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Protection of *C. elegans* from Anoxia by HYL-2 Ceramide Synthase

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Oxygen deprivation is rapidly deleterious for most organisms. However, *Caenorhabditis elegans* has developed the ability to survive anoxia for at least 48 hours. Mutations in the DAF-2/DAF-16 insulin-like signaling pathway promote such survival. We describe a pathway involving the HYL-2 ceramide synthase that acts independently of DAF-2. Loss of the ceramide synthase gene *hyl-2* results in increased sensitivity of *C. elegans* to anoxia. *C. elegans* has two ceramide synthases, *hyl-1* and *hyl-2*, that participate in ceramide biogenesis and affect its ability to survive anoxic conditions. In contrast to *hyl-2(lf)* mutants, *hyl-1(lf)* mutants are more resistant to anoxia than normal animals. HYL-1 and HYL-2 have complementary specificities for fatty acyl chains. These data indicate that specific ceramides produced by HYL-2 confer resistance to anoxia.

The molecular pathways underlying resistance of *Caenorhabditis elegans* to oxygen deprivation (*1*–*8*) appear to be conserved, at least in part, between vertebrates and invertebrates. These pathways differ in *C. elegans* according to the developmental stage and oxygen tension. For example, hypoxia-inducing factor 1 (HIF-1) is not required for resistance of either nematode embryos (*2*) or young adults (table S1) to anoxia. However, it stimulates survival of embryos to hypoxia (*9*). On the other hand, mutation of the *daf-2* gene, an insulin and insulin-like growth factor receptor–like gene, promotes survival of *C. elegans* to hypoxia or anoxia (*1*, *5*), an effect that is mediated by the glyceraldehyde-3-phosphate dehydrogenases GPD-2 and GPD-3 (*5*). In *C. elegans*, mutations have not been detected that cause a reduction in viability under anoxia in adult hermaphrodites (*5*). We sought to identify essential components of the pathways regulating the adaptation of *C. elegans* to anoxia.

We tested the ability of young adult wild-type N2 Bristol *C. elegans* (72 hours after the L1 stage grown at 20°C) to survive anoxia at 20°C (0.001% O_2). Survival rates were 99.5% ± 1.5% ($n > 5$), 81% ± 14.5% ($n > 5$), and 8.5% ± 9.5% ($n > 5$) after 24, 48, and 72 hours of anoxia, respectively (Fig. 1A). Animals that failed to survive anoxia became rodlike and showed the presence of propidium iodide–stained necrotic cells throughout their entire body (Fig. 1B).

In order to identify genes required for resistance to anoxia, we screened a library of mutants for animals with increased sensitivity to anoxia (table S1). After eliminating a role for other mutations in the initial mutant line, we characterized a mutation in the homolog of yeast longevity assurance gene 2 (*hyl-2* gene), *hyl-2(gnv1)* (*10*) (fig. S1), that conferred increased sensitivity to anoxia (Fig. 1C and fig. S2). The mutation consists of two consecutive base substitutions at positions 1297 and 1298 (CATCAT → CAATAT) that result in the conversion of $His^{168}His^{169}$ residues into $Gln^{168}Tyr^{169}$ in the Lag motif of HYL-2. Sensitivity to anoxia was also observed in *hyl-2(tm2031)* mutants carrying a deletion of the *hyl-2* gene (Fig. 1C). Both *hyl-2(gnv1)* and *hyl-2(tm2031)* mutants were indistinguishable from N2 animals and had a normal fecundity and life span (fig. S3). *hyl-2(gnv1)* mutants, as *hyl-2(tm2031)*, were also more sensitive to heat shock at 36°C (fig. S4A) but had normal responses to thermal stress at 30°C for 7 days and to hypotonic shock (fig. S4, B and C). Introduction in *hyl-2(gnv1)* animals of a green fluorescent protein (GFP)*::WT hyl-2* transgene under the control of the endogenous *hyl-2* promoter (fig. S5C) showed gene expression from the larval to the adult stage with strong expression in the gut, the posterior bulb of the pharynx, the hypoderm, and unidentified cells of the head and the tail (Fig. 2A). Expression of the transgene partially restored resistance to anoxia and heat shock in *hyl-2(gnv1)* animals (Fig. 2B and fig. S4D). Collectively these experiments indicate that *hyl-2* is required for adaptation of the nematode to anoxia.

hyl-2 belongs to a eukaryotic gene family known as longevity assurance genes (*Lass* genes, fig. S6). Several members encode dihydroceramide synthases for the de novo ceramide pathway (*11*) and are therefore called ceramide synthase (CerS) genes (*12*). These genes have sequence similarity in a domain called the Lag motif that is essential for enzyme activity (fig. S6) (*13*). In *Saccharomyces cerevisiae*, *LAG1* and *LAC1* are required for de novo ceramide synthase activity, and yeast lacking both genes are almost inviable. *C. elegans* has three ceramide synthase genes, *hyl-1*, *hyl-2*, and *lagr-1*. Simultaneous deletion of both *hyl-1* and *hyl-2* is lethal (*10*). Sphingolipids from *C. elegans* are somewhat different from their counterparts in other eukaryotes because they contain exclusively isosphingoid bases (*14*), which are presumably used by the dihydroceramide synthases from worms. Nevertheless, *hyl-1* can complement the loss of function of yeast *LAG1* and *LAC1* (*15*). To test whether HYL-2 is a ceramide synthase, we transformed *lag1Δlac1Δ* yeast with an expression vector carrying the cDNA of either *LAG1*, *hyl-1*, or *hyl-2*. All expression vectors rescued the growth phenotype of *lag1Δlac1Δ* strain (Fig. 2C), indicating that *hyl-2*, like *hyl-1*, is an ortholog of *LAG1*. Expression of *hyl-2(gnv1)*

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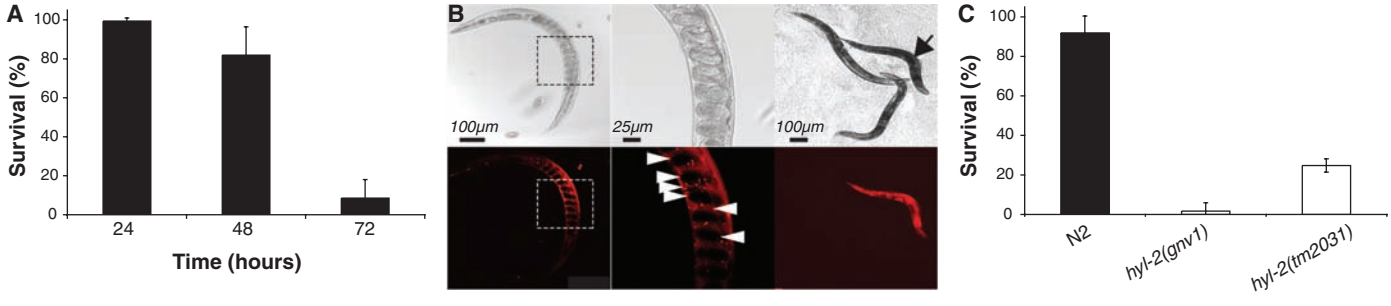

Fig. 1. The *hyl-2(gnv1)* mutation confers hypersensitivity to anoxia in *C. elegans*. (**A**) Survival of wild-type Bristol N2 animals exposed to anoxia for 24, 48, and 72 hours. Results are mean ± SD, $n > 5$. (**B**) Young adults N2 worms were stained with propidium iodide postanoxia and observed with a fluorescent microscope. After 16 hours of anoxia, dead worms displayed stained nuclei (white arrowhead) throughout the cell body (left and middle images). At 48 hours of anoxia, dead animals (top right image, black arrow) were intensively stained as a result of necrosis in all tissues, whereas living animals were unstained (top right image). (**C**) Survival of *hyl-2(gnv1)* and *hyl-2(tm2031)* mutants after 48 hours of anoxia (mean ± SD, $n > 5$).

cDNA in *lag1Δlac1Δ* yeast failed to rescue the lethal phenotype, demonstrating that His^{168}, His^{169}, or both are required for HYL-2 function (Fig. 2C). This result supports previous reports on the essential function of the evolutionarily conserved His residues of the Lag motif (*13*, *16*). Thus, the inability of *hyl-2* mutants to adapt to oxygen deprivation appears to result from a loss of function of the HYL-2 ceramide synthase.

In contrast to *hyl-2* deficient animals, *hyl-1(gk203)* and *hyl-1(ok976)* deletion mutants (fig. S5A) resisted anoxia better than N2 worms (Fig. 3, A and B) (*17*). We therefore tested whether HYL-1 could substitute for HYL-2 in *hyl-2*–deficient worms. We fused *hyl-1* to a sequence encoding GFP and expressed the construct under the control of *hyl-2* promoter. This ensured appropriate temporal and spatial expression of the *GFP::hyl-1* transgene (fig. S5D). Expression of the *GFP::hyl-1* transgene conferred a small protection against anoxia but was significantly less efficient than the *GFP::hyl-2* transgene (Fig. 3C). This finding was confirmed in three different transgenic lines, making it unlikely that suboptimal expression of the *GFP::hyl-1* transgene was responsible for the weak beneficial effect. Thus, although HYL-1 can complement growth defects in yeast as efficiently as does HYL-2, it cannot completely substitute for HYL-2 to confer strong resistance to anoxia. Thus, anoxia tolerance may require one or more ceramide species that are either specifically or preferentially synthesized by HYL-2.

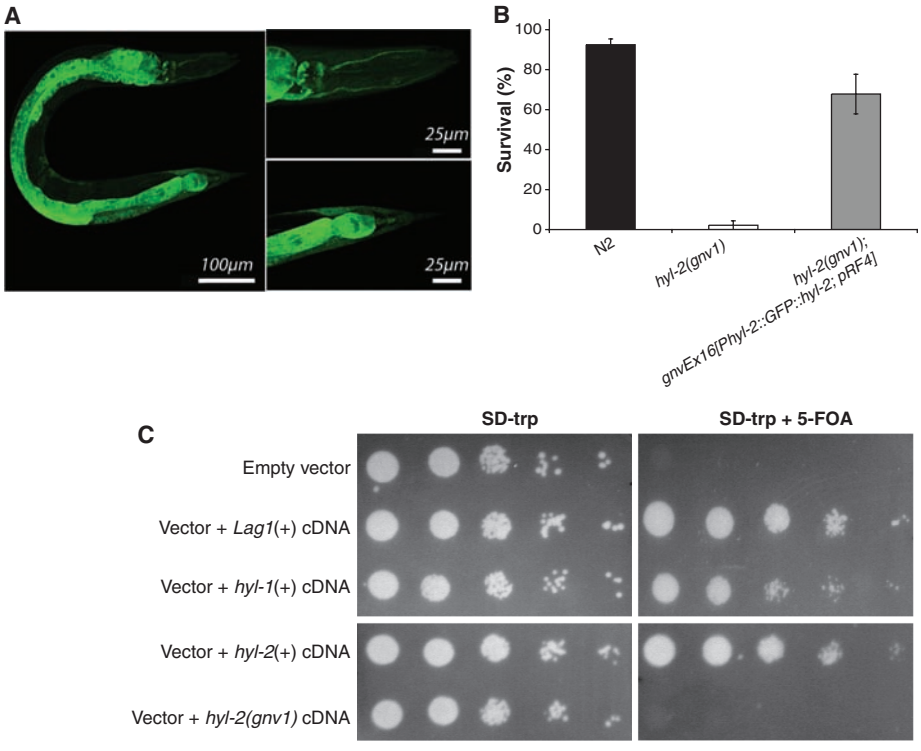

Fig. 2. Sensitivity to anoxia results from loss of function of HYL-2 ceramide synthase. (**A**) GFP staining of *hyl-2(gnv1)* adult animals expressing the transgene *GFP::hyl-2* under the control of the *hyl-2* promoter. (**B**) Survival after 48 hours of anoxia of *hyl-2(gnv1)* animals complemented with the transgene *GFP::hyl-2* under the control of the *hyl-2* promoter (mean ± SD, $n > 5$). (**C**) Functional complementation of mutant yeast cells by *C. elegans hyl-1* and *hyl-2* in *lag1Δlac1Δ S. cerevisiae* transformed with pRS424–*hyl-1*, pRS424–*hyl-2*, and pRS424–*hyl-2(gnv1)* (*TRP1* plasmid). The compound 5-FOA (5-fluorouracil-6-carboxylic acid monohydrate) is a counterselection for cells that carry the *URA3*-based plasmid with yeast *LAG1*. Therefore, only cells that have lost this plasmid can grow, making growth dependent on function of the heterologous ceramide synthase.

Dihydroceramide synthases combine a sphingoid base with a fatty acyl–coenzyme A (CoA) to form dihydroceramide, which is then desaturated by a dihydroceramide desaturase into ceramide in animals. In many organisms, multiple ceramide synthases are expressed, each displaying fatty acyl-CoA specificity to produce a diversity of ceramides differing in their fatty acyl chains (*18*–*20*). Ceramides are the precursors for sphingolipids, including sphingomyelins, which are present in *C. elegans* (*14*). We quantified the major ceramide (Cer) and sphingomyelin (SM) species of *C. elegans* N2, *hyl-1(ok976)*, and *hyl-2(gnv1)* animals by electrospray ionization mass spectrometry (ESI-MS). *hyl-1(ok976)* and *hyl-2(gnv1)* mutants expressed different types of Cers and SMs than N2 worms did (Fig. 4, fig. S7, and table S2). *hyl-2*–deficient worms [*hyl-2(gnv1)*] had fewer Cers and SMs with C_{20} to C_{22} fatty acyl chains and more with C_{24} to C_{26} fatty acyl chains than did N2 animals. In contrast, *hyl-1*–deficient worms [*hyl-1(ok976)*] expressed more C_{20} to C_{22} Cers and SMs than did N2 worms, but they contained the same or lesser amounts of C_{24} to C_{26} Cers and SMs. These data indicated that efficient synthesis of C_{20} to C_{22} Cers requires HYL-2 whereas that of C_{24} to C_{26} Cers is mainly dependent on HYL-1. To verify this, we measured dihydroceramide synthase activity in microsomes isolated from N2 and mutant worms with ^{3}H-sphinganine and acyl-CoA substrates of various lengths (fig. S8, A and C). Membranes from *hyl-2(tm2031)* mutants catalyzed synthesis of more C_{26} Cers, whereas membranes from *hyl-1(ok976)* mutants catalyzed synthesis of more C_{22} Cers. All membranes were equally active with use of C_{24}-acyl CoA (fig. S8B). These results are consistent with the ESI-MS results and confirm that the two Cer synthases have

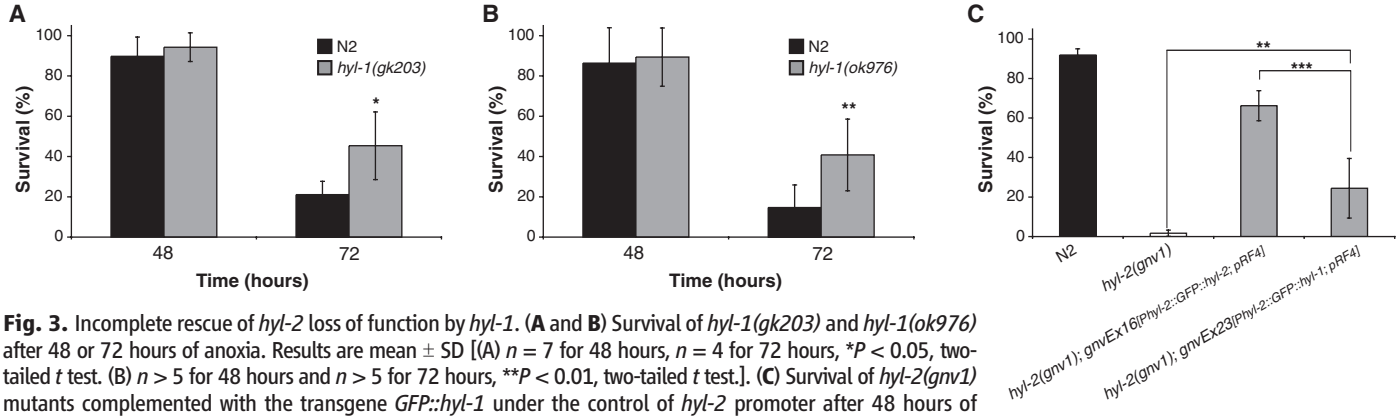

Fig. 3. Incomplete rescue of *hyl-2* loss of function by *hyl-1*. (**A** and **B**) Survival of *hyl-1(gk203)* and *hyl-1(ok976)* after 48 or 72 hours of anoxia. Results are mean ± SD [(A) $n = 7$ for 48 hours, $n = 4$ for 72 hours, $^*P < 0.05$, two-tailed *t* test. (B) $n > 5$ for 48 hours and $n > 5$ for 72 hours, $^{**}P < 0.01$, two-tailed *t* test.]. (**C**) Survival of *hyl-2(gnv1)* mutants complemented with the transgene *GFP::hyl-1* under the control of *hyl-2* promoter after 48 hours of anoxia. Results are mean ± SD, $n > 5$, $^{**}P < 0.01$, $^{***}P < 0.001$, analysis of variance (ANOVA) test.

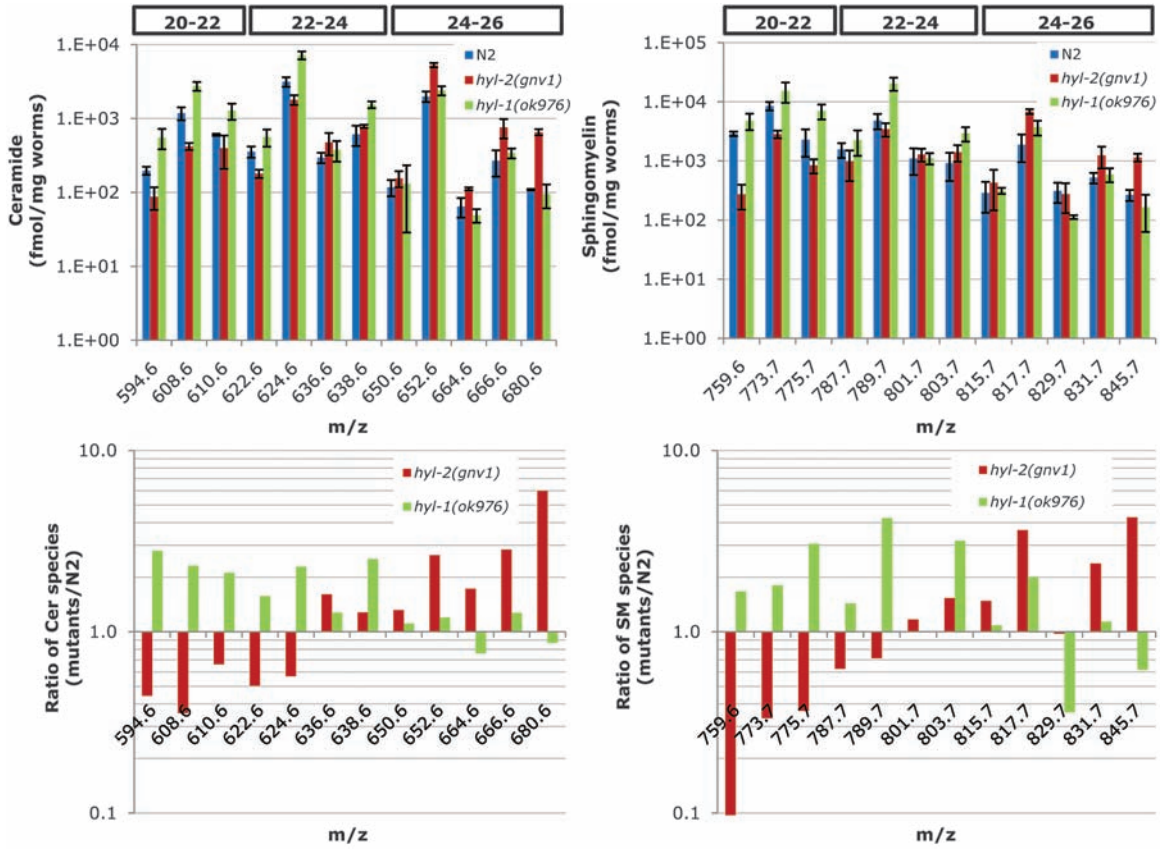

Fig. 4. Ceramide, sphingomyelin, and phosphatidylcholine (PC) quantification in N2, *hyl-2(gnv1)*, and *hyl-1(ok976)* worms. Cers, SMs, and PC species were quantified by ESI-MS (see fig. S7 for quantification of PC species). Cers and SMs were quantified after base hydrolysis of glycerophospholipids (*23*). Graphs of the amounts are shown on a logarithmic scale ± SD (n = 4). The amount of each lipid species found in mutant worms was divided by the amount found in wild-type Bristol N2 worms and shown in a graph below the corresponding graph of the total amounts. Acyl chain lengths corresponding to charge/mass (m/z) values are shown in boxes (see also table S2). The entire profiles of Cer and SM species of N2, *hyl-2(gnv1)*, and *hyl-1(ok976)* animals are different with P = 0.002 for Cers and P = 0.007 for SMs (multivariate ANOVA test).

different specificities for fatty acyl chains. They indicate that HYL-2 may promote survival of animals in anoxic conditions by producing C_{20} to C_{22} Cers and SMs. In support of this hypothesis, the *hyl-1(ok976)* mutant worms produced larger total amounts of Cers and SMs than did N2 and *hyl-2(gnv1)* mutants (fig. S9A). Moreover, expression of the *GFP::hyl-1* transgene under the control of the *hyl-2* promoter in *hyl-2(gnv1)* worms failed to restore normal amounts of C_{20} to C_{22} ceramides (fig. S9B), which could explain why the transgene did not restore normal resistance to anoxia in *hyl-2(gnv1)* animals.

Ceramides function in radiation-induced apoptosis of cells in the germ line (*21*). However, apoptosis appears not to account for the sensitivity of HYL-2–deficient animals to anoxia because lack of CED-3 caspase activity in *ced-3(n717);hyl-2(gnv1)* double mutants did not extend survival of these animals during anoxia (fig. S10). Sphingosine 1-phosphate, an anti-apoptotic derivative of ceramide, also appears not to function in resistance to anoxia because resistance of *sphk-1(ok1097)* animals, in which conversion of isosphingosine to isosphingosine 1-phosphate is prevented by a null allele of sphingosine kinase, was normal and those of *sphk-1(ok1097);hyl-1(ok976)* and *sphk-1(ok1097);hyl-2(tm2031)* double mutants were not modified (fig. S11, A and B). A block in ceramide synthase is expected to lead to an increase in isosphingoid bases, which are ceramide precursors. Therefore, we examined the amounts of isosphingoid bases in the *sphk-1(ok1097)* and *hyl* mutants by MS-ESI. Isosphingoid bases accumulated in *hyl-2* mutants and were less abundant in *hyl-1* mutants (fig. S11C). However, *sphk-1(ok1097)* worms accumulated even more isosphingoid bases than either *hyl* mutant but adapted to anoxia normally (fig. S11, A to C), suggesting that accumulation of isosphingoid bases did not influence survival under anoxic conditions.

The *daf-2/daf-16* insulin-like signaling pathway is involved in oxygen deprivation survival in *C. elegans* (*1*, *5*). We also found that *daf-2(e1370)* mutants survived 72 hours of anoxia better than did N2 worms (fig. S12A). To determine whether *hyl-2* and *daf-2* interact genetically, we generated *daf-2(e1370);hyl-2(gnv1)* double mutants and tested their sensitivity to 48 hours of anoxia. Resistance of the double mutants was significantly improved compared with that of *hyl-2(gnv1)* mutants, whereas it was decreased compared with that of *daf-2(e1370)* mutants (fig. S12B). Thus, it appears that, with respect to anoxia resistance, HYL-2 and DAF-2 are acting in parallel pathways that mutually influence each other.

We have shown that a dihydroceramide synthase, with a distinct substrate specificity, provides an important function in the anoxia response in *C. elegans*. Rather than their quantity, it is the chemical structure of ceramide species that seems to be important for resistance to anoxia. Ceramides have been reported previously to be effectors of kinases or phosphatases in various biological processes (*22*). It is most likely that the activity of key ceramide species during anoxia relies on interaction of ceramides with other molecules integrated in a cell survival pathway.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/324/5925/381/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 and S2
References

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Sequential Regulation of DOCK2 Dynamics by Two Phospholipids During Neutrophil Chemotaxis

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During chemotaxis, activation of the small guanosine triphosphatase Rac is spatially regulated to organize the extension of membrane protrusions in the direction of migration. In neutrophils, Rac activation is primarily mediated by DOCK2, an atypical guanine nucleotide exchange factor. Upon stimulation, we found that DOCK2 rapidly translocated to the plasma membrane in a phosphatidylinositol 3,4,5-trisphosphate–dependent manner. However, subsequent accumulation of DOCK2 at the leading edge required phospholipase D–mediated synthesis of phosphatidic acid, which stabilized DOCK2 there by means of interaction with a polybasic amino acid cluster, resulting in increased local actin polymerization. When this interaction was blocked, neutrophils failed to form leading edges properly and exhibited defects in chemotaxis. Thus, intracellular DOCK2 dynamics are sequentially regulated by distinct phospholipids to localize Rac activation during neutrophil chemotaxis.

Chemotaxis regulates a wide range of biological functions, including developmental morphogenesis, wound healing, and immune responses (*1*). During chemotaxis, filamentous actin (F-actin) polymerizes asymmetrically at the leading edge of the cell, providing the force necessary to extend membrane protrusions in the direction of migration (*1*, *2*). This morphologic polarity is regulated by Rac, a member of the small guanosine triphosphatases (GTPases) that cycle between inactive guanosine diphosphate (GDP)–bound and active guanosine triphosphate (GTP)–bound states (*3*). Rac is preferentially activated at the leading edge (*1*, *4*, *5*), which is achieved in part by regulating the subcellular localization of guanine nucleotide exchange factors (GEFs) (*1*, *3*). The GEFs contain a variety of localization motifs such as pleckstrin homology (PH) domains and the DOCK homology region (DHR)–1, both of which bind to phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (*3*, *6*), a lipid product of phosphoinositide 3-kinases (PI3Ks). Upon stimulation, PIP_3 transiently accumulates at the plasma membrane edge facing the highest level of chemoattractant (*1*, *2*). However, a functional leading edge is established even in neutrophils lacking PI3Kγ, the major generator of PIP_3 in this cell type (*7*, *8*). Thus, other factors may alternately suffice to localize Rac GEFs at the leading edge during neutrophil chemotaxis.

DOCK2 is a member of the CDM family of proteins (*Caenorhabditis elegans*, CED-5; mammals, DOCK180; and *Drosophila melanogaster*, Myoblast city) and is predominantly expressed in hematopoietic cells (*9*). Although DOCK2 does not contain the PH and Dbl homology domains typically found in GEFs, DOCK2 can bind to PIP_3 through its DHR-1 domain (*10*) and mediates the GTP-GDP exchange reaction for Rac by means of its DHR-2 domain (*11*, *12*). DOCK2 is a major Rac GEF that controls motility and polarity during neutrophil chemotaxis (*10*). In response to chemoattractants, neutrophils polarize and accumulate DOCK2 at the plasma membrane edge (fig. S1). To explore the mechanism controlling intracellular DOCK2 dynamics, we first analyzed the role of PIP_3 by crossing PI3Kγ$^{-/-}$ mice with mice that had been made by a "knock-in" strategy to express endogenous DOCK2 as a fusion protein with green fluorescent protein (GFP) (*10*, *13*). When neutrophils from DOCK2-GFP mice were stimulated in suspension with chemotactic factors such as *N*-formyl-Met-Leu-Phe (fMLP) and C5a, DOCK2 rapidly translocated to the plasma membrane at 15 s in the presence, but not in the absence, of PI3Kγ (fig. S2). Thus, the initial membrane translocation of DOCK2 is mediated by PIP_3. However, despite the absence of PI3Kγ expression, DOCK2 and F-actin nonetheless still accumulated preferentially at the pseudopod at later time points (fig. S2).

Phosphatidic acid (PA) is a negatively charged phospholipid that can function as a lipid anchor by binding directly to positively charged sites on effector proteins (*14*). In response to many types of external stimuli, signaling pools of PA are formed through hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) or phosphorylation of diacylglycerol (DAG) by diacylglycerol kinase (DGK) (*14*, *15*). Although PLD has been implicated in migratory responses of *Dictyostelium discoideum*, epithelial cells, and neutrophils (*16*–*18*), the mechanistic basis is largely unknown. To investigate whether subcellular localization of DOCK2 is influenced by PLD, GFP-tagged DOCK2 was expressed in human embryonic kidney (HEK) 293T cells with or without coexpression of PLD2, a PLD isoform that localizes primarily to the plasma membrane (*19*). DOCK2 was localized mainly in the cytosol when expressed alone; however, it readily accumulated at the plasma membrane when PLD2 was coexpressed (Fig. 1, A and B). In contrast, the catalytically inactive PLD2 mutant K758R in which Lys^{758} is replaced by Arg (*20*) failed to alter DOCK2 localization (Fig. 1, A and B). Thus, coexpressing PLD2 induces plasma membrane accumula-

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