Caenorhabditis elegans OSR-1 Regulates Behavioral and Physiological Responses to Hyperosmotic Environments

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ABSTRACT

The molecular mechanisms that enable multicellular organisms to sense and modulate their responses to hyperosmotic environments are poorly understood. Here, we employ *Caenorhabditis elegans* to characterize the response of a multicellular organism to osmotic stress and establish a genetic screen to isolate mutants that are osmotic stress resistant (OSR). In this study, we describe the cloning of a novel gene, *osr-1*, and demonstrate that it regulates osmosensation, adaptation, and survival in hyperosmotic environments. Whereas wild-type animals exposed to hyperosmotic conditions rapidly lose body volume, motility, and viability, *osr-1(rm1)* mutant animals maintain normal body volume, motility, and viability even upon chronic exposures to high osmolarity environments. In addition, *osr-1(rm1)* animals are specifically resistant to osmotic stress and are distinct from previously characterized osmotic avoidance defective (OSM) and general stress resistance *age-1(hx546)* mutants. OSR-1 is expressed in the hypodermis and intestine, and expression of OSR-1 in hypodermal cells rescues the *osr-1(rm1)* phenotypes. Genetic epistasis analysis indicates that OSR-1 regulates survival under osmotic stress via CaMKII and a conserved p38 MAP kinase signaling cascade and regulates osmotic avoidance and resistance to acute dehydration likely by distinct mechanisms. We suggest that OSR-1 plays a central role in integrating stress detection and adaptation responses by invoking multiple signaling pathways to promote survival under hyperosmotic environments.

WATER loss, due to conditions of high salinity and drought, affects growth and development of multiple organisms in their natural habitats. Cellular structure and protein function are highly dependent upon aqueous environments, and an imbalance in water homeostasis can cause irreversible damage with detrimental effects on organisms. To protect themselves against dehydration, diverse organisms (*e.g.*, bacteria, fungi, tardigrades, rotifers, nematodes, and desert resurrection plants) have developed survival strategies to detect and withstand cellular dehydration in hyperosmotic environments (Potts 1994; Clegg 2001; Crowe 2002). The synthesis and accumulation of compatible osmolytes (glycerol, trehalose, sucrose, and *myo*-inositol) is a universal osmoadaptive response of diverse organisms to cellular dehydration (Yancey *et al.* 1982) and is suggested to stabilize membranes and native proteins and regulate cell volume (Crowe *et al.* 1984; Welch and Brown 1996).

The eukaryotic response to osmotic stress has been extensively investigated in the budding yeast, *Saccharomyces cerevisiae*. In yeast, perturbations in the osmotic environment are detected by a two-component system (SLN1-YPD1-SSK1; Maeda *et al.* 1994; Posas *et al.* 1996) and other sensors, such as SHO1 (Maeda *et al.* 1995) and MSB2 (O'Rourke and Herskowitz 2002; O'Rourke *et al.* 2002), that regulate activation of the osmoadaptive HOG/p38 pathway (Brewster *et al.* 1993) and glycerol biosynthesis (Hohmann 2002).

In contrast to unicellular organisms such as yeast, much less is known about sensors, signal transduction pathways, and mechanisms that constitute the response to high osmolarity in multicellular organisms. In mammals, osmotic stress results in activation of an adaptive response that involves three different mitogen-activated protein (MAP) kinases (SAPKs), p38, JNK, and ERK5 (de Nadal *et al.* 2002). A novel osmosensing scaffold protein has been recently discovered and shown to activate the mammalian p38 MAPK pathway in osmotic stress conditions, in a manner strikingly similar to that seen in yeast (Uhlik *et al.* 2003). Yet another osmosensor is the VR-OAC receptor (TRPV4), which has been suggested to have a role in sensing systemic osmotic and hydrostatic pressure (Liedtke *et al.* 2000).

The nematode *Caenorhabditis elegans* has emerged as a powerful genetic model for studying osmosensation in multicellular organisms (Culotti and Russell 1978; Bargmann *et al.* 1990). In addition to adaptive responses (as described above for mammals and yeast),

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C. elegans worms have also developed mechanisms by which they avoid hyperosmotic environments. Mutants defective in sensing high osmolarity (OSM) have been described (Culotti and Russell 1978; Colbert *et al.* 1997; Hart *et al.* 1999). Unlike wild-type animals, OSM mutants fail to avoid environments of high osmolarity, due at least partly to defective function of the polymodal osmosensory ASH neurons (Colbert *et al.* 1997; Hart *et al.* 1999). However, the entire complement of molecular mechanisms by which multicellular organisms integrate responses that lead to detection, adaptation, and survival under osmotic stress remain poorly understood.

To address these issues, we have employed *C. elegans* as a model system and characterized its response to hyperosmotic environments. Here we report on the cloning and characterization of a novel gene, *osr-1*, and show that in *C. elegans*, OSR-1 regulates multiple aspects of the osmotic stress physiology via distinct genetic pathways to promote survival under hyperosmotic environments.

MATERIALS AND METHODS

Strains and culture conditions: *C. elegans* worms were cultured at 22° under standard growth conditions (Brenner 1974). Strains used in this study were Bristol strain N2 (wild-type), *osr-1 (rm1), unc-73(e936);dpy-5(e61), age-1(hx546), daf-16(mgdf50), osm-9 (ky10), ocr-2(ak47), osm-10(n1052), unc-43(n1186), sek-1(km4), nsy-1 (ky397), pmk-3(ok169), jnk-1(jk7), jkk-1(km2),* and *mek-1(ks54).*

Genetic screens for osmotic stress resistant mutants: Standard EMS mutagenesis (Brenner 1974) was performed on N2 strain hermaphrodites. The F_2 generation (~20,000 haploid genomes) was screened for mutants that maintained normal swimming behavior on NGM agar plates containing 500 mm NaCl (high-salt plates) for >15 min (acute motility assay). Putative mutants that showed normal motility in this assay were isolated for further analysis.

Mapping and cloning of *osr-1*: Standard mapping procedures (Brenner 1974) were used to map *osr-1* to chromosome I. We used three-factor mapping to identify *osr-1* on −0.2 map units (MU) between *dpy-5* and *unc-73*. Individual cosmids from the identified genetic interval were injected into *osr-1(rm1)* animals at 40 ng/μl with 100 ng/μl *myo-2::gfp* plasmid as a co-injection marker. Rescue experiments were performed with at least three independent transgenic lines.

Behavioral and stress resistance assays: Worms were placed on high-salt plates containing 500 mm NaCl and scored for their motility over a period of 10 min (acute motility assay) and after 1, 5, and 12 hr (chronic adaptation assay). Osmotic stress survival assays were performed on the high-salt plates seeded with *Escherichia coli* (OP50). To score for viability, worms were collected from the salt plates using a recovery buffer (300 mm NaCl in M9), transferred to regular NGM plates (50 mm NaCl), and allowed to recover overnight before scoring for viability. Osmotic avoidance behavior (OSM) was quantified as the percentage of worms that crossed a 2-cm ring of 4 m NaCl or 8 m fructose on an NGM agar plate, within 5 min. Nose touch response, 1-octanol repellent assays (Hart *et al.* 1999), and heat (Lithgow *et al.* 1995) and oxidative stress (Lee *et al.* 2003) experiments were performed as described previously. Statistical significance between mutants in the behavioral and stress assays was determined using the two-tailed *t*-test and by one-way ANOVA test.

Molecular biology: The functional *osr-1::gfp* operon construct and the tissue-specific expression constructs were generated using the Gateway system (Invitrogen, Carlsbad, CA; Walhout *et al.* 2000). The rescuing fragment (pASRM1) or the heterologous promoter::*osr-1* cDNA was inserted into a pEntry vector containing the SL2-*gfp* operon cassette. The promoters used were *dpy-7* (hypodermal), *vha-6* (intestine; Wang *et al.* 2002), and F25B3.3 (pan-neuronal; Altun-Gultekin *et al.* 2001). The full transcription unit of *osr-1* was determined using a partial expressed sequence tag (EST; yk563c9) obtained from Yuji Kohara and a SMART RACE cDNA kit (CLONTECH, Palo Alto, CA). Further details of plasmid construction are available on request.

RNAi experiments: RNAi experiments were performed using a previously described feeding method (Timmons *et al.* 2001). For *osr-1* RNAi, pAS1 was constructed by subcloning the *osr-1* cDNA (1932 bp) into the L4440 vector. For *pmk-1* RNAi, we used the pDK177 plasmid (Kim *et al.* 2002). Eggs were hatched on *E. coli* (HT115) carrying the L4440 expression vector (control groups), HT115 carrying pDK177 plasmid (*pmk-1*), or the plasmid pAS1 (*osr-1*) and allowed to grow for 3 days (adult stage). For knockdown of both *pmk-1* and *osr-1*, eggs were hatched on plates containing equal amounts of bacteria expressing dsRNA for both *pmk-1* and *osr-1*. Animals that were grown on the RNAi plates were exposed to 500 mm NaCl for 24 hr and scored for viability as mentioned above. Efficiency of the feeding RNAi was confirmed using the one-step RT-PCR kit (Invitrogen).

RESULTS

To establish a metazoan model system for osmotic stress signaling, we characterized the organismal response of *C. elegans* to hyperosmotic stress. When wild-type (N2 strain) animals were exposed to acute osmotic stress (10 min, 500 mm NaCl), we observed a complete loss of motility and reduction in body volume of the worms (Figures 1, A–D, and 2A). Remarkably, upon transfer to normal growth medium (50 mm NaCl), these animals recovered their original body size and regained normal motility within minutes (Figure 1, E–G). However, prolonged exposure of N2 worms to high salt concentrations severely affects motility and viability. After 5 hr of exposure to 500 mm NaCl, most N2 animals were immotile and those that were motile (43%; Figure 2B), exhibited a sluggish swimming behavior. This prolonged exposure to high osmotic stress was eventually lethal as after 24 hr 87% of the wild-type animals died (Figure 2C).

To identify genes mediating the osmotic stress responses in *C. elegans*, we designed a genetic screen to isolate mutants that are *o*smotic *s*tress *r*esistant (OSR) at 500 mm NaCl. Mutants that maintained normal motility on the high-salt plates for 15 min were isolated for further analysis. Among the 104 isolated mutant strains, we have identified four different complementation groups: *osr-1*, *osr-2*, *osr-3*, and *osr-4*. In this study we report on the cloning and characterization of *osr-1*.

***osr-1(rm1)* mutants are specifically resistant to osmotic stress:** We found that *osr-1(rm1)* animals have an enhanced ability to resist hyperosmotic stress (Figures 1

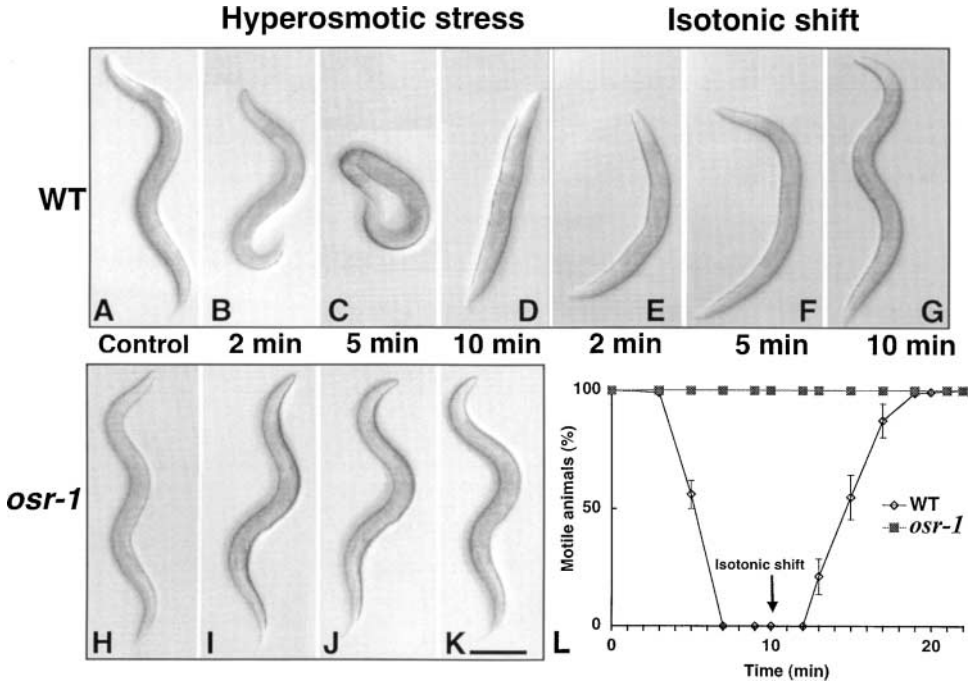

FIGURE 1.—Characterization of the *C. elegans* osmotic stress response. (A) Wild-type (N2 strain) worm in isotonic medium (50 mM NaCl). (B–D) Dehydration of an N2 worm during 10 min in osmotic stress. (E–G) Recovery of a shrunken N2 worm during 10 min in isotonic medium. (H–K) *osr-1 (rm1)* worm in acute osmotic stress. (L) Time course of percentage of motile N2 (◇) and *osr-1(rm1)* animals (■) in osmotic stress ($N > 500$ scored for each strain). Arrow indicates the time point at which the inactive N2 worms were shifted to isotonic medium while *osr-1(rm1)* animals remained under the salt stress. The N2 and *osr-1(rm1)* animals shown in this figure are of the same respective animal at different time points. Bar, 0.2 mm.

and 2). In contrast to N2 animals, *osr-1(rm1)* animals exhibit normal swimming behavior (Figure 1, H–K) when exposed to a transient osmotic stress (10 min, 500 mM NaCl) and remain viable (88%) even after 24 hr (Figure 2C). *osr-1(rm1)* animals are also resistant to osmotic stress caused by sorbitol, glucose, sucrose, and KCl, demonstrating that *osr-1(rm1)* animals have a general resistance to hyperosmotic stress (data not shown).

To determine whether the *osr-1(rm1)* mutation is specific to osmotic stress or confers resistance to multiple stresses, as is observed for long-lived mutants of the insulin-like signaling (ILS) pathway (KENYON *et al.* 1993; LARSEN 1993; DORMAN *et al.* 1995; LITHGOW *et al.* 1995; MURAKAMI and JOHNSON 1996; KIMURA *et al.* 1997; TISSENBAUM and RUVKUN 1998), we tested for the ability of *osr-1(rm1)* animals to survive heat-shock (35°) or oxidative stress (300 mM paraquat; Figure 2, D and E). In contrast to *age-1(hx546)* animals, *osr-1(rm1)* animals were sensitive to both stresses ($P < 0.05$). Moreover, *osr-1(rm1)* animals did not exhibit other phenotypes associated with general stress resistance as seen in other ILS pathway mutants, such as extended life span or constitutive dauer formation (data not shown). Thus, *osr-1(rm1)* animals are a new class of mutants that are resistant specifically to osmotic stress.

OSR-1 and AGE-1 have critical roles in resistance to chronic osmotic stress: We explored the possibility that the general stress resistant mutant, *age-1(hx546)*, might be also resistant to osmotic stress. However, as shown in Figure 2A, exposure of *age-1(hx546)* animals to 500 mM NaCl caused a complete loss of motility and reduction in body volume within 11 min, similar to wild-type animals. This demonstrates that *age-1(hx546)* animals are not preadapted to acute osmotic stress like *osr-1(rm1)* animals (Figure 2A). In addition, and in contrast to *osr-1 (rm1)* animals, *age-1(hx546)* animals have a wild-type phenotype for osmotic avoidance (Figure 2F). Interestingly, *age-1(hx546)* animals exhibited motility and viability similar to that of the *osr-1(rm1)* animals upon prolonged exposures to high osmotic environments (Figure 2, B and C). These results indicate that although AGE-1 does not regulate osmotic avoidance or resistance to acute osmotic stress, both OSR-1 and AGE-1 regulate survival under conditions of prolonged hyperosmotic stress.

***osr-1(rm1)* animals display an osmotic avoidance abnormality phenotype:** The observed osmotolerance of *osr-1(rm1)* animals led us to investigate whether OSR-1 is involved in detection of high osmolarity environments as was described previously for osmotic avoidance defective (OSM) mutants (CULOTTI and RUSSELL 1978; BARGMANN *et al.* 1990). *osr-1(rm1)* animals exhibited an osmotic avoidance abnormality phenotype as they fail to avoid regions of 4 M NaCl or 8 M fructose (Figure 2F and data not shown). The osmotic avoidance defect of *osr-1(rm1)* animals is quantitatively similar to that seen in the previously characterized *osm* mutants, such as *osm-9(ky10)* (COLBERT *et al.* 1997), *osm-10(n1052)* (HART *et al.* 1999) and *ocr-2(ak47)* (TOBIN *et al.* 2002).

Osmosensation in *C. elegans* is dependent on the integrity of osmosensory organs (amphids and phasmids) and function of the ASH neurons; the defective osmotic avoidance phenotype in *osm-9(ky10)*, *osm-10(n1052)*, and *ocr-2(ak47)* has been ascribed to impaired function of the ASH neurons (COLBERT *et al.* 1997; HART *et al.* 1999; TOBIN *et al.* 2002). On the basis of lipophilic dye staining, we could not detect any morphological abnor-

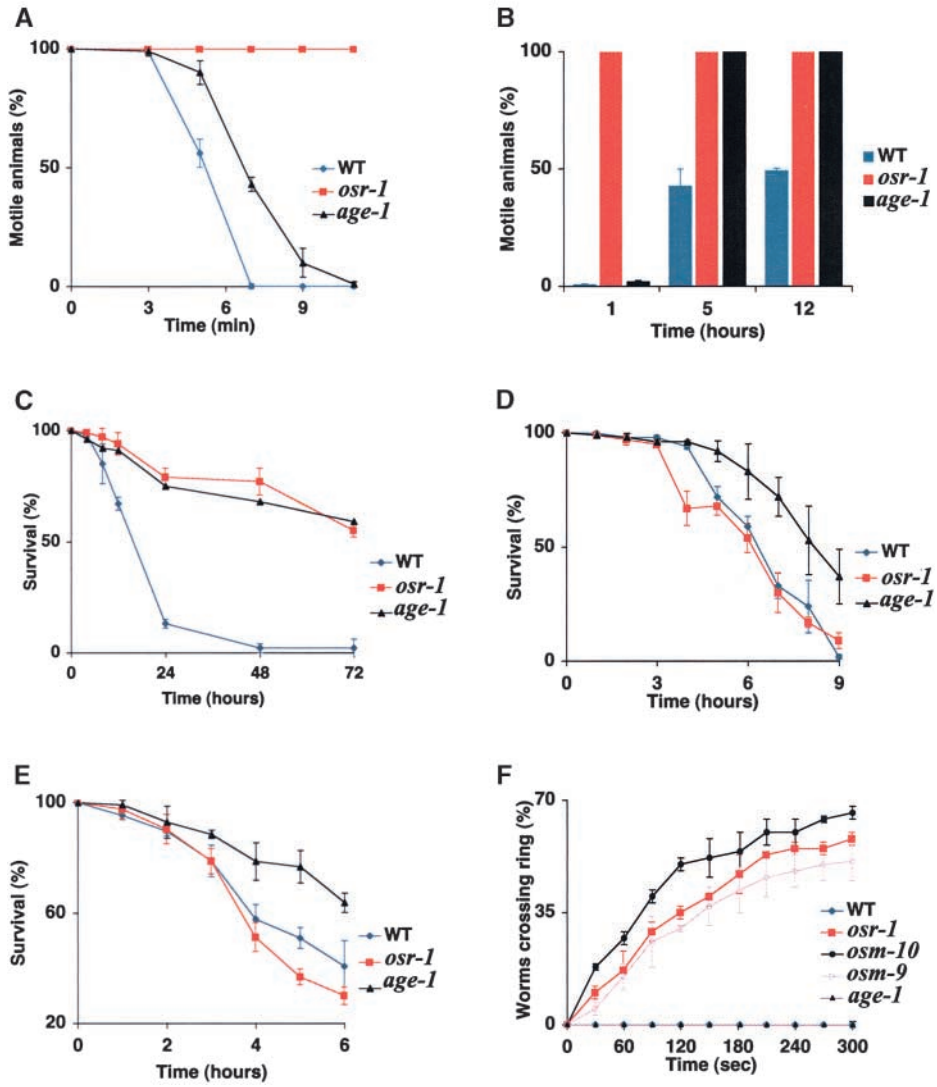

FIGURE 2.—*osr-1(rm1)* worms are specifically resistant to osmotic stress. (A and B) Motility assays of wild-type (N2 strain; blue diamond and blue bar), *osr-1(rm1)* (red square and red bar), and *age-1(hx546)* (black triangle and black bar) animals following (A) acute and (B) chronic exposure to 500 mM NaCl. (C) Survival of N2 (blue diamond), *osr-1(rm1)* (red square), and *age-1(hx546)* (black triangle) animals during 3 days of exposure in 500 mM NaCl. (D and E) Survival of N2 (blue diamond), *osr-1(rm1)* (red square), and *age-1(hx546)* (black triangle) animals exposed to either (D) heat shock (35°) or (E) oxidative stress (300 mM paraquat). (F) Osmotic avoidance assay of N2 (blue diamond), *osr-1(rm1)* (red square), *age-1 (hx546)* (black triangle), *osm-9(ky10)* (pink open circle), and *osm-10 (n1052)* (black solid circle) animals. Error bars represent standard error for three to five independent replicates at each time point. Each replicate contained 50–100 adult hermaphrodite animals that were tested.

malities in the osmosensory organs (amphids and phasmids) of *osr-1(rm1)* animals (A. SOLOMON and R. MORIMOTO, unpublished results). In addition, *osr-1(rm1)* animals have normal responses to nose touch and the repellent, 1-octanol (data not shown), which are also mediated by the polymodal ASH neurons via distinct signaling pathways (KAPLAN and HORVITZ 1993; HART *et al.* 1999). Although we cannot rule out that the ASH neurons are not impaired for osmosensation in *osr-1(rm1)* animals, our results demonstrate that at least the mechanosensory and chemosensory responses mediated by the same neurons are normal in these animals.

We have also found that, unlike *osr-1(rm1)* animals, other osmotic avoidance defective mutants, *osm-9(ky10)*, *ocr-2(ak47)*, and *osm-10(n1052)*, are not resistant to either acute or chronic osmotic stress. Also, none of these mutations affect osmotic stress tolerance in OSR-1 deficient animals (data not shown). This demonstrates that the osmosensory pathway defined by OSM-9, OCR-2, and OSM-10 does not mediate acute or chronic resistance to osmotic stress, and that the hyperresistance of *osr-1(rm1)* animals is independent of the pathways regulated by the OSM genes tested by us.

Positional cloning, sequence analysis, and expression patterns of *osr-1*: *osr-1* was cloned using standard two- and three-factor mapping and transformation rescue approaches (Figure 3A). Two cosmids, C32E12 and F56A3, with an overlapping region of 11 kb gave a full rescue of the *osr-1(rm1)* phenotypes. In this overlapping region, we identified a 4-kb minimal rescuing fragment (pASRM1) containing a single predicted gene, C32E12.3, that completely rescued all the *osr-1(rm1)* phenotypes. We found in *osr-1(rm1)* worms a G-to-A mutation at a predicted splice acceptor site in the transcription unit of C32E12.3 (Figure 3B). Introduction of this mutation in pASRM1 completely eliminated its ability to rescue the *osr-1(rm1)* animals' phenotypes. In addition, RNAi of *osr-1* in wild-type animals results in *osr-1(rm1)*-like phenotypes. We conclude that the *osr-1* gene corresponds to C32E12.3.

The *osr-1* gene encodes a predicted protein of 643 amino acids (Figure 3C) and lacks any domains that

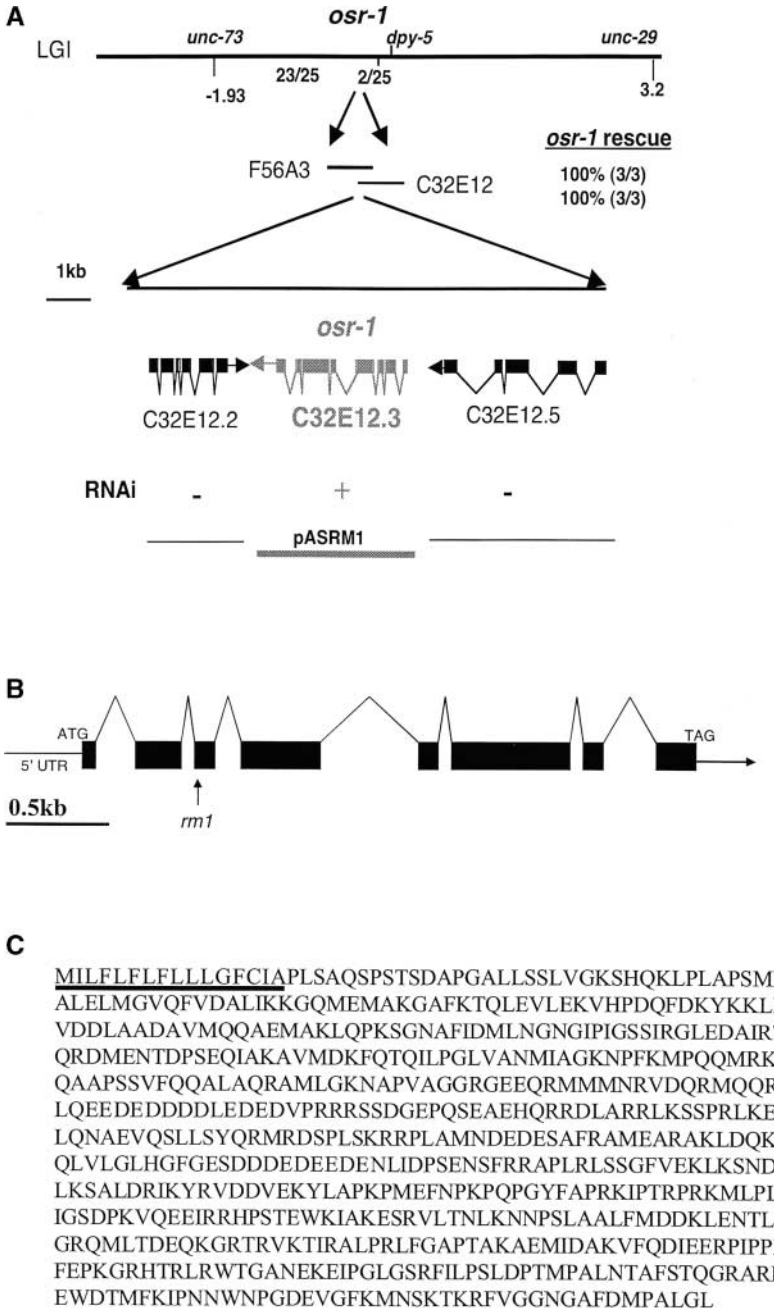

FIGURE 3.—Positional cloning of *osr-1*. (A) Genetic and physical maps of chromosome I. *osr-1* was mapped to ~ -0.2 MU between two cloned genes, *unc-73* and *dpy-5*. The numbers below the bar represent the recombination events that occurred between *dpy-5* and *osr-1* (2/25) and *unc-73* and *osr-1* (23/25). Numbers in parentheses show the fraction of transgenic lines that rescued *osr-1(rm1)* mutant animals. (B) The *osr-1* transcription unit. The full transcription unit of *osr-1* was determined using a partial EST (yk563c9) obtained from Yuji Kohara and using 5′ random amplification of cDNA ends experiments. Since the *osr-1* mRNA did not have SL consensus splice sequences, we introduced a stop codon in the 5′ untranslated region immediately in frame with the first putative ATG site in the rescuing construct pASRM1. Injection of this construct into *osr-1(rm1)* mutant animals gave a full rescue. (C) *osr-1* encodes a novel protein of 643 amino acids with a predicted N-terminal signal peptide (underlined).

would suggest a specific molecular function. Proteins with significant similarity to OSR-1 were found only in the genomes of *C. briggsae* (86% overall identity to CBE 12837) and the parasitic nematode *Strongyloides ratti* (48% identity in partial EST kt82b03.y1).

To identify the tissues in which OSR-1 is expressed and functions, we placed the rescuing fragment, pASRM1, in an artificial operon with green fluorescent protein (GFP). This construct fully rescued the *osr-1(rm1)* phenotypes and GFP fluorescence was seen in the hypodermis and intestine during all developmental stages (Figure 4, A and B). To test which tissues have functional importance, we performed rescue experiments using heterologous promoter constructs including hypodermal (P_{dpy-7}::*osr-1::gfp*), intestinal (P_{vha-6}::*osr-1::gfp*; WANG *et al.* 2002) and pan-neuronal ($P_{F25B3.3}$::*osr-1::gfp*) promoters (ALTUN-GULTEKIN *et al.* 2001). We found that only hypodermal expression of *osr-1* cDNA rescues the *osr-1(rm1)* phenotypes (Figure 4C).

OSR-1 genetically interacts with CaMKII and a conserved p38 MAPK cascade: To understand the mechanisms by which OSR-1 regulates osmotic stress responses in *C. elegans*, we tested the potential role(s) of the *C. elegans* homologs of HOG/p38 MAPK signaling pathway (BREWSTER *et al.* 1993) in mediating phenotypes seen in *osr-1(rm1)* animals. In *C. elegans*, there are multiple MAPK encoding genes implicated in development and resistance to abiotic and biotic stresses (KURZ and EWBANK 2003).

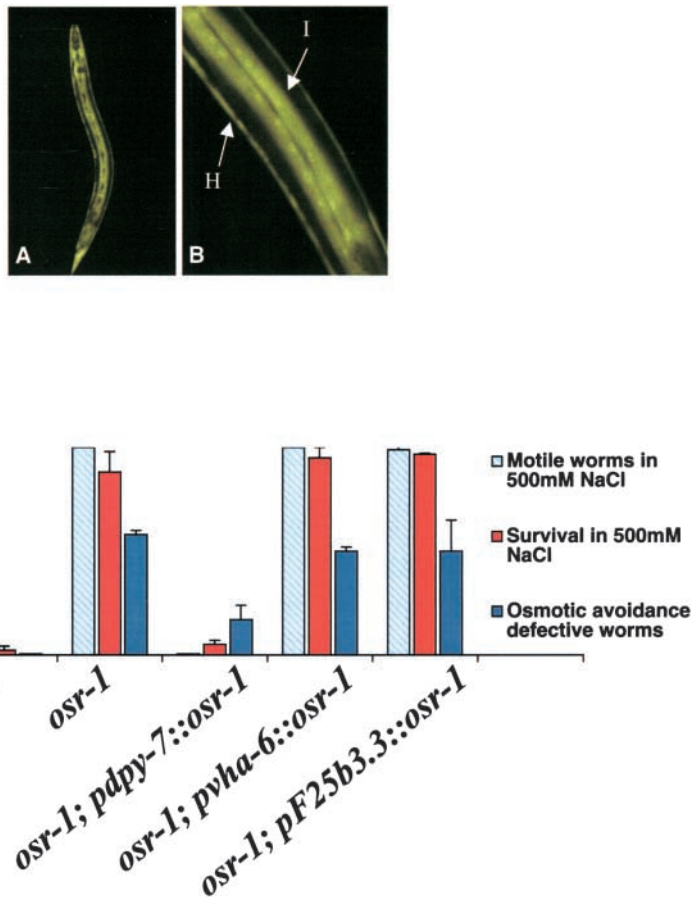

FIGURE 4.—OSR-1 is expressed in the hypodermis and intestine. (A) L3 stage of N2 animals showing expression of OSR-1 in the intestine and hypodermis. (B) Section from the midbody of the L3 stage animal. Arrows indicate hypodermis (H) and intestine (I). (C) Tissue-specific expression of *osr-1* cDNA in the hypodermis ($P_{dpy\text{-}7}$), intestine ($P_{vha\text{-}6}$), and nervous system ($P_{F25B3.3}$). Expression of *osr-1* cDNA under the hypodermal promoter was able to rescue all the *osr-1(rm1)* phenotypes. Error bars represent standard error for three independent replicates at each time point. Each replicate contained >50 adult hermaphrodite animals that were tested.

The Esp pathway (NSY-1/MAPKKK → SEK-1/MAPKK → PMK-1/p38/MAPK), has been shown to function in pathogen resistance (KIM *et al.* 2002), and the neuronal symmetry (Nsy) pathway (UNC-43/CaMKII → NSY-1 → SEK-1 → unknown MAPK) mediates asymmetric neuronal cell fate in AWC sensory neurons (SAGASTI *et al.* 2001; TANAKA-HINO *et al.* 2002).

Downregulation of *pmk-1*/p38 in *osr-1(rm1)* worms by feeding RNAi significantly reduced their ability to survive chronic osmotic stress (Figure 5A; $P < 0.05$). Similarly, null mutations, of NSY-1 and SEK-1, completely suppressed the ability of *osr-1* deficient animals to survive osmotic stress (Figure 5A). These results led us to investigate the potential relationship between OSR-1 and the most upstream component of the Nsy pathway, UNC-43. As shown in Figure 5A, UNC-43, the only identified CaMKII in *C. elegans* (REINER *et al.* 1999), was also required for viability under chronic osmotic stress.

The interactions between OSR-1 and PMK-1/p38 appear to be specific because OSR-1 does not interact with two other *C. elegans* p38 homologs (BERMAN *et al.* 2001), PMK-2 and PMK-3 (A. SOLOMON and R. MORIMOTO, unpublished results). Mutations in genes that mediate heavy metal resistance, including JNK-1, JKK-1 (VILLANUEVA *et al.* 2001), and MEK-1 (KOGA *et al.* 2000), also do not suppress the OSR-1 phenotypes (Figure 5A and data not shown). Because the forkhead transcription factor, DAF-16, promotes general stress resistance and longevity (MURAKAMI and JOHNSON 1996; OGG *et al.* 1997; HENDERSON and JOHNSON 2001), we also tested for the effect of *osr-1* RNAi in a null mutant of *daf-16* (OGG *et al.* 1997). As shown in Figure 5A, the ability of OSR-1 to regulate survival in hyperosmotic environments is not dependent on DAF-16. Taken together, our results indicate that OSR-1 functions upstream of or in parallel to UNC-43/CaMKII and a conserved p38 pathway (NSY-1 → SEK-1 → PMK-1) in promoting survival under chronic osmotic stress (Figure 5B).

We also investigated the role of the PMK-1/p38 pathway in regulating resistance to acute dehydration and osmotic avoidance defects seen in *osr-1(rm1)* animals. Interestingly, we find that *osr-1(rm1)*, *pmk-1*(RNAi) ($N = 175$); *nsy-1(ky379)*, *osr-1*(RNAi) ($N = 1259$); and *sek-1 (km4)*, *osr-1*(RNAi) ($N = 376$) animals remained resistant to the acute effects of osmotic stress by maintaining normal swimming behavior (100% motile animals after 10 min). In addition, these animals retain the OSM-like phenotype, as ~57% of these animals cross the 4 M NaCl ring (data not shown), similar to the phenotypes seen in *osm-9(ky10)* and *osr-1(rm1)* animals ($P > 0.05$; see Figure 2F).

DISCUSSION

In this study, we have used the osmotic stress response of *C. elegans* as a model for stress signaling in a multicel-

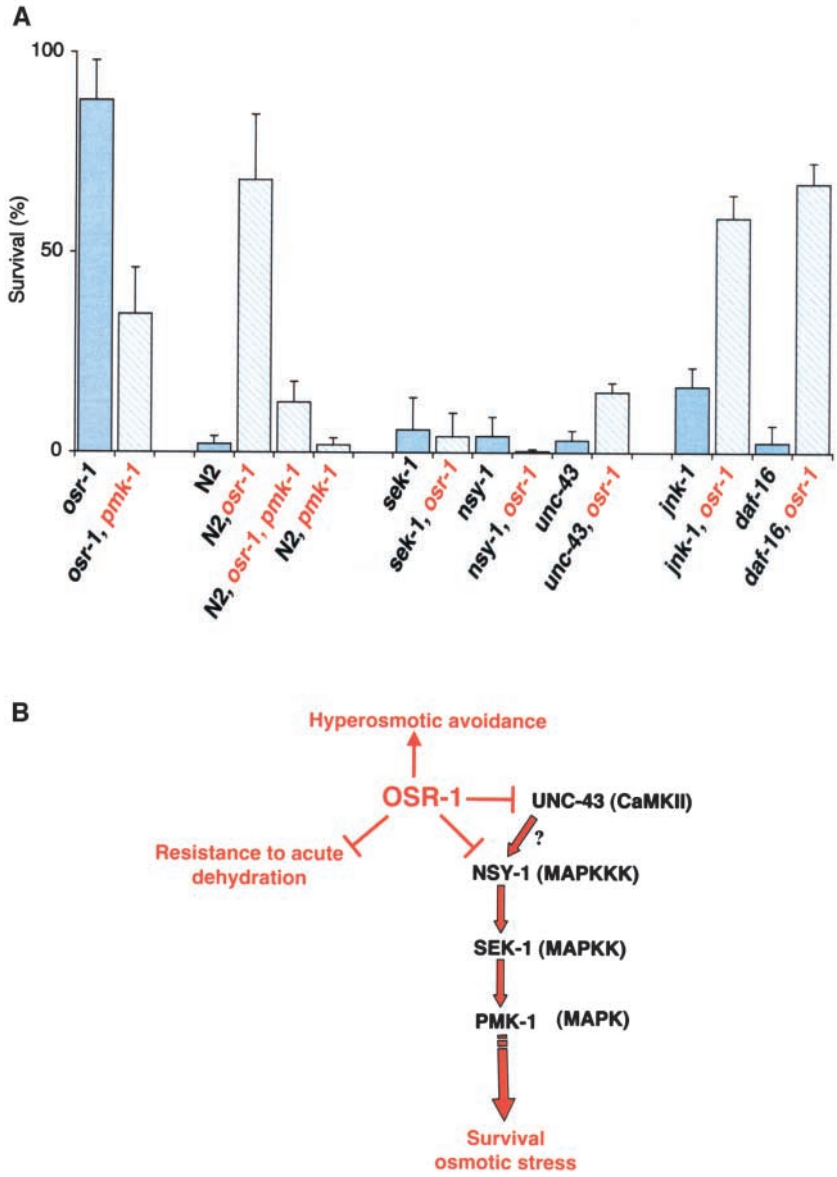

FIGURE 5.—OSR-1 genetically interacts with UNC-43/CaMKII and the conserved PMK-1/p38 MAPK signaling pathway to promote survival in osmotic stress. (A) Survival of N2, *osr-1(rm1)*, *sek-1(km4)*, *nsy-1(ky397)*, *unc-43(n1186)*, *daf-16(mgdf50)*, and *jnk-1(gk7)* mutant animals in 500 mM NaCl after 24 hr. Survival was also tested in the designated mutants with or without RNAi to *osr-1* and *pmk-1*. Progeny of each strain was grown on *E. coli* (HT115) carrying only the L4440 expression vector (solid blue bar; control group), HT115 carrying the plasmid pDK177 (for *pmk-1*), or the plasmid pAS1 (for *osr-1*), producing double-strand RNA (striped blue bar). On the *x*-axis, black text indicates genetic background while red text indicates the RNAi target gene. Error bars represent the standard error for three to five replicates. Each replicate contained >250 adult hermaphrodite animals. (B) Our model predicts that under normal conditions, OSR-1 functions to enable worms to avoid hyperosmotic environments and inhibits unidentified pathway(s), CaMKII, and a conserved p38 MAPK pathway. In high osmolarity, OSR-1 functions are probably inhibited, which in turn activates distinct signaling pathways to promote adaptation and survival.

lular organism. We established a novel genetic screen to identify genes that are critical regulators of the osmotic stress response in a whole animal and report the cloning and characterization of a novel gene, *osr-1*. On the basis of the phenotypes of *osr-1(rm1)* mutant animals, we suggest that OSR-1 regulates the ability of *C. elegans* to (i) detect and avoid high osmolarity, (ii) resist acute exposures to osmotic stress, and (iii) maintain viability upon prolonged exposure to osmotic stress. *osr-1(rm1)* animals are not resistant to heat and oxidative stress and do not display phenotypes associated with mutants of the ILS pathway, such as extended life span and constitutive dauer formation. Thus, OSR-1 specifically regulates osmotic stress responses in *C. elegans*.

How might OSR-1 regulate the different osmotic stress-induced responses in *C. elegans*? Osmotic upshifts in multiple organisms cause perturbations in membrane-dependent transport processes and activation of protein kinase cascades and lead to the accumulation of osmolytes, such as glycerol (HOHMANN 2002). Our genetic epistasis analysis demonstrates that OSR-1 regulates a conserved p38 MAPK pathway. This pathway has been shown to induce production of glycerol in yeast cells during osmotic upshifts (HOHMANN 2002). We also found that *osr-1(rm1)* animals accumulate high levels of glycerol (1455 ± 26 nmol of glycerol per milligram of protein; A. SOLOMON and R. MORIMOTO, unpublished results) even under normal growth conditions, while wild-type animals have undetectable levels. Since accumulation of glycerol has been previously implicated in osmotic stress tolerance in yeast and different species of nematodes including *C. elegans* (HOHMANN 2002; QIU and BEDDING 2002; LAMITINA *et al.* 2003), we hypothesize that activation of the p38 MAPK cascade and glycerol accumulation might be sufficient to explain the osmotic stress tolerance of *osr-1(rm1)* animals.

We demonstrate that *pmk-1* downregulation suppresses partially (>50%) the chronic osmotic stress re-

sistance phenotype of *osr-1(rm-1)* animals, and it does not affect resistance to acute dehydration and osmotic avoidance defects in these animals. These findings raise the intriguing possibility that OSR-1 regulates these responses via other, yet to be identified, mechanisms (Figure 5B). This is also supported by our observations that null mutants of the upstream component of the p38 pathway, SEK-1 and NSY-1, also do not suppress the osmotic avoidance or acute dehydration phenotypes of *osr-1* deficient animals.

For the observed acute resistance to osmotic stress seen in *osr-1(rm1)* animals, we suggest that it might be related to alterations in cuticle structure, as we find that mutations in cuticle collagen genes, DPY-10 and DPY-2 (Levy *et al.* 1993), result in an *osr-1(rm1)*-like resistance phenotype in acute and chronic osmotic stress conditions (A. Solomon and R. Morimoto, unpublished results). We are currently investigating potential genetic interactions between cuticle component genes and *osr-1* to test this idea further.

We additionally found that the *age-1(hx546)* mutants are also resistant to the effects of chronic exposure to osmotic stress but, unlike *osr-1(rm1)* animals, they are not osmotic avoidance defective or resistant to acute osmotic stress. We are currently investigating the possibility that OSR-1 and AGE-1 regulate resistance to chronic osmotic stress through overlapping mechanisms. In our studies, we isolated an additional 104 OSR-like mutants and have so far characterized four different complementation groups. Understanding the interrelationships among these genes, *age-1*, and *osr-1*-dependent pathways will reveal in greater detail how OSR-1 functions to regulate osmotic stress responses in *C. elegans*.

Osmotic avoidance defective phenotypes have been previously described for the OSM class of mutants (Culotti and Russell 1978). Mutations in OSM-9, OCR-2, and OSM-10 influence the function of the osmosensory ASH neurons (Colbert *et al.* 1997; Hart *et al.* 1999; Tobin *et al.* 2002). However, we have found no evidence for impaired ASH neuron function in *osr-1(rm1)* animals. Also, *osm-9(ky10)*, *ocr-2(ak47)*, and *osm-10(n1052)* mutant animals are not resistant to either acute or chronic osmotic stress; moreover, none of these mutations suppress the osmotic stress resistance of *osr-1* deficient animals (data not shown). These results demonstrate that the ASH neurons and the pathways mediated by the OSM genes (OSM-9, OSM-10, and OCR-2) play a limited role, if any, in the ability of OSR-1 to regulate resistance to osmotic stress.

Consistent with previous suggestions of an important role for the hypodermis in osmoregulation in *C. elegans* (Petalcorin *et al.* 1999), we found that only hypodermal expression of *osr-1* cDNA rescues the *osr-1(rm1)* phenotypes. However, we cannot rule out the possibility that the hypodermal promoter (*dpy-7*) is not completely off in neurons or other tissues. Weak expression in the nervous system, for example, could indeed explain the partial rescue of the osmotic avoidance phenotype of *osr-1(rm-1)* animals.

As seen in yeast and mammals (Han *et al.* 1994; de Nadal *et al.* 2002), the p38 MAPK signaling pathway has an important role in mediating osmotic stress tolerance in *C. elegans*. However, as is the case with other signaling pathways such as the RAS pathway, the upstream regulators of this conserved MAPK cascade may differ between metazoans and unicellular organisms like yeast. For *C. elegans*, we suggest that CaMKII regulates a p38 pathway in an OSR-1-dependent manner (Figure 5B); CaMKII has also been implicated in osmotic responses in mammalian kidney cells (Tokuda *et al.* 2002). In contrast, the HOG/p38 cascade in *S. cerevisiae* is regulated by a two-component system (SLN1-YPD1-SSK1; Maeda *et al.* 1994; Posas *et al.* 1996) and no orthologs of the two-component system have been identified yet in animals.

Our genetic data indicate that OSR-1 negatively regulates multiple osmotic stress responses in *C. elegans* probably via distinct mechanisms. We favor the interpretation that under normal conditions, OSR-1 functions to enable worms to avoid hyperosmotic environments. However, in hyperosmotic environments, OSR-1 functions are likely inhibited, which in turn activates distinct signaling pathways to promote adaptation and survival. Such a mechanism bears a striking resemblance to the reported inactivation of the response regulator, Ssk1, in the two-component system (SLN1-YPD1-SSK1) of *S. cerevisiae* during conditions of high osmolarity (Posas *et al.* 1996). We anticipate that further characterization of OSR-1 and the identification of its interacting partners and other *osr-1*-like genes will greatly facilitate the understanding of how metazoans integrate multiple responses and survive in hyperosmotic environments.

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