Protein Prospector v4.0.8. 'MS-Fit' program (http://prospector.ucsf.edu/prospector/4.0.8/html/msfit.htm accessed May 20, 2007)[14] was used to obtain PMF results. Spot 15 was difficult to resolve, and was found to contain overlapping proteins from both GST-1 and GST-10.

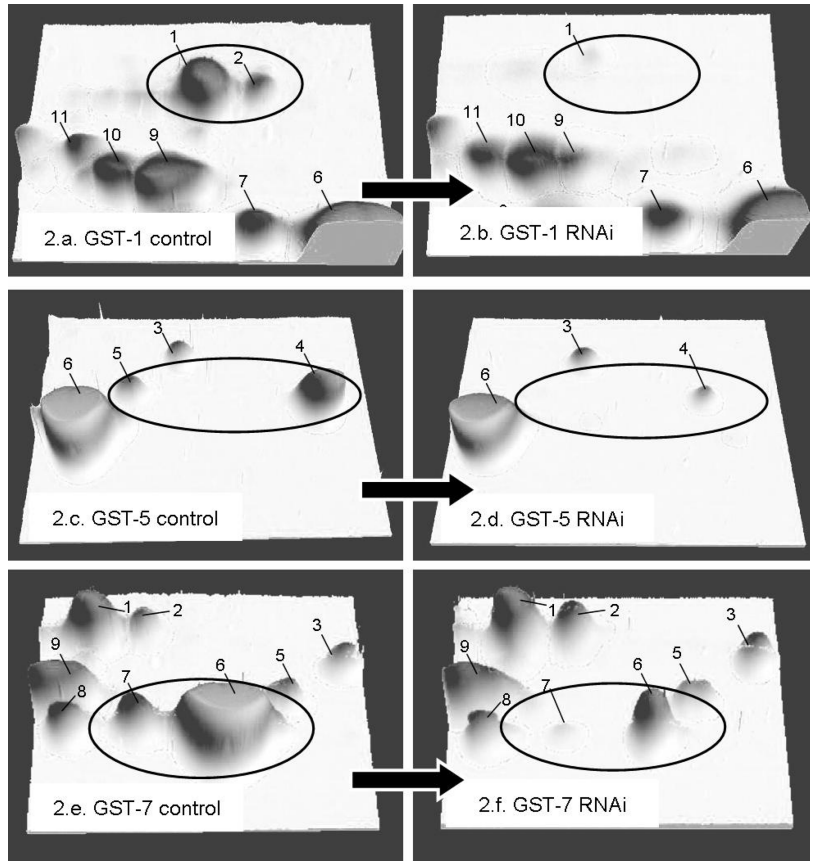

Figure 2. Representative three-dimensional profiles of the reduction in GSTs isolated from *C. elegans* N2 following RNAi exposure and resolved via 2-DE. The circled spots in each of the RNAi experiments represent the proteins from the targeted genes, with their corresponding 2-DE gel positions in the 'Control' experiment also shown. The effects of specific RNAi suppression are clear in comparison to the 'Control'. Spot identities are listed in Table 1. Table 2 and Figure 3 provide quantitative data.

in response to targeted GST knockdown ($p < 0.01$). This GST could now be further investigated at transcript level, immunologically, via additional protein isolation procedures and with alternative RNAi transcripts targeted to specific regions of the gene to explore the observed upregulation.

Optimization of a given protocol to promote effective RNAi within laboratories, and for specific genes and tissues remains a significant challenge for this still emerging and developing technique. Common problems still persist, however, are reported by many laboratories, and are described in some detail by the Fire laboratory within their online protocols (http://firelab.stanford.edu/ accessed (20.05.07)); for example, apparently unexplained variability of RNAi, tissue penetrance and subsequent suppression level as well as potential 'off-target' effects resulting in the nonspecific suppression of other gene products.[15] Thus, the final protein levels for the targeted genes and an appreciation of the response profile of nontarget genes is essential in comparing and grouping data sets prior to developing hypotheses or conclusions. Although several studies report evidence of varying levels of 'off-target' interference for some non-GST transcripts,[15] this has yet to be verified at a 2-DE proteomic level.

Previous genome-wide investigation of *C. elegans* revealed no observed significant impact on morphology or reproduction when GSTs 1, 5 and 7 were targeted.[4] Subsequent investigations showed that GST-1 RNAi may confer an increased susceptibility to pathogens,[16] and GST-5 RNAi may increase susceptibility to apoptosis.[17] However, visible changes in the phenotypic morphology of *C. elegans* were not evident and culture yields appeared unaffected in our investigations. GST subproteomic analysis in this study can now suggest the function of these particular GSTs is compensated for either by the very low level of protein remaining or by subtle alteration in regulation of another GST (genetic buffering).

RNAi is an emerging and developing technique, with research community initiatives underway to construct guidelines for the provision and sharing of data in order to allow robust interpretation and reproduction of experiments (e.g., Minimum Information About an RNAi Experiment (MIARE) http://miare.sourceforge.net/HomePage). We argue that a window of opportunity exists to include proteomic approaches within these largely transcript-based procedural guidelines. An open debate approach to the inclusion of validations in developing standard guidelines is needed to prevent stifling research and innovation in a field before it has matured.[18]

It has not been the focus of this case study to attempt to hypothesize upon the functional and biological significance of the changes in response levels of protein to RNAi. Rather, this report aims to promote the potential of the proteomic method of 2-DE in conjunction with fractionation of the proteome in assessment of RNAi technologies. This case study displays a subsection of proteins selected via affinity chromatography. There are other methods such as differential centrifugation, solubility and molecular size to fractionate complex protein mixtures in order to enrich for the protein(s) of interest. These methods do not require dedicated protein purification equip-

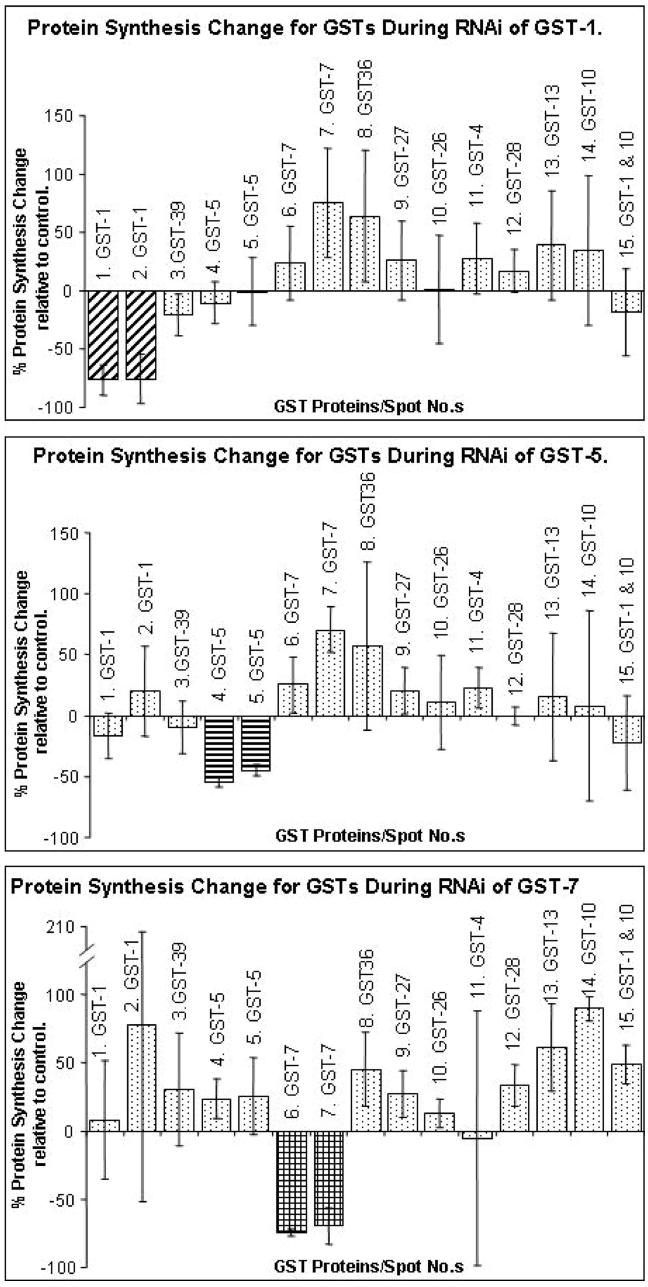

Figure 3. Mean percentage protein synthesis change across each RNAi experiment relative to controls. The reduction in protein for RNAi targeted genes is significantly different from overall change in nontargeted genes where variance was typically high (Student's *t*-test *p*-values of RNAi GST-1, 1.3×10^{-9}; RNAi GST-5, 7.7×10^{-12}; RNAi GST-7, 5.3×10^{-16}). Plasticity of protein synthesis response within nontargeted genes could suggest interactions and cascades may be initiated upon a disturbance of protein equilibrium and/or thresholds. Proteins of targeted genes are highlighted as hatched bars. 'Spot No.' refers to the 2-DE gel identification shown in Figure 1.

ment. Standard global 2-DE protein arrays would allow an opportunity to observe protein responses from off-targeted gene outcomes of RNAi.

The opportunity to identify and measure the response of proteins to targeted gene suppression provides a platform from which to design rational approaches to determine protein functions, redundancy, response cascades and interactions, as well as providing information upon any possible nonspecific

Table 2. Mean Percentage Proportion of Protein in total GSH-Affinity Preparations across Each RNAi Experiment[a]

		mean percentage proportion of protein (± standard deviation)			
spot no.	GST no.	RNAi of GST-1	RNAi of GST-5	RNAi of GST-7	control
1 and 2	GST-1	**4.3 (*3.4*)**	15.3 (*5.2*)	19.8 (*8.3*)	**17.5 (*9.2*)**
3	GST-39	1.7 (*0.3*)	2 (*0.6*)	2.8 (*0.7*)	2.1 (*0.4*)
4 and 5	GST-5	17.9 (*5.2*)	**9.8 (*4.8*)**	25 (*9.4*)	**20.4 (*9.3*)**
6 and 7	GST-7	36.1 (*8.6*)	36.4 (*8.4*)	**7.3 (*1.6*)**	**28.1 (*8.5*)**
8	GST-36	5 (*0.5*)	4.7 (*1.0*)	4.5 (*0.2*)	3.1 (*0.6*)
9	GST-27	9.9 (*3.7*)	9.1 (*2.1*)	10.2 (*4.7*)	7.7 (*2.5*)
10	GST-26	4.4 (*1.0*)	4.8 (*1.2*)	5.2 (*1.0*)	4.6 (*1.2*)
11	GST-4	2.1 (*0.7*)	2.1 (*0.9*)	1.4 (*1.2*)	1.7 (*1.0*)
12	GST-28	2.4 (*0.5*)	2.1 (*0.6*)	2.9 (*1.3*)	2.1 (*0.7*)
13	GST-13	9.7 (*1.5*)	8.1 (*3.9*)	11.7 (*2.7*)	7.5 (*3.0*)
14	GST-10	4.8 (*5.6*)	4 (*5.6*)	6.4 (*5.1*)	3.4 (*2.7*)
15	GST-1 and 10	1.7 (*1.2*)	1.7 (*1.2*)	2.8 (*1.2*)	1.9 (*30.5*)

[a] The extent of suppression for RNAi targeted genes is clear across all experiments (bold, underlined). The protein proportions of targeted genes are significantly different from their levels in control and RNAi experiments where they have not been subjected to RNAi (Student's *t*-test *p*-values for RNAi; GST-1, 1.4×10^{-3}; GST-5, 1.2×10^{-2}; GST-7, 3.6×10^{-6}). Suppression data for all apparent isoforms of a targeted gene have been grouped here. 'Spot no.' refers to the 2-DE gel identification in Figure 2.

effects. This is of particular value in the investigation of genes where little or no immediately observable phenotype occurs, and avoids the incorrect inclusion of nonsuppressed or differentially suppressed organisms into an interactive data set.

Proteomic RNAi validation quantitatively links altered protein levels of the targeted gene to observed phenotypic effects, with additional information relating to potentially interacting and responding protein networks. Therefore, we propose proteomic approaches should be incorporated into RNAi standard initiatives as they offer a superior level of information compared to transcript and immunological RNAi validation.

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Supporting Information Available: MALDI-ToF spectra and manually selected peak lists for each spot identified in Figure 1. Representrative view of MALDI-ToF spectrum, peaks selected and output from Protein Prospector v4.0.8. 'MS-Fit' program peptide mass fingerprint search (Figure S1). Three-dimensional image profiles of three biological replicates for each condition including controls (12 experiments in total) of GSTs isolated from *C. elegans* N2 following RNAi exposure and resolved via 2-DE (Figure S2). Percentage normalized protein proportions for each biological replicate (Tables S1 and S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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