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Claudins in *Caenorhabditis elegans*: Their Distribution and Barrier Function in the Epithelium

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Summary

Claudins (~23 kDa) with four transmembrane domains are major cell adhesion molecules working at tight junctions in vertebrates, where the intercellular space is tightly sealed (reviewed in [1–3]). We examined here the possible occurrence of claudin-like proteins in invertebrates, which do not bear typical tight junctions. Close blast searching of the *C. elegans* genome database identified four claudin-related, ~20-kDa integral membrane proteins (CLC-1 to -4), which showed sequence similarity to the vertebrate claudins. The expression and distribution of CLC-1 was then examined in detail by GFP technology as well as by immunofluorescence microscopy. CLC-1 was mainly expressed in the epithelial cells in the pharyngeal region of digestive tubes and colocalized with AJM-1 at their intercellular junctions. Then, to examine the possible involvement of CLC-1 in the barrier function, we performed RNA interference in combination with a tracer experiment: in CLC-1-deficient worms, the barrier function of the pharyngeal portion of the digestive tubes appeared to be severely affected. CLC-2 was expressed in seam cells in the hypodermis, and it also appeared to be involved in the hypodermis barrier. These findings indicated that multiple species of the claudin homologs, which are involved in the barrier function of the epithelium, exist in *C. elegans*.

Results and Discussion

In multicellular organisms, the barrier function of the epithelium is essential to isolate the internal from the external environment as well as to establish various compositionally distinct fluid compartments within the body [1–3]: for epithelial cellular sheets to function as a barrier, the leakage of solutes through the paracellular routes must be prevented. In vertebrates, tight junctions play a key role in this intercellular sealing, and, recently, two distinct types of tight junction-specific integral membrane proteins with four transmembrane domains, occludin (~65 kDa) and claudins (~23 kDa), have been identified [4–6]. Among them, it is now widely accepted that claudins are directly involved in intercellular sealing: claudins comprise a multigene family consisting of more than 20 members in humans/mice. In lower organisms such as insects, however, the integral membrane proteins responsible for intercellular sealing have not yet been identified [7].

Our first question was whether claudin (and occludin) homologs exist in *C. elegans*. We then tried a blast search of the wormpep database (Sanger Centre) with the known amino acid sequences of mammalian claudins (claudin-1 to -16) and occludin. As shown in Figure 1, four claudin-like molecules, which had a molecular mass around 20 kDa (CLC-1 to -4) and had four transmembrane domains and two conserved cysteine residues in the first extracellular loop, were found. No occludin-like sequence was found with similar searches. CLC-1 showed 25% identity to mouse claudin-6, CLC-2 exhibited 23% identity to mouse claudin-5, and CLC-3 was 26% identical to mouse claudin-4. CLC-4 was not detected through blast searches with known mammalian claudins, but it is similar (36% identity) to CLC-1. Considering that human claudins showed more than 70% identity to corresponding mouse claudins, more information was required to conclude that these proteins are *C. elegans* homologs of claudins.

We then focused on CLC-1 and examined in detail its expression and distribution in worms. First, we constructed a reporter that fuses GFP to the predicted last amino acid of the CLC-1 protein (CLC-1-GFP) and is driven by 1.24 kb of genomic sequence upstream of the endogenous CLC-1 gene, and the resultant construct was injected into the worms to isolate stable transformants. Intense GFP signals were detected as lines from the pharyngeal region of the digestive tubes (Figure 2) in addition to other types of cells (see Figure S1 in the Supplemental Data available with this article online). As shown in the DIC images (Figure 2A), the pharyngeal region of the digestive tubes can be subdivided into four portions: procorpus, metacorpus, isthmus, and terminal bulb. Among them, the procorpus and isthmus portions are elongated, which allowed us to examine the subcellular distribution of CLC-1-GFP in detail. In both portions, three parallel GFP-positive lines (occasionally one or two additional lines in the isthmus portion) were observed to run longitudinally and to surround the interior lumen. Close inspection revealed that each of these thick lines occasionally resolved into two thinner lines (Figure 2A). This distribution of CLC-1 was confirmed by whole-mount immunofluorescence staining with anti-

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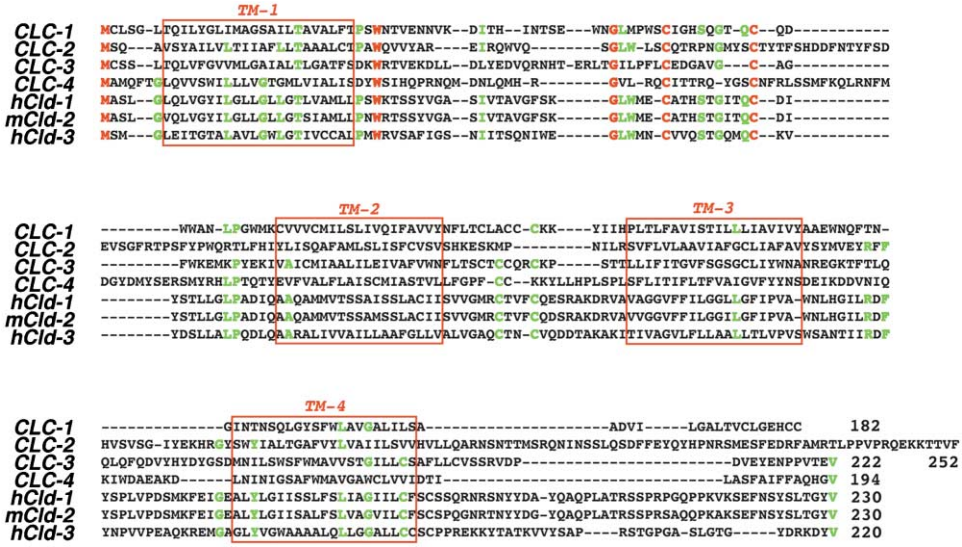

Figure 1. Amino Acid Sequence Alignment of Claudin Homologs of *C. elegans* with Vertebrate Claudins

C. elegans gene database (wormpep) was blast searched with mammalian claudin-1 to -16. CLC-1 (C09F12.1) was found by a search with mouse claudin-7 and human claudin-14. A blast search with several claudins, including mouse claudin-1, -6, and -7 and human claudin-7 and -9 detected CLC-2 (C01C10.1). CLC-3 (ZK563.4) was found by a blast with mouse claudin-10 and mouse/rat/cattle claudin-16. CLC-4 (T05A10.2) was not detected with blast searches with known mammalian claudins, but it has a homology (36% identity) with CLC-1. Four transmembrane domains were boxed (TM-1 to -4). Amino acid residues conserved in all claudins are colored red. Amino acid residues conserved in all vertebrate claudins and at least one of CLC-1 to -4 are colored green. Note that two cysteine residues are conserved in the first extracellular loop.

CLC-1 pAb, although the staining signals were not so intense (Figure 2B).

These findings suggested that CLC-1 is expressed in the epithelial cells of the pharyngeal region of the digestive tubes and is concentrated at the intercellular junctions located at the most apical portion of the lateral membranes. We then compared the distribution of CLC-1 with AJM-1, a general marker for intercellular junctions in *C. elegans* [8]: since *C. elegans* transformants stably expressing AJM-1-GFP were kindly provided, we exogenously expressed the DsRed fusion protein with CLC-1 (CLC-1-DsRed) in these worms. As shown in Figure 2C, CLC-1-DsRed appeared to be colocalized with AJM-1-GFP at least at the light microscopic level. Interestingly, no typical tight junctions have been observed in *C. elegans* by conventional chemical fixation [9]. We then observed the pharyngeal epithelial cells by high-pressure freezing followed by freeze substitution [10]. Freeze substitution is known to be superior to the conventional chemical fixation in terms of the preservation of fine structures, and the high-pressure freezing method allowed us to preserve the whole body of the worms without any ice crystal formation. The isthmus portion was made up of three large myoepithelial cells and three small marginal cells [11], although conventional chemical fixation has not been able to capture these cells in such a "relaxed" state (Figure 3A). In a single transverse section, six intercellular junctions were observed between adjacent cells, each pair of which was apposed closely. These images would easily explain the distribution of CLC-1-GFP, i.e., three thick GFP-positive lines, in the procorpus and isthmus portions. Interestingly, high-pressure freezing followed by freeze substitution for the first time identified a novel type of cell-to-cell junction localized more apically than the zonula adherens-like junctions (Figure 3B). This junction was clearly different from tight or septate junctions, but in this junction, the plasma membranes of adjacent cells were closely apposed ($<$10 nm). Although immunoelectron microscopy was unsuccessful in these worms when anti-CLC-1 pAb was used, these images led us to speculate that this is a good candidate for the localization of CLC-1. Consistently, AJM-1 was reported to be concentrated more apically than HMR-1/HMP-1/HMP-2 (cadherin/α-catenin/β-catenin) [12], but it would be premature to discuss further the detailed localization of CLC-1 compared to AJM-1 and cadherin/catenins at the electron microscopic level.

The next question is whether CLC-1 is involved in the permeability barrier of the epithelial sheets also in *C. elegans*. To address this question, we developed a simple tracer experiment system with *C. elegans*: when wild-type worms were incubated in a solution containing a high-molecular mass dye, TRITC-dextran (10 kDa), for 30 min, they drank the solution. Since the epithelium of the digestive tubes functions as a barrier, TRITC-dextran did not infiltrate the internal cavity of the body across the epithelial cell layers; thus, the interior lumen of the digestive tubes was clearly visible under a fluorescence microscope (see Figure 4B). We then attempted to suppress the expression of CLC-1 in worms by using the RNA interference method [13], and we applied this tracer experiment system to these worms. The effectiveness of the dsRNA of CLC-1 was confirmed by injecting it into one side of the gonads of the transformants expressing CLC-1-GFP: 47% of F1 worms (n = 351) became nega-

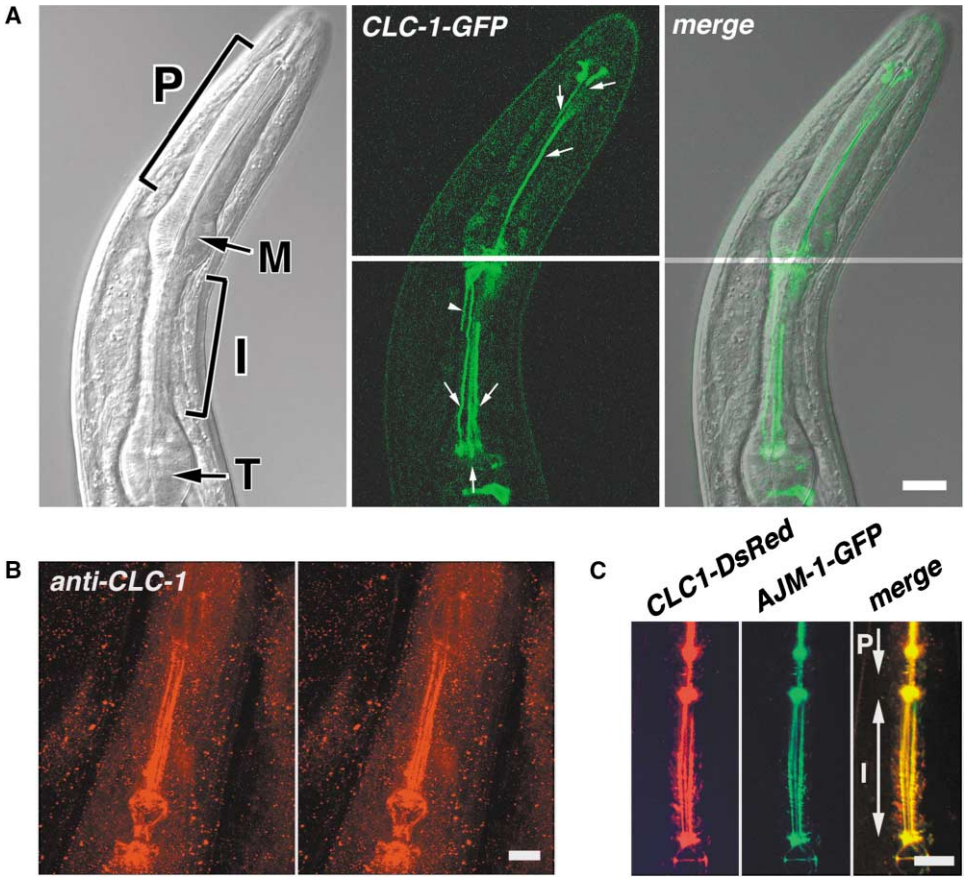

Figure 2. Localization of CLC-1 in *C. elegans*

(A) Transformants expressing CLC-1-GFP. CLC-1-GFP was driven by 1.24 kb of the genomic sequence upstream of the endogenous CLC-1 gene. The DIC image represents four portions of the pharyngeal region: procorpus (P), metacorpus (M), isthmus (I), and terminal bulb (T) (left panel). In both the procorpus (upper middle panel) and isthmus (lower middle panel) portions, three parallel GFP-positive lines (arrows) were observed to run longitudinally and to surround the interior lumen, as shown in the merged image (right panel). Occasionally, one or two additional lines were detected in the isthmus portion (arrowhead).

(B) Stereo pairs of immunofluorescence micrographs of the isthmus portion. The CLC-1 distribution shown in (A) was confirmed by whole-mount immunofluorescence staining with anti-CLC-1 pAb.

(C) Comparison of the distribution of CLC-1 with that of AJM-1, a junctional marker, in the isthmus portion. The DsRed fusion protein with CLC-1 (CLC-1-DsRed) was exogenously expressed in transformants stably expressing AJM-1-GFP. CLC-1 appeared to be colocalized with AJM-1-GFP. I, isthmus; P, procorpus.

The scale bars represent 5 μm.

tive for GFP signals. The effectiveness of this technique was also confirmed by semiquantitative RT-PCR (Figure 4A). Then, we applied the above tracer experiment system to worms in which the CLC-1 expression was suppressed: when dsRNA specific for CLC-1 was injected into one side of the gonads of the worms, in 62% of F1 worms, TRITC-dextran infiltrated the internal cavity of the body ($n = 282$), although the degree of infiltration varied among worms (Figure 4C). This infiltration was never observed in any F1 worms ($n = 97$) when the control dsRNA was injected (Figure 4B). Close analyses of the infiltration patterns (Figure 4C), including analysis of the computer-generated transverse sectional images (Figure 4D), favored the notion that the TRITC-dextran infiltrated through the paracellular pathway, not the transcellular pathway, of the pharyngeal myoepithelial/marginal cells. This finding suggests the direct involvement of CLC-1 in the barrier function in the simple epithelium in the pharyngeal region of *C. elegans*.

We also examined the expression and distribution of CLC-2 by using GFP technology: CLC-2-GFP was detected in hypodermal seam cells, which fuse to form a single elongated syncytium [14] (Figures S2Aa and S2Ab). However, in contrast to CLC-1-GFP, under the expression condition used in this study, GFP signals did not appear to be concentrated at the borders between the seam cell syncytium and surrounding hypodermal cells. When adult wild-type worms were whole-mount stained with anti-CLC-2 pAb, the seam cell syncytium appeared to be outlined to give two parallel lines, although these lines were thick and not very sharp (Figure S2Ac). Also, in some types of vertebrate cells, claudins are known to be distributed diffusely along lateral membranes without clear concentration at tight junctions, although they may "function" mainly at tight junctions [15, 16]. Therefore, at present, it is difficult to evaluate whether the diffuse distribution of CLC-2-GFP in seam cells is representative of that of endogenous CLC-2.

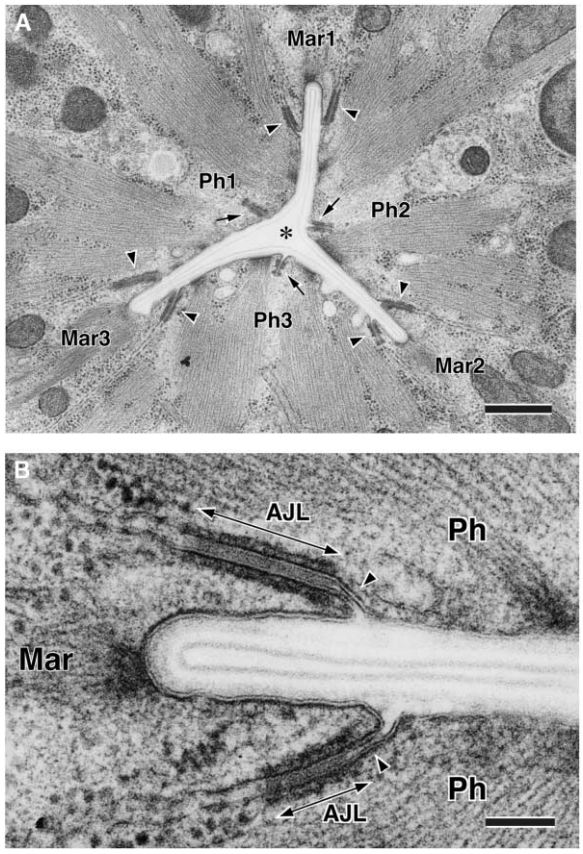

Figure 3. Quick-Freeze/Freeze Substitution Electron Microscopy of the Isthmus Portion of the Digestive Tube

(A) A low-power transverse sectional image. Samples were quickly frozen by using a high-pressure freezing machine and were freeze substituted. This portion consists of three pharyngeal myoepithelial cells (Ph1–Ph3) and three marginal cells (Mar1–Mar3). Six intercellular junctions (arrowheads) were observed, but at the resolution of light microscopy, they would be observed as three thick lines (see Figure 2). Asterisks indicate the lumen of the digestive tube. During development, two epithelial cells are fused to generate each myoepithelial cell. Arrows represent the residual intercellular junctions that originated from the two adjacent prefusion cells. Judging from Figure 2, these structures did not appear to contain CLC-1. The scale bar represents 0.5 μm.

(B) Intercellular junction at higher magnification. Note a specialized cell-to-cell junctional region (arrowhead) localized more apically than the zonula adherens-like junction (AJL). In this type of junction, plasma membranes of adjacent cells were closely apposed (<10 nm). The scale bar represents 0.1 μm.

Interestingly, in CLC-2-deficient worms produced by the RNA interference method, the hypodermal cell layers on the body surface became leaky against TRITC-dextran (Figure S2B): dye infiltration across the hypodermis was observed in 55% of F1 worms (n = 216). Thus, it is tempting to speculate that CLC-2 functions at cell-cell borders, and that CLC-2 is involved in the sealing mechanism between the seam cell syncytium and surrounding hypodermal cells.

In this study, through a blast search of the database, we found claudin-like sequences in *C. elegans* and showed that these molecules, at least CLC-1, can be regarded as *C. elegans* homologs of claudins not only structurally, but also functionally. We believe that a further detailed comparison between mammalian and *C. elegans* claudins will give new insight into the physiological implications of the barrier in multicellular organisms in general.

Supplemental Data

Supplemental Data including methodological details and figures are available at http://www.current-biology.com/content/supplemental.

Acknowledgments

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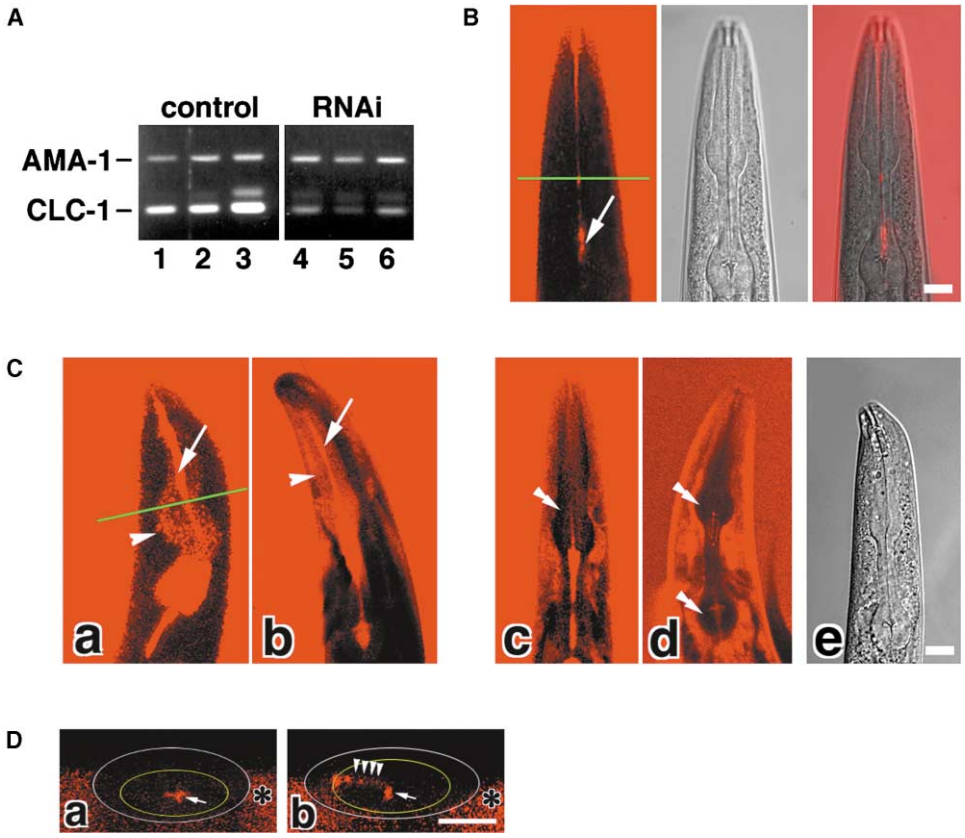

Figure 4. Permeability Assay for the Pharyngeal Region of the Digestive Tubes

The dsRNA of GFP (control) or CLC-1 was injected into one side of the gonads of the worms, and F1 worms were washed with M9 medium.

(A) Semiquantitative RT-PCR. RNA was extracted from independent sets of F1 worms (lanes 1–3, control; lanes 4–6, CLC-1 dsRNA-injected), and semiquantitative RT-PCR was performed by using the mixture of primers for CLC-1 and AMA-1 (RNA polymerase II, [17]; internal control). The expression of CLC-1 was clearly suppressed when the dsRNA of CLC-1 was injected.

(B) Control F1 worms were suspended in 0.01% TRITC-dextran (MW = 10,000, Molecular Probes) and rotated for 30 min at 25°C. The worms were collected by mild sedimentation and were observed under a fluorescent confocal microscope after weak anesthetization with 0.2% phenoxypropanol. In some samples, lysine-fixable tetramethylrohdamine-dextran (MW = 10,000, Molecular Probes) was used as a tracer, and samples were fixed with 10% formaldehyde before observation. In all of these control F1 worms (n = 97) that drank the dye solution, the outline of the interior lumen of the digestive tubes was clearly visualized without any leakage (arrow) (left panel). Middle panel, DIC image; right panel, merged image.

(C) When dsRNA specific for CLC-1 was injected into one side of the gonads followed by the tracer experiment, 62% of F1 worms exhibited dye infiltration (n = 282): the dye infiltrated the internal cavity of the body. The degree of infiltration varied among worms, but the pattern of infiltration was subclassified into two types: (a and b) type I and (c–e) II. In (a and b) type I, in addition to intense signals from the interior lumen of the digestive tubes (arrows), diffuse and granular signals were detected around the lumen (arrowheads). In (c–e) type II, intense signals were detected from the lumen as well as the body cavity and were excluded from the regions of epithelial cells themselves (double arrowheads).

(D) Computer-generated transverse sectional views of (a) control and (b) CLC-1-suppressed worm bodies at the level of green lines in (B) and (C), respectively. White and yellow oval lines represent the body surface and the basal level of the digestive epithelial cells, respectively. Asterisks, agar stained with dye; arrow, the lumen of the digestive tube. In (b) (type I in [C]), the dye did not appear to get into the cytoplasm of epithelial cells per se, but it did leak into the body cavity through their paracellular pathway (arrowheads). Images in Ca, Cb, and Db, as well as in Cc–Ce, strongly suggested that in the CLC-1-suppressed worms, intercellular sealing of the digestive tubes, not the transcellular barrier, was affected.

The scale bars represent 7 μm.

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