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A UPR-independent infection-specific role for a BiP/GRP78 protein in the control of antimicrobial peptide expression in *C. elegans* epidermis

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The nematode *C. elegans* responds to infection by the fungus *Drechmeria coniospora* with a rapid increase in the expression of antimicrobial peptide genes. To investigate further the molecular basis of this innate immune response, we took a two-dimensional difference in-gel electrophoresis (2D-DIGE) approach to characterize the changes in host protein that accompany infection. We identified a total of 68 proteins from differentially represented spots and their corresponding genes. Through class testing, we identified functional categories that were enriched in our proteomic data set. One of these was "protein processing in endoplasmic reticulum," pointing to a potential link between innate immunity and endoplasmic reticulum function. This class included HSP-3, a chaperone of the BiP/GRP78 family known to act coordinately in the endoplasmic reticulum with its paralog HSP-4 to regulate the unfolded protein response (UPR). Other studies have shown that infection of *C. elegans* can provoke a UPR. We observed, however, that in adult *C. elegans* infection with *D. coniospora* did not induce a UPR, and conversely, triggering a UPR did not lead to an increase in expression of the well-characterized antimicrobial peptide gene *nlp-29*. On the other hand, we demonstrated a specific role for *hsp-3* in the regulation of *nlp-29* after infection that is not shared with *hsp-4*. Epistasis analysis allowed us to place *hsp-3* genetically between the Tribbles-like kinase gene *nipi-3* and the protein kinase C delta gene *tpa-1*. The precise function of *hsp-3* has yet to be determined, but these results uncover a hitherto unsuspected link between a BiP/GRP78 family protein and innate immune signaling.

Introduction

The nematophagous fungus *Drechmeria coniospora* infects various species of nematodes. Its spores adhere to the surface of a worm, germinate and perforate the cuticle. The worm's body is then totally invaded by the fungus, rapidly causing death (reviewed by Engelmann and Pujol[1]). When *D. coniospora* infects *Caenorhabditis elegans* this triggers the expression of a large number of genes including those encoding antimicrobial peptides (AMPs) of the NLP family.[2-4] The induction of several *nlp* genes is dependent upon a protein kinase C delta (PKCδ)/p38 MAPK pathway that can be activated in the epidermis either by infection or by sterile wounding.[5] In both cases, signaling passes via TPA-1, a PKCδ that acts upstream of TIR-1, the nematode ortholog of SARM, and a MAPK cassette constituted of a MAP3K (NSY-1), MAP2K (SEK-1) and the p38 MAPK PMK-1. This then acts upstream of the STAT-like transcription factor STA-2 to regulate *nlp* gene expression.[6] The elements that contribute to signaling upstream of TPA-1 have only been partially characterized. Wounding and infection require G-protein signaling upstream of TPA-1, while infection specifically involves the Tribbles-like kinase NIPI-3.[5,7]

Part of the innate defenses against intestinal pathogens and toxins are also mediated by a p38 MAPK cascade that shares many but not all of the elements that act in the epidermis;[8-14] reviewed by Partridge et al.[15] and Coleman and Mylonakis.[16] Intestinal infection or exposure to bacterial toxins can also induce an unfolded protein response (UPR); this too is linked to the p38 pathway.[17,18]

The UPR in *C. elegans* is divided into constitutive and inducible pathways, the former being essential during development.[19,20] Part of the UPR involves activation of the endoribonuclease IRE-1 that leads to the production of an alternatively spliced isoform of the mRNA of the transcription factor XBP-1.[21] Compounds such as thapsigargin, dithiothreitol and tunicamycin that perturb endoplasmic reticulum (ER) homeostasis trigger a UPR and lead to IRE-1 activation. The subsequent production of the specific form of XBP-1 then leads to the expression throughout the organism of a large number of genes, many involved in metabolism, or the secretory pathway, including chaperones.[19-21]

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Feeding worms the bacterial pore-forming toxin Cry5B also activates IRE-1 and upregulates chaperone expression specifically in the intestine. This requires the p38 MAPK signaling cassette.[17] The Gram-negative bacterial pathogen *Pseudomonas aeruginosa* also induces IRE-1-mediated splicing of *xbp-1* mRNA in larvae and consequent chaperone gene expression, in a p38 MAPK-dependent manner.[18] As the UPR-deficient *xbp-1* mutants arrest as larvae when cultured on *P. aeruginosa*,[18] in this case, it was suggested that the ER cannot cope with the combined developmental and defense demands placed upon it (reviewed in Ewbank and Pujol[22]).

In addition to the UPR-mediated changes in protein maturation, turnover and trafficking, the innate immune response may also affect the activity, post-translational modification and subcellular localization of signal transduction proteins. These can be analyzed at a global level through proteomic approaches. Indeed, there have already been a number of informative studies addressing the changes in the proteome that accompany infection of *C. elegans* by several different bacterial pathogens.[23-25]

To extend our characterization of the response of *C. elegans* to *D. coniospora* we have now compared the proteomes of infected and control worms using two-dimensional difference gel electrophoresis (2D-DIGE). This fluorescence-based method allows two different protein samples tagged with two distinct fluorescent dyes to be run on the same gel, thereby improving comparative quantitation. We decided to focus on a single time point, early in the infection, with the hope of detecting changes in proteins involved in signal transduction, rather than finding proteins altered by the pathophysiological consequences of infection. We found that few changes were detected in whole extracts, but after fractionation we detected changes in many proteins. For one of these candidates, a *C. elegans* BiP/GRP78 homolog, we defined a novel role in the regulation of AMP gene expression.

Results

Protein fractionation reveals changes in the proteome induced by fungal infection. The infection of *C. elegans* by *D. coniospora* induces significant changes in gene expression within a matter of hours[26] (and unpublished data). We used a standard 2D-DIGE approach to identify alterations at the protein level in *C. elegans* after 5 h of infection with *D. coniospora*. As with any 2D gel approach, with DIGE it is appropriate to refer to changes in representation, rather than stating that a protein is more or less abundant, unless all protein spots are identified and quantified. With whole animal extracts (FT), when we used a narrow-range pH gradient for isoelectric focusing, although 890 protein spots were detected, we observed no differences between extracts of infected and control worms. With a broad-range pH gradient that allowed 1,478 spots to be resolved, just three differentially represented proteins were detected. Only one of these was present in sufficient quantities to allow identification; it corresponded to the galectin LEC-6 (see Materials and Methods for access to data). We therefore adopted a more laborious approach, separating the extracts into four fractions (F1, F2, F3 and FNS), and performing DIGE as above for each one. The fractionation allowed many more spots to be detected (9,246 in total), and revealed differences in intensity for 67 and 103 spots in the narrow- and broad-range pH gradient gels, respectively. All these spots were excised and analyzed by mass-spectrometry, leading to an identification of a protein from 98 spots (**Tables 1 and 2**). This clearly illustrates the interest of combining DIGE with a prior protein fractionation approach.

Table 1. Protein identification from narrow pH (4–7) gel

Fractions	F1	F2	F3	FNS	FT	Total
Number of detected spots	867	933	883	1,016	890	4,589
Number of differentially represented spots	12	14	13	28	0	67
Number of identified spots	4	6	7	26	0	43

Classification of differentially represented proteins. In some cases, the same protein was identified from more than one spot, either within the same fraction on the same gel, or from different fractions and/or gels. As a consequence, the 98 characterized spots corresponded to 67 individual proteins that were differentially-represented between infected and control worms (**Table 3**). We used WormMart[27] (WS220) to match each of the 68 identified proteins (LEC-6 and the 67 others) with its corresponding *C. elegans* gene (**Table 3**). They fall into many different structural and functional classes (**Table S1**). We therefore performed two complementary bioinformatic analyses to find common themes. We first used the KEGG database[28] to determine whether there was an over-representation of higher-level systemic functions within the list of 68 genes. The most populated categories (**Table 3**) were "implicated in a metabolic pathway" (13 genes), and "protein processing in endoplasmic reticulum" (8 genes). We then used EASE,[29] with our extensive in-house annotations culled from the *C. elegans* literature and referenced to WS220.[4] There were 24 functional classes identified as significantly enriched ($p < 0.001$, Fisher exact test; see Materials and Methods). Among these classes, 6 were related to the response of *C. elegans* to infection, with a further 10 linked to aging and stress-resistance, including to the insulin/DAF-2 pathway (**Tables 4 and S2**). Given the intimate connection between stress-resistance and susceptibility to infection, a part of the observed protein changes could thus be directly or indirectly associated with an innate immune response. The EASE analysis also revealed a potential connection with protein processing in the endoplasmic reticulum, as had been seen with KEGG.

Fungal infection in adults does not provoke the UPR. The proteins linked to protein processing in the endoplasmic reticulum included the calreticulin CRT-1, the protein disulphide

Table 2. Protein identification from broad pH (3–10) gel

Fraction	F1	F2	F3	FNS	FT	Total
Number of detected spots	1,588	1,238	1,083	1,638	1,478	7,025
Number of differentially represented spots	35	13	7	45	3	103
Number of identified spots	20	1	0	33	1	55

Table 3. List of identified proteins from spots with different intensities

Spot N° Range pH 4–7	Spot N° Range pH 3–10	Wormpep name	Worm base ID	Gene name
	F1-740	ZK455.1	WBGene00000040	*aco-1**
F2-401; FNS-17	FNS-677	C34E10.6	WBGene00000229	*atp-2**
FNS-489	FNS-1706	F47B10.1	WBGene00009812	*F47B10.1**
	FNS-1926	K10B3.7	WBGene00001685	*gpd-3**
	FNS-1965	F33H1.2	WBGene00001686	*gpd-4**
	FNS-1717	H28O16.1	WBGene00010419	*H28O16.1**
FNS-228		Y45G12B.1	WBGene00021562	*nuo-5**
F3-516		K12G11.3	WBGene00010790	*sodh-1**
FNS-455		Y49A3A.2	WBGene00013025	*vha-13**
F3-516		Y39G8B.1	WBGene00012722	*Y39G8B.1**
F2-821	F2-1119	Y69A2AR.18	WBGene00022089	*Y69A2AR.18**
FNS-401	FNS-1522	ZK829.4	WBGene00014095	*ZK829.4**
FNS-198		F40F9.6	WBGene00009583	*aagr-3*†*
FNS-463		Y38A10A.5	WBGene00000802	*crt-1*†*
FNS-315		C15H9.6	WBGene00002007	*hsp-3*†*
FNS-411		C07A12.4	WBGene00003963	*pdi-2*†*
	F1-776	Y113G7A.3	WBGene00004754	*sec-23*†*
FNS-239		T05E11.3	WBGene00011480	*T05E11.3*†*
F3-142		T14G8.3	WBGene00011771	*T14G8.3*†*
FNS-157		T24H7.2	WBGene00020781	*T24H7.2*†*
F1-463	F1-1375; F1-1389; FNS-739; FNS-1617	M03F4.2	WBGene00000066	*act-4*
F1-460		T25C8.2	WBGene00000067	*act-5*
	FNS-1450	B0334.3	WBGene00007143	*B0334.3*
F2-100		C08H9.2	WBGene00007463	*C08H9.2*
	F1-1246; FNS1717	C44B7.10	WBGene00016630	*C44B7.10*
	F1-1234	C07H6.5	WBGene00000479	*cgh-1*
	FNS-2317	T03E6.7	WBGene00000776	*cpl-1*
	FNS-2295	F58G1.4	WBGene00010266	*dct-18*
F3-526; F3-531	FNS-1996	C18A11.7	WBGene00001000	*dim-1*
FNS-684		F54H12.6	WBGene00018846	*eef-1B.1*
FNS-684		Y41E3.10	WBGene00012768	*eef-1B.2*
FNS-209	FNS-996; FNS-1002; FNS-1018; FNS-1019; FNS-1055	F25H5.4	WBGene00001167	*eef-2*
		F09B12.3	WBGene00008607	*F09B12.3*
		F57F4.4	WBGene00019017	*F57F4.4*
FNS-285; FNS-286; FNS-287; FNS-288		T21G5.3	WBGene00001598	*glh-1*
FNS-489		C26D10.2	WBGene00001840	*hel-1*
FNS-390		Y22D7AL.5	WBGene00002025	*hsp-60*
	F1-1012	F10C1.2	WBGene00002053	*ifb-1*
FNS-401		M6.1	WBGene00002056	*ifc-2*
	FNS-1051	C43C3.1	WBGene00002067	*ifp-1*
FNS-503		F57B9.6	WBGene00002083	*inf-1*
	F1-553; F1-579	K08H10.2	WBGene00010695	*K08H10.2*
	FNS-1284	Y71H2AM.19	WBGene00002244	*laf-1*

Table 3. List of identified proteins from spots with different intensities (continued)

Spot N° Range pH 4–7	Spot N° Range pH 3–10	Wormpep name	Worm base ID	Gene name
	F1-533; F1-579	K08H10.1	WBGene00002263	*lea-1*
	FT-1877	Y55B1AR.1	WBGene00002269	*lec-6*
	F1–1012	DY3.2	WBGene00003052	*lmn-1*
	FNS-1051	Y48C3A.7	WBGene00003119	*mac-1*
F2-821		Y69A2AR.30	WBGene00003161	*mdf-2*
F3-731		C36E6.3	WBGene00003369	*mlc-1*
	F1-362; F1-367	R07G3.3	WBGene00019940	*npp-21*
FNS-134	FNS-739	F54F2.1	WBGene00003929	*pat-2*
	F2-1199	R05G6.7	WBGene00019900	*R05G6.7*
F2-564; F3-516		F25H2.10	WBGene00004408	*rla-0*
F2-584		B0041.4	WBGene00004415	*rpl-4*
	F1-741	T22F3.3	WBGene00020696	*T22F3.3*
F2-456; FNS-463	F1-1115; FNS1669	K01G5.7	WBGene00006536	*tbb-1*
F2-401; FNS-455		C36E8.5	WBGene00006537	*tbb-2*
	FNS-1567	Y71H2AM.23	WBGene00007000	*tufm-1*
FNS-784		F40G9.3	WBGene00006715	*ubc-20*
F1-114; F3-97	F1-463; FNS-373	F11C3.3	WBGene00006789	*unc-54*
	F1-1234; F1-1246	F08B6.4	WBGene00006819	*unc-87*
	F1-908	Y54E10A.9	WBGene00006888	*vbh-1*
	FNS-534	K09F5.2	WBGene00006925	*vit-1*
	FNS-501; FNS-517; FNS-518	C42D8.2	WBGene00006926	*vit-2*
	FNS-518; FNS-532	F59D8.1	WBGene00006927	*vit-3*
	FNS-531; FNS-534	F59D8.2	WBGene00006928	*vit-4*
	FNS-1126; FNS-1129	K07H8.6	WBGene00006930	*vit-6*
FNS-831		Y48A6B.3	WBGene00012964	*Y48A6B.3*

*Proteins implicated in a metabolic pathway. †Protein processing in endoplasmic reticulum.

isomerase PDI-2 and HSP-3. HSP-3 and the closely related HSP-4 represent the worm's BiP/GRP78 homologs. All these proteins function in the ER to ensure the correct folding of nascent polypeptides and are important components of the UPR. Given the reported link between the UPR and resistance to bacterial toxins and infection,[17,18,22,30,31] we decided to investigate whether the UPR is involved in the host response to *D. coniospora* infection.

A direct measure of the activation of the UPR is provided by the detection of a specific UPR-associated alternatively spliced isoform of the transcription factor XBP-1. In contrast to the splicing of *xbp-1* observed when young adult worms were treated with the UPR-inducing drug tunicamycin, the alternatively spliced isoform of *xbp-1* was not detected following *D. coniospora* infection (**Fig. 1A**). Another indicator of the UPR is an increased expression of *hsp-3* and *hsp-4*. In *C. elegans*, the UPR is often monitored in vivo using a p*hsp-4*::GFP transgene reporter, which has a lower constitutive expression and higher level of induction during a UPR than p*hsp-3*::GFP.[32] In contrast to tunicamycin-treated worms, there was neither induction of an p*hsp-4*::GFP transgene reporter after infection (**Fig. 1B**), nor increase of the *hsp-4* transcript as measured by qRT-PCR (data not shown). This is consistent with previous genome-wide transcriptome studies that found that the expression of *hsp-4* (and *hsp-3*) was not significantly altered following infection with *D. coniospora*.[3,4] When worms carrying a p*nlp-29*::GFP transgene reporter were exposed to tunicamycin, strong GFP expression was observed in young larvae. This is consistent with a previous microarray study that reported the induction of a number of epidermal AMP genes, including *nlp-29*, in L2 larvae treated with tunicamycin.[20] A marked increase in reporter gene expression was also seen in young larvae carrying a p*nlp-30*::GFP reporter transgene. On the other hand, no induction of either of these reporters was seen in L4 or adult worms (**Fig. 1B** and data not shown). Similar results were obtained using the UPR-inducing agents dithiothreitol and thapsigargin (data not shown).

We also tested whether direct activation of effector genes in the epidermis would trigger a UPR. PMA activates the PKCδ TPA-1 that controls multiple AMP genes, including *nlp-29*.[7] It provokes very high levels of AMP gene expression within 4 h (unpublished results). Treating worms with PMA for 5 h did not lead to splicing of *xbp-1* nor to induction of *hsp-4* or the p*hsp-4*::GFP

Table 4. Functional classification by EASE of differentially represented proteins

Gene category	Infection	Stress	List hits	Population hits	Probability
Down ≥ 2x *daf-2* (D6); Halaschek-Wiener 2005		X	25	234	7.6E-31
Proteome changes *S. aureus*; Bogaerts 2010	X		17	109	7.2E-24
Differentially expressed proteins in *crt-1;cnx-1* vs N2 at 20°C; Lee 2006			7	13	9.0E-15
Protein expression; Kim 2001			14	446	4.7E-10
Proteome changes *Aeromonas h.*; Bogaerts 2010	X		7	64	2.8E-09
Down ≥ 2x dauer; Halaschek-Wiener 2005		X	6	36	2.9E-09
Differentially expressed proteins in *crt-1;cnx-1* vs N2 at 25°C; Lee 2006			4	12	7.2E-08
Up > 1.75x in *M. luteus* vs. *Pseudomonas* sp; Coolon 2009	X		6	69	1.7E-07
Glycoproteins GaL6 binding; Kaji 2007			9	287	8.0E-07
Heat shock; Kim 2001		X	4	25	1.8E-06
Cell structural, muscle; Kim 2001			9	332	2.6E-06
Down after organophosphorus pesticide chlorpyrifos + diazinon; Vinuela 2010		X	5	65	3.5E-06
Regulated down_*daf-2* mutant and RNAi,Class2,-IGF1; Murphy 2003		X	7	222	1.4E-05
Down > 1.75x in *Pseudomonas* spp vs *E. coli*; Coolon 2009	X		4	44	1.8E-05
Down after organophosphorus pesticide diazinon; Vinuela 2010		X	5	121	7.2E-05
Regulated down_Bt toxin, Cry5B; Huffman 2004		X	8	442	0.00017
Differentially expressed proteins in *crt-1* vs N2 at 20°C; Lee 2006			2	7	0.00026
Regulated down_Cadmium; Huffman 2004		X	7	388	0.00046
Energy generation; Kim 2001			4	104	0.00052
Up > 1.75x in *B. megaterium* vs. *Pseudomonas* sp; Coolon 2009	X		3	45	0.00055
Down ≥ 2x oxidative stress; Park 2009		X	2	13	0.00095
DNA synthesis; Kim 2001			7	440	0.00096
Up ≥ 2x by PA14 8h; Troemel 2006	X		5	233	0.00146
Overlap Between oxidative stress and aging—downregulated genes by oxidative stress; Park 2009		X	3	66	0.00169

transgene reporter (**Fig. 1A**, data not shown). The expression of many epidermal genes, including some AMPs, is strongly upregulated by osmotic stress.[3,33] Although exposure to high salt did induce a p*nlp-29*::GFP transgene reporter as expected, it did not cause a measurable increase in p*hsp-4*::GFP expression (**Fig. 1B**). Thus, neither fungal infection nor the strong induction of gene expression by PMA or salt provokes a UPR, and conversely a UPR does not trigger the expression of anti-fungal immune effectors in adult *C. elegans*.

***hsp-3* regulates *nlp-29* AMP gene expression.** While the results described above suggested that the UPR did not play a direct role in the antifungal innate immune response, the representation of a number of ER-resident proteins is modulated by infection. This led us to assay directly the role of the corresponding genes in the regulation of *nlp-29* by RNAi. While several of the tested genes had an effect (results not shown), *hsp-3* stood out for its strong effect, essentially totally blocking the induction of p*nlp-29*::GFP normally observed upon infection in adult worms (**Fig. 2A**). A similar abrogation of reporter gene expression was seen in an *atf-6* mutant, but not in a *pek-1* mutant background (**Fig. S1**).

The mRNA sequence of the second BiP/GRP78 gene in *C. elegans*, *hsp-4*, is highly similar to that of *hsp-3* (1472/1873 nucleotides identical, including several contiguous stretches of more than 21 nucleotides) and would thus be predicted to be targeted by the *hsp-3* RNAi construct. At the same time, there is a reciprocal control of *hsp-3* and *hsp-4*, such that a decrease in *hsp-3* expression normally leads to an increase in the level of *hsp-4*, and vice versa.[34] As RNAi with *hsp-4* did block p*nlp-29*::GFP induction upon infection in adult worms (data not shown), we sought to discriminate between the two genes using available null mutants. We observed a strong reduction in p*nlp-29*::GFP expression only in an *hsp-3* mutant, not in an *hsp-4* mutant background (**Fig. 2B**). Attempts to establish a *hsp-3*;*hsp-4* strain were confounded by the fact that homozygous double mutants were sterile. When we inactivated *hsp-3* by RNAi in the *hsp-4* mutant background, the adult worms were sterile, and the induction of p*nlp-29*::GFP expression upon infection was blocked (data not shown).

In *C. elegans*, fertility and pathogen resistance are interlinked, via the FOXO transcription factor DAF-16,[35,36] which also plays a role in the UPR.[37] We therefore assayed the effect of *hsp-3* RNAi on p*nlp-29*::GFP expression in a *daf-16* mutant background. Loss of *daf-16* had no effect on the abrogation of p*nlp-29*::GFP expression provoked by *hsp-3* RNAi, or by RNAi with the STAT-like transcription factor *sta-2,* previously characterized for its role

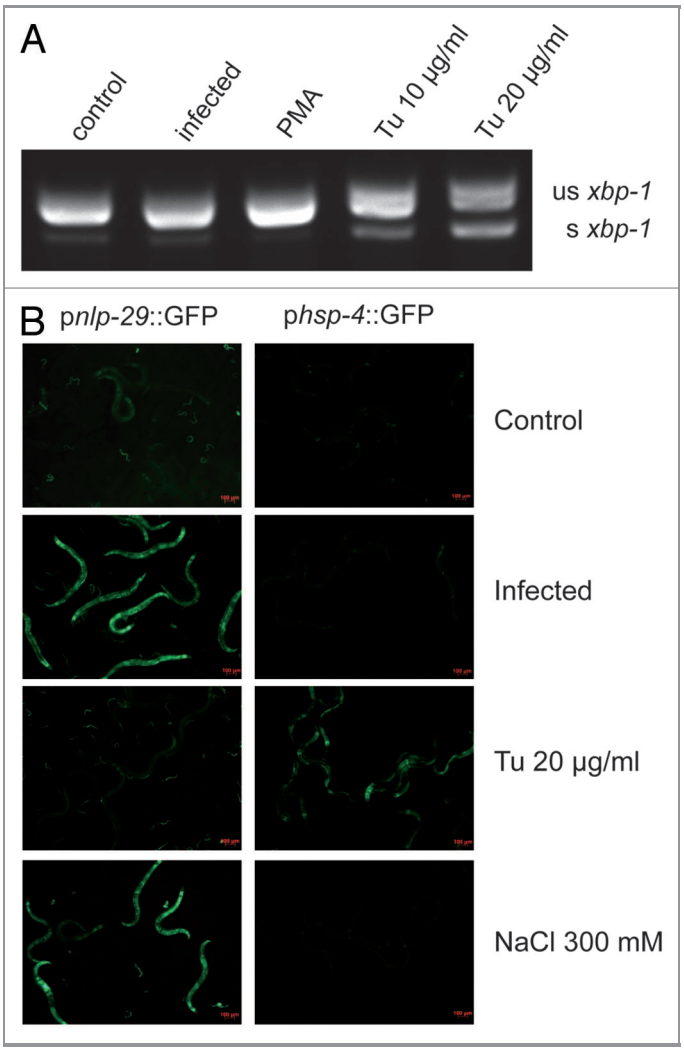

Figure 1. Fungal infection of adult worms does not induce a UPR. (A) RT-PCR analysis of *xbp-1* splicing. Under standard culture conditions (control), a 220 bp amplicon from an unspliced (us) *xbp-1* transcript is detected, together with very low levels of a 197 bp amplicon from a spliced (s) transcript. The abundance of this smaller band does not increase after infection with *D. coniospora* (infection) or PMA treatment (PMA), but is clearly increased upon UPR-induction with tunicamycin (Tu). (B) The green fluorescence in transgenic worms carrying a p*nlp-29*::GFP (strain IG274; left column) or a p*hsp-4*::GFP (IG1320; right column) reporter was observed after infection, exposure to tunicamycin, or high salt. While infection and osmotic stress induced high level of p*nlp-29*::GFP expression, tunicamycin induced p*hsp-4*::GFP.

in *nlp-29* expression[6] (**Fig. 2A**), indicating that the effect of *hsp-3*, and of *sta-2*, is independent of *daf-16*.

We then determined the specificity of the effect of *hsp-3* on reporter gene expression. In clear contrast to the near-complete block of p*nlp-29*::GFP expression after infection, in an *hsp-3* mutant the induction of the reporter gene was at least as strong as in the wild-type background when triggered by PMA, salt or wounding. In the *hsp-4* mutant, however, no effect was seen under any of the experimental conditions (**Fig. 2B**). These results underline the specific role *hsp-3* plays in regulating p*nlp-29*::GFP only after infection, and place *hsp-3* genetically upstream of, or parallel to, the PKCδ TPA-1.

***hsp-3* acts downstream of *nipi-3* to regulate *nlp-29* AMP gene expression.** The only previously known component of the innate immune signaling pathways that regulates *nlp-29* expression specifically upon infection is the Tribbles-like kinase *nipi-3*. Overexpression of *nipi-3* leads to an induction of p*nlp-29*::GFP.[5] This induction was blocked in the *hsp-3* mutant background, placing *hsp-3* genetically downstream of *nipi-3* (**Fig. 3A**). Consistent with this result, *hsp-3* did not block the increased expression of p*nlp-29*::GFP provoked by an activated form of GPA-12 that triggers TPA-1 independently of NIPI-3[7] (**Fig. 3B**). Together these results indicate that *hsp-3* acts between *nipi-3* and *tpa-1* to control the expression of *nlp-29* upon fungal infection (**Fig. 4**).

Discussion

Much of our previous characterization of the innate immune response of *C. elegans* to *D. coniospora* has been focused on the host transcriptional changes that accompany infection. In Drosophila, the expression of many components of immune signaling pathways are themselves highly regulated upon infection.[38,39] In contrast, none of 18 genes known to influence *nlp-29* expression, including the p38 MAPK cascade components *nsy-1*, *sek-1* and *pmk-1*, show a marked change in their expression level after infection.[4] In a previous DIGE-based pilot study, we identified RACK-1 as a factor involved in the regulation of anti-fungal defenses.[7] In an attempt to identify additional candidates, we extended the approach and undertook a comprehensive proteomic study of the changes that accompany fungal infection.

A number of other comparative gel-based proteomic studies have been performed using *C. elegans*[40,41] including two looking at protein changes upon bacterial infection of the intestine.[23,24] It is striking that certain proteins, such as ACT-4, SODH-1, VHA-13 and PDI-2, appear in almost every published list. This may reflect an intrinsic bias in the approach, since the measured expression level[4] for the genes corresponding to the proteins that we identified as differentially represented was very significantly higher than that of genes in general, (71.7% $>$ 5 dcpm vs. 6.6% for all transcripts; $p < 0.001$ binomial test). It may also result from the fact that all these analyses used whole-animal extracts, potentially masking tissue-specific biologically relevant variations in protein abundance, and underlines the interest for developing efficient and simple methods to allow protein extraction from a specific *C. elegans* tissue.

If a particular spot on a gel increases or decreases in intensity, one cannot always infer that the total level of the corresponding protein was changed. For example, post-translational modifications may render a protein more difficult to extract, so that spot intensity does not reflect protein abundance. Similarly, post-translational modifications may also lead to an alteration of the sub-cellular localization of a protein, which may cause a protein to be found in different extraction fractions, and thereby affect spot intensity. As many proteins give rise to multiple spots, generally because of post-translational modifications, only if all the spots for

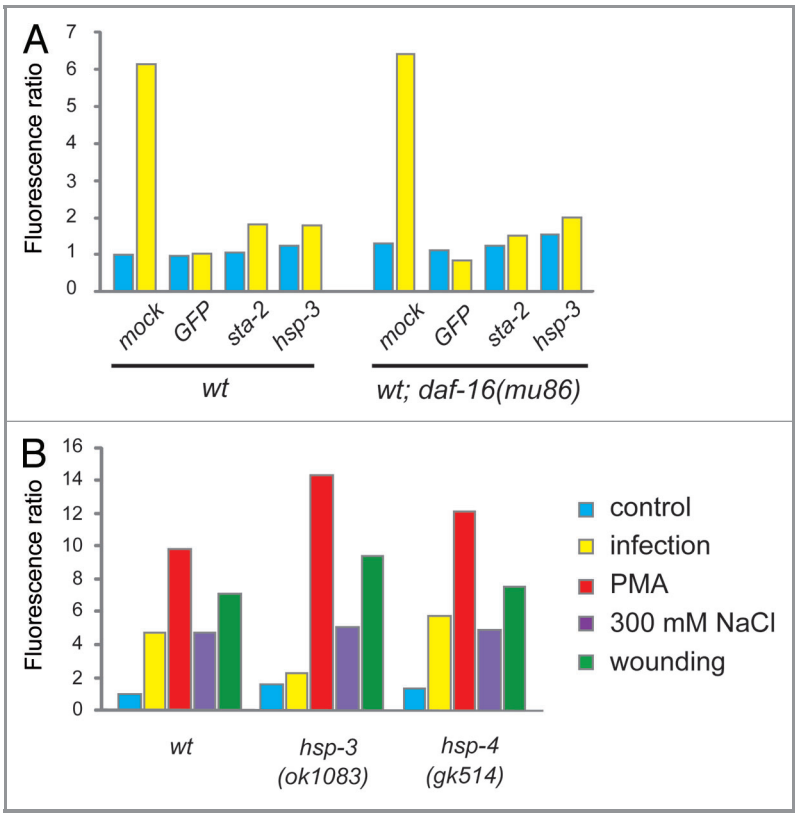

Figure 2. A specific role for *hsp-3* in the regulation of *nlp-29*. (A) Quantification of the effect of control (*K04G11.3*), *GFP*, *hsp-3* and *sta-2* RNAi on p*nlp-29*::GFP expression in a wild-type or *daf-16*(*mu86*) mutant background. For reasons given elsewhere,[5] in this and the subsequent graphs, error bars are not shown. Data are representative of three independent experiments. (B) Quantification of p*nlp-29*::GFP expression in *hsp-3*(*ok1083*) and *hsp-4*(*gk514*) mutant backgrounds following different treatments. In all cases, quantification was with the COPAS Biosort. The normalized average ratio of green to red fluorescence is shown. The analysis was restricted to worms with a TOF above 450. The number of worms analyzed here and in subsequent figures is given in the **Supplemental Material**.

a given protein were identified and quantified from each fraction would one be able to quantitate protein abundance. The development of alternative methods, such as metabolic labeling coupled to mass spectrometry holds considerable promise for profiling changes during pathogenic challenge in *C. elegans*.[25] In the meantime, caution needs to be exercised when analyzing gel-based proteomic results. For this reason, until we have further functional evidence for a role in innate immunity for the various candidate proteins we identified, any discussion of a putative role would be premature.

The exception is HSP-3, which clearly has a specific function in regulating AMP gene expression. The *hsp-3* gene is expressed at a high-level and is unchanged by infection,[4] so the total level of HSP-3 may not change upon infection. We have not established the change in HSP-3 (e.g., degradation, phosphorylation, etc.) that leads to a change in intensity of the corresponding spot. It is noteworthy that in an *atf-6* mutant background there is a marked reduction of p*nlp-29*::GFP expression after infection. This may reflect a role for ATF-6 in regulating HSP-3 levels.

During development, *hsp-3* has an unambiguous role in the UPR.[19,42] The data presented here indicate that the immune function of *hsp-3* is independent of its function in the UPR. There is, however, evidence for a link between the UPR and AMP gene regulation in larvae. As mentioned above, a number of genes, including *cnc-4*, *fipr-26*, *nlp-28* and *nlp-29* are induced in L2 larvae after treatment with tunicamycin, apparently independently of *xbp-1*.[20] But although tunicamycin does provoke upregulation of p*nlp-29*::GFP in larvae, *D. coniospora* infection does not induce p*hsp-4*::GFP expression either in larvae or adults. Further, this UPR-induced expression of p*nlp-29*::GFP is independent of the p38 MAPK pathway, as it is observed in *pmk-1* mutant background, as well as in a *tpa-1* and *nipi-3* mutant backgrounds (data not shown), and overall, there is only a minimal overlap between the genes upregulated by tunicamycin and *D. coniospora* infection.[3,20] So the relationship between anti-fungal innate immunity and the UPR is not straightforward.

It is interesting, nonetheless, to speculate on how HSP-3 might exert its influence on AMP expression. There are several plausible models that are based on the idea that although genetically *hsp-3* is positioned between *nipi-3* and *tpa-1*, it seems unlikely that it plays a direct role in signal transduction. HSP-3 might be needed to ensure the correct intracellular localization of NIPI-3, itself, or of a protein that acts downstream of NIPI-3 and upstream of TPA-1. It may therefore be worthwhile to look at NIPI-3 localization in wild-type and *hsp-3* mutant worms. The presence of two almost identical BiP/GRP78 proteins in the nematode is intriguing, as mammals, for example, only have one. It is conceivable that HSP-3 has a UPR-independent function outside the ER. Interestingly, one of the areas of sequence divergence between the two proteins is at the C-terminus; where HSP-3 has the ER retention signal KDEL sequence, HSP-4 has HDEL.

There are many examples of heat shock proteins playing a more or less direct role in innate immune responses.[43] For example, they can function as endogenous danger signals to indicate cell stress and tissue damage to the immune system. As another example, the conserved SGT1/HSP90 complex binds NLR proteins, and modulates innate immune signaling in plants and animals,[44] although it should be noted that there are no obvious NLR proteins in *C. elegans*. A study of the intracellular localization of HSP-3, and of the consequences of artificially expressing it in the cytoplasm could be merited. It is interesting to note that in an *hsp-3* mutant there is some residual induction of the *nlp-29* reporter gene, but this is fully abolished if the mutants are subject to RNAi against *hsp-4* (C.C., unpublished observations). On the other hand, we have shown that loss of *hsp-4* function alone has no effect on *nlp-29* reporter gene expression. This suggests that *hsp-4* can partially compensate for the absence of *hsp-3*.

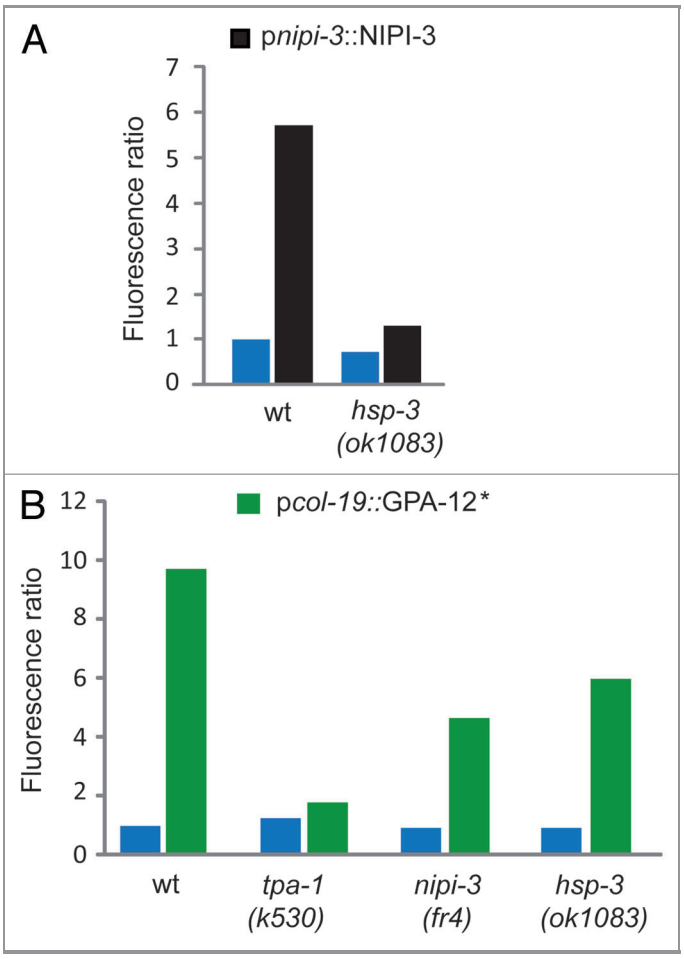

Figure 3. *hsp-3* acts genetically downstream of *nipi-3* but not of *gpa-12*. (A) p*nlp-29*::GFP reporter expression was quantified in *wt* and *hsp-3* (*ok1083*) mutant worms with (black bars) or without (blue bars) copies of a transgene containing *nipi-3* under the control of its own promoter. (B) Quantification of p*nlp-29*::GFP reporter expression in *wt*, *tpa-1*(*k530*), *nipi-3*(*fr4*) and *hsp-3*(*ok1083*) mutant worms with (green bars) or without (blue bars) copies of a transgene containing a gain-of-function (*) allele of *gpa-12* under the control of the epidermis-specific *col-19* promoter. Both p*nipi-3*::NIPI-3 and p*col-19*::GPA-12* transgenes provoke a robust *nlp-29* upregulation in the absence of infection in adult worms. Quantification was with the COPAS Biosort. The normalized average ratio of green fluorescence to time of flight (TOF) is shown. The analysis was restricted to worms with a TOF between 450 and 650.

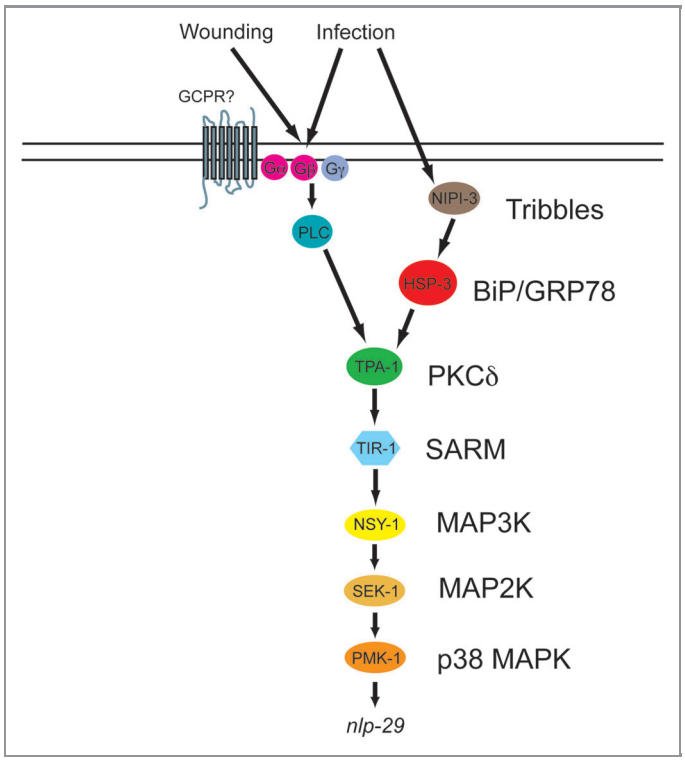

Figure 4. Model of the control of *nlp-29* expression. Signals perceived upon *D. coniospora* infection and injury are transduced by a PKCδ - p38 MAPK pathway to regulate the expression of *nlp-29*. HSP-3 functions between NIPI-3 and the PKCδ TPA-1. Many other known regulatory elements, including the OSM-11/WNK-1/GCK-3 pathway[48] and the recently described pseudokinase NIPI-4[49] have been omitted for the sake of clarity.

It has been shown that during *C. elegans* development, the activation of a p38 MAPK pathway that follows intestinal infection with the *P. aeruginosa* strain PA14 causes a UPR.[18] This is believed to be a consequence of the increased expression of innate immune effectors that overload the protein folding machinery in the ER. The results we have presented here show that infection of adult *C. elegans* by *D. coniospora* and the resultant induction of a large number of defense proteins in the epidermis does not provoke a UPR. This might reflect a relatively low constitutive activity of the secretory pathway in the epidermis, and therefore a buffering capacity in adult animals to cope with the consequences of infection. It will be interesting to dissect further the complex interplay between developmental, physiological (e.g., production of digestive enzymes in the intestine) and induced processes that put stress on the ER, both in *C. elegans* and other organisms. Additional study is also required to understand fully the UPR-independent role of BiP/GRP78 in innate immunity in *C. elegans* and to determine whether it might play any such role in other organisms.

Materials and Methods

Strains and culture condition. Worms were grown and maintained on nematode growth medium (NGM) and cultured with the *E. coli* strain OP50, as described.[45] The *hsp-3*(*ok1083*), *hsp-4*(*gk514*), *daf-16*(*mu86*), *tpa-1*(*k530*), *atf-6*(*ok551*), *pek-1* (*ok275*) mutants were obtained from the Caenorhabditis Genetics Center (CGC). The strain SJ17 (*xbp-1*(*zs12*) *III; zsIs4*[*phsp-4*::GFP] *V*)[21] was the kind gift of Dr Eric Chevet.

Reporter gene constructs and transgenic lines. IG274 (*wt; frIs7*[p*nlp-29*::GFP, p*col-12*::DsRed] *IV*) is described elsewhere.[5] IG981 [*hsp-3*(*ok1083*) *X; frIs7 IV*], IG982 [*hsp-4*(*gk514*) *II; frIs7 IV*] and IG1161 [*daf-16*(*mu86*) *I; frIs7 IV*], IG983 [*atf-6*(*ok551*) *II; frIs7 IV*], IG1424 [*pek-1*(*ok275*) *II; frIs7 IV*], were obtained by crossing the mutants *hsp-3*(*ok1083*), *hsp-4*(*gk514*), *daf-16*(*mu86*), *atf-6*(*ok551*) and *pek-1*(*ok275*) with IG274. The strain IG1320 (*wt; zsIs4*[p*hsp-4*::GFP] *V*) was obtained by backcrossing the

strain SJ17 with N2. The strain IG1363 (*wt; frEx486*[(p*col-19::*GPA-12*), *pNP21*(*pBunc-53*::GFP)]) was the kind gift of Dr Nathalie Pujol. The strains IG1361 [*tpa-1*(*k530*) *frIs7 IV; frEx486*], IG1364 [*hsp-3*(*ok1083*) *X; frIs7 IV; frEx486*] and IG1365 [*nipi-3*(*fr4*) *X; frIs7 IV; frEx486*] were obtained by crossing respectively *tpa-1*(*k530*), *hsp-3*(*ok1083*) and *nipi-3*(*fr4*) with IG1363.

Splicing of *xbp-1*. N2 worms were grown and maintained on NGM plates with OP50. When they reached the young adult stage, worms were infected with *D. coniospora* by transferring them to NGM/OP50 plates previously spread with a dense suspension of spores. These had been freshly harvested in M9 buffer. Otherwise uninfected young adult worms were tranferred onto NGM/OP50 plates containing 10 µg/mL or 20 µg/mL tunicamycin (Sigma) or 1 µg/mL PMA (Sigma). After 5 h worms were harvested and RNA extracted with Trizol as described.[46] Reverse transcription used High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and PCR analyses were performed as described.[17] Samples were normalized by Q-PCR as described[26] with *eft-2*[17] as an internal control.

RNAi. All RNAi feeding experiments were performed essentially as described,[47] using clones from the Ahringer library. All RNAi clones were sequence verified before use. The experiments were performed with worms cultured on OP50 until the L2 stage.

Infection, wounding, osmotic stress, PMA stress. Infection, and wounding were performed as described.[5] For exposure to osmotic stress, PMA and tunicamycin, compounds were added to NGM plates to a final concentration of 300 mM for NaCl, 1 µg/mL for PMA and 10 or 20 µg/mL for tunicamycin. Worms were grown and maintained on NGM plates with OP50. When they reached the young adult stage, worms were transferred onto the appropriate modified NGM/OP50 plates. Similar conditions were used to assay the induction of GFP expression in the strains IG274 and IG1320 (shown in **Fig. 1B**), except images were taken after only 5 h.

Biosorter. The quantification of fluorescent reporter gene (GFP) expression was performed with the COPAS Biosort (Union Biometrica), essentially as described.[26] Generally, animals were analyzed for length (time of flight), optical density (extinction), green fluorescence, and red fluorescence (if appropriate).

Protein extraction. A synchronized population of L4 IG274 worms was infected with *D. coniospora*. After 5 h, when there was a clear induction of GFP, indicative of the innate immune response to a productive infection, worms were harvested by washing plates with M9 buffer. Worms were pelleted by decantation, washed twice in the M9 buffer, then twice with PBS buffer. Proteins were extracted from a pellet of 500 µL of worms either by sonication in 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5, containing a phosphatase inhibitor cocktail (Roche), or using the 2D fractionation kit (Amersham) according to the manufacturer's instructions.

Protein fractionation, labeling, gel electrophoresis and identification. Full experimental details are publically available at http://miapegeldb.expasy.org/experiment/118. The comprehensive set of analytical data from this study is available at the World-2DPAGE database http://world-2dpage.expasy.org/repository/0042.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/virulence/article/20384

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