Insulinoma-Associated Protein IA-2, a Vesicle Transmembrane Protein, Genetically Interacts with UNC-31/CAPS and Affects Neurosecretion in *Caenorhabditis elegans*

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IA-2 (insulinoma-associated protein 2), a major autoantigen in type 1 diabetes, is a receptor-tyrosine phosphatase-like protein associated with the membrane of secretory granules of neural and endocrine-specific cells. Loss of IA-2 activity in the mouse results in reduced insulin release and additional phenotypes, consistent with a general effect on neurosecretion and hormone release. To gain further insight into the cellular mechanisms of IA-2 function, we have studied the *Caenorhabditis elegans* homolog, CeIA-2 encoded by the *ida-1* gene. Using two independent putative null alleles of *ida-1*, we demonstrate that animals lacking CeIA-2 activity are viable and exhibit subtle defects. Genetic studies of mutants in *ida-1* and several genes involved in neurosecretory vesicle cargo release and signaling highlight two roles for CeIA-2. First, CeIA-2 has a specific and novel genetic interaction with UNC-31/CAPS, a protein that has been shown in other systems to regulate dense-core vesicle cargo release. Second, loss of CeIA-2 activity enhances weak alleles in the insulin-like signaling pathway. These results suggest that CeIA-2 may be an important factor in dense-core vesicle cargo release with parallels to insulin signaling in mammals.

Key words: autoantigen; diabetes; insulin; synaptic transmission; dense core vesicle; protein tyrosine phosphatase; PTP; aldicarb; dauer; *C. elegans*

Introduction

Vesicle trafficking in neuroendocrine cells and neurons is essential for proper hormone signaling and neurotransmission. The two prominent neurosecretory vesicle types are synaptic vesicles (SVs) and dense-core vesicles (DCVs). SVs mediate the release of fast-acting neurotransmitters such as glutamate and acetylcholine, whereas DCVs mediate the release of hormones, neuropeptides, and biogenic amine neurotransmitters. Many components involved in SV trafficking have been identified, providing a rich cellular and molecular understanding of this pathway in neurons. Several of these factors have subsequently been shown to also regulate DCV cargo release (Gerber and Sudhof, 2002). However, there is some evidence that DCV trafficking involves some components not involved in SV trafficking. For example, a calcium-activated protein for secretion (CAPS) seems to be a specific regulator of DCV fusion to the plasma membrane (Walent et al., 1992; Grishanin et al., 2002). It is important to determine the

identity and function of additional DCV pathway components to fully understand important cellular processes, such as hormone release.

IA-2 (insulinoma-associated protein 2, also known as ICA-512) and its paralog, IA-2 β (phogrin), are among the components that have been implicated in DCV trafficking (Lan et al., 1994; Kawasaki et al., 1996; Lu et al., 1996). IA-2 and IA-2 β are transmembrane protein-tyrosine phosphatase-like (PTP) proteins belonging to an evolutionarily conserved family (Cai et al., 2001). Although highly related to PTPs, IA-2 family members lack phosphatase activity because of amino acid substitutions in the catalytic domain (Magistrelli et al., 1996; Wishart and Dixon, 1998; Cai et al., 2001). These amino acid substitutions are evolutionarily conserved, suggesting that the lack of enzymatic activity is a critical feature of these proteins (Wishart and Dixon, 1998).

IA-2 and IA-2 β have been localized to the membrane of DCVs in neurons and peptide-secreting endocrine cells, including pancreatic β -cells (Lan et al., 1994; Solimena et al., 1996; Pouli et al., 1998). Both proteins have been shown to undergo calcium-dependent phosphorylation in response to secretagogues, and IA-2 interacts with the plasma membrane cytoskeleton protein spectrin (Berghs et al., 2000; Ort et al., 2001). In IA-2-deficient mice, only 50–60% of the normal amount of insulin was secreted from isolated pancreatic islets by glucose stimulation *in vitro* (Saeki et al., 2002). Moreover, in IA-2-deficient mice, blood glucose levels were significantly elevated in glucose tolerance tests

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(Saeki et al., 2002). These findings suggest that IA-2 family members may play an important role in the regulated secretion of insulin. IA-2 is also a major autoantigen associated with type 1 diabetes, thereby providing diagnostic information for disease, often before the onset of clinical symptoms (Lan et al., 1996; Notkins, 2002).

We have turned to the nematode *Caenorhabditis elegans* as a model system to aid in our understanding of the function of IA-2 family members. Previous studies have shown that *C. elegans* contains a single gene (*ida-1*) related to IA-2 and IA-2 β , and it is expressed predominantly in the nervous system (Cai et al., 2001; Zahn et al., 2001). *C. elegans* is a genetically tractable system with a simple nervous system (302 neurons) that has been extensively described at the cellular and ultrastructural levels. *C. elegans* studies have made major contributions to our understanding of SV trafficking, providing genetic and molecular characterization of key components (Rand and Nonet, 1997; Chalfie and Jorgensen, 1998). It seems likely that *C. elegans* will serve an equally important role in understanding DCV trafficking and may provide important information on IA-2 function in mammalian neurosecretion.

As a first step in the functional analysis of the *C. elegans* IA-2 protein (CeIA-2), we have characterized phenotypes associated with putative null alleles of the gene *ida-1*. We show that animals lacking CeIA-2 function are viable but have subtle defects attributable to deficits in acetylcholine release from the presynaptic membrane. Using double-mutant combinations between *ida-1* and a variety of genes involved in SV and DCV trafficking and cargo release, we provide evidence that CeIA-2 primarily interacts with factors in the DCV pathway. *ida-1* has a specific and novel genetic interaction with uncoordinated mutants of the gene *unc-31*, encoding the *C. elegans* homolog of CAPS, a protein that regulates DCV cargo release in mammals and *Drosophila*. Moreover, mutants of *ida-1* preferentially enhance the dauer phenotype of weak alleles in the insulin-like signaling pathway. These results suggest that CeIA-2 is required for proper DCV cargo release with striking parallels to insulin signaling in mammals.

Materials and Methods

Strains. Strains used were as follows: wild-type N2, *ida-1(ok409)* III, *ida-1(tm334)* III, *unc-18(e81)* X, *unc-31(e169)* IV, *unc-31(e928)* IV, *unc-64(e246)* III, *unc-104(e1265)* II, *daf-2(e1370)* III, *daf-7(e1372)* III, *daf-11(m47)* V, *daf-12(rh273)* X, *daf-28(sa191)* V, *rab-3(js49)* II, and *aex-3(sa5)* X. A pair of primers (5'-CTAATCGACACTGGTCTCAC-3' and 5'-CGTTCCATTGAGCCTCAATC-3') was designed and sent to the *C. elegans* gene knock-out group at the Oklahoma Medical Research Foundation for screening of *ida-1* mutants. A deleted allele, designated *ida-1(ok409)* III, was isolated and kindly provided to us. The second allele, *ida-1(tm334)* III, was kindly provided by Dr. Shohei Mitani's (Tokyo Women's Medical University School of Medicine, Tokyo, Japan) laboratory. These two mutants were outcrossed with N2 a minimum of four times, and then one or both alleles were crossed into each of the following genetic backgrounds to create double mutants: *unc-18(e81)* X, *unc-31(e169)* IV, *unc-31(e928)* IV, *unc-64(e246)* III, *unc-104(e1265)* II, *daf-2(e1370)* III, *daf-7(e1372)* III, *daf-11(m47)* V, *daf-12(rh273)* X, *daf-28(sa191)* V, *rab-3(js49)* II, and *aex-3(sa5)* X. Double mutants were confirmed by phenotype, molecular genotyping of specific alleles, or both. For making double mutants between the linked alleles, F2 homozygotes for the visible phenotype were selected, and the presence of the *ok409* or *tm334* allele was determined by PCR. Double homozygotes were then selected from siblings. The *ida-1* genotype was confirmed by use of a single worm PCR (Williams, 1995) with three primers, two of which (5'-CACCGAGGATCGACTATTC-3' and 5'-TTCTTGGAGGAAGTTCTGG-3') flank the deletion break points and one (5'-GAAGTCTCGGACG GTCAGCTTAAC-

3') located within the deletion. Flanking primers yielded a PCR product of 365-bp for the *ok409* allele or 1543-bp for the *tm334* allele; the wild-type allele yielded a 2077 bp product. The exact deletion points of *ok409* and *tm334* were confirmed by DNA sequencing.

Locomotion assays. Standard locomotion assays were performed as previously described (Miller et al., 1999). Briefly, the locomotion rate was quantified by counting body bends of young adult animals on thin lawns of *Escherichia coli* strain OP50 bacteria. The assay was set up by placing single late larval stage 3 (L3) or early L4 larva on each of 10 locomotion assay plates. Plates were then placed at room temperature for 24 hr, during which time the larva matured to young adults. To count body bends, a plate was placed on the dissecting microscope for 30 sec, and then body bends were counted for 3 min. A body bend was counted each time the tip of the tail passed through the maximum or minimum amplitude. After all 10 plates had been counted, the process was repeated two more times on the same set of animals. Mean body bends per minute were calculated for each animal, and these means were used to calculate the mean and SE of the entire set of 10.

Dauer formation. Adults were allowed to lay eggs on plates for 3 hr at room temperature, and progeny were scored after 48 hr at 26°C (Ailion and Thomas, 2000). Dauers were distinguished by the distinctive body shape, darkly pigmented intestine, and a constricted pharynx. Partial dauers, which were included in the dauer category for scoring, lacked at least one of these characteristics. Incubation temperature is a critical variable in dauer assays. Each set of assays included all of the relevant strains, and the actual ambient incubator temperature surrounding the plates was monitored with a digital thermometer (Barnant Co., Barrington, IL).

Pharmacological assays. Acute effects of aldicarb and levamisole on mutants were assessed in a paralysis assay as described (Nonet et al., 1993). Sensitivity to aldicarb was examined by transferring 50 adult animals to plates containing aldicarb and assaying the time course of paralysis. Animals were considered paralyzed if they appeared hypercontracted and failed to move even if prodded with a platinum wire. Chronic effects of aldicarb on mutants were quantified in a growth assay as previously described (Nguyen et al., 1995; Miller et al., 1999). Single L1 larva were placed on individual culture plates containing 0, 10, 25, 50, 75, 100, 200, 300, or 400 μ M aldicarb and grown at room temperature ($\sim$ 22°C) for 96 hr. Growth was then stopped by putting the plates at 4°C. For each concentration, the number of progeny was counted as a percentage of the number of progeny produced on the no-drug control. Triplicate assays were performed, and an N2 control set was included with each set of strains to allow comparisons between different sets of assays.

Transgenic lines. Wild-type *C. elegans* received injections of plasmid constructs or cosmid DNA at concentrations of 50–100 μ g/ml along with 50 μ g/ml pRF4 *rol-6(d)* as a transformation marker (Mello and Fire, 1995); pRF4 was injected alone at 50 μ g/ml as a control for Roller effects. At a minimum, two stably transformed strains were established for each test construct. A CeIA-2::green fluorescent protein (GFP) translational fusion construct in the pPD95.69 vector was driven by a 3282-bp 5' upstream sequence of CeIA-2 as described before (Cai et al., 2001). An integrated line of the CeIA-2::GFP reporter construct, designated KM246, was generated by irradiation of one of the stable extrachromosomal array strains (Egan et al., 1995). The integrated line was backcrossed two times to lessen the frequency of second site mutations caused by the irradiation used for integration. The CeIA-2::GFP cDNA was also driven by a 3265 bp 5' upstream sequence of *acr-2* (acetylcholine receptor subunit) for expression of the CeIA-2::GFP fusion protein in VA, DA, VB, DB, DA, IL1, RMD, and PVQ motor neurons (Squire et al., 1995; Nurrish et al., 1999). The *acr-2* promoter was PCR-amplified with primers (5'-CAGTTGACTGCAGAAGTCA-GAGCTGTGGAAGTCG-3' and 5'-GACCGTCTTGGCCATGAAAACG-CGCTCCTTCCTG-3') and confirmed by sequencing.

Antibody staining. Immunocytochemistry was performed using a modification of the protocol of Finney and Ruvkun (1990), as described by Nonet et al. (1993). Antisera against synaptotagmin (antibody 1095; kindly provided by M. Nonet, Washington University School of Medicine, St. Louis, MO) and RAB-3 (kindly provided by K. Iwasaki, National Institute of Bioscience and Human Technology, Ibaraki, Japan) were used as described (Iwasaki and Toyonaga, 2000). Antisera against sero-

tonin were obtained from H. Steinbusch (Maastricht University, Maastricht, the Netherlands) and performed using a whole-mount procedure as described in a detailed protocol (<http://home.sandiego.edu/~cloer/loerlab/anti5htshort.html>; Loer and Kenyon, 1993). Anti-GFP antibodies were obtained from Clontech (Palo Alto, CA). Antibodies were detected using rhodamine- or FITC-conjugated donkey anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA).

Results

A punctate axonal pattern of CeIA-2::GFP colocalizes with synaptotagmin

Previous studies of *ida-1* expression relied on transcriptional fusions with a GFP reporter gene (Cai et al., 2001; Zahn et al., 2001). Expression was almost exclusively seen in neurons, although the number of expressing neurons varied depending on the promoter element used. A 3.3 kb upstream promoter element gave widespread expression, with most or all neurons showing some level of expression (Cai et al., 2001). A 7.6 kb upstream promoter element gave a more restricted pattern of expression limited to ~30 neurons (Zahn et al., 2001).

To address the subcellular distribution of IA-2, we generated a translational fusion between the complete CeIA-2 cDNA and GFP driven by a 3.3 kb upstream promoter region of *ida-1*. Multiple independent transgenic strains showed very similar patterns of expression; CeIA-2::GFP was easily detected in a restricted set of ~13 neurons. Most of these neurons were a subset of those reported by Zahn et al. (2001). CeIA-2::GFP was strongest in the ALA neuron and easily detectable in the VCs, HSNs, and PHCs. A lower level of GFP was also detected in the uv1 secretory cells (Fig. 1). Some, but not all, of these expressing neurons have previously been shown by electron microscopic studies to contain DCVs (White et al., 1986). However, not all DCV-containing neurons expressed this GFP reporter gene. We believe that the pattern of GFP expression from this reporter gene likely under-represents the normal CeIA-2 distribution because of previous transcriptional reporter results and very faint GFP that can be observed in additional neurons under certain conditions (see below).

To decrease the level of mosaic expression typically observed for extrachromosomal transgenes, and for further genetic studies, we generated an integrated transgenic strain (*P_{ida-1}*CeIA-2::GFP, KM246), harboring this reporter gene. The expression pattern of the integrated strain was similar to that seen with extrachromosomal arrays. Despite integration, there was still a degree of mosaic expression, most notably in the VC motor neurons.

A striking aspect of the *ida-1* translational GFP reporter expression was the subcellular distribution of GFP. *P_{ida-1}*CeIA-2::GFP was observed in neuronal cell bodies and in puncta along the length of axons of neurons in which it was expressed (Fig. 1). A punctate distribution of other neuronal components, such as synaptobrevin and synaptotagmin, has been shown to reflect accumulation of fusion protein-tagged vesicles at synapses (Nonet, 1999). To determine whether the *P_{ida-1}*CeIA-2::GFP axonal pattern was coincident with synapses, we stained animals expressing this CeIA-2::GFP transgene with an antibody against synaptotagmin. As shown in Figure 1B, the punctate GFP reporter pattern in axons colocalized with synaptotagmin staining, suggesting clustering of CeIA-2::GFP to synaptic regions.

Characterization of two putative null *ida-1* alleles

We obtained two deletion alleles of *ida-1* (*ok409* and *tm334*) to study the effect of loss of CeIA-2 activity in *C. elegans*. We sequenced PCR products amplified from both mutant animals to characterize the nature of each deletion (Fig. 2). The *ok409* allele

Figure 1. CeIA-2 GFP reporter gene expression. *A*, GFP fluorescence image of a transgenic animal harboring the *P_{ida-1}*CeIA-2::GFP reporter gene. Very strong expression is detected in the ALA, VC, HSN, and PHC neurons as labeled. Lower-level expression is seen in the uv1 neurosecretory cells of the vulva. Dashed lines indicate the outline of the animal, with the head at the bottom and the tail in the top right corner. The punctate pattern of the *P_{ida-1}*CeIA-2::GFP reporter in axons is most easily observed in the solitary lateral axons of ALA, one of which is visible in the focal plane shown. The boxed region of the head expression pattern of the ALA neuron is shown at a higher magnification in the inset. Arrowheads indicate the punctate pattern of GFP observed in most axons of GFP-positive neurons. *B*, Coincidence of CeIA-2::GFP puncta and Synaptotagmin-labeled synapses. Puncta (arrows) in a lateral ALA axon segment just posterior to the pharynx are shown. These puncta are visualized by CeIA-2::GFP expression (far left) and are revealed as synapses by anti-synaptotagmin antibody staining (far right). The center panel merges the two outside images with anti-synaptotagmin in red and CeIA-2::GFP in green with overlap in yellow. Asterisks mark the ventral and dorsal nerve cords that are slightly out of the focal plane and that are heavily labeled by anti-synaptotagmin staining.

resulted from a 1713 bp deletion with break points in introns 3 and 7. The deleted *ida-1(ok409)* gene would encode a protein product from only exons 1–3 because of a frame shift and stop codon resulting from the predicted mRNA splicing. The *tm334* allele resulted from a 534 bp deletion with break points in exons 3 and 4 (Fig. 2). The deleted *ida-1(tm334)* gene would encode a protein from only exons 1, 2, and most of 3 because of a frame shift and stop codon two amino acids downstream of the deletion break point. We have used reverse transcription-PCR to confirm the predicted mRNA products and premature stop codons for each mutant. Both deletion alleles are predicted to encode similarly truncated CeIA-2 proteins that lack the C-terminal two-thirds of the protein, including the transmembrane domain and the protein-tyrosine phosphatase-like domain. Both *ida-1* alleles are assumed to be nulls based on the molecular characterization.

Loss of CeIA-2 activity results in subtle visible phenotypes

Animals homozygous for *ida-1(ok409)* or *ida-1(tm334)* are viable and fertile. Assays for rate of development and chemotaxis appeared normal in the homozygous mutants. Homozygous *ida-*

Figure 2. Characterization of *ida-1* deletion alleles. The top diagram represents the Celia-2 protein with certain domains marked relative to their corresponding exons: signal peptide (SP), transmembrane domain (TM), and protein-tyrosine phosphatase-like core (PTP). The bottom diagram shows the genome structure of the *ida-1* gene, which consists of 12 exons (filled boxes). The approximate locations of the deleted regions for the alleles *ok409* and *tm334* are indicated by brackets below the exons. Both deletions result in a premature stop codon attributable to a frame shift after the deletion break points, and each would lack all protein encoded downstream of exon 3, including the TM and PTP domains. The predicted truncated protein product resulting from either deletion is indicated at the N-terminal end by hatched lines. Single-animal PCR products from the *ida-1* gene of wild-type animals and homozygous mutants are shown.

1(ok409) and *ida-1(tm334)* animals had slightly reduced brood sizes of 70 and 90%, respectively, compared with wild-type animals. Because both alleles are predicted nulls, the more severe brood size reduction observed for *ok409* may be attributable to strain background differences between the two alleles. We were able to detect two subtle motor program defects in *ida-1* mutant animals affecting locomotion and defecation. We compared the mean locomotion rates of wild-type and the two *ida-1* mutant alleles by counting body bends of the animals (Miller et al., 1999). The locomotion rate of the mutants was slower than that of the wild type and became progressively worse with age (Table 1). The locomotion rate of *ida-1* mutants was 70% of that of wild type for day 1 adults, 50% of that of wild type for day 2 adults, and 25% of that of wild type for day 3 adults. Both *ida-1(ok409)* and *ida-1(tm334)* mutants also had a minor defecation motor program defect affecting the muscle contractions leading to the expulsion of waste. In wild-type animals, defecation cycles have been well characterized, and each cycle ends with the near simultaneous contraction of the posterior body wall and enteric muscles of the animal, resulting in an expulsion (Thomas, 1990). An examination of defecation in *ida-1* mutants revealed that the expulsion step of the cycle was missing in ~10% of the defecation cycles (data not shown). The subtle motor program defects observed in *ida-1(ok409)* and *ida-1(tm334)* mutants was consistent with a mild and general problem in nervous system function.

Loss of CeIA-2 results in impaired synaptic transmission

The neuronal expression of *ida-1* and the minor motor defects of *ida-1* mutants led us to investigate synaptic transmission in *ida-1* mutant animals. Aldicarb, an acetylcholinesterase inhibitor, has been commonly used to assess acetylcholine release and presynaptic function. To test whether *ida-1* mutants have defects in synaptic transmission, we used two different aldicarb assays. First, we used a short-term assay in which we measured the rate of paralysis induced by treatment of adult animals with 0.5 mM aldicarb for 4 hr at room temperature. Second, we used a growth rate assay in which we counted the number of progeny from

parent animals after 96 hr of growth on plates containing various concentrations of aldicarb (Miller et al., 1999). For both assays, *ida-1* mutants were compared with wild-type animals and two mutants shown previously to have an aldicarb resistance phenotype, *unc-31* (encoding the CAPS homolog) and *unc-64* (encoding the syntaxin homolog).

In the short-term aldicarb test, 97% of wild-type animals were paralyzed after 4 hr on plates containing 0.5 mM aldicarb. In contrast, only 50% of *ida-1(ok409)* or *tm334* mutants were paralyzed in the same assay. This compared with 25% paralysis for *unc-31(e169)* and only 5% paralysis for *unc-64(e246)*. In the aldicarb growth rate assay, *ida-1(ok409)* or *tm334* mutants tolerated moderately higher concentrations of aldicarb than did wild-type animals. This is reflected by an increased population rate for *ida-1(ok409)* or *tm334* compared with wild type at all aldicarb concentrations up to 200 μ M (Fig. 3A). In comparison, *unc-31(e169)* and *unc-64(e246)* tolerated even higher concentrations of aldicarb than did *ida-1(ok409)* or *tm334*. These results demonstrated that *ida-1* mutants are aldicarb-resistant; that is, they have reduced acetylcholine release compared with wild-type animals. The aldicarb resistance of *ida-1* mutants is modest and at the lower end of the hierarchy of tested mutant strains: wild type < *ida-1(ok409)* or *tm334* < *unc-31(e169)* < *unc-64(e246)*. The aldicarb effects of the *ida-1(ok409)* or *tm334* mutants appeared to be attributable to neuronal events because levamisole, an agonist of postsynaptic acetylcholine receptors, failed to distinguish between wild-type and *ida-1* mutant animals (data not shown).

To confirm that the aldicarb resistance was caused by the *ida-1* deletions, we attempted to rescue the defect with either the integrated, full-length *ida-1* cDNA fused to GFP (*P_{ida-1}CeIA-2::GFP*) or the cosmid B0244 containing the wild-type *ida-1* gene. We found that both DNA constructs reduced the aldicarb resistance of *ida-1(ok409)* mutants (Fig. 3B). The cosmid B0244 gave full rescue of aldicarb resistance and actually showed enhanced sensitivity to the drug. This mild yet reproducible enhanced sensitivity to aldicarb might have been attributable to overexpression of *ida-1* from the cosmid. The *P_{ida-1}CeIA-2::GFP* construct rescue was partial and may have reflected a limited expression profile of this reporter gene (described above) compared with the endogenous *ida-1* gene, the mosaic nature or dose of reporter gene expression, or a combination of these factors.

Aldicarb resistance has been commonly attributed to decreased acetylcholine release, presumably from SVs of motor neurons (Miller et al., 1996). We were interested to test whether the mild aldicarb resistance of *ida-1* mutants could be rescued by expression of the CeIA-2 cDNA in the cholinergic motor neurons. We made transgenic strains harboring the CeIA-2::GFP fusion protein under the control of a 3.2 kb promoter region of the *acr-2* gene. This promoter has been shown to be specifically expressed in the A- and B-type cholinergic motor neurons of the ventral nerve cord (Nurrish et al., 1999), and that pattern was observed in our transgenic lines (data not shown). *P_{acr-2}CeIA-2::GFP* transgenic strains showed absolutely no rescue of the *ida-1(ok409)* aldicarb resistance phenotype, using either short or long-term assays (data not shown). This result suggested that loss of CeIA-2 activity in cholinergic motor neurons is not the cause of the aldicarb resistance.

Loss of CeIA-2 does not affect the axonal distribution of SV membrane-associated proteins

To determine how *ida-1* mutations impair presynaptic activity, we examined *ida-1* mutants using antibodies specific for two SV membrane-associated proteins, SNT-1 (synaptotagmin; Nonet et

Table 1. Analysis of behaviors of *ida-1* mutants

Strain	Body bends (times/min) (<i>n</i> = 10)			Expulsion (%) (<i>n</i> = 20)	Brood size (<i>n</i> = 10)	Life span (d) (<i>n</i> = 25)
	Day 1	Day 2	Day 3			
Wild type	17.0 ± 4.8	14.9 ± 4.1	12.7 ± 2.8	99 ± 1	290 ± 30	23 ± 4
<i>ok409</i>	11.6 ± 6.5*	7.3 ± 6.2**	3.6 ± 2.1**	80 ± 5*	185 ± 25*	16 ± 5*
<i>tm334</i>	10.6 ± 3.7*	6.4 ± 2.8**	3.3 ± 1.8**	85 ± 5*	222 ± 27*	21 ± 6

Values are mean ± SE.

***Statistically significant differences between wild type and mutants (*p < 0.05, **p < 0.01).

Figure 3. Aldicarb resistance of *ida-1* mutants compared with other strains using a growth assay (see Materials and Methods). The population growth rate is shown on the y-axis, and the aldicarb concentration is shown on the x-axis in all figures. A 100% growth rate is defined as the number of progeny produced from a starting population of L1 larvae over a 96 hr period in the absence of aldicarb. Curves are representative of the average of duplicate experiments. *A*, The aldicarb resistance of *ida-1(ok409)* or *tm334* is compared with that of *unc-31(e169)* or *unc-64(e246)*. *B*, The aldicarb resistance of *ida-1(ok409)* is partially rescued by expression of the wild-type *ida-1* cDNA (*CeIA-2::GFP*). Rescue data shown for the cDNA construct from two independent transgenic lines harboring the cDNA clone are combined. The aldicarb phenotype was fully reversed in transgenic animals harboring the wild-type *ida-1* gene on the cosmid B0244. The control is an *ida-1(ok409)* strain harboring the marker plasmid pRF4 (*rol-6*) used for generating transgenic lines that confers a Rol phenotype. *C*, The aldicarb resistance of *unc-64(e246)* is enhanced by *ida-1(ok409)* or *tm334*. *D*, The aldicarb resistance of both the hypomorphic *unc-31(e169)* and putative null *unc-31(e928)* alleles is suppressed by *ida-1(ok409)* or *tm334*.

al., 1993) and RAB-3 (Iwasaki et al., 1997), kindly provided by the Nonet and Iwasaki laboratories, respectively (Fig. 4). In wild-type animals (Fig. 4*A*) and *ida-1* mutants (Fig. 4*B*), anti-SNT-1 antiserum stained only the synapse-rich axons and did not stain cell bodies, suggesting that SNT-1 is correctly localized to synaptic terminals in the *ida-1* mutants. Anti-RAB-3 antiserum stained predominantly axons in wild-type animals (data not shown) and *ida-1* mutants (Fig. 4*C*), although faint staining of occasional cell bodies was visible. As a control for our immunolocalization of RAB-3, we used the *aex-3(sa5)* mutant (Fig. 4*E*) and replicated the previously reported accumulation of RAB-3 in cell bodies of neurons (Iwasaki et al., 1997). These results suggested that there was no gross abnormality in SV vesicle distribution in *ida-1* mutants.

It was possible that the bundled axons of the ventral nerve cord were preventing us from noticing, by antibody staining, more subtle aspects of SV distribution in *ida-1* mutants. To visu-

alize the distribution of these vesicles at the single-cell level, we used an SNB (synaptobrevin) GFP reporter driven by the *str-3* promoter, kindly provided by the laboratory of C. Bargmann (University of California, San Francisco, CA). This reporter gene is expressed in only the two ASI neurons in the head and GFP puncta reflect the accumulation of vesicles in the seven to nine synapses of each axon (Crump et al., 2001). Introduction of this reporter gene into the *ida-1(ok409)* or *tm334* mutant background failed to reveal any SV localization defects (Fig. 4*G*). Together, these results suggest that *ida-1* is not essential for proper transport or localization of SVs in axons, at least at the level of resolution provided by these assays.

As a complement to these studies, we asked whether the neuronal cell body and punctate axonal pattern of the *P_{ida-1}CeIA-2::GFP* reporter gene was altered in any one of several mutants affecting either vesicle transport (UNC-104/kinesin) or cargo release (UNC-31/CAPS, UNC-64/Syntaxin, and UNC-18/Munc-18). We crossed our *P_{ida-1}CeIA-2::GFP* strain into *unc-31(e169)*, *unc-64(e246)*, *unc-18(e81)*, and *unc-104(e1265)* mutants and observed the reporter gene expression pattern. In all cases except *unc-104(e1265)*, reporter gene product distribution within individual neurons was indistinguishable from that observed in wild-type animals (data not shown). For *unc-104(e1265)*, we observed very faint cell body staining of a small number of neurons in the head that are not visible in a wild-type background

(data not shown). Neurons that strongly express the reporter gene showed no detectable difference in the distribution of the GFP signal within individual neurons. We interpreted the faint GFP signals in *unc-104(e1265)* animals harboring the *P_{ida-1}CeIA-2::GFP* reporter gene as an accumulation of normally weak GFP in the cell body of neurons. In a wild-type background, this low-level GFP is undetectable because of its distribution along the axons. This suggested that UNC-104/kinesin might play a minor role in the axonal distribution of the *P_{ida-1}CeIA-2::GFP* reporter product.

A specific and novel interaction between *ida-1* and *unc-31/CAPS*

To better understand the function of *CeIA-2*, we explored possible genetic interactions between mutants in *ida-1(ok409)* or

tm334) and several genes encoding factors involved in neurosecretory vesicle trafficking and cargo release. This included genes affecting SV docking and fusion (*unc-18*, *rab-3*, and *unc-64/syntaxin*), the RAB-3 nucleotide exchange factor AEX-3 (*aex-3*), and the DCV fusion factor UNC-31/CAPS (*unc-31*). In addition to the distinctive phenotypes of each mutant, they are all aldicarb-resistant. Therefore, we assayed aldicarb resistance of each of these mutants alone and in combination with the *ida-1* alleles. Of the tested mutants, only *unc-31/CAPS* and *unc-64/syntaxin* had detectable genetic interactions with *ida-1(ok409)* or *tm334*). Double mutants between *unc-64(e246)* and *ida-1(ok409)* or *tm334*) had an enhanced aldicarb resistance phenotype (Fig. 3C). No other double mutant showed enhanced aldicarb resistance (data not shown), although *aex-3(sa5)* mutants alone have such high resistance to aldicarb that enhancement might not be detected in these assays. In contrast, double mutants between *unc-31(e169)* and *ida-1(ok409)* or *tm334*) had an aldicarb resistance phenotype that was indistinguishable from that of *ida-1(ok409)* or *tm334*) alone; that is, *ida-1* was epistatic to *unc-31* (Fig. 3D). To confirm this genetic interaction, we made double mutants between *ida-1(ok409)* and the putative null deletion allele *unc-31(e928)*. As with the previously tested hypomorphic allele, *ida-1(ok409)* was epistatic to *unc-31(e928)* for aldicarb resistance (Fig. 3D). Loss of *ida-1* did not suppress the overt Unc phenotype of either *unc-31* allele, although the body bend assay showed a slight improvement in double mutant combinations (data not shown). These results demonstrated that *ida-1* acts genetically in an *unc-31* pathway, suggesting a role for CeIA-2 in DCV trafficking, cargo release, or both.

A distinctive phenotype of *unc-31* mutants is the accumulation of serotonin in the cell bodies of serotonergic neurons (Desai et al., 1988), presumably reflecting the direct effect of impaired release of this DCV-specific neurotransmitter. To determine whether *ida-1* mutants affected serotonin release, either alone or in combination with *unc-31* mutants, we stained mutants with anti-serotonin antibodies. As previously reported (Desai et al., 1988), there is an easily detectable increase of serotonin staining in the head neurons of *unc-31* mutants. For *ida-1* mutants, we focused on the serotonin-containing neurons having high levels of *P_{ida-1}CeIA-2::GFP* reporter gene expression, namely, the HSN neurons and uv1 cells. The serotonin staining results for these cells in *ida-1* mutants were clearly not as robust as observed for the head neurons of *unc-31* mutant animals. Although there was a tendency for *ida-1* mutants to have higher levels of serotonin staining in the HSN and uv1 cells, the assays were not quantitative, and the variability from animal to animal made it difficult to draw any conclusion. Staining of *ida-1; unc-31* double mutants revealed the same increase in serotonin levels in the head neurons as observed for *unc-31* mutants alone.

ida-1 interacts genetically with genes in the dauer pathway

Under ideal conditions, the *C. elegans* life cycle comprises embryogenesis, four larval stages (L1–L4), and adulthood. However, in

Figure 4. Localization of SNT-1, RAB-3, and SNB-1 in *ida-1* mutants. Wild-type (*A*) and *ida-1(ok409)* (*B*) animals were stained with anti-synaptotagmin (SNT-1) antisera. SNT-1 immunoreactivity in both strains was similar, with axons uniformly labeled, such as those of the ventral nerve cord (shown). RAB-3 antibody staining is shown alone (top) or as a merged image with DAPI staining (bottom) for *ida-1(ok409)* (*C*, *D*) or *aex-3(sa5)* (*E*, *F*). As previously reported, RAB-3 accumulates in the neuronal cell bodies of *aex-3(sa5)* mutants (*E*, *F*, arrows). No cell body staining is observed in *ida-1(ok409)* (*C*, *D*) or wild-type (data not shown) animals. *G*, Synapses in a single ASI neuron were visualized with the *P_{str-3}*SNB-1::GFP reporter transgene (Crump et al., 2001). A merged image of *P_{str-3}*SNB-1::GFP fluorescence and bright-field optics focusing on one ASI axon in the nerve ring of an *ida-1(ok409)* mutant animal is shown with a schematic diagram of a single ASI neuron shown below. As in wild-type animals (data not shown), seven to nine GFP puncta, reflecting individual synapses, were seen along the ASI axon in *ida-1(ok409)*. Similar staining with the antibodies or SNB-1::GFP was observed for *ida-1(tm334)* (data not shown).

response to overcrowding, starvation, or elevated temperatures, an alternative life cycle that is suited to dispersal is adopted in which L2 larvae enter diapause and become dauer larvae. On sensing improved conditions, the dauer larva exits diapause, molting into the L4 stage to resume the life cycle. At least three genetically distinct pathways regulate dauer formation in *C. elegans* (Kimura et al., 1997; Patterson and Padgett, 2000). Two of these pathways, transforming growth factor β (TGF- β)-like and cGMP, act through a downstream nuclear hormone receptor encoded by *daf-12*. The third pathway is an insulin-like signaling pathway that acts through the forkhead-related factor encoded by *daf-16*. Several of the insulin-like pathway factors also interact genetically with *daf-12*, suggesting that the dauer decision reflects a complex integration of signaling from multiple inputs in a variety of cells.

Previous studies have demonstrated that *unc-31* and *unc-64* share a common dauer-related phenotype, namely, that both mutations result in an enhanced rate of dauer formation at elevated temperatures (Iwasaki et al., 1997). These genes have been placed genetically in the insulin-like signaling pathway for dauer formation (Ailion et al., 1999). We tested whether *ida-1(ok409)* or *tm334*) mutants, either alone or in combination with *unc-31* and *unc-64* mutants, had a dauer phenotype. We let animals lay embryos for 2–3 hr and then shifted those embryos to an elevated temperature and scored the number of dauer larvae formed after 3 d. As observed by others previously, the exact temperature is critical in determining the percentage of animals that form dauers (Ailion and Thomas, 2000), and care was taken to precisely measure the temperature of plates within the incubators.

At 26°C, wild-type and *ida-1(ok409)* or *tm334*) animals rarely, if ever, formed dauers. In contrast, both *unc-31(e169)* and *unc-64(e246)* mutants formed dauers at 26°C with rates of 38 and 35%, respectively, in our assays. Double mutants between *unc-*

Figure 5. Effect of *ida-1* mutants on the percentage of dauer formation of *unc-64* and *unc-31* mutants. At 26°C, wild-type and *ida-1(ok409)* animals show no dauer formation, whereas ~35% of *unc-64(e246)* animals form dauers. Dauer formation of the *unc-64(e246)* strain was enhanced to >92% in the double mutants with *ida-1(ok409)*. At 26°C, ~37% of *unc-31(e169)* or 80% of *unc-31(e928)* animals form dauers. Dauer formation was dramatically suppressed in double mutants with *ida-1(ok409)* and either the hypomorphic *unc-31(e169)* allele or the putative null allele *unc-31(e928)*. Similar results were observed for *ida-1(tm334)* (data not shown). Values that differ significantly are indicated (* $p < 0.01$).

64(e246) and *ida-1(ok409 or tm334)* showed dramatic enhancement of the dauer phenotype at 26°C, producing between 90 and ~98% dauers (Fig. 5). The opposite effect was observed in *unc-31(e169); ida-1(ok409 or tm334)* double mutants. The dauer phenotype of *unc-31(e169)* was completely suppressed in the *ida-1* mutant background; no dauers were formed by the double-mutant animals at 26°C (Fig. 5). Because the *unc-31(e169)* mutant is a hypomorphic allele, loss of dauer formation in the *ida-1(ok409 or tm334); unc-31(e169)* double mutants might have reflected an increase in UNC-31 activity in the absence of CeIA-2. To test this genetically, we made double mutants between *ida-1(ok409 or tm334)* and the putative null *unc-31(e928)* allele. As observed with the hypomorphic *unc-31* allele, *unc-31(e928); ida-1(ok409 or tm334)* double mutants dramatically reduced the percentage of dauers (<5%) formed at 26°C (Fig. 5). These results demonstrated that a low level of UNC-31 activity is not the underlying cause of the suppression of dauer formation in *ida-1; unc-31* double mutants. The ability of *unc-31* to affect dauer formation is dependent on *ida-1* activity.

We were interested to know whether the effects of *ida-1* mutations on dauer formation were restricted to the insulin-like signaling pathway. We therefore made double-mutant combinations between *ida-1(ok409)* and mutants of genes affecting other branches of the genetic pathway for dauer formation. The three genes we tested were *daf-7*, *daf-11*, and *daf-12*. The *daf-7* gene encodes a member of the TGF- β superfamily (Ren et al., 1996; Schackwitz et al., 1996). Double mutants between the *daf-7* temperature-sensitive loss-of-function allele *e1372* and *ida-1(ok409)* resulted in a slight enhancement of dauer formation at 22.5°C (40%; $n = 105$) compared with *daf-7(e1372)* alone (28%; $n = 120$). The *daf-11* gene encodes a guanylate cyclase, and the *m47* allele is a predicted null (Birnbay et al., 2000). The *ida-1(ok409) daf-11(m47)* double mutants also showed a slight enhancement of dauer formation at 25°C (92%; $n = 147$) compared with *daf-11(m47)* alone (80%; $n = 134$). To test interactions between *ida-1* and *daf-12*, we used a constitutive dauer (*daf-c*) allele, *rh273*. There was no significant difference in the rate of dauer formation at any temperature tested in *ida-1(ok409); daf-12(rh273)* double mutants (38%; $n = 133$) compared with *daf-*

Figure 6. Effect of *ida-1* mutants on the percentage of dauer formation of *daf-2* and *daf-28* mutants. At 22.5°C, wild-type, *ida-1(ok409)*, and *daf-2(e1370)* animals show almost no dauer formation; 10% of *daf-28(sa191)* form dauers. Both *ida-1(ok409)* and *ida-1(tm334)* (data not shown) mutants significantly enhance either *daf-2(e1370)* or *daf-28(sa191)* dauer formation. Values that differ significantly are indicated (* $p < 0.01$).

12(rh273) alone (40%; $n = 186$). Interpretation of the *daf-12* interaction results is complicated by the difficulty in scoring the partial and transient dauers generated by this *daf-12* allele and the complex genetic interactions between *daf-12* and all branches of the genetic pathway of dauer formation. The results from *ida-1* double mutants with *daf-7* and *daf-11* are consistent with, but do not prove, a role for *ida-1* that is predominantly restricted to the insulin-like signaling branch of the dauer formation pathway.

***ida-1* alleles enhance weak alleles of multiple genes in the insulin-like signaling pathway**

Many of the components of the *C. elegans* insulin-like signaling pathway have been characterized, and there are numerous parallels to insulin signaling in mammals. In *C. elegans*, the binding of ligand (e.g., DAF-28) to the insulin receptor-like factor encoded by the *daf-2* gene has been shown to act through phosphatidylinositol (PI) 3-kinase (*age-1*), Akt/PKBs (*akt-1* and *akt-2*), phosphoinositide-dependent kinases (*pdk-1* and *pdk-2*), and other factors to regulate the action of the forkhead transcription factor DAF-16 (*daf-16*), which, in turn, regulates dauer formation (Paradis and Ruvkun, 1998; Paradis et al., 1999; Pierce et al., 2001; Li et al., 2003). The genetic interactions we found between *ida-1* and two genes, *unc-31* and *unc-64*, in the insulin-like branch of the dauer pathway prompted us to assay additional genes in this branch. We made double mutants between *ida-1(tm334 or ok409)* and a *daf-2* allele (*e1370*) or a *daf-28* allele (*sa191*) and assayed the percentage of dauers formed at 22.5°C compared with each mutant alone. The *daf-2(e1370)* allele is hypomorphic, whereas *daf-28(sa191)* appears to be antimorphic (Li et al., 2003). At 22.5°C, wild-type animals and *ida-1(tm334 or ok409)* mutants formed no dauers, whereas *daf-2(e1370)* mutants formed 0.5% dauers, and *daf-28(sa191)* mutants formed 10% dauers (Fig. 6). In comparison, *ida-1(tm334 or ok409) daf-2(e1370)* double mutants had an enhanced rate of dauer formation, with a level of 30%. Similarly, the *ida-1(tm334 or ok409); daf-28(sa191)* double mutants had an enhanced rate (e.g., 50 vs 10%) of dauer formation compared with either single mutant (Fig. 6). These results are consistent with a role of CeIA-2 in the insulin-like signaling branch of the dauer regulation pathway.

Discussion

CeIA-2 genetically interacts with UNC-31/CAPS

IA-2 is a transmembrane protein-tyrosine phosphatase implicated in hormone neurosecretion. We find that a *C. elegans* IA-2::GFP translational reporter gene is present in both the cell body of neurons and in a punctate axonal distribution that correlates with a synaptic marker. We have begun to genetically dissect the role of CeIA-2 in neurosecretion during *C. elegans* development using putative null alleles of *ida-1*, the gene encoding CeIA-2. *ida-1* mutants have subtle motor program defects and a mild aldicarb resistance. Double mutant combinations between *ida-1* and previously characterized genes involved in SV or DCV exocytosis suggest a specific role for CeIA-2 in DCV cargo release.

The most surprising result of our studies was the epistatic relationship between *ida-1* and *unc-31* mutants. Both the aldicarb resistance and dauer phenotypes of *unc-31* mutants are dependent on the presence of wild type *ida-1* activity. To our knowledge, *ida-1* is the first gene described capable of suppressing these *unc-31* mutant phenotypes. This genetic interaction suggests that CeIA-2 modulates, directly or indirectly, the activity of UNC-31/CAPS.

CAPS is one of the rare factors suggested to function specifically in DCV exocytosis. CAPS appears to be able to interact with both the plasma membrane and DCVs, suggesting it may physically bridge these two substrates. Recent reports demonstrated that mammalian CAPS associates with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] on the plasma membrane via its pleckstrin homology domain and also targets to DCVs via its distal C-terminal sequences (Martin and Kowalchuk, 1997; Grishanin et al., 2002). After docking of DCVs to the plasma membrane, the synthesis of PI(4,5)P₂ and PI 4-phosphate induced by PI 4-kinases and PI 5-kinases are thought to account for at least part of the requirement for Mg-ATP in the priming process (Hay et al., 1995; Wiedemann et al., 1996; Olsen et al., 2003). Therefore, interactions with both DCVs and the plasma membrane may play an important role in the mechanism by which CAPS facilitates the release of DCV cargo. The binding determinants of CAPS on DCVs have not been identified. Given our results showing a genetic interaction between *unc-31* and *ida-1*, it would be interesting to examine whether the PTP domain-containing cytosolic region of CeIA-2 interacts with the distal C-terminal sequences of UNC-31/CAPS or with a common effector molecule.

Studies of *Drosophila* CAPS provide further support that this factor is a critical and specific regulator of DCV exocytosis. In *Drosophila*, null *dcaps* mutants have severe movement defects and die late in embryogenesis (Renden et al., 2001). Examination of synaptic terminals revealed a greater than threefold increase in DCVs in these mutants suggesting an essential function for CAPS in vesicle fusion and cargo release. Importantly, these studies also demonstrated an accumulation of SVs at neuromuscular junctions in *dcaps* mutants (Renden et al., 2001; Richmond and Broadie, 2002). This SV phenotype was shown to be non-cell-autonomous and secondary to an upstream defect, most likely impaired release of DCV cargo in neurosecretory cells modulating the activity of motor neurons.

These observations in *Drosophila* provide an excellent framework in which to interpret our own results with *ida-1* in *C. elegans*. *ida-1* mutant alleles have a mild aldicarb resistance phenotype, suggesting a deficit in acetylcholine release from SVs. As in *Drosophila*, we suggest that the aldicarb resistance of *ida-1* null mutants reflects a secondary consequence of impaired DCV function in upstream neurons. This conclusion is consistent with

our inability to rescue the aldicarb phenotype of *ida-1(ok409)* when expression of a rescuing transgene is restricted to the A- and B-type cholinergic motor neurons. Three other mutants of putative DCV-specific proteins in *C. elegans* have reduced acetylcholine release, resulting in an aldicarb resistance phenotype. These are UNC-31/CAPS (Iwasaki et al., 1997; Berwin et al., 1998; Renden et al., 2001; Munoz and Riddle, 2003) and two peptide hormone-processing enzymes, egg laying-defective (EGL)-21/carboxypeptidase E and EGL-3/proprotein convertase 2 (Jacob and Kaplan, 2003). It seems likely that impaired DCV cargo release in upstream neurosecretory cells leads to aldicarb resistance in all of these mutants. The molecular mechanism(s) underlying DCV cargo release and its modulation by CeIA-2 and UNC-31/CAPS is not revealed by our genetic studies. However, our studies suggest that understanding the molecular relationship between CeIA-2 and UNC-31/CAPS will be critical to understanding fully the mechanism of DCV exocytosis.

CeIA-2 and the regulation of insulin-like signaling in *C. elegans*

Dauer formation is regulated by at least three pathways in *C. elegans*: insulin-like signaling, TGF- β -like signaling, and cGMP. These pathways have been well studied genetically allowing us to explore interactions between *ida-1* and dauer pathway components. We find that *ida-1* shows the strongest genetic interactions with mutants in the insulin-like signaling pathway. This includes the genes encoding UNC-31/CAPS, UNC-64/syntaxin, the insulin-like receptor DAF-2, and insulin-like ligand DAF-28 (Ailion et al., 1999; Li et al., 2003). The ability of *ida-1* mutants to enhance, albeit weakly, loss-of-function alleles in the TGF- β (*daf-7*) and cGMP (*daf-11*) branches of the dauer pathway is also consistent with a role for CeIA-2 that is primarily restricted to insulin-like signaling. Although *ida-1* mutants do not have a dauer phenotype on their own, CeIA-2 appears to be a modifier of processes regulated by genes in the insulin-like signaling pathway of dauer formation.

Our results suggesting that CeIA-2 functions in the *C. elegans* insulin-like signaling pathway are strikingly similar to those described for IA-2 function in mammals. Mouse knock-outs of the gene encoding IA-2 result in elevated blood glucose levels in glucose tolerance tests (Saeki et al., 2002). Moreover, cultured pancreatic β cells from these knock-out mice have a 40–45% reduction in insulin release after glucose stimulation. In contrast to the single IA-2 family member found in invertebrates, mice have two related genes, IA-2 and IA-2 β (phogrin). In mice, neither knock-out alone nor in combination eliminates insulin release (J. Kubosaki and A. L. Notkins, unpublished data). As in the worm, then, it appears that neither IA-2 nor IA-2 β is essential for insulin secretion, and, although impaired under stressful conditions, limited insulin signaling can occur in mammals and *C. elegans* in the complete absence of IA-2/IA-2 β and CeIA-2, respectively.

The identification of CeIA-2 as a component involved in DCV cargo release adds to our general understanding of DCV trafficking. Future studies will be needed to transform our genetic results into molecular detail. For example, what factors interact directly with CeIA-2, and what is the molecular mechanism by which CeIA-2 facilitates DCV cargo release? Exploitation of *C. elegans* genetics will be an important adjunct to these future studies because it will allow us to identify additional, and perhaps novel, components regulating DCV cargo release.

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