Gap Junction Channel Protein Innexin 2 Is Essential for Epithelial Morphogenesis in the *Drosophila* Embryo

Reinhard Bauer, Corinna Lehmann, Julia Martini, Franka Eckardt, and Michael Hoch*

Institut für Molekulare Physiologie und Entwicklungsbiologie der Universität Bonn, Abt. für Molekulare Entwicklungsbiologie, 53115 Bonn, Germany

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Direct communication of neighboring cells by gap junction channels is essential for the development of tissues and organs in the body. Whereas vertebrate gap junctions are composed of members of the connexin family of transmembrane proteins, in invertebrates gap junctions consist of Innexin channel proteins. Innexins display very low sequence homology to connexins. In addition, very little is known about their cellular role during developmental processes. In this report, we examined the function and the distribution of *Drosophila* Innexin 2 protein in embryonic epithelia. Both loss-of-function and gain-of-function *innexin 2* mutants display severe developmental defects due to cell death and a failure of proper epithelial morphogenesis. Furthermore, immunohistochemical analyses using antibodies against the Innexins 1 and 2 indicate that the distribution of Innexin gap junction proteins to specific membrane domains is regulated by tissue specific factors. Finally, biochemical interaction studies together with genetic loss- and gain-of-function experiments provide evidence that Innexin 2 interacts with core proteins of adherens and septate junctions. This is the first study, to our knowledge, of cellular distribution and protein–protein interactions of an Innexin gap junctional channel protein in the developing epithelia of *Drosophila*.

INTRODUCTION

In both vertebrates and invertebrates, the direct communication of cells is mediated by gap junctions, which consist of clusters of intercellular aqueous channels that allow the exchange of ions and small molecules between the cytoplasm of closely apposed cells (Goodenough *et al.*, 1996; Kumar and Gilula, 1996). Single gap junction channels consist of two hemi-channel subunits, with one hemi-channel contributed by each participating cell. In vertebrates, each hemi-channel represents a hexameric protein complex, which is composed of members of the connexin family of transmembrane proteins (Willecke *et al.*, 1991, 2002). Most mammalian cells express more than one connexin isoform and assemble homomeric or heteromeric hemi-channels (connexons), leading to the formation of homotypic or heterotypic gap junctions (Jiang and Goodenough, 1996; Diez *et al.*, 1999; He *et al.*, 1999; Locke *et al.*, 2000; Cottrell *et al.*, 2002). Consistent with a role in inter- and intracellular signaling processes, the connexins 32, 36, 43, and 46 are targeted to lipid rafts, which are cholesterol–sphingolipid-rich microdomains that function as membrane trafficking and signal transduction platforms (Schubert *et al.*, 2002). Electron microscopy has revealed that gap junction channels, which are formed by head-to-head-docking of hemi-channels from neighboring cells, are arranged into tightly packed plaques that may consist of hundreds or thousands of channels; forming structures of several microns in diameter (Goodenough *et al.*, 1996; Lauf *et al.*, 2002). Studies in tissue culture

have shown that connexin assembly and plaque formation is enabled by calcium-dependent core components of adherens junctions, the E-cadherins (Musil *et al.*, 1990; Jongen *et al.*, 1991; Meyer *et al.*, 1992; Fujimoto *et al.*, 1997; Xu *et al.*, 2001; for review, see Yeager *et al.*, 1998).

Connexin homologues have not been identified in invertebrates (Adams *et al.*, 2000). Instead, a separate protein family, called Innexins, which are structurally and functionally analogous to connexins, were shown to be the key components of gap junction channels (for review, see Phelan and Starich, 2001). The analysis of the few *innexin* mutants have been as yet identified in *Drosophila* and *Caenorhabditis elegans* has demonstrated an essential role for these gap junctional channel proteins in electrical signal transmission. Mutations in the *C. elegans unc-7* and *unc-9* genes result in loss-of-coordination phenotypes (Starich *et al.*, 1996; Barnes and Hekimi, 1997). In addition, *eat-5* mutants display feeding defects that result from impaired of the electrical coupling between pharynx muscles (Starich *et al.*, 1996). Similarly, mutations in the *Drosophila shaking-B* and *ogre* genes interfere with transmission at electrical synapses of the giant fiber system (Phelan *et al.*, 1996, 1998) and reduce optical ganglia size (Watanabe and Krankel, 1990), respectively. The germline-specific *Drosophila innexin 4* gene was recently shown to play a role in the survival of early germ cells during gametogenesis (Tazuke *et al.*, 2002). We have previously shown that *innexin 2* is required to control foregut morphogenesis in response to the Wingless/WNT signaling cascade (Bauer *et al.*, 2002). Whereas the mRNA expression patterns of the *Drosophila innexin* genes have been extensively studied by in situ hybridization during oogenesis and embryogenesis, virtually no studies exist on protein distribution, trafficking, assembly, turnover, or interactions with other cellular proteins. Here, we present the first study of

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* Corresponding author. E-mail address: m.hoch@uni-bonn.de.

gap junction protein distribution in *Drosophila* epithelial cells. We demonstrate an essential function for Innexin 2 for epithelial morphogenesis, and we provide evidence of interaction between Innexin 2 and other junctional core proteins in polarized epithelial cells.

MATERIALS AND METHODS

Cloning and Transgene Production

An *innexin* 2 fragment corresponding to the full-length *innexin* 2cDNA was subcloned into the pP[UAST] transformation vector (Brand and Perrimon, 1993) to generate the P[UAS-*innexin* 2] transgene. To generate a *inx* 2-GFP fusion construct a polymerase chain reaction (PCR) fragment coding for full-length *innexin* 2 was cloned via *EcoRI*/*Bam*HI in frame with the eGFP coding sequence of the pMJ Green vector (gift from K. Willecke, Universität Bonn, Bonn, Germany). The *innexin* 2-GFP fusion fragment was excised using *EcoRI*/*Not*I and inserted in the pP[UAST] to get P[UAS-*innexin* 2-GFP] transgenic flies. The nucleotide sequence of both constructs was confirmed by sequencing. The pP[UAS-*innexin* 2] and pP[UAS-*innexin* 2-GFP] constructs were introduced into *w*⁻ flies by P element-mediated transformation (Rubin and Spradling, 1982).

Genetic Manipulation of Flies

We used standard techniques for fly manipulation. The P allele *kropf*^{P16} that was used in this study is a transcript-null allele (Bauer *et al.*, 2002). The amorphic alleles *arm*^{Y35} and *shg*² were a gift from E. Knust. *cor*⁵ was a gift from A. Fehon, Duke University, Durham, NC. Homozygous mutant *kropf*^{P16} embryos were identified by the absence of green fluorescent protein (GFP) expression derived from the FM7, *GAL4-Kr::UAS-GFP* balancer. High levels of ectopic Innexin 2 were induced by crossing the *UAS-innexin* 2 lines with the ubiquitously driving maternal V32-Gal4 strain (gift from D. St. Johnston) or the *paired-Gal4* effector strain (Xiao *et al.*, 1996) that mediates expression in a pair-rule pattern in blastoderm embryos (Xiao *et al.*, 1996). *UAS-p35* was obtained from the Bloomington Stock Center, Indiana University, Bloomington, IN. To express p35 together with *innexin* 2, *w*; *UAS-innexin* 2/+; *UAS-p35*/+ males were crossed to *w*; V32-GAL4 virgin females. The effect of overexpression was analyzed in the progeny by performing anti-Innexin 2 staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Germ line clones of *kropf*^{P16} mutants were induced as described by Chou and Perrimon (1996) by using BL#1844 as FRT line and BL#1813 as ovo^{D1} line (stocks were obtained from the Bloomington Stock Center).

Immunohistochemistry

For antibody staining, embryos were fixed in 4% formaldehyde, 100 mM phosphate buffer. Otherwise, antibody staining was performed as described previously (Bauer *et al.*, 2002). Primary antibodies were as follows: anti-Innexin 1 (rabbit, 1:50), anti-Innexin 2 (rabbit, 1:50), anti-Coracle (guinea pig, 1:1000; a gift from R. Fehon), anti-Armadillo (1:10; Hybridoma Bank), anti-Crumbs (1:10; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-DE-cadherin (1:50; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GFP (1:50; Santa Cruz Biotechnology). Secondary antibodies were as follows: Alexa Fluor 488 (1:200; MoBiTec, Goettingen, Germany), Alexa Fluor 546 (1:400; MoBiTec), Cy3 (1:200, mouse; Dianova, Hamburg, Germany), Cy2 (1:100, mouse; Dianova), Cy3 (1:200, rabbit; Dianova), and Cy5 (1:100, mouse; Dianova). Fluorescent images were recorded using a Leica TSP2 confocal microscope (Leica, Wetzlar, Germany), and images of multilabeled samples were acquired sequentially on separate channels. All images were processed with Adobe Photoshop software.

Antibody Generation

Polyclonal antisera were generated in rabbits (Davids Biotechnology, Regensburg, Germany) against Innexin 1- and Innexin 2-specific oligopeptides conjugated to KLH via a N-terminal cysteine. The peptides used were FAKQVEPSKHDRK representing amino acids 348–362 of Innexin 1 and KLRLHLLLRARSRLA representing amino acids 297–310 of Innexin 2. IgGs were purified from immune serum by affinity purification.

TUNEL Staining

Fixed embryos were subjected to TUNEL assay by using the in situ cell death detection kit (TM Red; Roche Diagnostics, Mannheim, Germany). The assay was performed as described previously (Booth *et al.*, 2000).

In Vitro Translation, Immunoprecipitation, and Immunoblotting

Full-length Armadillo and epitope-tagged Innexin 2 fusion protein (6 copies of myc epitope recognized by c-Myc 9E10 mAb) were independently transcribed and translated in the presence of [³⁵S]methionine at 30°C for 2 h in a

50-μl mixture by using a TNT[®]-coupled rabbit reticulocyte lysate (Promega, Madison, WI) according to the manufacturer's instructions. To analyze the interaction of Armadillo and Innexin 2, the in vitro-translated reactions were mixed and immunoprecipitated. Immunoprecipitation was performed using the immunoprecipitation starter pack (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instruction. To prevent nonspecific binding of proteins to protein G-Sepharose, preclearing of the in vitro lysate was performed in the presence of protein G-Sepharose according to the manufacturer's instructions. Subsequently, antibody incubation and precipitation of the immune complexes from precleared in vitro lysate was done in phosphate-buffered saline (PBS) containing 5% bovine serum albumin in a final volume of 400 μl. The immunoprecipitation samples were incubated for 2 h either with anti-Arm monoclonal antibody (N-27 A1), or with mouse monoclonal anti-c-Myc 9E10 (c-Myc 9E10; Santa Cruz Biotechnology). Protein G-Sepharose (40 μl) was added to each mixture and incubated for 90 min. Beads were washed five times with excess PBS and two times with wash buffer (50 mM Tris, pH 8), resuspended in SDS-PAGE sample buffer, and boiled for 3 min. Samples were run on SDS-gel. The gel was dried and analyzed by autoradiography. To perform coimmunoprecipitation analysis using embryonic extracts, *Drosophila* embryos (0–24 h) were collected, rinsed with water, dechorionated with 50% bleach for 3 min, and rinsed with 0.1% Triton X-100. For the following immunoprecipitation, the *Drosophila* embryos were homogenized either in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris [pH 8.0]) or immunoprecipitation (IP) buffer (CHAPS-buffer; Laing *et al.*, 2001) containing PBS, 1% Triton X-100, 0.5% CHAPS, 0.1% SDS, and protease inhibitors (Roche Diagnostics). Cleared supernatant was incubated for 4 h with anti-Innexin 2 antibody, preimmune serum, and control IgG or with the control anti-c-Myc antibody followed by the addition of Sepharose beads as described above. The beads were washed three times with excess IP buffer (RIPA- or CHAPS-buffer) and three times with excess PBS. Immunoblots were blocked with nonfat dry milk in Tris-buffered saline plus 0.05% Tween. Washes were done in Tris-buffered saline plus 0.05% Tween without milk. Bound antigen was detected by enhanced chemiluminescence (Amersham Biosciences). The antibodies for immunoblotting were used at the following concentrations: anti-Armadillo (1:300; Hybridoma Bank), anti-DE-cadherin (1:300; Santa Cruz Biotechnology), and anti-Innexin 2 (1:300).

Yeast Two-Hybrid Analysis and Plasmid Construction

For the construction of the bait vector, the full-length cytoplasmic loop sequence of Innexin 2 (DNA fragment corresponding to amino acids 131–179) was cloned into the CytoTrap bait vector pSOS (Stratagene, LA Jolla, CA). An *innexin* 2 PCR product was generated using the LD 11658 clone as template together with the following primers: 5'-ggg atc ccg aag tcc tgg gaa ggc gga-3' (5' primer), and 5'-cga gct cgt cgg aag gcg tag aaa tt-3' (3' primer). Subsequently, the Innexin 2 PCR amplification product was cloned in frame with hSOS into the bait vector via the 5'-*Bam*HI and 3'-*Sac*I restriction sites.

The CytoTrap Screen was performed according the CytoTrap manual (Stratagene) instructions. After the testing of the Innexin 2CL/hSOS fusion protein for autoactivation, 100 μg of an embryonic *Drosophila melanogaster* cDNA library (catalog no. 200444; Stratagene) together with 100 μg of the bait construct were transformed into the yeast *Saccharomyces cerevisiae* temperature-sensitive mutant strain *cdc25H*. This strain contains a point mutation at amino acid residue 1328 of the *CDC25*-gene, which prevents growth at 37°C, but allows normal growth at a permissive temperature (25°C). The CytoTrap system is based on the ability of the hSOS, to complement the *cdc25* defect and to activate the yeast Ras signaling pathway (for details, see Broder *et al.*, 1998). The localization of hSOS to the plasma membrane occurs through the interaction with the target proteins, which are localized to the plasma membrane by a fused myristylation sequence. After first screening procedures and incubation at 37°C for 5 d, 17 potential interacting target clones were isolated. Five of them were confirmed in a rescree, among them a clone coding for the intracellular part of the adhesion protein DE-cadherin (aa 1350–1570). Target clone 2 was tested for lack of autoactivation by retransformation of clone 2 together with the pMyr Vector into the *cdc25H* strain.

RESULTS

A Specific Innexin 2 Antibody

As a first step toward a functional analysis of Innexin gap junction proteins in the development of tissues and organs in the *Drosophila* embryo, we generated an anti-Innexin 2 antibody against a peptide containing the C-terminal amino acids 297–310 of Innexin 2 (see MATERIALS AND METHODS; Figure 1A). This antibody recognized the 43-kDa protein band of in vitro-translated Innexin 2 protein and a band of the same size in embryonic extracts of wild-type embryos (Figure 1B). To test the specificity of the antibody, we overexpressed an Innexin 2-GFP construct in a pair rule pattern

Figure 1. Innexin 2 antibody production. (A) Predicted structure of Innexin 2 with four transmembrane domains, two extracellular loops, an intracellular cytoplasmic loop, and the intracellular N- and C-terminal domains. The amino acid sequence and the localization of the Innexin 2 peptide used for antibody production is indicated. (B) Western blot using anti-Innexin 2 antibody; in vitro translated (IVT) Innexin 2 and embryonic protein extract of 0- to 24-h wild-type embryos. (C–E) Expression of *UAS-innexin 2-GFP* by using a *paired-Gal4* driver line. Embryos were stained with anti-Innexin 2 in red and anti-GFP in green. The staining pattern is completely overlapping further confirming the specificity of the Innexin 2 antibody. (F–K) Staining of a stage 16 *kropf*^{P16} mutant embryo by using anti-Innexin 2 (red) and anti-Armadillo (green). Confocal images in F–H and I–K depict epidermal and salivary gland tissue, respectively.

Figure 2. Innexin 2 localization in the epidermis of *Drosophila* wild-type embryos. (A–C) Coimmunostaining of Innexin 2 (red) and Crumbs (green) in a stage 14 wild-type embryo. (D–F) Double staining of a stage 14 embryo monitoring expression of Innexin 2 (red) and Armadillo (green). Note the overlap of Armadillo with Innexin 2. (G–I) Innexin 2 (red) and DE-cadherin (green) double staining of a stage 14 embryo. Note the overlap of DE-cadherin with Innexin 2. (J–L) Coimmunostaining of Innexin 2 (red) and Coracle (green) in a stage 14 wild-type embryo. Shown is a magnification of the epidermal cells as compared with the perspective in F and C to better visualize the cellular distribution of Innexin 2 compared with the Coracle domain (see text for further details). (M–P) Triple staining of an early stage 15 embryo by using anti-Innexin 2 (red), anti-Coracle (green), and anti-Armadillo (blue) antibodies. Note the distribution of Innexin 2 apical to the Coracle domain, but overlapping with Armadillo in the epidermal cells.

in every other segment of the epidermis by using *paired-Gal4/UAS-innexin 2-GFP* transgenic flies and performed immunostaining by using the Innexin 2 antibody. As shown in Figure 1, C–E, the antibody detects the stripe pattern in the ectopic expression experiment. In contrast, anti-Innexin 2 antibody staining was not detected in the epidermis (Figure 1, F–H) nor in other epithelial organs such as the salivary glands (Figure 1, I–K) of late zygotic *kropf* mutants, whereas other proteins such as the β -catenin homolog Armadillo are expressed. Consistent with these *in vivo* results, protein extracts of zygotic *kropf* mutants, which are *innexin 2* transcript null (Bauer *et al.*, 2002), reveal a successive decay of maternal Innexin 2 protein from stage 12 onwards. Finally, no protein could be detected in late zygotic mutants (our unpublished data), further demonstrating the specificity of the antibody.

Dynamics of Innexin 2 Protein Localization in Epithelial Cells of Developing Embryos

Previous studies have shown that in the early embryonic stages when the segmentation pattern is determined, *innexin 2* mRNA is ubiquitously distributed in the embryo (Curtin *et al.*, 1999; Stebbings *et al.*, 2000; Bauer *et al.*, 2002; Stebbings *et al.*, 2002).

With the beginning of gastrulation, *innexin 2* transcripts become refined to a segmental pattern. In contrast, Innexin 2 was distributed rather ubiquitously during germ-band extension stage in epidermal epithelial cells, most likely due to the stability of maternally deposited protein compared with the *innexin 2* RNA. Using confocal microscopy, we were able to determine that Innexin 2 is found in a punctate pattern in the plasma membrane and to some extent also in the cytoplasm of the epithelial cells (Figure 2, C and F). Notably, this kind of protein distribution is characteristic of the vertebrate connexin gap junction proteins; they oligomerize to hexameric units that traffic in vesicles through the cytoplasm to the plasma membrane where they integrate and display mobility as hemi-channels (for reviews, see Evans and Martin, 2002a; Goodenough and Paul, 2003).

To more precisely determine the localization of Innexin 2 channel proteins along the apico-basal axis of the epithelial cells of the embryo, we performed coimmunostaining with membrane and cell polarity markers. Anti-Crumbs antibody (Figure 2, A–C) was used to visualize apical membrane localization; anti-Armadillo (Figure 2, D–F) and anti-DE-cadherin antibodies (Figure 2, G–I) were used to mark the

Figure 3. Cellular distribution of Innexin 1 and 2 in *Drosophila* wild-type embryos. Innexin 2 (red, A–F) and Innexin 1 (red, G–L) distribution. Confocal images of stage 16 salivary glands (sg) (A–C and G–I) and of stage 16 hindgut (hg) epithelia (D–F and J–L). Anti-Coracle (green) was used as a marker for septate junctions and anti-Armadillo (blue) for adherens junctions. For further information, see the text.

adherens junctions on the apico-lateral side of epithelial cells. The *DE*-cadherin transmembrane protein and the cytoplasmic protein Armadillo, which encodes the *Drosophila* homolog of β -catenin, are structural components of adherens junctions that mediate cell-cell adhesion and communication (Peifer, 1993; Cox *et al.*, 1996, 1999; Pai *et al.*, 1996; Peifer and Polakis 2000). Anti-Coracle antibody (Figure 2, J–L) was used to label the septate junctions on the lateral region of epithelial cells (Tepass and Hartenstein, 1994; for review, see Tepass *et al.*, 2001). Coracle is a septate junction-associated protein and a member of the Protein 4.1 superfamily of cytoplasmic proteins (Fehon *et al.*, 1994) that includes Protein 4.1, and the Ezrin, Radixin, and Moesin proteins (Takeuchi *et al.*, 1994).

Investigating the distribution of Innexin 2 proteins along the apico-basal axis of epidermal cells revealed an accumulation of Innexin 2 signals in the apico-lateral membrane domain. The expression domain was apical to that of Coracle (Figure 2, J–L), but colocalized with Armadillo and *DE*-cadherin (Figure 2, D–F, G–I; triple staining in M–P). It is of note, however, that we also found Innexin 2 signals in lateral and baso-lateral positions, which may reflect the mobility of the protein in the plasma membrane as suggested for vertebrate connexin hemi-channels (Figure 2C; F; Lauf *et al.*, 2002). In contrast to the apico-lateral accumulation of Innexin 2 in the epidermis, in salivary gland cells the protein accumulated in the baso-lateral region, below the septate junctions as shown by anti-Innexin 2/anti-Coracle/anti-Armadillo/triple staining (Figure 3, A–C). Finally, in hindgut epithelial cells we found an accumulation of Innexin 2 in the Coracle expression domain in the lateral membrane region of the cells (Figure 3, D–F). In contrast to the variable tissue/organ positioning of Innexin 2 along the apico-basal axis of

epithelial cells, we found an accumulation of Innexin 1 in the baso-lateral region for both the salivary gland and the hindgut (Figure 3, G–I and J–L, respectively).

Innexin 2 Is Required for Epithelial Morphogenesis

To investigate whether Innexin 2 plays an essential role during epithelial development, we analyzed the phenotype of *kropf* mutants. As shown previously, *kropf* is required for zygotic viability because approximately one-third of the homozygous *kropf* mutant animals die during the early larval stages due to a malformation of the proventriculus organ (Bauer *et al.*, 2002). The other two-thirds of the *kropf* mutants are embryonic lethal. Cuticle preparations demonstrated that these embryos often displayed large holes in the epidermis, predominantly in the head region with occasional segment defects accompanied by a reduction in the size of the embryos (Bauer *et al.*, 2002; our unpublished data). Monitoring the expression of the segment polarity gene engrailed in stage 11 wild-type and *kropf* mutant embryos reveals that the formation of epidermal segments seems to be initiated normally in a large fraction of the mutant embryos (Figure 4, A–D). However, we observed strong morphogenetic defects during germband retraction and at later stages of embryonic development. In extreme cases, we found an almost complete loss of the foregut epithelium and a strong reduction of the hindgut and Malpighian tubule tissues as revealed by anti-Crumbs antibody staining (Figure 4, E–H).

The variability of the phenotype and the mRNA expression pattern during oogenesis suggested a maternal contribution of *innexin 2*. We therefore produced germline mosaics to generate mutants lacking both the maternal and zygotic *innexin 2* expression (see MATERIALS AND METHODS; Chou and Perrimon, 1996). We found that in these embryos,

Figure 4. Epithelial defects and cell death in *kropsf* mutants. Anti-Engrailed antibody staining of stage 10 (A and B) and stage 13 (C and D) wild-type (A and C) and *kropsf*^{P16} mutant embryos (B and D). The segmental pattern of *engrailed* is not dramatically altered; however, note the prominent foregut (B, arrowhead) and the midgut defects (D, arrowhead). *kropsf*^{P16} mutant embryos are much smaller than wild-type embryos. Anti-Crumbs antibody staining of stage 10 (E and F) and stage 13 (G and H) wild-type (E and G) and *kropsf*^{P16} mutant embryos (F and H). Note that many epithelial tissues that invaginate during embryonic development, including the foregut (arrowhead), the hindgut (arrow), the Malpighian tubules, the salivary glands, and the tracheal system, are strongly reduced in size (H compared with G). Cuticle preparation and TUNEL staining of a wild-type embryo (I and L), a maternal and zygotic *kropsf* null mutant (J and M), a maternal null, zygotic heterozygous *kropsf* mutant (K and N). Armadillo (blue) was used in L and N to outline the embryos' morphology. For further details, see text. (O–Q) Ubiquitous expression of *innexin 2* by using the maternal *V32Gal4* driver and the *UAS-innexin 2* effector strains. Innexin 2 in red and Armadillo in green. (R–V) Comparison of wild-type embryos and embryos expressing ubiquitously *innexin 2* and *p35* by applying the maternal *V32Gal4* driver together with the *UAS-p35* and the *UAS-innexin 2* effector lines. (R–U) Confocal images showing immunostaining of Armadillo (green) in wild-type stage 7 embryos (R and S) and stage 7 embryos overexpressing *innexin 2* and *p35* (T and U). Note the multilayering and an irregular cell shape (U, arrowheads; compare with S). For further information, see text. (V) TUNEL assay of the same embryo as described in U. Note that apoptosis is absent indicating *p35* activity.

epithelial tissues and organs were severely affected and the cuticle failed to form (Figure 4J, compare with 4I). TUNEL staining indicated that extensive cell death occurred in these embryos (Figure 4M, compare with 4L). This phenotype is consistent with the role of gap junctions in adhesion and cell-to-cell communication, both of which are required for the integrity of cell layers and the morphogenesis of tissues and organs (for reviews, see Starich and Phelan, 2001; Evans

and Martin, 2002a). The epithelial defects and cell lethality were rescued to a large extent when one paternal copy of *innexin 2* was added back to the maternal null background (maternal null, zygotically heterozygous; Figure 4, K and N). The latter embryos showed a mutant phenotype very similar to the zygotic one, displaying large holes in the head region and showing a variable spectrum of segment defects (Figure 4K). Misexpression of Innexin 2 by using the maternal driver

line V32-Gal4, which is known to mediate very high levels of ubiquitous expression in early blastoderm embryos, also resulted in massive cell death in all epithelia of the embryo (Figure 4, O–Q); all epithelial tissues disintegrated, the cells rounded up, and the cell polarity markers lost their membrane localization. V32Gal4/UAS-*innexin 2* misexpression in the background of ubiquitous p35 activity, which results in the absence of cell death, produced multilayering and a variation of cell size in the embryos (Figure 4, T–V, compare with WT in R and S). In summary, the loss and gain-of-function data strongly suggest that correct levels of Innexin 2 are essential for both the survival of epithelial cells and to ensure proper morphogenesis of epithelial tissues.

Interaction of Innexin 2 and Other Junctional Proteins in Epithelial Cells of the Embryo

To further examine the role of Innexin 2 in epithelial cells, we determined its distribution in mutants for Coracle, DE-cadherin, and Armadillo. Coracle is a septate junction-associated protein required for dorsal closure, salivary gland morphogenesis, and cuticle formation during embryonic development (Fehon *et al.*, 1994; Lamb *et al.*, 1998). In the salivary glands of amorphic *coracle*⁵ mutants in which the septate junctions are disrupted but cell polarity is not affected (Lamb *et al.*, 1998), Innexin 2 accumulated in the apical region compared with the baso-lateral region of wild-type stage 15 embryos (Figure 5, A–C, compare with Figure 3, A–C). In amorphic *armadillo*^{YD35} mutants, we found increased levels of Innexin 2 in the cytoplasm (Figure 5, D–F). Similarly, Innexin 2 accumulated in the cytoplasm (Figure 5, G–I) of zygotic *shotgun* (DE-cadherin) mutants, which show disorganization of epithelial tissues in the head and the ventral epidermis (Uemura *et al.*, 1996; Tepass *et al.*, 1996). It is known that in the latter mutants, the level of membrane-bound Armadillo is decreased and its cytoplasmic level is slightly increased (Tepass *et al.*, 1996; Uemura *et al.*, 1996). Consistent with this finding and with our previous observation that Innexin 2 and Armadillo colocalize in the epidermis, we observed an extensive overlap of Innexin 2 and Armadillo signals in the cytoplasm of *shotgun* mutant cells in the ventral epidermis (Figure 5I, compare with Figure 2F). In zygotic *kropf* mutants in which the head region is most heavily affected, we detected the loss of expression (Figure 5J, asterisk) or the mislocalization of Coracle, Armadillo, and DE-cadherin in many cells (Figure 5K; our unpublished data). The affected cells often showed a “rounded up” phenotype and seemed to have lost polarity (Figure 5, J–L), further supporting the essential role of Innexin 2 for the maintenance of epithelia. It is of note, however, that zygotic *armadillo*, *shotgun*, and *kropf* mutants contain significant amounts of respective maternal proteins that contributed substantially to the maintenance of the general epithelial architecture and of the apico-basal polarity in many cells of the embryo; it is known that mutant effects can only be observed in a subset of tissues (Tepass *et al.*, 1996; Uemura *et al.*, 1996).

To further test the role of Innexin 2 for cell polarity, we performed misexpression experiments by using the UAS-Gal4 system (Brand and Perrimon, 1993) and examined the localization of Innexin 2 and of the various junction markers. Because we obtained massive cell death when using the maternal V32-Gal4 line to drive the UAS-*innexin 2* effector construct, we switched to the use of the *paired*-Gal4 driver line that mediates misexpression from blastoderm stage onwards in a pair-rule pattern (Gutjahr *et al.*, 1993; Xiao *et al.*, 1996). For these experiments, we used UAS-*innexin 2* or UAS-*innexin 2*-GFP effector lines to monitor misexpression.

In stage 14 wild-type embryos, Armadillo, DE-cadherin, and Coracle are rather uniformly expressed (Figure 6, A–C; our unpublished data). On misexpression of *innexin 2* or *innexin 2*-GFP, we found an accumulation of Armadillo (Figure 6, D–F and G–I) or Coracle (Figure 6, J–L) in the *paired* domains in every other segment, whereas expression of these proteins in the interstripe regions was unchanged. In summary, our loss and gain-of-function analyses provide evidence for interactions between Innexin 2 and other junctional core components.

Biochemical Interaction Studies with Innexin2 and Other Junctional Proteins

The genetic loss- and gain-of-function experiments suggest the possibility that Innexin 2 and the other junctional proteins may be part of multiprotein complexes required for epithelial development. To further examine this possibility we performed a molecular screen for interaction partners of Innexin 2 by using the yeast Ras-recruitment system (see MATERIALS AND METHODS; Broder *et al.*, 1998; Köhler and Müller, 2002). This screening system is a modification of the canonical yeast two-hybrid system in that it enables the study of membrane-associated proteins interacting with cytoplasmic proteins fused to Ras (see MATERIALS AND METHODS; Broder *et al.*, 1998). When using the cytoplasmic loop of Innexin 2 as bait to screen a 0- to 24-h embryonic *Drosophila* library, we isolated 17 putative target clones of which five were verified in the rescreen. Among the five clones isolated were a PDZ-domain protein, β -tubulin (homologues of both are known interaction partners for Connexin 43; Lehmann and Hoch, unpublished data; for review, see Evans and Martin, 2002a) and one clone encoding the cytoplasmic domain of DE-cadherin (Figure 7A; aa 1350–1570). The intracellular domain of DE-cadherin is known to contain the binding site for Armadillo, which in turn binds to α -catenin linking the complex to the actin cytoskeleton (Kemler, 1993). To further test whether an Innexin 2/DE-cadherin-protein interaction occurs in vivo in the fly embryo, we prepared extracts of 0- to 24-h wild-type embryos and performed coimmunoprecipitation analysis. As shown in Figure 7B, DE-cadherin was specifically coimmunoprecipitated by anti-Innexin 2 antibody and vice versa (Figure 7C), indicating that the two proteins may also directly interact in vivo. We also tested for an interaction of Innexin 2 with Armadillo. Because we were unable to isolate Armadillo clones with yeast two-hybrid screens by using the cytoplasmic loop or the C-terminal domain of Innexin 2 as bait, we tested for protein-protein interactions in vitro. We performed in vitro translation reactions to generate full-length Armadillo and c-Myc-tagged Innexin 2 proteins, respectively. Both translation reactions were subsequently mixed, incubated and immunoprecipitated with either anti-Armadillo or anti-c-Myc antibodies (see MATERIALS AND METHODS). As shown in Figure 7D, anti-c-Myc antibody coprecipitated Armadillo and anti-Armadillo antibody coprecipitated Innexin 2. To examine whether an interaction between these proteins occurs in vivo in the fly embryo, we prepared extracts of 0- to 24-h wild-type embryos and assayed for protein interactions by coimmunoprecipitation. As shown in Figure 7E, Armadillo was specifically coimmunoprecipitated by anti-Innexin 2 antibody and vice versa (Figure 7F), indicating that the two proteins may directly interact both in vitro and in vivo. Similarly, anti-Innexin 2 antibody also specifically coimmunoprecipitated Coracle when extracts of 0- to 24-h wild-type embryos were used (our unpublished data). In summary, our immunohistochemical, biochemical, and genetic data provide evidence

Figure 5. Defect mutants for *innexin 2* and other junctional components. (A–C) Innexin 2 (red) and Armadillo (blue) staining in stage 16 *coracle* mutants. Note the mislocalization of Innexin 2 compared with WT salivary glands of the same stage (see Figure 3, A–C). (D–F) Confocal images showing coimmunostaining of Innexin 2 (red) and Coracle (green) in the epidermis of stage 10 *armadillo* mutants. (G–I) High magnification of a coimmunostaining of Innexin 2 (red) and Armadillo (green) of the ventral epidermis of stage 10 *shotgun* (*DE-cadherin*) mutants. Note the strong overlap of Innexin 2 and Armadillo signals. (J–L) Confocal images showing coimmunostaining of Coracle (green) and Armadillo (blue) in the head region of zygotic stage 10 *kropic* mutants. Asterisk (*) marks loss of Coracle expression. For further information, see text.

that the gap junctional channel protein Innexin 2 directly interacts with other junctional core proteins.

DISCUSSION

The assembly and the cellular distribution of the junctional complement of a particular cell is a dynamic process that is

essential for the proper differentiation of cells and tissues during development (Tepass and Hartenstein 1994; Nagafuchi 2001). The role of gap junctions for cellular communication and adhesion is well known from ultrastructural analysis in both vertebrates and invertebrates. Whereas extensive studies in the last decade have elucidated the role of connexin gap junction proteins in vertebrates (for review,

Figure 6. Innexin 2 overexpression experiments. (A–C) Uniform localization of Innexin 2 (red), Coracle (green), and Armadillo (blue) in the epidermis of stage 13 wild-type embryos. (D–F) stage 15 *pairedGal4/UAS-innexin 2-GFP* embryo. Anti-Innexin 2 is in red and anti-Armadillo is in green. Note the overlapping expression pattern of Innexin 2-GFP and Armadillo. (G–I) Stage 13 *pairedGal4/UAS-innexin 2* embryo. Anti-Innexin 2 is depicted in red and anti-Armadillo is in blue. Note the overlapping expression of Innexin 2 and Armadillo. (J–L) Immunostaining of a stage 13 embryo by using a *paired-Gal4* driver line to induce Innexin 2 expression. Innexin 2 (red); Coracle (green). Note the enrichment of Coracle in the ectopic Innexin 2 stripes, compared with wild-type (A–C).

see Evans and Martin, 2002a,b), it has only recently become clear that invertebrates gap junctions are composed of Innexins, which display very low sequence similarity to the connexins (for review, see Starich and Phelan, 2001). Here, we present the first study on the distribution of gap junction proteins in developing epithelia of the *Drosophila* embryo, and we demonstrate a function of Innexin 2 for the formation of epithelia. Furthermore, our immunohistochemical and biochemical studies provide evidence that Innexin 2 may interact with other junctional core proteins in polarized epithelial cells.

Innexin 2 Is Essential for Epithelial Morphogenesis

kropf mutant embryos lacking both the maternal and zygotic *innexin* contribution fail to develop epithelial tissues, and TUNEL staining demonstrates extensive cell death. Embryos, which are maternal null and contain one copy of zygotic *innexin 2* mutants, show a milder phenotype in which many tissues go on to develop. However, in these embryos cells round up and lose polarity, which most likely produces the large cuticular holes and the variable spectrum of segment defects observed in these embryos (Figure 4). These embryos also display strong

defects in epithelial tissue and organ formation. Similarly, we find severe morphogenesis defects of epithelial tissues and organs in zygotic *kropf* mutants (Figure 4, A–H). Embryos in which Innexin 2 has been extensively overexpressed (by using V32Gal4) are embryonic lethal and display defects such as multilayering and irregular cell shapes (Figure 4, R–V); as in the loss-of-function mutants, cells round up and lose polarity when the Innexin 2 level is increased in the cells. Similar, but not as drastic effects are obtained upon mild overexpression of Innexin 2 by using drivers such as daughterless-Gal4 (Bauer and Hoch, unpublished data); the epidermal phenotype of these embryos is similar to the one of *kropf* mutant embryos. Our genetic loss- and gain-of-function experiments thus clearly demonstrate that establishing and maintaining a proper Innexin 2 level is critical for the survival of epithelial cells and for proper morphogenesis of tissues and organs in the body. This is consistent with our earlier findings of Innexin 2 controlling morphogenesis of the foregut during proventriculus development (Bauer *et al.*, 2002). Cells in tissues and organs have to coordinate their activities by communicating directly with each other during development. Gap junctions that link the cytoplasm of

Figure 7. Innexin 2 interacts biochemically with DE-cadherin and Armadillo. (A) CytoTrap Screen by using the full-length cytoplasmic loop of Innexin 2 as a bait. (B, C, E, and F) Embryonic extracts of 0- to 24-h wild-type embryos were used for immunoprecipitation with either Innexin 2-, DE-cadherin-, or Armadillo-antibody. Controls were performed either with preimmune serum (B), control IgG (E), or anti-c-Myc (C and F). (D) Immunoprecipitation with in vitro-translated (IVT) Myc-Innexin 2 (Myc-Inx2) or Armadillo. Input lanes depict one third of in vitro translated Myc-Innexin 2 and Armadillo used in the immunoprecipitation reactions. Immunoprecipitation was performed in the presence of anti-c-Myc- or anti-Armadillo-antibody. Asterisks (*) denote immunoprecipitated proteins.

neighboring cells ensure the integration of metabolic activities in interacting cellular networks. Furthermore, ultrastructural analysis has shown an important role of gap junctions in cell adhesion. In neighboring attached cells each gap junction channel is formed by head-to-head docking of two partner hemi-channels, producing gap junction channels that associate to form large clusters of plaques. It is thus likely that the deleterious effects observed in the Innexin 2 loss- and gain-of-function situations occur through changes in cell adhesion and cell-to-cell communication.

Innexin 2 Distribution in Epithelial Cells

Our immunohistochemical data determining the distribution of Innexins 1 and 2 show that both proteins are localized in a punctate pattern in the plasma membrane

and to some extent in the cytoplasm of the epithelial cells (Figures 2 and 3). A fraction of both proteins is distributed along the entire apico-basal axis of epithelial cells. However, in the case of each Innexin, we observed an accumulation of the proteins in specific membrane domains, depending on the Innexin and the type of tissue. Innexin 2 accumulates in the apico-lateral membrane domain in the epidermis, in the lateral domain in the hindgut, and in a baso-lateral position in the salivary glands (Figures 2 and 3). In contrast, we find an accumulation of Innexin 1 in the baso-lateral region for both the salivary gland and the hindgut (Figure 3). We conclude from these findings that the accumulation of Innexins in specific membrane domains of epithelial cells is likely to be regulated by tissue-specific factors during the development of epithelia in the fly embryo. A punctate pattern and localization in

the cytoplasm and the membrane also occurs in vertebrate connexins. They are cotranslationally integrated into the endoplasmic reticulum and pass through the Golgi apparatus via vesicles to the plasma membrane (Falk, 2000). Live imaging analysis in vertebrate tissue culture cells has shown that unopposed hemi-channels dynamically move within the plasma membrane until they dock end to end with partners in neighboring attached cells to generate gap junction channels. Gap junction channels laterally associate with each other to form large gap junction plaques (Lauf *et al.*, 2002; Schubert *et al.*, 2002). It is therefore possible that the punctate distribution of Innexins 1 and 2 in the membranes may reflect the mobility of hemi-channels in the plasma membrane and that accumulation of Innexin signals may reflect the association of gap junctions into plaques.

Interaction of Innexin 2 with Other Junctional Proteins

In the epidermis, Innexin 2 protein accumulates in the apicolateral membrane domain and colocalizes with Armadillo and DE-cadherin. It is of note that this applies only to a fraction of the Innexin 2 protein pool. We find that in mutants for both zygotic *armadillo* and *DE-cadherin*, the Innexin 2 localization is altered, and in Innexin 2 overexpression experiments, Armadillo and DE-cadherin are organized into Innexin 2 pattern. Further evidence for an interaction of the proteins is provided by the yeast two-hybrid analysis and the immunoprecipitation both in vitro-translated and embryonic extracts. Armadillo and DE-cadherin are core components of adherens junctions, which are multiprotein complexes mediating cell-cell adhesion and communication (fore reviews, Tepass, 1999; Nagafuchi, 2001). DE-cadherin belongs to a large family of membrane proteins that mediate Ca^{2+} -dependent homophilic adhesion (Tepass, 1999). It possesses a highly conserved cytoplasmic domain that binds to the cytoplasmic protein β -catenin/Armadillo, thereby forming a cadherin-catenin complex. β -Catenin is bound, in turn, by α -catenin to link the actin cytoskeleton to the (D)E-cadherin/ β -catenin complex (Tepass, 1999; Nagafuchi, 2001). Whether the interaction of Innexin 2 with DE-cadherin and Armadillo plays a major or minor role in epithelial cells (given that only a portion of Innexin 2 protein colocalizes with the adherens junction proteins) and at which step of the biogenesis of Innexin 2-containing gap junctions this interaction may occur is not known at this point. However, it has been demonstrated that the spatial association between Gap and adherens junctions is an important factor in the maturation of vertebrate and invertebrate tissues (Tepass and Hartenstein, 1994; Angst *et al.*, 1997). Furthermore, adherens junction assembly was demonstrated to be a prerequisite for the formation of gap junction plaques in fibroblasts (Ko *et al.*, 2000). A direct interaction of the Armadillo homologue β -catenin with connexin 43 was shown to occur in cardiac myocytes (Ai *et al.*, 2000). Tissue culture studies and the analysis of neural crest cell migration in the mouse embryo have shown that the assembly and function of gap junctions is directly controlled by E- and N-cadherins, respectively (Musil *et al.*, 1990; Jongen *et al.*, 1991; Meyer *et al.*, 1992; Fujimoto *et al.*, 1997; Xu *et al.*, 2001). Recent analysis of connexin trafficking has suggested that adherens junction formation at high Ca^{2+} -concentration leads to the formation of actin cables that directly or indirectly transport connexins from the cytoplasm to the cell-cell contact membranes via the Golgi apparatus (Hernandez-Blazquez *et al.*, 2001). Our results are thus consistent with findings in vertebrates that suggest a close link between gap junction and adherens junction biogenesis.

Our experiments further indicate that Coracle, a homologue of the vertebrate membrane-skeleton Protein 4.1, may be another Innexin 2 interaction. Coracle is a key component of septate junctions in the lateral membrane domain of epithelial cells and tissues (Fehon *et al.*, 1994; for review, see Tepass *et al.*, 2001). It has been suggested that septate junctions provide some of the functions ascribed to tight junctions in vertebrate tissues (Tepass and Hartenstein, 1994). Interactions of connexins with the tight junction proteins ZO1 (Giepmans and Moolenaar, 1998; Toyofuku *et al.*, 1998) and occludin (Kojima *et al.*, 1999) have been described and occur in hepatocytes. Although we have no evidence that the interaction of Innexin 2 with Coracle is direct, we believe that Coracle and Innexin 2 are part of multiprotein complexes that can be immunoprecipitated from embryonic extracts. Tissue and cell type-specific interaction partners for gap junction channels that may modulate the distribution, trafficking, maturation, signaling activity, and other biological functions of gap junction proteins may thus be a common feature for vertebrates and invertebrates.

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