Deletion of the Intestinal Peptide Transporter Affects Insulin and TOR Signaling in Caenorhabditis elegans*

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The mammalian intestinal peptide transporter PEPT1 mediates the uptake of di- and tripeptides from the gut lumen into intestinal epithelial cells and acts in parallel with amino acid transporters. Here we address the importance of the PEPT1 orthologue PEP-2 for the assimilation of dietary protein and for overall protein nutrition in Caenorhabditis elegans. pep-2 is expressed specifically along the apical membrane of the intestinal cells, and in pep-2 deletion mutant animals, uptake of intact peptides from the gut lumen is abolished. The consequences are a severely retarded development, reduced progeny and body size, and increased stress tolerance. We show here that pep-2 cross-talks with both the C. elegans target of rapamycin (TOR) and the DAF-2/insulin-signaling pathways. The pep-2 mutant enhances the developmental and longevity phenotypes of daf-2, resulting, among other effects, in a pronounced increase in adult life span. Moreover, all aspects of a weak let-363/TOR RNA interference phenotype are intensified by pep-2 deletion, indicating that pep-2 function upstream of TOR-mediated nutrient sensing. Our findings provide evidence for a predominant role of the intestinal peptide transporter for the delivery of bulk quantities of amino acids for growth and development, which consequentially affects signaling pathways that regulate metabolism and aging.

In all organisms the uptake of amino acids is mediated by a multitude of integral cell membrane carriers that transport amino acids either in free or in peptide-bound form. The two mammalian peptide transporters PEPT1 and PEPT2 are proton-dependent rhogenic carriers and have been grouped into the POT (proton-coupled oligopeptide transporter) superfamily, which is also called peptide transporter family (1). They transport short-chain peptides but also a variety of pharmacologically important compounds including selected β-lactam antibiotics and angiotensin-converting enzyme inhibitors, as well as antiviral and antineoplastic drugs (2, 3). In contrast, these transporters cannot transport larger peptides or free amino acids (4). PEPT1 is localized to apical membranes of intestinal epithelial cells where it mediates the uptake of di- and tripepti
dides following the digestion of dietary proteins. In kidney, PEPT1 is also found in epithelial cells of the proximal tubule where it may contribute to the reabsorption of peptides after glomerular filtration (5).

The Caenorhabditis elegans genome contains two homologues of the human pep1 and pep2 genes, designated pep-2 and pep-1 (aka opt-2 and opt-1, encoding CPTB and CPTA, respectively) (6). Although the deduced transporter proteins reveal only modest amino acid similarities with the mammalian proteins, the in vitro transport characteristics of the C. elegans and mammalian orthologues in Xenopus laevis oocytes are very similar (6). The C. elegans pep-2 gene encodes a protein with 36.9% amino acid sequence identity compared with the human PEPT1 and represents the low affinity/high capacity isomorph of proton-coupled peptide transporters. Based on its role in intestinal absorption of peptide-bound amino acids, the PEP-2 protein may be important for overall protein nutrition of the organism.

Whole body protein nutrition is linked to the available amino acid pool that regulates metabolic and reproductive adaptations through the partially interconnected insulin/IGF1 and TOR/S6K signaling pathways. TOR senses the cellular amino acid pool and contributes to a signaling cascade that regulates transcription, translation, and protein degradation (7). A partial loss of TOR function in Drosophila reduces growth, and flies deficient in the p70S6K gene (DS6K, a downstream target of TOR) are extremely delayed in development and are smaller in size (8). More recently, let-363, a C. elegans homologue of TOR, was identified and characterized (9). let-363 loss-of-function mutants exhibit a developmental arrest and death at the L3 larval stage (9). Furthermore, it was shown that TOR function affects the life span and may interact with the DAF-2/insulin-signaling pathway in C. elegans (10). TOR has been implicated in the insulin/IGF network based on cell culture experiments (11, 12), and in Drosophila TOR is required for the growth-stimulating effect of the phosphatidylinositol 3-kinase pathway (13). In both Drosophila and mammals, the levels of dietary protein or amino acid availability also affect the insulin-signaling pathway to control metabolism and growth (14–16). In addition, the aging process was shown to be regulated hormonally by this evolutionarily conserved signaling pathway (17–19). In C. elegans, the daf-2 gene encodes an insulin/IGF receptor, and the downstream components include the AGE-1/phosphatidylinositol 3-kinase, PDK-1/PDK1, the AGC kinases AKT-1/2 and SGK-1, PKB, DAF-18/PTEN, and the forkhead transcription factor DAF-16/FKHR1/FOXO (20). Extensive

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1 The abbreviations used are: IGF, insulin-like growth factor; TOR, target of rapamycin; AMCA, Nε-7-amino-4-methylcoumarin-3-acetic acid; S6K, ribosomal protein S6 kinase; GFP, green fluorescent protein; RNAi, RNA interference.
studies revealed that the DAF-2 pathway regulates aging, re-
production, lipid metabolism, and dauer formation independ-
ently of one another (15, 21–23).

Here we describe the phenotypic alterations in *C. elegans*
caused by the deletion of the intestinal peptide transporter
**pep-2**. Our analysis clearly identifies intestinal peptide absor-
ption as a key process in body protein homeostasis that affects
both the TOR and the insulin-signaling pathways.

**EXPERIMENTAL PROCEDURES**

*C. elegans Strains*—The strains used were as follows: N2 Bristol
(wild type), DA455: *eat-2(ad453)i*, CB1370: *daf-2(e1370)III*, DR26: *daf-
16(m26fI), DH1033: bIs1[+2;GFP,pFRA,sqt-1(sce103)]; BR2748: bIs1[+
itor-2;GFP,pFRA,sqt-1(sce103)]; pep-2(lg601)X, DR2686: *daf-2(e1370)III*,
pep-2(lg601)X, DR2689: *del-16(m26fI);pep-2(e1370)III*; pep-2(lg601)X, BR3061: *del-16-
(m26fI);del-2(e1370)III*; pep-2(lg601)X. DR1908: mIs1([rol-6(su1006),
daf-7p::GFP]), BR3062: mIs1([rol-6(su1006),daf-7p::GFP];pep-2(lg601)X,
BR2743: pep-1(g501)IV and BR2744: pep-1(g501)IV; pep-2(lg601)X. To isolate
the *pep-1* and *pep-2* deletion mutants, a UV/trimethyl psoralen
mutagenized *C. elegans* library was screened by PCR with gene-specific
primers. The mutant allele *lg601* was back-crossed with N2 wild type
animals seven times before analysis. *pep-2(lg601)* is recessive and re-
present a strong loss-of-function allele that could be rescued by a wild
type transgene. In the *pep-1* mutant allele *lg501*, a 2.5-kb deletion
removes 1269 bp of the promoter sequence, the translational start
codon, and the first six exons of the gene (bp 18289–20835 on cosmid
C06G12).

**β-Ala-Lys-AMCA Staining**—Mixed staged animals were washed off
of agar plates with M9 buffer. Equal amounts of worms were incubated
in a 1 mM β-Ala-Lys-AMCA solution (in M9) for 2–3 h followed by
at least four additional washing steps with M9. As a control, worms
were incubated in M9 buffer for the same time period. Pictures were taken
with an AxioPlan 2 (Zeiss) using AxioVision 3.0 software.

**Assays for Developmental and Behavioral Phenotypes**

**Body Length Measurements**—Synchronized wild type and pep-
2(lg601) animals were collected 0–5 days after L4 moulting. Pictures of 20
individual worms were taken with Axioplan 2, and the precise body
length was measured with AxioVision 3.0 software.

**Postembryonic Development**—5–15 adult hermaphrodites were placed
on fresh plates for egg laying. After 2–3 h they were removed,
and at least 16 worms of the progeny were singled onto individual
plates. The F1 animals were monitored every 2 h until they laid the first
egg.

**Yolk Protein Distribution**—Yolk protein distribution was analyzed by
visual inspection of GFP expression in DH1032 bIs1[[+2;GFP,
pFRA,sqt-1(sce103)]; BR2746: bIs1[[+2;GFP,pFRA,sqt-1(sce103)];
pep-2(lg601)X.

**Life Span**—Life span assays were performed as described (25), except
that adult hermaphrodites were allowed to lay eggs for 8–10 h. Animals
were scored dead once per hour. For assays at 25 °C, the animals were grown
to 15 °C until the L4 moulting and then shifted to 25 °C. We used the L4
moulting at t = 0 for life span analysis.

**Stress Resistance and Other Assays**—The assays for heat stress
(35 °C) and oxidative stress (paraquat) resistance were performed as
described previously (26), except that on plates containing 150 mM
paraquat animals were scored once a day. Self-bred size, embryonic
development, deferation, and pharyngeal pumping assays were per-
formed as described previously (26). All analyses used animals fed with
Escherichia coli OP50.

**Amino Acid Supplementation**

For amino acid supplementation, 300 μl of amino acids (mixture 1:1
of minimum Eagle’s medium amino acids (50×) without 1-glutamine
(Ivortinogen)) were added on top of the agar (35-mm plates) seeded with
E. coli. Fresh plates were prepared each day during the experiment.

**TOR (let-363) RNA Interference (RNAi) Experiments**

RNAi through-feeding experiments were performed as described pre-
viously (27). Two independent sources of TOR/RNAi vectors were used.
The PCR product of cDNA from yk18c10 was cloned into vector
pPD129.35, resulting in a plasmid (gift of A. Gartner, Max-Planck-Institute
for Biochemistry, Martinsried, Munich) that induced only a weak let-363
phenotype. A plasmid inducing a strong let-363/TOR phenotype had
already been described previously (9, 10). For developmental and intes-
tinal phenotypes, animals at the L4 stage were placed on RNAi-induc-
ing plates and allowed to lay progeny. Adults were removed or trans-
ferred to new RNAi-producing plates. First and third generation
progeny grown on RNAi plates were scored for a TOR phenotype and
yielded identical results. Life span experiments were performed accord-
ing to Vellai et al. (10).

**Culturing Worms for Protein Carbonyl Determination**

Hermaphrodites of wild type and mutants were grown on NGM agar
plates seeded with *E. coli OP50*. Eggs were collected by sodium hypo-
chlorite treatment and allowed to hatch by being incubated overnight
at 20 °C in M9 buffer. Newly hatched L1 larvae were cultured on 150-mm
agar plates. To prevent progeny production, 5-fluoro-2-deoxyuridine
d(FdUR, Sigma) was added to the agar at a final concentration of 40 μM
after the animals had reached adulthood.

**Protein Carbonyl Measurement**

Worms were washed off of the agar plates with M9 buffer. Living
animals were collected because they floated on sucrose, washed several
times with M9 buffer, resuspended with 5 mM EDTA, and frozen at
−80 °C until use. Protein extracts were made by pounding and sonifi-
cation. Their protein carbonyl content was measured as previously
described (28). Protein concentration was determined by the Bio-Rad
Protein Assay (Bio-Rad Laboratories). Three to nine separate determi-
inations were used to calculate the mean ± S.E. for each different
age group.

**RESULTS**

**PEP-2 Functions as the Peptide Transporter in Intestinal
Cells**—We characterized a 1.7-kb deletion mutant, pep-
2(lg601), which lacks 257 bp of the promoter, the translational
start codon, and the first six exons of the *pep-2* gene (Fig. 1A).
Even if *PEP-2* were expressed from this mutant allele, it would
lack the N-terminal six transmembrane domains required for
substrate binding and transport (29) and therefore *pep-
2(lg601)* most likely represents a strong loss-of-function or null
allele. To demonstrate that the *pep-2(lg601)* mutant strain has
lost its capability for transport of di- and tripeptides, animals
were exposed to the fluorescent dipeptide conjugate β-Ala-Lys-
AMCA that was previously shown to be a representative sub-
strate of PEPT1 (30). Efficient uptake of the reporter molecule
into intestinal epithelial cells of wild type animals was indi-
cated by a strong fluorescence of all intestinal cells, whereas
the gut lumen lacked staining suggesting complete and rapid
intestinal peptide absorption (Fig. 1B). When *pep-2(lg601)* an-
imals were exposed to β-Ala-Lys-AMCA, the fluorescence was
detectable only in the gut lumen, indicative of a normal inges-
tion but lack of absorption; we never observed fluorescence
(other than the normal gut epifluorescence) inside of the epili-
ethelial cells (Fig. 1B). We conclude that *PEP-2* is the only
functional transporter in the intestine of *C. elegans* that is
capable of transporting the dipeptide-conjugate β-Ala-Lys-
AMCA representative for di- and tripeptides (30) across the apical
membrane into the intestinal cells.

**Loss of PEP-2 Function Affects Growth and Development—
Deletion of the pep-2 gene results in a decreased body size and
a markedly reduced brood size (Fig. 2, A and D, and Table I), a
similar phenotype to that reported for animals treated with
opt-2 RNAi (31). Moreover, the postembryonic development, in
which the animals depend on external food supply, is delayed
1.8-fold in pep-2 mutants at 15 and 20 °C (Fig. 2B and Table I),
whereas embryonic development that takes place in a protec-
tive eggshell is not significantly affected (Fig. 2C). In addition,
the reproductive life span is extended from 5 days (wild type) to
9 days (Fig. 2E). Unlike in Egl (egg-laying-defective) animals,
the eggs are laid at approximately the same developmental
stage as in wild type animals (28–56 cell stage). This suggests that
the production, rather than the retention, of eggs in the uterus
appears to be the limiting factor in pep-2 animals. Fertilized eggs in the uterus of pep-2(lg601) displayed the same

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level of vitellogenin::gfp (vit-2::gfp) fluorescence as those of wild type animals (Fig. 2F), indicating a similar yolk concentration in mature eggs. These data indicate that receptor-mediated endocytosis of yolk is not affected in pep-2 mutant animals. However, because the production of individual eggs is severely retarded, the reduced availability of amino acid nitrogen most likely results in a delay of de novo protein synthesis. Deletion of pep-2, however, does not cause lethality, and the additional deletion of pep-1 (orthologue of hPepT2) did not enhance the pep-2 phenotype (Fig. 2G). Transgenic expression of pep-2 rescued both β-Ala-Lys-AMCA uptake (Fig. 1B) and the developmental defects (Fig. 2B). Therefore, the expression of the pep-2 genomic region in mutant animals is sufficient to restore the function of the transporter. This data provide further support for PEP-2 being the only uptake system for di-/tripeptides in the intestine.

Loss of PEP-2 Cannot Be Compensated by Amino Acid Supplementation of the Food Source—The developmental defects observed in pep-2 mutants could be caused by impaired food intake, a limited availability of amino acids as energy substrates resulting in caloric restriction, or a more specific effect by an insufficient supply of amino acids for maintaining protein homeostasis. We have measured pharyngeal pumping and defecation as two of the behavioral parameters recognized as valid indicators of sufficient food consumption (32). Both parameters are indistinguishable in pep-2 and wild type animals (data not shown; Ref. 31). Therefore, malabsorption of dipeptides and the associated lower intake of amino acids appear to be the sole determinants of the developmental defects.

To assess whether amino acid deprivation in the pep-2 mutants can be overcome by the supplementation of free amino acids in the food, an amino acid mixture was added to the standard E. coli food source. In wild type animals, this amino acid supplementation did not affect brood size (Fig. 2D). In contrast, in pep-2(lg601) animals, it resulted in a partial but significant suppression of the brood size defect (Fig. 2D) and only a modest reduction of generation time (data not shown). These data suggest that amino acids from the supplement...
Intestinal Peptide Transport and Growth Regulation

Table I
Developmental phenotypes in pep-2 and daf-2 mutants

| Genotype                             | Postembryonic development* (hours) | N2   | No. of progeny* (a) | N2   |
|--------------------------------------|-----------------------------------|------|-------------------|------|
| Wild type                            | 67.1 ± 1.6 (48)                   | 100  | 316.6 ± 4.1 (54)  | 100  |
| daf-16(m26)                          | 74.1 ± 0.8 (37)                   | 110  | 292.6 ± 7.3 (28)  | 92   |
| pep-2(lg601)                         | 117.5 ± 2.6 (664)                 | 175  | 109.3 ± 1.9 (79)  | 35   |
| daf-16(m26);pep-2(lg601)             | 114.0 ± 2.1 (16)                  | 170  | 70.9 ± 4.6 (10)   | 22   |
| daf-2(e1370)                         | 108.8 ± 4.9 (20)                  | 162  | 210.0 ± 5.4 (30)  | 66   |
| daf-16(m26);daf-2(e1370)             | 78.7 ± 1.4 (37)                   | 117  | 260.8 ± 4.2 (18)  | 82   |
| daf-2(e1370);pep-2(lg601)            | 148.7 ± 6.1 (21)                  | 222  | 51.6 ± 1.1 (32)   | 16   |
| daf-16(m26);daf-2(e1370);pep-2(lg601)| 125.1 ± 2.2 (24)                  | 186  | 113.6 ± 2.6 (20)  | 36   |

*Values are means ± S.E. with number of animals shown in parentheses.

Delivered via the amino acid transporters cannot compensate for the loss of PEP-2-mediated peptide transport under normal feeding conditions. Therefore, PEP-2 activity is critical for amino acid availability and homeostasis in the organism.

pep-2(lg601) Interacts with the DAF-2 Insulin-signaling Pathway—C. elegans animals with reduced activity of the insulin/IGF receptor (DAF-2) also display developmental and reproductive defects (33). In our experiments, both daf-2(e1370) and pep-2(lg601) mutants showed a similarly delayed postembryonic development and reduced brood sizes (Table I). In daf-2;pep-2 double mutants, these phenotypic aspects are even more pronounced than in either of the single mutants (Table I). Only the phenotype caused by daf-2(e1370) was suppressed by daf-16(m26). In the daf-2;pep-2 double and daf-16;daf-2;pep-2 triple mutants, the phenotype of pep-2(lg601) was not affected by daf-16(m26). Thus, neither growth retardation nor reduced brood size of the pep-2 animals can be suppressed by daf-16 (Table I). These results suggest that pep-2 acts in parallel to daf-2 in a pathway that does not depend on DAF-16 function.

The consequences of a restricted amino acid intake in adult worms have not been addressed previously, but it is known that a reduced caloric intake, or the perturbation of the underlying signaling pathways that sense energy availability, prolongs life span (34). The reduced dietary availability of amino acids in pep-2 mutants may be interpreted as a reduced intake of calories, and therefore we tested whether the adult life span is altered in this mutant. As a control we used eat-2(ad453) (35), a calorically restricted mutant that exhibits an extended life span as compared with the wild type (25). eat-2 was shown to also act in parallel to the insulin-like signaling pathway, a major contributor to the regulation of life span in C. elegans. Whereas we observed that both eat-2(ad453) and daf-2(e1370) animals showed a longevity phenotype at 20 or 25 °C as reported previously (25, 36), the life span of pep-2(lg601) animals was not altered at 20 °C and was even slightly shorter at 25 °C (Table II, Fig. 3A). Similar results have been reported recently after RNAi treatment of pep-2 (31, 37). Therefore, the reduced amino acid availability does not account for a caloric restriction that is severe enough to alter the adult life span substantially. In addition, unlike food deprivation, which can induce a dauer phenotype, pep-2(lg601) at 25 °C did not show an increase in dauer formation in the presence of food as compared with wild type.

To assess whether there exists a cross-talk between the dietary intake of amino acids and daf-2-controlled longevity, we analyzed life span alterations in the daf-2(e1370);pep-2(lg601) double mutant strains, in which both pathways are perturbed. Surprisingly, the life span of daf-2(e1370) can be extended drastically in a pep-2 mutant background by around 60% when compared with the daf-2 single mutant (Fig. 3A, Table II). In addition, this longevity effect of pep-2(lg601) was completely suppressed in a daf-16;daf-2;pep-2 triple mutant (Fig. 3B, Table II). Thus, pep-2 deletion affects both daf-16-dependent and -independent outputs.

Stress Resistance Is Enhanced in pep-2(lg601)—All long lived mutants in the daf-2 signaling pathway are hyperresistant to oxidative stress, heat, or UV stress (38–40). To assess whether there is an increased stress resistance in the pep-2(lg601) mutant background, we tested the animals for heat tolerance (35 °C) and resistance to oxidative stress (paraquat) (Fig. 3, C and D). Both assays showed an increased stress resistance of pep-2(lg601) mutant animals, which cannot be suppressed by daf-16(m26). In addition, the stress resistance of daf-2(e1370) mutant animals was significantly increased in the pep-2 mutant background. Most strikingly, under conditions (150 mM paraquat) that kill 100% of the pep-2(lg601) animals within 7 days and 90% of the daf-2(e1370) mutants within 10 days, no lethality was observed among daf-2;pep-2 mutants (Fig. 3D).

Oxidative stress is known to damage proteins by the introduction of carbonyl groups, and the age-dependent accumulation of protein carbonyl appears to mirror the life span as shown in a variety of C. elegans mutants (28). The relevance of dietary amino acid supply on the protein oxidation level during the aging process has also been demonstrated in mammals (rats), where a low protein intake in the absence of a caloric restriction markedly reduces the concentration of protein carbonyls (41). Based on the observed connections between protein metabolism and insulin signaling, the expansion of longevity in daf-2;pep-2 double mutants could result from a decreased amount of oxidative damaged proteins in pep-2(−/−) animals. To test this hypothesis, we analyzed the protein carbonyl content in pep-2(lg601), daf-2(e1370), and daf-2(e1370);pep-2(lg601) mutants during aging. At days 19/20 post-hatching, daf-2;pep-2 animals had lower protein-carbonyl levels (3.83 ± 0.11 nmol/mg protein) than wild type animals (5.01 ± 1.00) or the pep-2 (5.44 ± 1.85) and daf-2 (5.55 ± 1.52) single mutants. When analyzed in aged animals (at days 30–40, when all wild type and pep-2 single mutants have already died), daf-2;pep-2 double mutants also had a significantly lower level of protein-carbonyl (2.84 ± 1.57 nmol/mg protein) than daf-2 (5.01 ± 0.71 nmol/mg protein). In summary, these results suggest that whereas the pep-2-dependent restricted amino acid availability is insufficient to promote longevity as long as the DAF-2 signaling pathway is intact, the pep-2-dependent restricted amino acid availability is capable of amplifying the daf-2 aging phenotype by reducing the generation of oxidized protein species or by further enhancing the degradation of the oxidatively damaged proteins.

pep-2(lg601) Affects the TOR Signaling Pathway—Elimination of C. elegans TOR (let-363) results in delayed development, eventually leading to developmental arrest and death at the L3 larval stage (9). We observed that the phenotypes of two different let-363(RNAi)-inducing constructs that we used (see “Experimental Procedures”) were distinct, and generally weaker than the mutant, which allowed us to test epistasis...
survival curves for animals at 25 °C enhanced in the daf-2;pep-2 double mutant background. The majority of let-363(RNAi_weak) larvae displayed a weak enlargement of the intestinal lumen (Fig. 4A) corresponding to the phenotype reported previously (9). All aspects of the let-363(RNAi_weak) phenotype were strongly enhanced in a pep-2(lg601) background (Fig. 4B). The double mutants developed much slower and had a substantially enlarged intestinal lumen, which contained undigested bacteria in the luminal fluid (Fig. 4B) as described previously for the let-363 null mutant (9). In addition, 100% (n > 250) of the double mutant animals arrested at L2 or L3 larval stages.

Feeding of either let-363 RNAi constructs was sufficient to extend life span, confirming recent reports from C. elegans and Drosophila (10, 42). pep-2(lg601) mutant background further increased this longevity effect of let-363(RNAi_weak) significantly (p < 0.0001; Fig. 4D, Table II) but was not able to affect the let-363(RNAi_strong) phenotype (Fig. 4C, Table II). These data are consistent with pep-2 being genetically located upstream of let-363/TOR and indicate that the obvious restriction of amino acid availability in the pep-2 mutant affects the TOR pathway.

**DISCUSSION**

The function of the mammalian peptide transporters has been studied extensively in vitro, but the contribution of PEPT1 to overall amino acid absorption in the gut and its role in protein nutrition in vivo is unknown. We show here that PEP-2 is the only functional intestinal peptide transporter in C. elegans, whereas PEP-1, the functional homologue of the mammalian PEPT2, is not involved in the absorption of dietary peptides in C. elegans. The pep-2 deletion mutant strain we have analyzed here displays strong developmental and reproductive defects consistent with a specific reduction in intestinal uptake of food-derived peptides as the prime source of amino acids in periods of high amino acid demand. An additional supply of free amino acids by the diet caused only a mild amelioration of the phenotype, emphasizing the indispensable role of intestinal peptide intake to ensure normal growth and development. These data greatly expand on a recent report describing the pep-2 RNAi phenotype (31).

The insulin/IGF signaling pathway is known to be affected by the availability of dietary protein or amino acids in both Drosophila (16) and mammals (14, 15). Recent microarray analyses indicate that pep-2 is down-regulated in a daf-2(−) mutant background (37). Consistent with being negatively con-

**TABLE II**

| Genotype | Mean life span[a] | Maximum life span | N[b] |
|----------|-------------------|-------------------|------|
|          | days              | days              |      |
| 20 °C    |                   |                   |      |
| Wild type| 13.6 ± 0.2        | 25                | 500  |
| e1370    | 18.3 ± 0.9        | 36                | 50   |
| pep-2(lg601) | 14.2 ± 0.2  | 26                | 250  |
| daf-2(e1370) | 21.8 ± 1.0    | 52                | 150  |
| daf-16(m26) | 10.4 ± 0.2     | 20                | 300  |
| daf-2(e1370);pep-2(lg601) | 31.9 ± 1.3    | 71                | 150  |
| daf-16(m26);pep-2(lg601) | 9.3 ± 0.2     | 20                | 300  |
| let-363(RNAi_weak) | 18.3 ± 0.6  | 30                | 100  |
| let-363(RNAi_weak); pep-2(lg601) | 22.2 ± 0.4  | 34                | 100  |
| 25 °C    |                   |                   |      |
| Wild type| 11.0 ± 0.3        | 21                | 200  |
| pep-2(lg601) | 9.0 ± 0.3       | 18                | 200  |
| daf-2(e1370) | 24.3 ± 0.7     | 46                | 197  |
| daf-16(m26) | 8.9 ± 0.3       | 17                | 100  |
| daf-16(m26);pep-2(lg601) | 10.9 ± 0.5    | 23                | 100  |
| daf-16(m26);pep-2(lg601) | 7.4 ± 0.3       | 17                | 100  |
| daf-2(e1370);pep-2(lg601) | 38.3 ± 0.9     | 77                | 187  |
| daf-2(e1370);pep-2(lg601) | 9.8 ± 0.5       | 26                | 100  |
| let-363(RNAi_strong) | 15.4 ± 0.4    | 22                | 100  |
| let-363(RNAi_weak); pep-2(lg601) | 15.8 ± 0.3  | 23                | 100  |

[a] Values are means ± S.E.
[b] Total number of animals.

**Fig. 3.** pep-2 interacts with the DAF-2 insulin-like signaling pathway. The effects on life span and heat stress in wild type (●), pep-2(lg601) (○), daf-2(e1370) (■), daf-2(e1370);pep-2(lg601) (▲), daf-16(m26) (♦), daf-16(m26);pep-2(lg601) (△), and daf-16(m26);daf-2(e1370) (△). A, the survival curves at 25 °C show that pep-2(lg601) animals have a slightly reduced adult life span compared with wild type, but the adult life span of daf-2(e1370) mutant animals is extended significantly in the pep-2(lg601) mutant background. B, survival curves for animals at 25 °C. Any life span extension is suppressed in a daf-16(−) background. C, survival curves of young adult animals incubated at 35 °C. The heat tolerance is increased in pep-2(lg601) animals, and daf-2(e1370);pep-2(lg601) double mutants exhibit an even higher heat tolerance compared with daf-2(e1370) single mutants. D, survival curves of young adult animals on 150 mM paraquat at 20 °C. pep-2(lg601) shows an increased resistance to oxidative stress, which is not suppressed in a daf-16(−) mutant background. The increased stress resistance of daf-2(e1370) animals is dramatically enhanced in the daf-2;pep-2 double mutant.

with pep-2. The majority of let-363(RNAi_weak) larvae developed into reproductive adults that displayed only a slightly reduced brood size (259.5 ± 18.2 compared with 322.3 ± 14.5 of wild type at 20 °C), whereas 15% of the adult animals became sterile. In addition, let-363(RNAi_weak) larvae displayed a weak enlargement of the intestinal lumen (Fig. 4A) corresponding to the phenotype reported previously (9). All aspects of the let-363(RNAi_weak) phenotype were strongly enhanced in a pep-2(lg601) background (Fig. 4B). The double mutants developed much slower and had a substantially enlarged intestinal lumen, which contained undigested bacteria in the luminal fluid (Fig. 4B) as described previously for the let-363 null mutant (9). In addition, 100% (n > 250) of the double mutant animals arrested at L2 or L3 larval stages.

Feeding of either let-363 RNAi constructs was sufficient to extend life span, confirming recent reports from C. elegans and Drosophila (10, 42). pep-2(lg601) mutant background further increased this longevity effect of let-363(RNAi_weak) significantly (p < 0.0001; Fig. 4D, Table II) but was not able to affect the let-363(RNAi_strong) phenotype (Fig. 4C, Table II). These data are consistent with pep-2 being genetically located upstream of let-363/TOR and indicate that the obvious restriction of amino acid availability in the pep-2 mutant affects the TOR pathway.

**DISCUSSION**

The function of the mammalian peptide transporters has been studied extensively in vitro, but the contribution of PEPT1 to overall amino acid absorption in the gut and its role in protein nutrition in vivo is unknown. We show here that PEP-2 is the only functional intestinal peptide transporter in C. elegans, whereas PEP-1, the functional homologue of the mammalian PEPT2, is not involved in the absorption of dietary peptides in C. elegans. The pep-2 deletion mutant strain we have analyzed here displays strong developmental and reproductive defects consistent with a specific reduction in intestinal uptake of food-derived peptides as the prime source of amino acids in periods of high amino acid demand. An additional supply of free amino acids by the diet caused only a mild amelioration of the phenotype, emphasizing the indispensable role of intestinal peptide intake to ensure normal growth and development. These data greatly expand on a recent report describing the pep-2 RNAi phenotype (31).

The insulin/IGF signaling pathway is known to be affected by the availability of dietary protein or amino acids in both Drosophila (16) and mammals (14, 15). Recent microarray analyses indicate that pep-2 is down-regulated in a daf-2(−) mutant background (37). Consistent with being negatively con-
phenotype? Restricted amino acid availability could also reduce expressed independently of involved in amino acid and nucleotide synthesis may also be signaling, ensure normal growth, development, and reproduction. DAF-16 is inactive, resulting in an inconspicuous life span.

Middle panel
Interactive with DAF-2/DAF-16 insulin signaling.

Reduced amounts of oxidative damaged proteins are not sufficient to enhance life span, since the genetic program for longevity elimination of di-/tripeptide transport reduces intracellular amino acid levels, affects TOR function, and results in reduced growth, development, and reproduction. Reduced amounts of oxidative damaged proteins are not sufficient to enhance life span, since the genetic program for longevity elimination of di-/tripeptide transport reduces intracellular amino acid levels, affects TOR function, and results in reduced growth, development, and reproduction. Reduced amounts of oxidative damaged proteins are not sufficient to enhance life span, since the genetic program for longevity elimination of di-/tripeptide transport reduces intracellular amino acid levels, affects TOR function, and results in reduced growth, development,

Right panel
An additional mutation in daf-2 enhances defects in growth, development, and reproduction in parallel to pep-2. In addition, DAF-16 is activated and executes the longevity program. pep-2 mutant background further enhances longevity due to decreased amount of oxidatively damaged proteins. aa, amino acid; Ins, insulin-like molecule; odp, oxidatively damaged proteins.

trolled by daf-16, our data show that the developmental defects of pep-2(-) animals (reduced brood size and extended generation time) as well as increased stress tolerance are not suppressed in a daf-16 mutant background. However, there is also strong evidence that the regulatory processes initiated by the restricted availability of amino acids (pep-2) and by the DAF-2-mediated signal transduction pathways act in parallel to regulate life span. Such a model is further corroborated by the finding that daf-16, which also is controlled via daf-16-dependent insulin signaling, is not affected in pep-2 mutants. Moreover, these results are consistent with previous results suggesting that other targets of the daf-2 pathway involved in amino acid and nucleotide synthesis may also be expressed independently of daf-16 (43).

How does pep-2 contribute to the DAF-2-mediated longevity phenotype? Restricted amino acid availability could also reduce substrate delivery for mitochondrial respiration, yet reduced respiratory chain activity generally results in a phenotype significantly different from that of pep-2-deficient animals (44). On the other hand, life span is correlated to oxidative stress, which is known to damage proteins by the introduction of carbonyl groups, and, indeed the age-dependent accumulation of protein carbonyl mirrors the life span effects of a variety of C. elegans mutants (28). pep-2 single mutants revealed an increased stress resistance that is independent of functional daf-16 and, in combination with daf-2, showed a strikingly high tolerance when challenged with oxidative stress. In addition, the long-lived daf-2;pep-2 double mutants accumulated less oxidatively damaged proteins than daf-2 single mutants, consistent with a correlation between both effects. We, therefore, hypothesize that limited amino acid availability is a contributing factor to protein carbonyl accumulation during aging. This
has also been observed in rats, where a low protein intake in the absence of a caloric restriction also markedly reduced the concentration of protein carbonyls (41). The effects of reduced amino acid availability on the aging processes in pep-2 mutant animals becomes unmasked only in a sensitized background with impaired insulin signaling and requires an active DAF-16 transcription factor.

The pep-2 mutant phenotype in C. elegans is reminiscent of the phenotype observed in other organisms deprived of amino acids or after mutations in components of the TOR pathway. In Drosophila, a partial loss of TOR function reduces growth, and flies deficient in the p70S6K genes are extremely delayed in development and are smaller in size (8). The inactivation of the S6K gene in mice also results in a reduced body size (45). We here show that all phenotypic aspects of a weak TOR RNAi mutant can be strongly amplified by pep-2(lg601), whereas a let-363 RNAi construct resulting in a strong phenotype is epistatic to pep-2 with respect to longevity. We conclude that pep-2 acts upstream of let-363/TOR in the same genetic pathway and that alterations in amino acid availability affect TOR activity.

We have demonstrated that pep-2 is active in the intestine, which in C. elegans represents the adipose tissue of the animal, comparable with the fat body of Drosophila (46, 47). This organ was recently shown to act as a nutrient sensor in C. elegans that alterations in amino acid availability affect TOR activity. Maintenance of the cellular amino acid pool is a critical determinant for proper development, growth, and reproduction. Moreover, the intestine has also been observed in rats, where a low protein intake in the absence of a caloric restriction also markedly reduced the concentration of protein carbonyls (41). The effects of reduced amino acid availability on the aging processes in pep-2 mutant animals becomes unmasked only in a sensitized background with impaired insulin signaling and requires an active DAF-16 transcription factor.

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Deletion of the Intestinal Peptide Transporter Affects Insulin and TOR Signaling in *Caenorhabditis elegans*

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