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Original Article

Tissue-specific distribution and variation of the channel-forming protein ductin during development of *Drosophila melanogaster*

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ABSTRACT Ductins represent membrane channel proteins which are supposed to form both proton channels in V-ATPases and connexon channels in gap junctions. In order to localize and characterize these proteins in different tissues of *Drosophila*, we applied indirect immunofluorescence microscopy and immunoblots, using antisera prepared against *Drosophila* ductin and against *Nephrops* ductin. Previously, these antisera have been shown to recognize, in ovarian follicles and young embryos of *Drosophila*, the ductin monomer of 16 kDa and a putative dimer of 29 kDa. Moreover, both anti-ductin sera label antigens in plasma membranes and in the cytoplasm and block, when microinjected, cell-cell communication via gap junctions. In the present study, comparing several embryonic, larval and adult tissues, the anti-ductin sera were found to recognize antigens with various locations in cells of the midgut, the salivary gland, the nervous system, the muscles and the epidermis. For example, in midgut cells, antigens were labeled mainly in apical plasma membranes and in the apical part of the cytoplasm, while in salivary-gland cells, labeling was found throughout the plasma membranes and the cytoplasm. We conclude that putative gap junctions were revealed in the salivary gland, the nervous system and the epidermis, while plasma membrane-associated putative V-ATPases were detected in the midgut, the salivary gland and the muscles. Moreover, V-ATPases associated with cytoplasmic vesicles were found in almost every tissue. On immunoblots of homogenates from various tissues, the anti-ductin sera specifically labeled bands of 16, 21 and 29 kDa. When comparing these bands using peptide mapping with V8 protease, we found that they represent closely related proteins. Therefore, either different ductins or modifications of a single ductin appear to be present in different cellular regions, cell types and developmental stages of *Drosophila*.

KEY WORDS: *gap junction, immunocytochemistry, membrane, peptide mapping, V-ATPase.*

Introduction

In recent years, several lines of evidence have led to the conclusion that ductins represent highly conserved multifunctional proteins which form various types of membrane channels, e.g. proton channels in vacuolar-type proton pumps (subunit c) and connexon channels in gap junctions (for reviews, see Finbow and Pitts, 1993, 1998; Finbow *et al.*, 1995). Depending on their function, ductins reside in the membranes in two opposite orientations: In gap junctions, the C- and N-termini and the putative loop 2 are located on the cytoplasmic side, whereas they are located either vacuolar or extracellular in V-ATPases (Dunlop *et al.*, 1995).

Using two polyclonal anti-ductin sera, immunocytochemically and on immunoblots, we localized and characterized ductins in various tissues of *Drosophila*. One of the antisera was raised against ductin from isolated gap junctions of the lobster *Nephrops norvegicus* (Buultjens *et al.*, 1988), whilst the other was prepared against a nonapeptide at the N-terminus of ductin from *Drosophila* melanogaster (Bohrmann and Lämmel, 1998). In previous studies, using light and electron microscopy, these anti-ductin sera have been shown to bind to antigens located in the plasma membranes and in the cytoplasm of *Drosophila* ovarian follicles and, in microinjection experiments, they were found to block gap-junction mediated communication between oocyte and follicle cells. On immunoblots of ovary extracts,

Abbreviations used in this paper: AD16, affinity-purified anti-*Drosophila* ductin serum; apAN2, affinity-purified anti-*Nephrops* ductin serum; BSA, bovine serum albumin; NIS, non-immune serum; NmAbs, non-immune monoclonal antibodies; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel-electrophoresis; V-ATPase, vacuolar-type proton pump.

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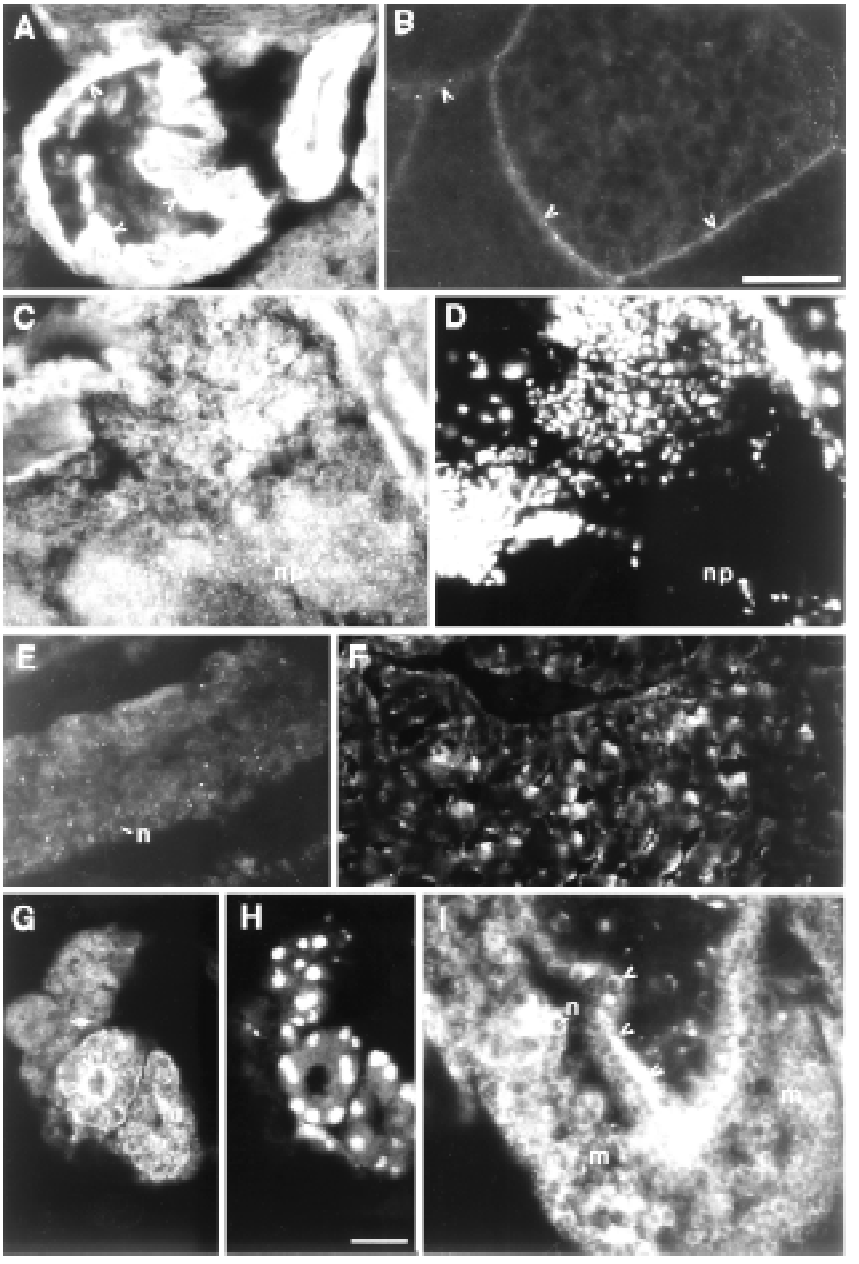

Fig. 1. Indirect immunofluorescence staining with apAN2 *(cf. Table 1).* **(A)** *Adult midgut: intense labeling in the apical cytoplasm and at the apical plasma membranes (<, brush border).* **(B)** *Larval salivary gland (whole mount): punctate and diffuse labeling in the cytoplasm and at the lateral plasma membranes (<).* **(C)** *Adult brain supraesophageal ganglion: punctate and diffuse labeling, especially in the neuropile (np).* **(D)** *DAPI staining of cryosection C, showing the nuclei.* **(E)** *Embryonic ventral nerve cord: punctate and diffuse labeling; n, nucleus.* **(F)** *Indirect flight muscles (cross section): labeling in the cytoplasm and at the plasma membranes.* **(G)** *Adult salivary duct: labeling in the cytoplasm and at the plasma membranes, especially on the apical side of the cells (<).* **(H)** *DAPI staining of cryosection G, showing the nuclei.* **(I)** *Embryonic midgut: intense labeling in the apical cytoplasm (<); labeling also in mesodermal cells (m); n, nucleus. Bars in B (B,E,F) and H (A,C,D,G-I) represent 20 μm.*

both antisera recognize the 16 kDa monomer and a 29 kDa putative dimer of ductin (Bohrmann, 1993; Bohrmann and Braun, 1999).

Also in *Drosophila* embryos, proteins of 16 and 29 kDa are labeled by the antisera, and a similar immunocytochemical pattern as in follicles is observed. When microinjected, both antisera block gap-junctional communication, resulting in a disturbance of morphogenetic movements during gastrulation and, as a consequence, in specific defects of the larval cuticle (Bohrmann and Lämmel, 1998). In the embryonic, larval and adult tissues compared in the present study, both antisera labeled closely related proteins with different molecular masses and cellular locations. Obviously, throughout development, different isoforms or modifications of ductin are prevailing in different tissues, either as components of putative gap junctions or V-ATPases.

Results

Indirect immunofluorescence microscopy

On whole mounts and cryosections of various embryonic, larval and adult *Drosophila* tissues, the *Nephrops* antiserum apAN2 and the *Drosophila* antiserum AD16 recognized cytoplasmic as well as membranous antigens (Figs. 1,2). In many cell types, especially in regions of cell-cell contact, a punctate labeling pattern was observed at the plasma membranes and in the cytoplasm. Often, the staining was very intense in the cortex of the cells, predominantly in the apical cortex of epithelial cells, and at the apical plasma membranes. Whilst punctate membrane labeling in areas of cell-cell contact is presumed to represent gap-junction structures, diffuse or continuous membrane labeling in apical and basal regions of epithelial cells, as well as labeling in cytoplasmic vesicles, is presumed to represent V-ATPase molecules. Diffuse labeling of lateral membranes, however, might either represent homogeneously dispersed gap-junction particles or V-ATPase particles. The immunocytochemical results summarized in Table 1 are specific, since in control preparations, either incubated without primary antibodies or with various NmAbs (see Materials and Methods), no staining was observed (cf. Bohrmann, 1993; Bohrmann and Braun, 1999).

In cells of the midgut, the salivary gland, the nervous system, the muscles and the epidermis, with both apAN2 and AD16 labeling was found throughout all analysed stages of development. In the midgut, the apical cytoplasm and the apical brush border were intensely stained (Figs. 1 A,I, 2 C,D,I) while, in the salivary gland, punctate as well as diffuse staining throughout the cytoplasm and at the apical and lateral plasma membranes was observed (Figs. 1 B,G,H, 2 E,H). In the nervous system, especially in the neuropile, both antisera resulted in an intense punctate as well as diffuse labeling in the cytoplasm, and apAN2 also at the plasma membranes (Figs. 1 C-E, 2 A,B). In the muscles and in the epidermis, both antisera revealed antigens in the cytoplasm and, especially apAN2, also at the plasma membranes (Figs. 1F, 2 F,G). The labeling patterns in epithelia of young embryos have been described previously (Bohrmann and Lämmel, 1998).

A detailed light and electron microscopic analysis of the patterns obtained with apAN2 and AD16 (and with additional antibodies) in the

ovary is presented elsewhere (Bohrmann and Braun, 1999). In short (Table 1): In the follicle cells, both antisera resulted in a punctate as well as diffuse labeling in the cytoplasm and at the plasma membranes. Especially in regions of cell-cell contact, with apAN2 a prominent punctate pattern was obtained at the membranes. In the nurse cells, both antisera labeled antigens in the cytoplasm and at the plasma membranes, whilst in the oocyte, very intense labeling of the yolk spheres, the cytoplasm and the oolemma was observed.

Taken together, the patterns obtained with both affinity-purified anti-ductin sera were largely similar, although, in some tissues, apAN2 clearly revealed a punctate pattern at the lateral plasma membranes, whilst AD16 showed more intense staining in cytoplasmic vesicles (Table 1). Immunocytochemically, apAN2 seems to preferentially bind to ductin as a component of gap junctions, whereas AD16 preferentially binds to ductin as a component of V-ATPases (cf. Bohrmann and Braun, 1999). Therefore, it is reasonable to conclude that putative gap junctions were revealed in the epidermis of the embryo; in the salivary gland, the nervous system and the epidermis of the larva; and in the salivary gland, the nervous system and the ovary of the adult. Moreover, plasma membrane-associated putative V-ATPases were detected in the midgut, the salivary gland, the muscles and the ovary, while vesicle-associated V-ATPases were found in almost every tissue.

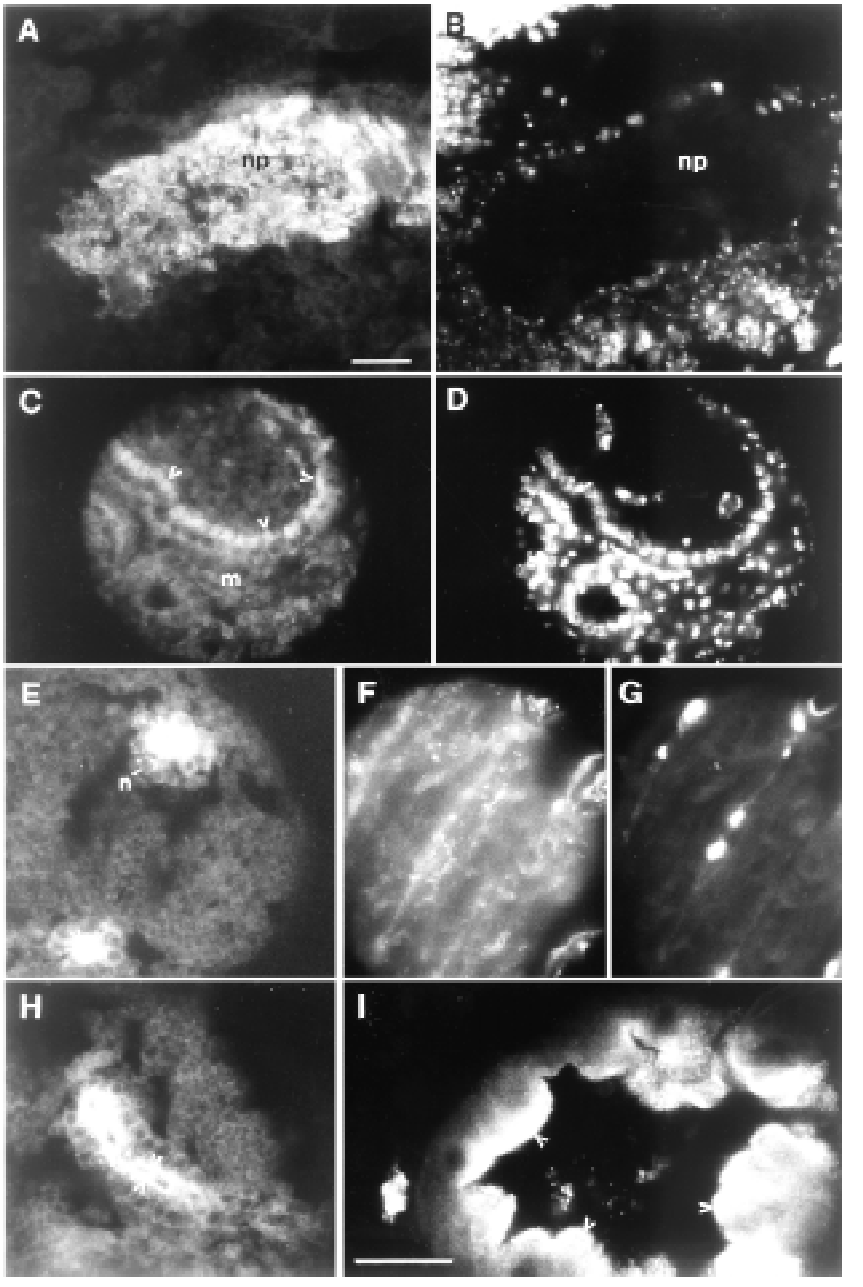

Fig. 2. Indirect immunofluorescence staining with AD16 *(cf. Table 1).* **(A)** *Larval brain: punctate and diffuse labeling in the neuropile (np).* **(B)** *DAPI staining of cryosection A, showing the nuclei.* **(C)** *Embryonic midgut: intense labeling in the apical cytoplasm (<); m, mesodermal cells.* **(D)** *DAPI staining of cryosection C, showing the nuclei.* **(E,H)** *Embryonic salivary gland (E, cross section, H, longitudinal section): punctate and diffuse labeling in the cytoplasm and at the plasma membranes, especially on the apical side of the cells (<); n, nucleus.* **(F)** *Larval muscle: punctate and diffuse labeling.* **(G)** *DAPI staining of cryosection F, showing the nuclei.* **(I)** *Adult midgut: intense labeling in the apical cytoplasm and at the apical plasma membranes (<, brush border). Bars in A (A-H) and I, resperesent 20 μm.*

Immunoblots

In homogenates of various embryonic, larval and adult *Drosophila* tissues, both apAN2 and AD16 specifically recognized more than one protein. Figure 3 shows representative examples of our immunoblots. On control blots, either incubated without primary antibodies or with various NmAbs (see Materials and Methods), no staining was obtained (cf. Bohrmann, 1993; Bohrmann and Braun, 1999). Throughout oogenesis, large amounts of a 29 kDa protein were detected with both anti-ductin sera: in previtellogenic follicles (up to stage 7), in nurse-cell and oocyte fragments of vitellogenic follicles (stage 10), and in mature eggs (stage 14). This protein, which appears to be a dimeric cytoplasmic form of ductin, is synthesized in excess in the nurse cells and accumulates in the oocyte. In membrane-enriched fractions of ovary cells, also the 16 kDa monomer was detected with apAN2 (Bohrmann and Braun, 1999). With AD16, in addition to the monomer and the putative dimer, a protein of 21 kDa was detected. In the embryo, the 16 kDa and the 29 kDa protein were labeled by both antisera, the 29 kDa protein being prevalent. In the larval thorax (consisting mainly of muscles), the 21 kDa band was very prominent, whereas the bands of 16 and 29 kDa appeared weaker (especially with apAN2). In the larval brain, the 16 kDa monomer and small amounts of the 29 kDa putative dimer were detected, while in the salivary gland, besides the 16 kDa band, the bands of 21 kDa (apAN2) and 29 kDa (AD16) were clearly labeled. In the adult head (consisting mainly of nervous tissue), the proteins of 21 and 29 kDa were detected, and in the adult thorax (consisting mainly of muscles), the 16 kDa band (apAN2) and the bands of 21 and 29 kDa (AD16) were labeled.

Taken together, besides some minor bands, which are considered to either represent proteolytic fragments or non-specific binding of the antibodies, proteins of 16, 21 and 29 kDa were consistently labeled by both affinity-purified anti-ductin sera, although in part only

by one of them. Therefore, we analysed the molecular relationship between these three proteins in closer detail.

Peptide mapping by limited proteolysis

For the investigation of protein relationships, various peptide mapping techniques have been developed (reviewed in Andrews, 1990; Carrey, 1990). For our purpose, partial digestion with *Staphylococcus aureus* V8 protease in SDS gels (Cleveland *et al.*, 1977) appeared to be best suited. The peptide maps were visualized by Coomassie blue staining, by silver staining, and by immunoblot analysis, respectively. In order to optimize the results, we varied the protein amount in the gel slices, the protease concentration in the sample wells, and the incubation time in the stacking gels. We found the qualitative appearance of the peptide band patterns to be largely insensitive to the hydrolysis conditions. However, when comparing the patterns obtained with different staining methods, several differences were found. Figure 4 shows representative examples of the peptide maps.

By means of immunoblots, the larval 21 kDa protein and the ovarian 29 kDa protein were identified on Coomassie-blue stained gels and cut out exactly. Although rather large amounts of the two proteins were subjected to limited proteolysis, Coomassie-blue staining always resulted in relatively faint peptide maps showing only a few bands. However, the fragment patterns of both proteins were rather similar (Fig. 4A), and the 29 kDa protein led to distinct fragments of 21 kDa and 16 kDa, while the map of the 21 kDa protein showed a 16 kDa band. Silver staining is more sensitive than Coomassie-blue staining and resulted in peptide maps showing many fragments, especially in the low molecular-mass range. The fragment patterns of both analysed proteins were largely identical (Fig. 4B). Since smaller protein amounts were applied, the 21 kDa and the 29 kDa protein disappeared almost completely. Besides several other common fragments, a 16 kDa fragment was detected in both maps, and the map of the 29 kDa protein also showed a 21 kDa fragment. Immunological detection of the fragment patterns with the anti-ductin serum AN2 confirmed that the 21 kDa and 29 kDa bands had been cut out correctly (Fig. 4C). This method resulted in peptide maps showing a small quantity of strong bands. Since the antiserum AN2 is supposed to

TABLE 1

LABELING OBTAINED WITH THE ANTI-DUCTIN SERA APAN2 AND AD16 IN EMBRYONIC, LARVAL AND ADULT TISSUES OF *DROSOPHILA*

	apAN2			AD16		
	Embryo	Larva	Adult	Embryo	Larva	Adult
Midgut	**aC**	(C)	**aC,aM**	**aC**	(C)	**aC,aM**
Salivary gland	aC	**C*,M***	**C*,M***	**aC*,aM***	C*,M*	C*,M*
Nervous system	**C***	C*,M*	**C*,M***	C	**C***	C*
Muscles	(C)	C*	**C*,M**	(C)	**C***	C
Epidermis[1]	C,M*	C*,M*	(C)	C,M	C	(C)
Ovary[2]	—	—	F:C*,M*	—	—	F:C*,aM
	—	—	N:C*,M	—	—	N:C*
	—	—	O:C*,M	—	—	O:C*,M

bold: see also Figs. 1 and 2; a, apical; C, cytoplasm; F, follicle cells; M, plasma membranes; N, nurse cells; O, oocyte; *, punctate (in addition to diffuse) labeling; ( ), weak labeling; —, not analysed; [1], see Bohrmann and Lämmel (1998); [2], see Bohrmann and Braun (1999).

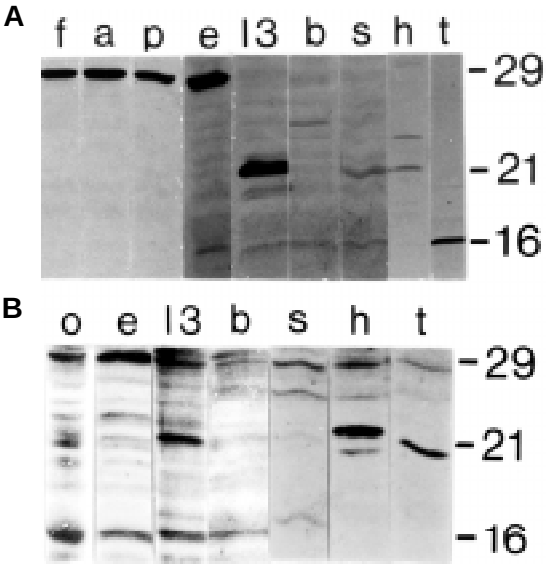

Fig. 3. Immunoblots of homogenates from various organs, body parts and developmental stages, showing specific binding of the anti-ductin sera apAN2 (A) and AD16 (B). *The bands recognized by both affinity-purified antisera are marked on the right (M_r x 10^3). o, adult ovary; f, previtellogenic ovarian follicles (up to stage 7); a, anterior part (nurse cells) of stage-10 follicles; p, posterior part (oocyte) of stage-10 follicles; e, embryo and first larval instar; l3, thorax of third larval instar; b, brain of third larval instar; s, salivary gland of third larval instar; h, adult head; t, adult thorax.*

recognize epitopes near the C-terminus and/or in loop 2 of *Drosophila* ductin (Bohrmann and Lämmel, 1998; Bohrmann and Braun, 1999), only a few proteolytic fragments containing these epitopes can be expected (Carrey, 1990). Although common fragments were labeled in both lanes, a 16 kDa fragment was strongly labeled in the map of the 21 kDa protein only. The *Nephrops*-ductin lane showed the 16 kDa monomer as well as the 29 kDa dimer. We were not able to analyse the fragment pattern of the 16 kDa band since, under the applied experimental conditions, the monomer did not show fragmentation (cf. Finbow *et al.*, 1984).

Taken together, the peptide maps obtained with the different staining methods show that the 21 kDa protein and the 29 kDa protein are closely related with each other as well as with the 16 kDa protein. These results strongly support the hypothesis that the ovarian 29 kDa protein is a maternal cytoplasmic precursor of the 16 kDa protein ductin, most likely a dimer (cf. Bonafede *et al.*, 1995; Bohrmann and Lämmel, 1998; Bohrmann and Braun, 1999). Moreover, the 21 kDa protein appears to be another modification of ductin, present in various tissues of *Drosophila*.

Discussion

In previous studies, ductins have been found to be components of both V-ATPases and gap junctions (e.g. Finbow *et al.*, 1994; Dunlop *et al.*, 1995). In arthropods, searches for homologues of connexins, that form connexon channels in gap junctions of vertebrates, have been unsuccessful, whereas ductins appear to be the predominant gap-junction proteins (John *et al.*, 1997). However, ductins might not be the only connexon-forming proteins in arthropods, since also 40 kDa proteins have been

observed in gap junctions of *Drosophila* and *Heliothis* (Ryerse, 1995). Moreover, members of the innexin family have been found to be components of gap-junction structures in *Drosophila* and *Schistocerca* (e.g. Phelan *et al.*, 1998; Curtin *et al.*, 1999; Ganfornina *et al.*, 1999; Todman *et al.*, 1999; Zhang *et al.*, 1999). On the other hand, these proteins, having no homologies with connexins or with ductins, might be accessory elements which facilitate the formation of gap junctions (Lo, 1999). Such a function, however, has also been attributed to connexins (Finbow and Pitts, 1993, 1998) and to ductins (Saito *et al.*, 1998).

In the present study, we analysed the tissue-specific distribution and variation of ductins in *Drosophila*. Since V-ATPases and gap junctions are known to reside in distinct cellular regions, antisera raised against ductins can be used for the localization of both structures. The antiserum AD16 has been prepared against a peptide at the N-terminus of *Drosophila* ductin, a part of the molecule that is known to be largely species-specific (Dow *et al.*, 1992). AD16 only contains antibodies specific for epitopes in this region, since it has been affinity-purified against the peptide used for immunization. According to database searches and experiments performed with tissues from other species, the epitopes recognized by AD16 are specific for *Drosophila* ductin (unpublished observations). Since, in the gap-junction form of ductin, after fixation the AD16 epitopes appear to be less accessible than *in vivo* to the antibodies (Bohrmann and Lämmel, 1998; Bohrmann and Braun, 1999), we conclude that our immunolocalization with AD16 primarily reveals the distribution of V-ATPases. The affinity-purified antiserum apAN2 is directed against ductin epitopes on the cytoplasmic side of gap junctions from *Nephrops* (Buultjens *et al.*, 1988). This antiserum has been found to cross-react with gap-junction proteins from *Drosophila* and from other species (reviewed in Finbow and Pitts, 1993). However, in V-ATPases of cytoplasmic vesicles, the apAN2 epitopes of *Drosophila* ductin, which are presumed to be located near the C-terminus and/or in loop 2, appear to be less accessible to the antibodies (Bohrmann and Lämmel, 1998; Bohrmann and Braun, 1999). In the embryonic, larval and adult tissues compared in the present study, both anti-ductin sera showed identical labeling in specific regions of the plasma membranes and the cytoplasm in cells of the midgut and the salivary gland. Thus, we conclude that AD16 and apAN2 labeled identical ductin molecules in these tissues. On the other hand, in the nervous system, the muscles and the epidermis, both antisera recognized cellular antigens in part differently. In these tissues, apAN2 appears to preferentially label gap junctions, whilst AD16 preferentially labeled V-ATPases.

Electron-microscopically, gap junctions have been observed - although seldom in part - in almost every embryonic tissue of *Drosophila* (Tepass and Hartenstein, 1994). Using antisera against ductin, gap junctions are detectable beginning with gastrulation (Bohrmann and Lämmel, 1998). In the present study, we revealed putative gap junctions in the salivary gland, the nervous system and the epidermis. In the ovary, gap junctions have been detected immunocytochemically, using light and electron microscopy, in the oolemma, in apical and lateral follicle-cell membranes, and in nurse-cell membranes. In the nurse cells, large amounts of ductin molecules are synthesized and stored in the oocyte as maternal precursors for later use during development (Bohrmann, 1993; Bohrmann and Haas-Assenbaum, 1993; Bohrmann and Braun, 1999).

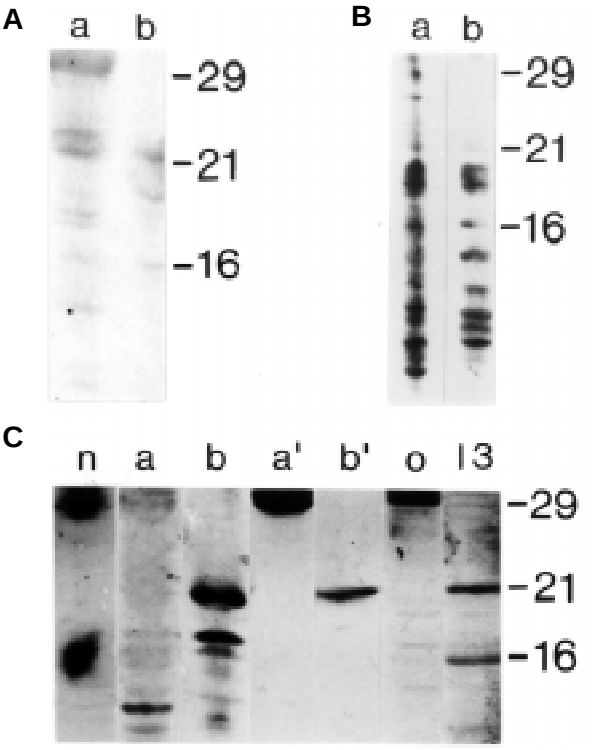

Fig. 4. Peptide maps of the 29 kDa and the 21 kDa protein, obtained using partial digestion with V8 protease. (A) *Coomassie blue staining,* **(B)** *silver staining and* **(C)** *immunoblot with anti-ductin serum AN2; bands of special interest are marked on the right (M_r x 10^3). a, map of 29 kDa protein; b, map of 21 kDa protein; a', isolated ovarian 29 kDa protein; b', isolated larval 21 kDa protein; n, Nephrops norvegicus ductin (16 kDa monomer and 29 kDa dimer); o, adult ovary; l3, thorax of third larval instar.*

V-ATPases are required for the acidification of many types of organelles (for reviews, see Nelson, 1992; Finbow and Harrison, 1997). In the *Drosophila* ovary, V-ATPases are presumed to generate the low pH of lysosomes or small vesicles, in which ligand-receptor complexes become dissociated, whilst during embryogenesis, V-ATPases are likely to be necessary for the processing of yolk (Bohrmann and Braun, 1999). V-ATPases coupled to secondary active antiport mechanisms appear to energize transport processes across plasma membranes in many species (for reviews, see Wieczorek, 1992; Harvey and Wieczorek, 1997). In insects, V-ATPases have been detected in the plasma membranes of several cell types: for example, in the midgut of *Manduca* (Jäger *et al.*, 1996), *Heliothis* (Pietrantonio and Gill, 1997) and *Aedes* (Gill *et al.*, 1998); in Malpighian tubules of *Formica* (Garayoa *et al.*, 1995), *Heliothis* (Pietrantonio and Gill, 1995), *Drosophila* (Dow *et al.*, 1997) and *Aedes* (Gill *et al.*, 1998); in salivary glands of *Periplaneta* (Just and Walz, 1994); and in ovarian follicles of *Manduca* (Janssen *et al.*, 1995), *Hyalophora* (Wang and Telfer, 1998) and *Drosophila* (Bohrmann and Braun, 1999). In the present study, plasma membrane-associated putative V-ATPases were detected in cells of the midgut, the salivary gland and the muscles, whereas vesicle-associated V-ATPases were found in almost every analysed cell type.

On immunoblots, both AD16 and apAN2 labeled proteins of 16, 21 and 29 kDa. Although it is not possible to exactly correlate one protein band with a particular immunocytochemical result, it is

obvious that the 29 kDa band is associated with strong cytoplasmic staining, while the 21 kDa and 16 kDa bands are primarily associated with punctate cytoplasmic and membrane labeling. We further analysed these bands using peptide mapping by limited proteolysis with V8 protease, a method that has widely been used to uncover molecular relationships (Andrews, 1990; Carrey, 1990). The peptide maps revealed that the three proteins are closely related with each other, and we conclude that the 29 kDa protein is a dimeric form of ductin. In several other species, aggregation of ductin has also been observed (Finbow *et al.*, 1992, 1994; Pietrantonio and Gill, 1995), the cytoplasmic oligomers being assembled and stored in the endoplasmic reticulum (Dunlop *et al.*, 1995).

Besides the ductin gene *Vha16* (Meagher *et al.*, 1990; Finbow *et al.*, 1994), three additional ductin genes, some of which may be pseudogenes, have recently been identified through the *Drosophila* genome project (*Vha16-2*, *Vha16-3* and *Vha16-4*; Dow, 1999). According to our database searches, the antiserum AD16 only recognizes epitopes in the protein derived from the gene *Vha16*, and not in the proteins derived from the other genes, whilst the epitopes recognized by apAN2 are not known. Therefore, either different ductins or post-transcriptional/post-translational modifications of a single ductin, that are differently recognized by the antisera, appear to be used for different purposes in different cellular regions, cell types and developmental stages.

Materials and Methods

Tissue preparation

Drosophila melanogaster Oregon R flies were reared at room temperature on standard food and anaesthetized with carbon dioxide. Body parts and organs (head, thorax, ovary) were isolated in Robb's saline (Robb, 1969; Bohrmann, 1991) using tweezers and tungsten needles. Anterior parts (head and thorax) and organs (brain, salivary glands) were isolated from third instar larvae. Embryos of different stages were collected, washed and dechorionated with diluted bleach using standard methods (Wieschaus and Nüsslein-Volhard, 1986).

Antibodies

For immunocytochemical and immunoblot analysis, we used the following antisera: (1) the chicken antiserum AD16 raised against the 16 kDa protein ductin from *Drosophila melanogaster* (Bonafede and Bohrmann, 1996; Bohrmann and Lämmel, 1998; Bohrmann and Braun, 1999), (2) the rabbit antiserum AN2 raised against gap-junction proteins of the lobster *Nephrops norvegicus* (Buultjens *et al.*, 1988; Leitch and Finbow, 1990), and (3) AN2 affinity-purified against *Nephrops* ductin (apAN2). A rabbit non-immune serum (NIS) as well as several anti-*Manduca* V-ATPase mAbs (Klein *et al.*, 1991; Jäger *et al.*, 1996), that gave negative results on *Drosophila* ovary preparations (NmAbs; cf. Bohrmann and Braun, 1999), were used as controls. AN2, apAN2 and NIS were kindly provided by M. E. Finbow (Glasgow, UK), whilst the NmAbs were gifts from U. Klein (München, Germany). AD16 was prepared by nanoTools (Denzlingen, Germany) against the maleimide-activated KLH-coupled nonapeptide Met-Ser-Ser-Glu-Val-Ser-Ser-Asp-Asn(-Cys) from the N-terminus of *Drosophila* ductin, and affinity-purified using a peptide-affinity column (sequence of the *Vha16* gene according to Meagher *et al.*, 1990, and Finbow *et al.*, 1994).

Indirect immunofluorescence preparations

For the immunostaining of cryosections, the body parts and organs were fixed for 30 minutes at 4°C in 4% formaldehyde dissolved in phosphate buffered saline (PBS). Embryos were fixed in 4% formaldehyde dissolved in PBS/heptane and vitelline envelopes were removed using methanol (Bohrmann and Lämmel, 1998). After washing in PBS, the tissues were embedded in a cryosectioning medium, frozen and cut into sections of 7-10 µm, which then were treated with Tween-20/PBS, NH_4Cl/PBS and skimmed milk powder/PBS (for details, see Bohrmann and Braun, 1999). Thereafter, the preparations were incubated overnight at 4°C in 0.5% bovine serum albumin (BSA)/PBS (control), in various NmAbs of different dilutions with 0.5% BSA/PBS (controls), in AD16 (diluted 1:20), and in apAN2 (diluted 1:200), respectively. With these dilutions optimal results were obtained. Following incubations in the appropriate biotinylated rabbit anti-chicken or goat anti-rabbit antibodies (Dianova, Hamburg, Germany), Streptavidin-Texas Red and DAPI were applied as described previously (Bohrmann, 1993). The preparations were mounted in 0.5% propylgallate/0.5 M Tris, pH 9, and photographed on Kodak T-Max film (400 ISO) using a Zeiss epifluorescence microscope.

For the immunostaining of whole mounts, the larval and adult organs and the embryos were fixed and treated as described above. Thereafter, they were washed in PBS, blocked with 2% BSA/0.1% Triton X-100/PBS and incubated overnight at 4°C in 0.5% BSA/0.1% Triton X-100/PBS (control), in various NmAbs of different dilutions with 0.5% BSA/0.1% Triton X-100/PBS (controls), in AD16 (diluted 1:20), and in apAN2 (diluted 1:200), respectively. Following incubations in the appropriate biotinylated secondary antibodies as well as in Streptavidin-Texas Red and DAPI, the preparations were mounted and photographed as described above. Each experiment was performed at least three times.

Immunoblotting

Immunoblots were performed as described previously (Bohrmann, 1993). The following samples were prepared: body parts of adult flies and third instar larvae; adult and larval organs; embryos and first instar larvae; previtellogenic ovarian follicles; nurse cell and oocyte fragments of stage-10 follicles (cf. Bohrmann and Gutzeit, 1987), and *Nephrops* gap-junction preparations (kindly provided by M. E. Finbow). The samples were homogenized and sonicated in SDS sample buffer containing 0.1 mM phenylmethylsulphonyl fluoride. Dissolved proteins were, either directly or after brief boiling, separated using 15% SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with skimmed milk powder/PBS and the membranes were incubated in 1% BSA/PBS (control), in various NmAbs of different dilutions with 1% BSA/PBS (controls), in NIS (diluted 1:200; control), in AD16 (diluted 1:50), in apAN2 (diluted 1:200), and in AN2 (diluted 1:200), respectively. With these dilutions optimal results were obtained. Subsequently, the appropriate biotinylated secondary antibodies (see above), Streptavidin-peroxidase and H_2O_2/chloronaphthol were applied, and photographs were taken on Agfaortho film (12 ISO). Each experiment was performed at least three times.

Peptide mapping by limited proteolysis

In order to analyse the molecular relationship of proteins, we used the method for peptide mapping described by Cleveland *et al.* (1977). Individual protein bands (identified by immunoblot analysis) were cut out of 15% SDS gels that were stained with Coomassie brilliant blue R-250. The gel slices were then placed in sample wells of 15% SDS gels having longer stacking gels, wherein partial digestion of the proteins was carried out using *Staphylococcus aureus* V8 protease (endoproteinase Glu-C; Sigma, Deisenhofen, Germany). After electrophoresis, the peptide maps were visualized either by Coomassie blue staining, by silver staining according to Heukeshoven and Dernick (1988), or by immunoblot analysis as described above, and photographed on Agfaortho film (12 ISO). In different experiments, the protein amount in the gel slices, the protease concentration in the sample wells, and the incubation time in the stacking gels, respectively, were varied (cf. Cleveland *et al.*, 1977; Andrews, 1990; Carrey, 1990). Each experiment was performed at least three times.

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