sqv mutants of Caenorhabditis elegans are defective in vulval epithelial invagination

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ABSTRACT By screening for mutations that perturb the invagination of the vulva of the Caenorhabditis elegans hermaphrodite, we have isolated 25 mutations that define eight genes. We have named these genes sqv-1 to sqv-8 (squashed vulva). All 25 mutations cause the same vulval defect, an apparent partial collapse of the vulval invagination and an elongation of the central vulval cells. Most sqv mutations also cause an oocyte or somatic gonad defect that results in hermaphrodite sterility, and some sqv mutations cause maternal-effect lethality. We propose that the sqv genes affect a pathway common to vulval invagination, oocyte development, and embryogenesis.

The movement and folding of epithelial cell layers are basic processes in morphogenesis (1). For example, amphibian gastrulation is initiated by the invagination of endodermal cells (2), neurulation involves the inward folding of ectodermal cells to form a tube that will become the spinal cord and brain (3), and the development of the vertebrate eye requires the optic vesicle to bend inward to form a cup that ultimately will become the retina (4).

There are several general models of how an epithelium might initiate inward folding or invagination (5–7). In models for sea urchin gastrulation (8), Drosophila gastrulation (9–12), and vertebrate neurulation (13), cells in the invaginating epithelium individually undergo cytoskeletal changes that result in constriction of their apical surface relative to their basal surface and consequent bending inward of the epithelium. Other models (14–16) propose that changes in cell-cell adhesion drive invagination. In the simplest such model (14), an increase in adhesiveness between cells in the invaginating epithelium favors an increase in the extent of contact between them and, consequently, an increase in their height. If the basal surfaces of the cells remain adherent to a substrate, causing the basal surface area to remain the same, this increase in cell height is accommodated by a decrease in apical surface area and consequent inward folding of the epithelium.

A third type of model, suggested for sea urchin gastrulation, proposes that changes in the extracellular matrix drive invagination (17). Cells that are to invaginate deposit a new hygroscopic layer of extracellular matrix between their apices and an older less hygroscopic matrix. The greater hydration of the new matrix layer causes it to swell and increase in surface area relative to the old matrix, driving the bilayer to bend inward and causing the underlying epithelial sheet to bend as well. Although each of these models of invagination is based on a single cellular mechanism, it is certainly possible that multiple mechanisms can be coordinated during invagination and that different examples of invagination involve different mechanisms to different extents.

Most analyses of epithelial invagination have been limited to manipulating epithelia in vitro, either mechanically or by the addition of chemical reagents, and to defining the expression patterns of molecules proposed to be involved. Over the past few years, the analysis of Drosophila mutants defective in gastrulation has identified an in vivo role for G-protein signaling (10, 11) and Rho-dependent cytoskeletal changes (12) in this process. To identify additional molecules involved in vivo in epithelial invagination, we have begun a genetic analysis of this process in the nematode Caenorhabditis elegans.

The C. elegans body is enclosed by a single layer of epithelial cells, which underlie a collagenous cuticle (18). During the third (L3) and fourth (L4) larval stages, the descendants of the vulval precursors P5.p, P6.p, and P7.p, a specialized set of outer epithelial cells, invaginate and create a tube that connects the outer epithelium to the layer of epithelial cells that enclose the uterus (19). This vulval tube allows the adult hermaphrodite both to lay eggs and to receive sperm from males. The intercellular signaling pathways that direct P6.p to undergo a so-called primary pattern of cell division and P5.p and P7.p to undergo secondary patterns of division have been studied extensively (20, 21). During the final round of vulval cell divisions, the primary descendants and some secondary descendants detach from the cuticle, allowing the vulval sheet to bend inward and the cells within it to rearrange their cell-cell contacts. Because vulval invagination can occur in the absence of most other nearby cells, including the vulval muscles (T.H., unpublished observations) and the somatic gonad (22), it is likely that the mechanical force required is intrinsic to the epithelium or its extracellular matrix, consistent with models for other invaginations (see above), although the primary descendants must be in contact with a cell in the gonad, the anchor cell, for the invagination to have the correct shape and to attach to the uterus (22–24). In this paper we describe the results of a screen for mutations that affect vulval invagination. The molecular characterization of three of the genes defined by these mutations is described in ref. 25.

MATERIALS AND METHODS

Genetics. Strains were cultured as described (26) and, unless indicated otherwise, were grown at 20°C. Wild type refers to the N2 strain. Most mutations and chromosomal rearrangements mentioned are described in refs. 27 or 28. Exceptions are let-253(n2412) (M. Labouesse, personal communication), lin-12(n302 n865) (29), and ndf40 (30).

Mutagenesis with ethyl methanesulfonate, genetic mapping, and complementation tests (all sqv mutations appeared to be completely recessive; data not shown) were performed by standard methods (26, 31). Alleles of sqv-3 failed to complement the deficiency ndf40, alleles of sqv-7 failed to complement the deficiency mnDf30, and alleles of sqv-8 failed to complement the deficiency mnDf29.

Abbreviations: sqv, squashed vulva; DIC, differential interference contrast.

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Phenotypic Characterization. Electron microscopy of individual animals was performed as described (32), except that sections were cut laterally (from left to right or from right to left) rather than from nose to tip. Wild-type and mutant larvae were stained with MH27 antibodies (33) by the method described in ref. 34, and the immunofluorescence was examined with a Bio-Rad MRC-500 confocal microscope. sqv-1(n2849), sqv-2(n3027), sqv-3(n2841), sqv-4(n2840), sqv-5(n3039), sqv-6(n2845), sqv-7(n2844), and sqv-8(n2822) mutants were examined by Nomarski differential interference contrast (DIC) microscopy at the L4 stage for the presence of HSN neurons and at the adult stage for vulval muscle contractions; for each of these mutants, HSNs and vulval contractions were observed, although the observed contractions did not result in the release of eggs. Artificial insemination was performed by the method described in ref. 35. Unlike sqv-1 to sqv-7 hermaphrodites, sqv-8 hermaphrodites can produce viable progeny when mated with or artificially inseminated with sperm from wild-type males (data not shown; ref. 36). To test whether sqv-8(n2822) animals exhibited a paternal effect like that reported for sqv-8(mn63) animals (36), we mated N2 males with sqv-8(n2822) hermaphrodites, mated the resulting male cross-progeny with sqv-8(n2822) hermaphrodites, and examined whether any progeny from the latter were Sqv. Because none were Sqv, we retested the sqv-8(mn63) allele in the same way. We were unable to reproduce the finding reported in ref. 36. We did notice that sqv-8 hermaphrodites mated with wild-type males produced a small number (<10%) of progeny that were dumpy, often twisted or rolling, and often bulging at the tail or midbody (data not shown); some did not reach adulthood. We therefore suggest that sqv-8 causes a maternal-effect lethality that can be rescued (sometimes only partially) by providing a wild-type sqv-8 gene in the zygote.

RESULTS

Isolation of Mutations that Perturb Vulval Invagination. To identify genes involved in vulval invagination, we undertook a genetic screen. We mutagenized the wild-type strain N2, transferred F1 progeny to individual plates, and, using a dissecting microscope, examined the F2 broods for animals with vulval defects. With this scheme, mutations that additionally might cause recessive sterility or maternal-effect lethality could be recovered in heterozygous siblings. F2 hermaphrodites were examined at the mid-L4 stage, after the space was absent or abnormal, indicating that the vulval cells that were dumpy, often twisted or rolling, and often bulging at the tail or midbody (data not shown); some did not reach adulthood. We therefore suggest that sqv-8 causes a maternal-effect lethality that can be rescued (sometimes only partially) by providing a wild-type sqv-8 gene in the zygote.

Previously identified mutation, spe-2(mn63) (36), which we found also causes a defect in vulval invagination. Although spe-2(mn63) hermaphrodites previously were reported to have a sperm defect (36), we were unable to identify such a defect either with the mn63 allele or a new allele, n2822 (see Materials and Methods), and additionally found that sperm from an n2822 homozygous hermaphrodite can produce viable cross-progeny (see below). With the permission of R. Herman (personal communication) we therefore have renamed this gene sqv-8.

Because the sqv mutations were not rare, the phenotypes they cause are recessive to wild type, and the vulval phenotypes caused by those mutations tested (all alleles of sqv-3, sqv-7, and sqv-8) are similar whether the mutations are homozygous or hemizygous, it is likely that these mutations cause a loss of gene function.

Mutations in sqv-1 to sqv-8 Cause a Partial Collapse of the Vulval Invagination and Affect Both Primary and Secondary Vulval Cell Descendants. All 25 newly isolated mutations and mn63 appear to cause identical vulval phenotypes, as judged by Nomarski DIC microscopy. Before the final round of vulval cell divisions, both wild-type and sqv mutant vulval cells lie along the ventral cuticle in a plane with the surrounding outer epithelium, hyp7 (Fig. 2 A and B). In the wild type, during the final round of vulval cell divisions, a defined subset of the vulval cells detaches from the cuticle and begins to invaginate, leaving the plane of the surrounding epithelium and creating a space between the apices of these vulval cells and the cuticle (Fig. 2C). In the sqv mutants, however, although the appropriate cells appear to detach from the cuticle, the resulting invagination space is considerably reduced in size (Fig. 2D). This abnormality persists, and later the height of the mutant vulval invagination appears to be slightly decreased relative to that of the wild type, suggesting that the mutant invagination may be partially collapsed (Fig. 2 E and F). The vulval phenotype caused by homozygous or hemizygous alleles of sqv-3, sqv-7, and sqv-8 (the only mutations tested as hemizygous) appeared to be identical and hence may correspond to the null phenotype of these genes. Three mutations, sqv-2(n2821), sqv-2(n2826), and sqv-7(n2839), cause a slightly weaker vulval defect than that depicted in Fig. 2 (i.e., a smaller reduction in the size of the invagination space) and therefore may cause only a partial loss of gene function.

To confirm that the Sqv vulval phenotype was not the result of abnormal vulval cell lineages, we directly observed the pattern of vulval cell divisions in at least one hermaphrodite of

![Diagram](https://example.com/diagram.png)

**Fig. 1.** Locations of sqv-1 to sqv-8 on the genetic map. The positions of sqv-1 to sqv-7 are based on the three-factor data in Table 1 and on linkage analysis. The position of sqv-8 was determined by Sigurdson et al. (36), who identified its first mutant allele (the "1 old" allele). A list of alleles is provided in Table 2.
when animals of either lin-12 genotype were also homozygous for a sqv mutation [sqv-3(n2841)] their vulval invagination spaces were severely reduced in size, indicating that invaginating vulval cells derived from both the primary and secondary lineages are affected by the Sqv mutant phenotype. It remains possible, however, that only particular descendants from each type of lineage are affected.

The Vulval Invagination Space Is More Electron Dense and the Central Invaginating Vulval Cells Are More Elongated in a sqv Mutant Than in the Wild Type. To examine directly the invaginating vulval cells at the early L4 stage, when the difference between wild-type and sqv mutant vulvae is first detectable by Nomarski DIC microscopy, we prepared serial sections of N2 and sqv-3(n2842) mutant hermaphrodites for electron microscopy. sqv-3(n2842) appears to cause a vulval defect identical to that of all other sqv mutants except sqv-2(n2821), sqv-2(n2826), and sqv-7(n2839) (see above) and was chosen arbitrarily. At this stage, the central wild-type vulval cells have detached from the cuticle and surround an invagination space of minimal electron density (Fig. 3A). The central vulval cells of the sqv-3(n2842) mutant also were detached from the cuticle but surrounded an invagination space that is not only considerably reduced in size but also more electron dense than that of the wild type (Fig. 3B), a difference that was observed in several N2 and sqv-3(n2842) animals. The increased electron density may reflect a qualitative difference in the composition of the mutant extracellular space or simply may be the result of concentrating wild-type material into a smaller volume.

To compare the arrangement and shapes of the wild-type and sqv-3 vulval cells, we traced their plasma and nuclear membranes as well as the cuticle in electron micrographs of our serial sections; four such tracings are shown in Fig. 3 C–F. Cell identifications were made on the basis of the known arrangement of vulval nuclei in each of the following genotypes: sqv-1(n2819), sqv-2(n2826), sqv-3(n2841) unc-69(e587), sqv-4(n2840), sqv-5(n3039) unc-75(e950), sqv-6(n2845), sqv-7(n2844) unc-4(e120), and sqv-8(mn63) unc-4(e120). In every case this pattern was wild type (data not shown). In addition, we have observed that these and the remaining 18 sqv mutations do not appear to affect the number and gross arrangement of the vulval nuclei at the mid- to late L4 stage (data not shown).

To determine which invaginating vulval cells are affected by the Sqv mutant phenotype, we took advantage of the cell lineage transformations caused by loss-of-function and gain-of-function mutations in the gene lin-12. In lin-12(n302 n865) animals no vulval precursor cells undergo a secondary pattern of divisions and instead P5.p, P6.p, and, often, P7.p undergo primary patterns of divisions, whereas in lin-12(n137) animals no vulval precursor cells undergo a primary pattern of divisions, and instead P3.p to P8.p undergo secondary patterns of divisions (29). We found that

![Fig. 3](image-url)
Fig. 4. 

The sqv Mutant Vulval Cells Form a Partially Functional Adult Vulval Tube. As wild-type vulval cells invaginate they reorganize their cell-cell contacts around the invagination space and fuse in a specific pattern that results in a stack of seven toroidal cells. The dorsal-most toroid is attached to the uterine epithelium, the ventral-most toroid remains attached to the outer epithelium, and the central hole of the stack becomes the vulval tube of the adult. We stained mid-L4 stage wild-type and mutant animals with a mAb, MH27, that recognizes the desmosomal connections between epithelial cells in C. elegans; in the wild-type, this antibody decorates each ring of contact between adjacent vulval toroids, as well as the vulval attachments to the uterus and outer epithelium and the connections between epithelial cells in the uterus. The MH27 antibody also recognizes a pattern of rings in sqv-3(n2842) animals, indicating that despite their abnormal invagination, the sqv vulval cells form toroids.

By the adult stage, the wild-type and sqv mutant vulvae appeared similar by Nomarski DIC microscopy (Fig. 4A). The MH27 antibody also recognizes a pattern of rings in sqv-3(n2841) vulvae in a pattern of stacked rings, indicating that, as in the wild type, the invading sqv-3 vulval cells can form toroids, although we did not ascertain whether their number and connection to the uterus were precisely correct. Arrowheads indicate the axes about which the vulval rings are arranged. The sqv-3 animal shown is somewhat twisted back on itself, causing the bright gonadal staining to the left of the vulva to appear closer and therefore be visible in B and also accounting for the two almost vertical bands of staining not present in A.

as judged by Nomarski DIC microscopy, and it was possible in this way to assign cell identities in the sqv-3 vulva consistent with its having a grossly normal arrangement of cells. However, a comparison of roughly equivalent wild-type and sqv-3 sections shows that the central mutant vulval cells (in particular, cells F, E, and D) are abnormally elongated, extending into and reducing the size of the invagination space. This general difference also was observed in other N2 and sqv-3(n2842) animals examined, although in these cases we did not follow the three-dimensional shapes of the cells through electron microscopy sections.

laid some eggs, and sqv-8 hermaphrodites could receive sperm from mating males (see Materials and Methods), mutant alleles of most sqv genes caused a defect in laying eggs (Table 1), resulting in older adult animals that were visibly bloated with unlaid eggs and often had protruding vulvae. Because the other cells required for egg laying, the HSN neurons and vulval muscles, appeared to be present and functional in these mutants (see Materials and Methods), their egg-laying defect is likely to be the result of the sqv vulval defect. Although sqv-5 hermaphrodites did not accumulate unlaid eggs in their uteri, they also laid few, if any, eggs, suggesting that they simply produced few oocytes (see below).

The sqv Mutations Cause Hermaphrodite Sterility, and At Least Some sqv Genes Are Required for Embryogenesis. Nearly all the mutations we isolated, including at least one allele each of sqv-1 to sqv-8, caused a severe reduction in hermaphrodite fertility (Table 2). The three mutations that had a weak or no effect on brood size are those that also caused weaker vulval defects (see above). We determined the stages at which progeny of mutant hermaphrodites arrest and found that most sqv-1 to sqv-8 mutants produced at least some eggs that failed to undergo cytokinesis. We did not determine whether these one-cell eggs underwent fertilization. They may have been unfertilized, because, like unfertilized oocytes from spe-1(mn47) unc-4(e120) hermaphrodites, they were dissolved by bleach (41), but they appeared to retain their shapes better than spe-1 oocytes and so may have a partial eggshell. In artificial insemination experiments we found that sperm from sqv-1(n2819), sqv-2(n3038), sqv-3(n2842), sqv-4(n2840), sqv-5(n3039), sqv-6(n2845), sqv-7(n2844), and sqv-8(n2822) hermaphrodites were able to produce adult cross-progeny when injected into eT1 hermaphrodites, indicating that the sterility of sqv-1 to sqv-8 hermaphrodites was caused by a defect in the mutant oocytes or somatic gonad. In addition, when the uterus of sqv-1 to sqv-8 mutants were dissected to assay egg-laying ability (see Table 1), a number of small oocyte-like cells were released along with oocytes/eggs of normal size, indicating that these mutants may have a defect in oocyte formation.

Some sqv mutants additionally produced eggs that arrested during embryogenesis, suggesting that the corresponding genes are required for embryonic development (Table 2). sqv-8 mutants in particular produced a large proportion of eggs that progressed beyond the one-cell stage before arresting. Although sqv-8(n2822) behaves genetically like a strong loss of sqv-8 function (Table 3), it remains possible that stronger losses of sqv-8 gene function might result in a fertility defect that resembles that of sqv-1 to sqv-7 mutants.

Table 1. sqv hermaphrodites retain eggs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average number of eggs in uterus</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>18.7</td>
<td>10–25</td>
</tr>
<tr>
<td>sqv-1(n2849)</td>
<td>35.1</td>
<td>25–45</td>
</tr>
<tr>
<td>sqv-2(n3037)</td>
<td>29.1</td>
<td>12–63</td>
</tr>
<tr>
<td>sqv-3(n2841)</td>
<td>31.3</td>
<td>10–59</td>
</tr>
<tr>
<td>sqv-4(n2840)</td>
<td>25.0</td>
<td>14–47</td>
</tr>
<tr>
<td>sqv-5(n3039)</td>
<td>3.2</td>
<td>1–5</td>
</tr>
<tr>
<td>sqv-6(n2845)</td>
<td>32.1</td>
<td>17–71</td>
</tr>
<tr>
<td>sqv-7(n2844)</td>
<td>34.7</td>
<td>21–87</td>
</tr>
<tr>
<td>sqv-8(n2822)</td>
<td>44.0</td>
<td>26–63</td>
</tr>
</tbody>
</table>

Fifteen mid-L4 stage hermaphrodites of each genotype were aged for 41 hr and individually dissected to release eggs from the uterus. Uteri of all mutant genotypes often contained oocyte-like cells less than half the size of normal oocytes; these cells were not counted. sqv-5 animals produced few oocytes. At least one allele of sqv-1, n2819, also may reduce the number of oocytes produced, because homozygotes did not retain a significant number of eggs in this assay but also laid few, if any, eggs. The remaining sqv-7 alleles were not tested in this assay.
DISCUSSION

We have isolated 25 mutations that result in a partial collapse of the vulval invagination and elongation of the central invaginating cells. These mutations appear to result in a loss of gene function and define eight genes, which we have named sqv-1 to sqv-8. These mutations do not prevent the formation of vulval toroids and a partially functional adult vulva, indicating that there must be other as yet unidentified genes involved in this process.

Any of several simple models could explain the sqv mutant phenotype. In all of these models the sqv genes are required to execute invagination efficiently, while other genes cause the changes in cell-cell contacts and the cell fusions required for the formation of vulval toroids.

One possibility is that the loss of the sqv genes decreases the rigidity of the vulval epithelium, reducing its ability to support itself over a large invagination space and causing it instead to form a collapsed invagination over a smaller space. Such a decrease in rigidity could result from a defect within the vulval cells themselves, for instance in the cytoskeleton or in the strength of the adhesion among these cells, or from a defect in the rigidity of the extracellular matrix adjacent to the apices of the vulval cells and lining the invagination space. The plausibility of such a model is supported by the observation that when the mid-L4 vulval epithelium is artificially subjected to increased outward pressure, it can collapse and resemble an L4 Sqv vulva: such a collapse has been observed when the cuticle adjacent to the mid-L4 invagination space is punctured (P. Sternberg, personal communication) and presumably occurs because the vulval epithelium is not sufficiently rigid to support the internal hydrostatic pressure of the worm in the absence of the cuticle. This observation also would be consistent with a...
model in which a leaky cuticle might cause the Sqv vulval defect; however, the cuticle of sqv-3(n2842) hermaphrodites appeared normal based on electron microscopy.

A second possibility is that the expansion of the invagination space, presumably by the accumulation of water, is an active process that requires sqv gene function.

A third possibility is that the Sqv vulval phenotype results from an inappropriate or increased adhesion between the apices of the vulval cells and abnormal material deposited in the mutant invagination space (we observed that the region between the sqv-3(n2842) vulval apices and cuticle was abnormally electron dense). If such material were not exposable and acted as a glue between the cuticle and the vulval cell apices, then as the basal side of the vulval epithelium bends away from the cuticle the central vulval cells would become stretched, resulting in the Sqv phenotype.

A fourth possibility is that the sqv phenotype results from an abnormally increased adhesiveness among the mutant vulval cells themselves. Such an increase would favor an increase in the extent of vulval cell-cell contact and therefore an elongation of the vulval cells, as is seen in the sqv mutants. Because, as proposed in ref. 14, such an increase in adhesiveness could drive invagination, it is also possible that this increase normally might occur during wild-type vulval invagination and simply be abnormally high in the sqv mutants.

In addition to causing a defect in vulval invagination, loss of sqv gene function results in a severe reduction in hermaphrodite fertility. sqv-1 to sqv-8 mutants appeared to produce some abnormally small oocytes and at least some eggs that fail to undergo cytokinesis and may not be fertilized, suggesting that the sqv genes may be required for aspects of oocyte development, function, and/or fertilization. In addition, sqv-1, sqv-2, sqv-3, sqv-4, sqv-7, and sqv-8 mutants produced eggs that arrested during embryogenesis, suggesting that these genes are required for embryonic development (it also might be possible to isolate sqv-5 and sqv-6 alleles that cause this phenotype). Such arrested animals often were abnormally shaped: in particular, sqv-8 embryos with well-developed pharynges often were less elongated than and lumpy in comparison with wild-type embryos at a similar stage of pharyngeal development. Hatched embryos of several sqv genotypes also were observed to be lumpy and poorly elongated (the “misshapen” embryos in Table 2). Finally, some sqv-8/+ progeny of sqv-8 hermaphrodites were dumpy, often twisted or rolling, and often bulging at the tail or midbody (see Materials and Methods). Because a normal, elongated body shape is derived in part from cell shape changes in the outer epithelium during embryogenesis, we suggest that at least some of the sqv genes may affect other aspects of epithelial morphogenesis in addition to vulval invagination.

We thank Robert K. Herman for, among other things, allowing us to rename the spe-2 gene, and the Caenorhabditis Genetics Center for providing the spe-8(mn63) unc-4(e120)/mnc1 strain. This work was supported by Public Health Service Research Grant GM24663. H.R.H. is an Investigator of the Howard Hughes Medical Institute.