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Amalgam is a ligand for the transmembrane receptor neurotactin and is required for neurotactin-mediated cell adhesion and axon fasciculation in *Drosophila*

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Neurotactin (NRT), a member of the cholinesterase-homologous protein family, is a heterophilic cell adhesion molecule that is required for proper axon guidance during *Drosophila* development. In this study, we identify amalgam (AMA), a member of the immunoglobulin superfamily, as a ligand for the NRT receptor. Using transfected Schneider 2 cells and embryonic primary cultures, we demonstrate that AMA is a secreted protein. Furthermore, AMA is necessary for NRT-expressing cells both to aggregate with themselves and to associate with embryonic primary culture cells. Aggregation assays performed with truncated NRT molecules reveal that the integrity of the cholinesterase-like extracellular domain was not required either for AMA binding or for adhesion, with only amino acids 347–482 of the extracellular domain being necessary for both activities. Moreover, the NRT cytoplasmic domain is required for NRT-mediated adhesion, although not for AMA binding. Using an *ama*-deficient stock, we find that *ama* function is not essential for viability. Pupae deficient for *ama* do exhibit defasciculation defects of the ocellar nerves similar to those found in *nrt* mutants.

Keywords: amalgam/axon fasciculation/cell adhesion/*Drosophila*/neurotactin

Introduction

Development of the nervous system is dependent on a series of coordinated cellular movements and specific cell–cell interactions. The specificity of these interactions is regulated by extracellular guidance cues and corresponding cell surface receptors. In recent years, a number of molecules involved in these heterophilic and homophilic recognition processes have been characterized. These include the netrins and their receptors (DCC and UNC5 protein families), semaphorins and their receptors (neuropilins and plexins) and ephrins and their receptors (the Eph class of tyrosine kinase receptors), to name a few (for a review see Tessier-Lavigne and Goodman, 1996).

Another class of molecules that share a common conserved domain structure is the serine esterase-like family of membrane proteins. Vertebrate members include rat thyroglobulin (Di Lauro *et al*., 1985) and neuroligin-1 (NL-1) and NL-2, which are rat neuronal cell surface proteins that act as ligands for β-neurexins (Ichtchenko *et al*., 1995). Neurexins are highly polymorphic cell surface molecules that are candidates for mediating cell recognition between neurons. Among invertebrates, this serine esterase-like family of proteins includes glutactin (GLT), a *Drosophila* glycoprotein localized to basement membranes (Olson *et al*., 1990), gliotactin (GLI), a *Drosophila* transmembrane protein expressed on peripheral glia and required to form the blood–nerve barrier (Auld *et al*., 1995), and *Drosophila* neurotactin (NRT), a transmembrane heterophilic cell adhesion molecule (Barthalay *et al*., 1990; De la Escalera *et al*., 1990; Hortsch *et al*., 1990; Darboux *et al*., 1996).

NRT was identified initially in a screen for monoclonal antibodies that specifically labeled antigens expressed in the developing nervous system (Piovant and Léna, 1988; Barthalay *et al*., 1990; De la Escalera *et al*., 1990; Hortsch *et al*., 1990). This molecule is a 135 kDa cell surface glycoprotein that has a 500 amino acid extracellular domain related to the cholinesterase family, and a 324 amino acid cytoplasmic domain. During development, NRT is transiently expressed at the surface of neuronal and epithelial cells during embryonic and larval stages but is not expressed in adult tissues, suggesting that NRT serves a specific function during development. This function was demonstrated by the isolation of loss-of-function *nrt* mutants that show subtle defects in axon guidance in embryonic and post-embryonic development (Speicher *et al*., 1998). These defects are increased when *nrt* mutations are combined with mutations in a number of other cell adhesion/cell recognition molecules, such as *neuroglian* (*nrg*). Consistent with these genetic studies, NRT has been shown to function as a heterophilic cell adhesion molecule. Schneider 2 (S2) cells expressing the NRT protein are able to interact with dissociated embryonic primary culture cells in an NRT-dependent manner (Barthalay *et al*., 1990).

These observations clearly suggest the existence of an NRT ligand or receptor. One obvious requirement for two proteins to function as receptor and ligand is to be co-localized in the same tissues during development. Among the proteins whose expression pattern has been described, amalgam (AMA) is one possible candidate. The *ama* gene is located within the Antennapedia complex and the

protein exhibits amino acid similarities to vertebrate neural cell adhesion molecules and other members of the immunoglobulin superfamily. The 333 amino acid AMA protein consists of a signal sequence, three immunoglobulin-like domains and a short C-terminal region (Seeger *et al*., 1988).

Accumulation of AMA is first observed during early stage 8 of embryogenesis, shortly after the formation of the three germ layers during gastrulation. At this stage, NRT is already expressed throughout the ectoderm and mesoderm. During germ band extension, AMA begins to be expressed within a row of midline cells that appear to be a subset of mesectodermal cells; NRT is expressed by midline cells and also more generally by the ectoderm layer (Seeger *et al*., 1988). During embryonic stages 11 and 12, from the fully extended germ band through germ band shortening, neuroblasts undergo a series of asymmetric cell divisions to produce ganglion mother cells that in turn divide symmetrically, generating two neurons. NRT is expressed ubiquitously in the ectoderm layer, outlining the epithelial cells and the developing neuroblasts and their progeny (Piovant and Léna, 1988). The AMA expression pattern is more restricted: AMA is not found on the neuroblasts; however, high levels of protein are found on their progeny (Seeger *et al*., 1988). Both proteins are expressed in a subset of mesodermal derivatives including the fat body and the dorsal vessel. By stage 13 of embryogenesis, mature neurons are extending axons along stereotyped pathways forming the segmentally repeated arrays of commissural and longitudinal axon bundles or fascicles. During these stages, AMA and NRT accumulation is seen within the fat body and throughout the central nervous system (CNS) on both neuronal cell bodies and their axons (Piovant and Léna, 1988; Seeger *et al*., 1988; De la Escalera *et al*., 1990; Hortsch *et al*., 1990). During early stages of peripheral nervous system (PNS) development, NRT is expressed weakly on axon pathways that connect the ventral, lateral and dorsal clusters, while AMA expression is not detectable. However, in later stage embryos, both proteins concentrate on external sensory organ precursors. Overall, the patterns of AMA and NRT accumulation during embryogenesis are strikingly similar.

In this study, we investigate whether AMA is a ligand for the NRT receptor. Using S2 cells as an experimental system, we demonstrate that AMA is secreted into the culture medium and that AMA-containing media can promote NRT-expressing S2 cells to aggregate with themselves. Furthermore, we find that AMA mediates the association of NRT-expressing S2 cells with dissociated embryonic primary culture cells, the original assay that defined the heterophilic adhesion function of NRT. Finally, we show that *ama* function is not essential for embryonic and adult development; however, *ama*-deficient pupae do exhibit defects in fasciculation of the ocellar pioneer nerves similar to those described for *nrt* mutants.

Results

AMA is a secreted protein

We previously demonstrated that NRT is a transmembrane protein whose extracellular domain is able to bind a ligand(s). Heterotypic binding assays utilizing embryonic cells obtained from gastrula stage embryos or transfected S2 cells expressing NRT protein (Barthalay *et al*., 1990) indicated that an NRT ligand(s) is present on the surface of embryonic cells. This NRT ligand was also found as a soluble form, since auto-aggregation of NRT-expressing S2 cells could be induced with a 100 000 *g* supernatant prepared from embryonic extracts (Darboux *et al*., 1996).

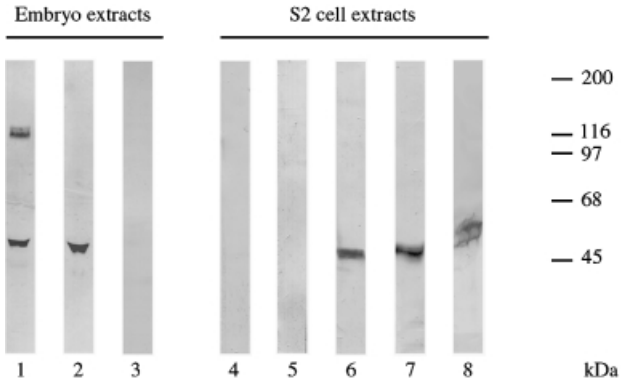

Fig. 1. AMA is a secreted protein. Wild-type and *ama*-deficient embryos were collected at gastrula stage and then mechanically dissociated into single cells. Soluble and membrane fractions were prepared by differential centrifugation at 14 000 and 100 000 *g*. SDS–PAGE and western blot analysis were performed on the wild-type 100 000 *g* pellet (lane 1) and on the supernatant of wild-type (lane 2) and *ama*-deficient embryos (lane 3). Neither S2 cells (lane 4) nor the culture medium (lane 5) displays detectable amounts of AMA protein. AMA transfectants (lane 6) and the corresponding culture medium (lane 7) accumulate significant levels of AMA protein. Expression of the AMA-TM construct was evaluated by SDS–PAGE and western blot analysis (lane 8). All blots were revealed with anti-AMA antisera.

Fractionation experiments using embryo extracts showed that AMA was present in soluble fractions. Western blot analysis with AMA-specific antisera indicated that extracts prepared from embryonic cells contained immunoreactive polypeptides of ~45 kDa within the membrane fraction (Figure 1, lane 1) and in the 100 000 *g* supernatant (Figure 1, lane 2). Higher molecular weight bands were only observed in the membrane fraction pellet and could be related to AMA molecules trapped in protein complexes. The *ama* gene encodes a protein with an N-terminal signal sequence and a weakly hydrophobic C-terminal domain. Immunostaining of whole-mount embryos suggests that AMA is a membrane-associated protein, although the weakly hydrophobic C-terminal domain is unlikely to tether AMA directly to the membrane (Seeger *et al*., 1988).

In order to confirm further that AMA is a secreted protein, we transfected S2 cells with a plasmid construct encoding the *ama* cDNA under the control of an inducible metallothionein promoter. After induction with divalent cations, products were immunodetected by western blot analysis of whole-cell extracts (Figure 1, lane 6). The culture medium in which AMA transfectants had grown contained soluble AMA protein (Figure 1, lane 7), while the culture medium where non-transfected cells had grown did not (Figure 1, lane 5). Non-transfected S2 cells expressed a very weak level of endogenous AMA protein (Figure 1, lane 4). Apparently this low level of expression is not sufficient to promote auto-aggregation of S2 cells transfected with *nrt* cDNA (Barthalay *et al*., 1990; Darboux *et al*., 1996). Taken together, these data indicate

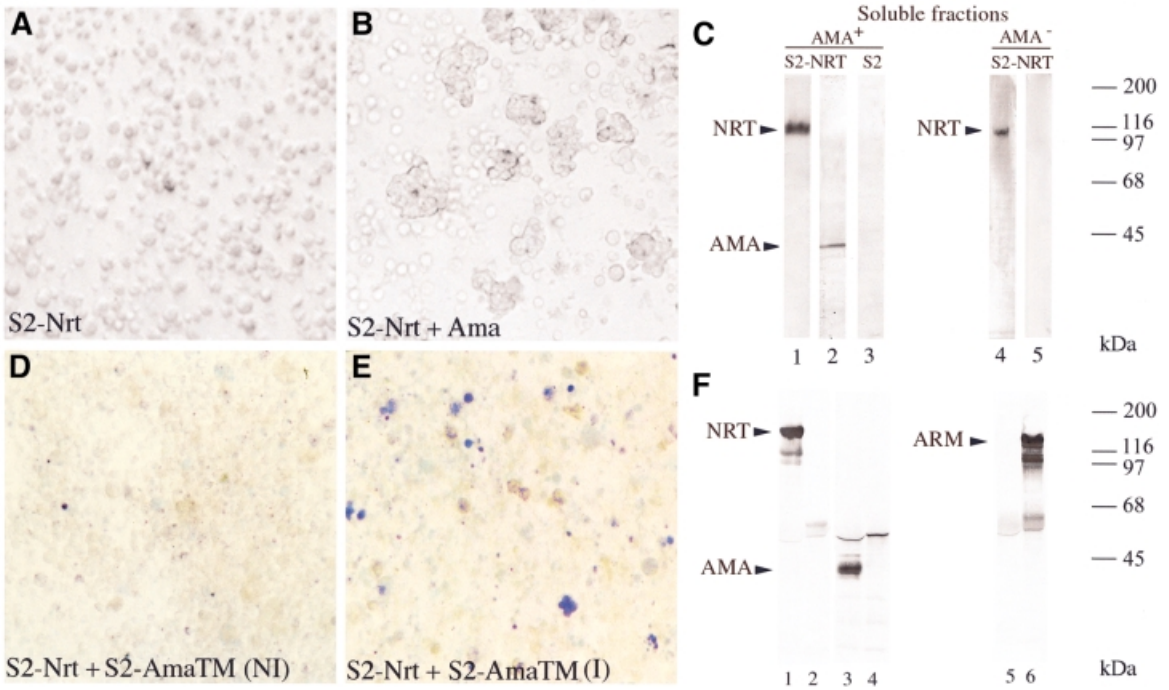

Fig. 2. AMA is required for aggregation of NRT transfectants. NRT transfectants expressing high levels of NRT protein did not aggregate spontaneously (**A**); however, if they were incubated with the 100 000 *g* supernatant, they formed homotypic aggregates (**B**). Protein analysis of the corresponding aggregates or cells is shown in (**C**). Soluble fractions used as a source of ligands were obtained from either wild-type (lanes 1–3) or *ama*-deficient (lanes 4 and 5) embryos. NRT transfectants were incubated with the wild-type soluble fraction to allow aggregation. Aggregates were analyzed by SDS–PAGE/western blotting with anti-NRT antibodies (lanes 1–4) or anti-AMA antibodies (lanes 2–4). NRT transfectants remained as single cells when they were incubated with AMA-deficient soluble fraction. Untransfected S2 cells (lane 3) did not bind AMA protein; however, staining for a longer time shows a very weak signal due to the endogenous AMA expression. S2 AMA-TM transfectants were grown to confluence on slide flasks and exposed to bivalent cations to induce AMA-TM expression. Incubation at 25°C for 1.5 h allowed full AMA-TM expression, then the cell monolayer was overlaid with a suspension of methylene blue-stained NRT transfectants (Barthalay *et al*., 1990). After a 15 min incubation and several gentle washes, the slides were examined by microscopy. (**E**) A field with blue NRT-expressing cells bound on unstained AMA-TM transfectants (I: induced). (**D**) The same experiment where expression of AMA-TM was not induced (NI). (**F**) A co-immunoprecipitation assay. Anti-NRT antibodies (lanes 1, 3 and 5) or anti-ARM antibodies (lanes 2, 4 and 6) were added to embryo lysates that were incubated with protein A–Sepharose. Immunoprecipitates were analyzed on SDS–PAGE, then blotted onto nitrocellulose and probed with anti-NRT (lanes 1 and 2), anti-AMA (lanes 3 and 4) and anti-ARM antibodies (lanes 5 and 6).

that AMA is a secreted, soluble protein that can associate with the cell surface.

AMA is necessary for NRT-mediated aggregation

To determine whether AMA plays a role in NRT-mediated heterophilic adhesion, NRT transfectants, which were not able to aggregate by themselves (Figure 2A), were incubated in culture medium containing secreted AMA protein. We observed aggregate formation similar to that in the experiment where the 100 000 *g* embryonic extract supernatant was used as a soluble fraction (Figure 2B) containing ligand activity (Darboux *et al*., 1996). Moreover, when a soluble fraction was prepared from embryos deleted for the *ama* gene (soluble protein fractions obtained from wild-type and *ama*-deficient embryos and assayed for the presence of AMA protein are shown in Figure 1, lanes 2 and 3), NRT transfectants did not aggregate (data not shown). To determine whether AMA interacts specifically with NRT, we conducted a sort of S2 cell pull-down assay. Untransfected and transfected S2 cells expressing NRT were incubated with soluble protein fractions prepared from either wild-type or *ama*-deficient embryos. These S2 cells were then pelleted and total cellular proteins were analyzed by western blot analysis with NRT- and AMA-specific antibodies. NRT-expressing S2 cells were able to pull-down AMA from wild-type embryonic extracts (Figure 2C, lanes 1 and 2), while control S2 cells did not (Figure 2C, lane 3). Not surprisingly, no AMA-immunoreactive material was found associated with NRT-expressing S2 cells that were incubated with soluble protein fractions prepared from *ama*-deficient embryos (Figure 2C, lanes 4 and 5). These results showed that AMA is necessary for NRT-mediated adhesion. A molecular association between AMA and NRT was demonstrated by a co-immunoprecipitation assay (Figure 2F). This association is specific since AMA is not detected in a control protein complex obtained with anti-armadillo (ARM) antibodies.

The results presented above suggested that a membrane-anchored form of AMA might interact directly with NRT-expressing cells and facilitate heterophilic aggregation. To test this hypothesis, a transmembrane form of AMA (AMA-TM) was generated. AMA-TM was created by fusing the entire *ama* open reading frame to the transmembrane and cytoplasmic domain of the *Drosophila* NRG protein (see Materials and methods). When plated onto plastic slide flasks, the AMA-TM S2 transfectants were able to bind methylene blue-stained

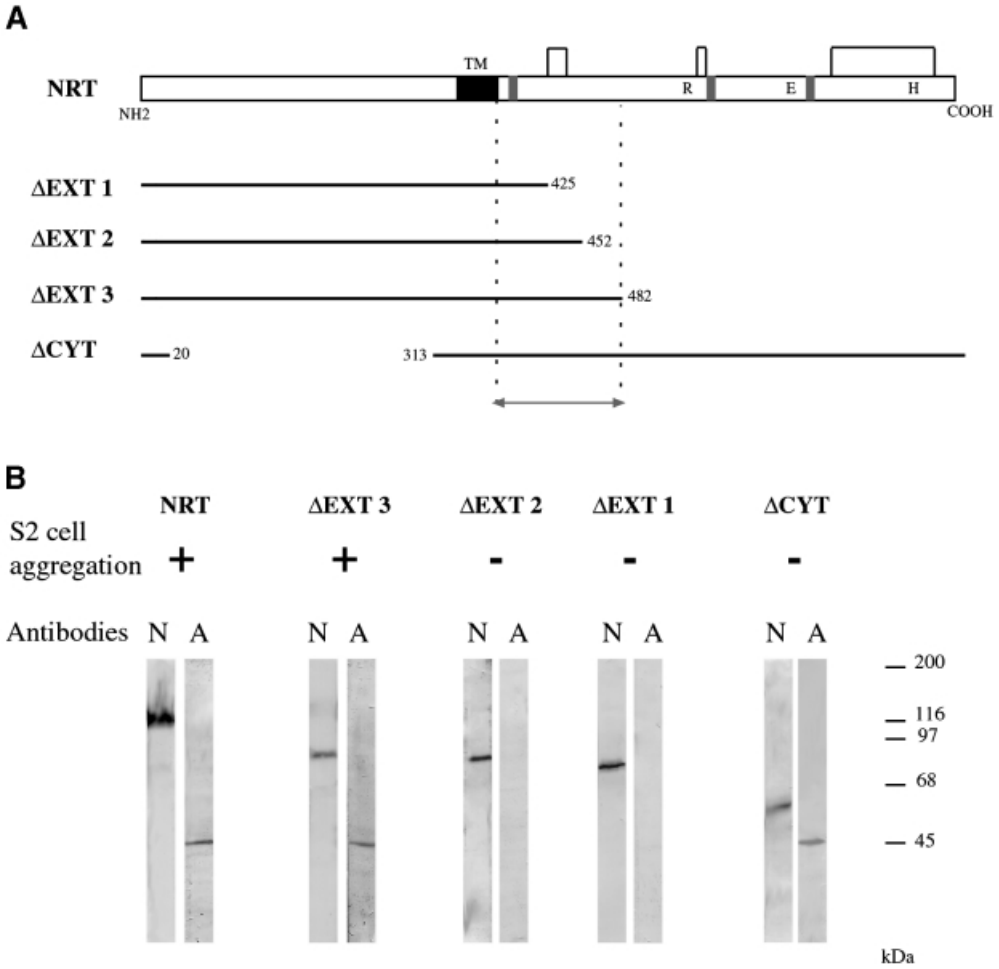

Fig. 3. Interactions between AMA and truncated NRT proteins. (**A**) Schematic representations of full-length NRT and cDNA constructs introducing different deletions of the extracellular domain (ΔEXT1, ΔEXT2 and ΔEXT3; see Darboux *et al*., 1996) or the cytoplasmic domain (ΔCYT; see Materials and methods). Lines represent the wild-type molecule or truncated NRT molecules; shaded rectangles show the three LRE motifs. The double arrow indicates the His347–His482 domain. (**B**) These various transfectants were incubated with culture medium containing secreted AMA and scored for aggregation (the results are indicated by a '+') and for the binding of AMA to NRT-expressing S2 cells. For that purpose, extracts of aggregated or non-aggregated cells were analyzed by SDS–PAGE/western blotting. Lanes stained with anti-NRT or anti-AMA antisera are marked by N or A, respectively.

NRT-expressing S2 cells (Figure 2E). This binding did not occur if AMA-TM expression was not induced by divalent cations (Figure 2D). This result suggests that AMA-TM transfectants are functionally equivalent to the embryonic cells used in the binding assay originally described by Barthalay *et al*. (1990) and that the AMA membrane-bound form may also bind the NRT molecule. We also observed that AMA-TM-expressing S2 cells could form large aggregates in our typical cell aggregation assay (data not shown). Thus, it would appear that AMA protein can interact with itself in addition to its interaction with NRT.

Binding of AMA to NRT-expressing cells does not require the NRT extracellular domain integrity

NRT is a type II transmembrane protein inserted in the lipid bilayer by a single hydrophobic region composed of 22 amino acids that separates the N-terminal cytoplasmic domain (323 amino acids) from the C-terminal extracellular domain (500 amino acids). By expressing truncated NRT proteins in S2 cells and using a soluble fraction prepared from embryonic extracts that promote cell aggregation, we have been able to localize a region within the extracellular domain between His347 and His482 (Figure 3B) that is essential for the adhesive function of NRT (Darboux *et al*., 1996). In order to determine if this *in vitro* recognition process requires only AMA, the same experiments were repeated by replacing the crude extract with culture medium containing secreted AMA protein. The transfected molecules are schematized in Figure 3A and the results of the aggregation assays are summarized in Figure 3B. Consistent with our previous results, we found that NRT molecules that were truncated downstream of residues Pro452 (ΔEXT2) or Arg425 (ΔEXT1) were inactive, while truncation downstream of His482 (ΔEXT3) generated a molecule that possessed the same adhesive properties as the full-length NRT. Simultaneously with these assays, aliquots of cells or aggregates were analyzed on SDS–PAGE, and AMA binding to transfectants was evaluated by western blot analysis. The results shown in Figure 3 indicated that only ΔEXT3 transfectants were able to form aggregates, and protein analysis demonstrated that these cells bound AMA while ΔEXT1 and ΔEXT2 transfectants stayed as single cells and no AMA binding was detected. Among the series of

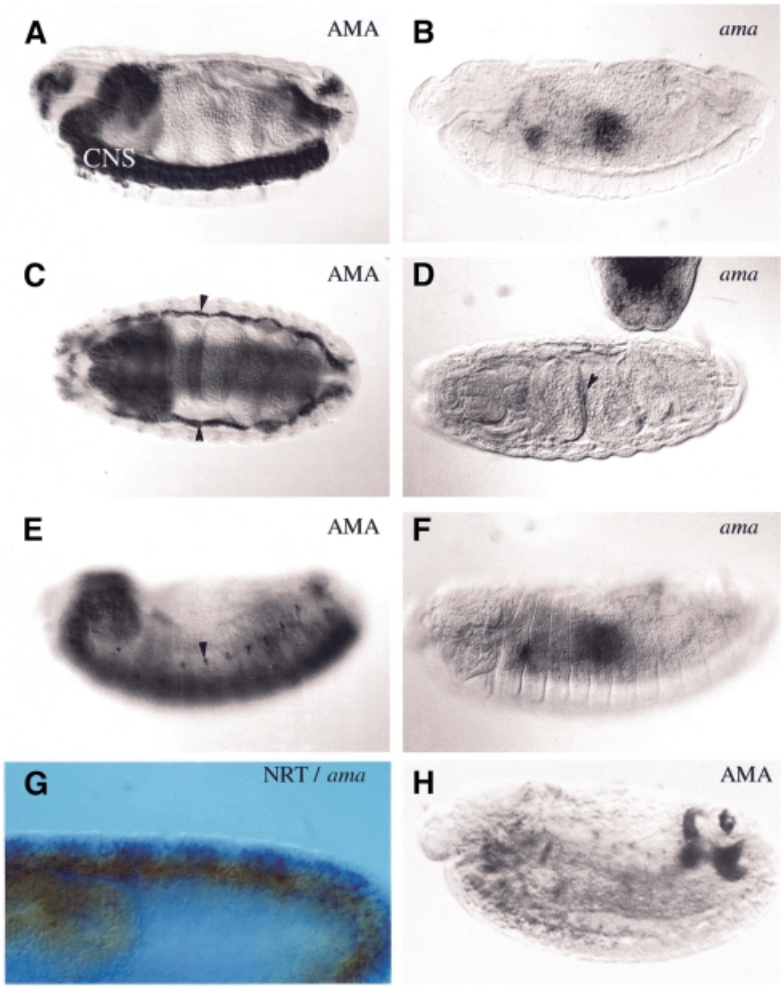

Fig. 4. Differences are apparent in the accumulation patterns of *ama* RNA and protein during embryonic development. Patterns of AMA protein (**A**, **C** and **E**) and RNA (**B**, **D** and **F**) accumulation in stage 13–15 embryos. (**G**) *ama* RNA accumulation (blue) and NRT protein expression (brown) in a stage 10 embryo. (**H**) AMA protein localization in an *nrt* mutant embryo. In the absence of NRT, specific patterns of AMA protein accumulation are not observed. In all panels, embryo orientation is anterior right and dorsal up. (C) and (D) are dorsal view. In (C), the arrowheads indicate fat body and in (D) the arrowhead points to a weak signal in the first gut constriction.

truncated molecules that were analyzed (ΔEXT1, ΔEXT2 and ΔEXT3), we found that the presence of AMA correlates with the capacity to form aggregates. This suggests that at least *in vitro*, AMA is the major component involved in this NRT-mediated cell–cell recognition process.

The NRT cytoplasmic domain is required for aggregation

The role of the NRT cytoplasmic domain in aggregation was investigated by using a construct designated ΔCYT. The ΔCYT molecule lacks the 293 amino acid cytoplasmic domain including five putative phosphorylation sites, leaving intact a short terminal sequence for correct initiation of translation as well as sequences near the transmembrane domain for proper membrane insertion and orientation (Figure 3A). The ΔCYT construct was used to transfect S2 cells, and protein expression was analyzed by western blot analysis. The apparent molecular weight of the ΔCYT molecule (60 kDa) was consistent with the predicted size of the glycosylated extracellular domain (522 amino acids). The correct translocation of the ΔCYT was investigated further by mild papain digestion of the transfectants. This treatment released a polypeptide whose molecular weight (55 kDa; data not shown) was compatible with the expected accessibility of the extracellular domain to proteases. These observations indicate that the removal of 293 amino acids from the cytoplasmic domain did not impair efficient insertion of NRT into the membrane or the overall stability of the NRT protein.

ΔCYT-expressing transfectants did not form aggregates in the presence of the culture medium containing secreted AMA protein, although the controls (full-length NRT transfectants) aggregated efficiently. This result demonstrates that the NRT cytoplasmic domain was necessary for cells to aggregate. Interestingly, AMA binding to ΔCYT transfectants was detected (Figure 3B). This demonstrates that AMA binding to the NRT extracellular domain alone is not sufficient to promote aggregation and that the NRT cytoplasmic domain is also required for NRT-mediated aggregation.

ama RNA and protein localization during embryogenesis

Since the data from S2 cell transfection experiments showed that AMA was secreted into the culture medium,

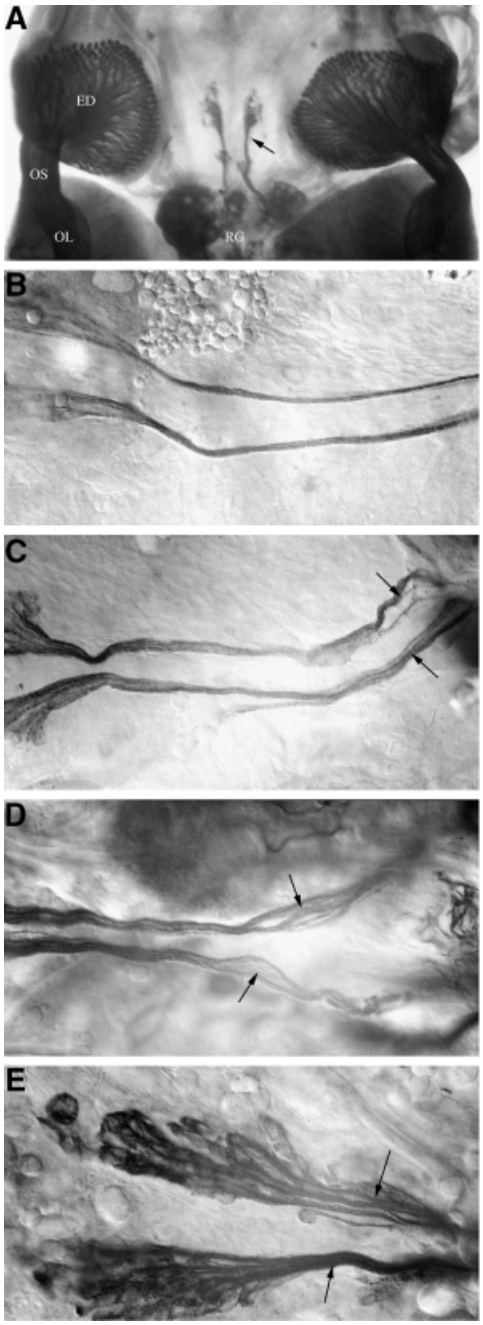

Fig. 5. *ama*-deficient pupae exhibit defects in ocellar pioneer nerve fasciculation. (**A**) Projection of ocellar pioneer axons (arrow) into the extracellular matrix in wild-type pupae before head eversion as visualized with monoclonal antibody 22C10. Axons from each ocellus extend in parallel fascicles to the brain (bottom of the image) through the ring gland (RG). Axons originating from photoreceptor cell precursors within the eye disc (ED) project into the optic lobe (OL) through the optic stalk (OS). (**B**) A higher magnification of the ocellar axon tracts in wild-type pupa. (**C–E**) Examples of ocellar axon defasciculation phenotypes in different regions of this pathway from *ama*-deficient pupae.

one might expect to find differences between *ama* RNA and protein expression within tissues during development. For *nrt*, there is a precise correspondence between patterns of *nrt* RNA and protein localization (De la Escalera *et al*., 1990; Hortsch *et al*., 1990). In the case of *ama*, there were interesting differences between RNA and protein accumulation profiles. Some of these differences are highlighted in Figure 4. During early stages of embryogenesis, there was a strong correlation between patterns of *ama* RNA and protein expression. For example, high levels of both *ama* RNA and protein were observed in the mesoderm invagination during early stages of germ band extension (data not shown and Seeger *et al*., 1988). During later stages of embryogenesis, differences between the patterns of RNA and protein accumulation become apparent. By stage 14, AMA protein is found predominantly within the CNS (Figure 4A), while *in situ* hybridizations showed no RNA expression within this tissue (Figure 4B). The same observation was made for the fat body, which accumulates large amounts of AMA (Figure 4C). Once again, low levels of *ama* RNA were detected in this tissue (Figure 4D). By stage 14, weak *ama* RNA signals were found around the gut (Figure 4F), but never accumulated in discrete areas such as the external sensory organs in the PNS where AMA protein is concentrated (Figure 4E). Clearly, there were regions where AMA protein accumulation did not correspond to high levels of *ama* transcripts, suggesting that AMA protein turnover is low.

The overall patterns of AMA protein accumulation during embryogenesis are interrelated with patterns of *nrt* RNA and protein expression (Seeger *et al*., 1988; De la Escalera *et al*., 1990; Hortsch *et al*., 1990). Differences in the patterns of *ama* RNA and NRT expression were observed. For instance, in a stage 10 embryo double stained for *ama* RNA and with anti-NRT antibody, differences were apparent (Figure 4G). *ama* transcripts, which identify cells producing the ligand, were located more apically than NRT-expressing cells. Clearly, many cells that express high levels of *ama* transcript did not express NRT protein. The normal pattern of AMA protein accumulation was also dependent upon the presence of NRT. In *nrt* mutant embryos, the pattern of AMA protein expression is clearly aberrant (Figure 4H) as compared with wild type (Figure 4A).

Absence of AMA protein causes defasciculation in the ocellar pioneer nerves

Given the requirement for AMA for NRT-mediated adhesion, additional *in vivo* approaches were undertaken in order to question whether AMA and NRT were involved in the same aspects of neural development. Like the *nrt* gene, *ama* is not essential for development. Adults that are deficient for either *ama* or *nrt* can be generated. In these studies, we analyzed *ama* requirements in *ama*-deficient pupae, since the most reliable phenotype for mutations in *nrt* was found to be defasciculation of the ocellar pioneer nerves (Speicher *et al*., 1998). During early pupal development, ocellar pioneer axons extend in the extracellular matrix (ECM) that covers the internal side of the prospective head without contacting the epithelium. This choice of ECM versus epidermis is crucial for normal pathfinding of these axons (for a full description of the ocellar nerve development see Garcia-Alonso *et al*., 1996). Lack of *ama* results in defasciculation of the normally tightly associated ocellar pioneer axons (Figure 5C, D and E). These defects occurred frequently: the penetrance was found to be 87% exhibiting defasciculation (21/24) compared with the wild-type background where defects

were observed in only 8% (1/13) of the individuals. The presence of any single split within the fascicles was considered to be a defasciculation defect. These data were similar to those published by Speicher *et al*. (1998) for a null mutation in *nrt*. As in the case of *nrt* mutants, this phenotype is predominant in pupae that did not go through the head eversion process. Despite these frequent defasciculation abnormalities, ocellar pioneer axons usually reach their brain targets. In contrast to *nrt* mutants, we did not observe any association with the epidermis or connections with the neighboring mechano-receptor axons in *ama*-deficient pupae.

Discussion

The serine esterase-like family of membrane proteins performs a variety of functions during development. Proteins in this family share a conserved domain structure, the serine esterase domain, although many members of this family lack certain key amino acids that are required for serine esterase enzymatic activity. Therefore, it is likely that this domain structure has evolved and is utilized for other processes in addition to its enzymatic function. For instance, a growing body of evidence suggests that the acetylcholine-hydrolyzing enzyme acetylcholinesterase (AChE) may have diverse functions, including a role in neurite outgrowth (Layer and Willbold, 1995). Another member of this protein family, rat NL-1, is a ligand for β-neurexins (Ichtchenko *et al*., 1995). These results and others suggest that AChE and other members of this serine esterase-like family of proteins play important roles in cell–cell interactions and signaling.

Drosophila NRT was shown by Barthalay *et al*. (1990) to function as a heterophilic cell adhesion protein in various cell culture assays, suggesting for the first time that a cholinesterase domain could be part of a recognition/signaling transmembrane receptor-like protein. Further studies delineated a specific region of the cholinesterase-like domain located near the membrane that is involved in this recognition process (Darboux *et al*., 1996). In this study, we demonstrate that AMA, a member of the immunoglobulin superfamily (Seeger *et al*., 1988), is a ligand for the NRT protein. We establish that AMA, which encodes three immunoglobulin-like domains and no clear membrane-anchoring sequences, is a secreted protein. We find that (i) AMA is able to promote the aggregation of NRT-expressing S2 cells; (ii) AMA interacts specifically with NRT; and (iii) AMA is necessary for the interaction of NRT-expressing S2 cells with dissociated embryonic primary culture cells, the original assay that established the heterophilic adhesion properties of NRT. The nearly identical patterns of AMA and NRT protein accumulation throughout embryogenesis are consistent with a receptor–ligand relationship *in vivo*. Furthermore, the finding that AMA protein fails to exhibit its normal pattern of accumulation in an *nrt* mutant is compatible with NRT acting as the key receptor for AMA during embryogenesis. Our observations that during embryogenesis cells that accumulate AMA on their surface are sometimes different from the cells that are expressing *ama* transcripts are intriguing. Certainly these differences can be explained easily for a secreted protein such as AMA. However, the functional significance, if any, of these disparities between spatial patterns of protein expression versus protein accumulation is unknown.

How does AMA facilitate NRT-mediated adhesion? Observations that an artificially generated membrane-anchored form of AMA can mediate homophilic aggregation in the absence of NRT indicate that AMA can interact with itself. This may suggest one mechanism whereby AMA could function as a linker between NRT molecules on opposing cell surfaces. NRT would bind AMA, which would bind to a second AMA molecule that could interact with NRT on the surface of a second cell. Thus, AMA would serve as a linker protein between NRT-expressing cells. Alternatively, AMA could alter the NRT protein upon binding such that it could now recognize in a homophilic manner other NRT molecules. Further studies will be required to distinguish between these different models.

The binding of AMA to NRT alone is not sufficient for cell aggregation. The finding that the NRT cytoplasmic domain is essential for adhesion indicates that important events are taking place in the cytoplasm that are critical for this adhesion process. The amino acid sequence of the NRT cytoplasmic domain does not reveal any clear motifs such as kinase or phosphatase domains. However, it does encode several putative phosphorylation sites, and we have found that NRT is actually phosphorylated on serine and threonine residues (S.Gorde and M.Piovant, unpublished results). Interestingly, NRT is enriched at sites of cellular contacts in neuronal and epithelial tissues and within NRT transfectant aggregates (Barthalay *et al*., 1990). This recruitment to specific regions of the membrane suggests that NRT may bind to cytoskeleton components. NRT-mediated cell adhesion might be considered as a two-step process: (i) binding of the ligand (AMA) to the extracellular domain, a step that can occur without the NRT cytoplasmic domain; and (ii) stabilization and strengthening of the interaction through clustering of NRT to sites of cell contact and interactions dependent upon the NRT cytoplasmic domain. This latter step was not achieved with ΔCYT transfectants, demonstrating the critical role of the NRT cytoplasmic domain in this process. While we have yet to define the precise molecular basis for the essential role that the NRT cytoplasmic domain plays in aggregation, there are certainly examples of other adhesion proteins that also require their cytoplasmic domain for adhesion activity. For example, the essential role of cadherin cytoplasmic domains for their homophilic adhesive function has been well documented (Nagafuchi and Takeichi, 1988; Jaffe *et al*., 1990). The central role of the cytoplasmic domain could also explain the ability of the NRT cyto-domain to convert the *Drosophila* AChE into a cell adhesion molecule (Darboux *et al*., 1996).

The isolation and characterization of *nrt* mutants have demonstrated that NRT is required for outgrowth, fasciculation and guidance of axons (Speicher *et al*., 1998). Mutations in *nrt* produce quite subtle and variable defects in axon guidance within the embryonic CNS (Speicher *et al*., 1998). Speicher and colleagues have shown that these phenotypes are greatly enhanced when *nrt* mutations are combined with mutations in other cell adhesion or signaling molecules such as *nrg* (Bieber *et al*., 1989), *derailed* (Callahan *et al*., 1995) and *kekkon-1*

(Musacchio and Perrimon, 1996). These results have led to the suggestion that *nrt* plays a cooperative or partially redundant role in axon guidance. One of the most robust phenotypes of *nrt* mutants alone is disruption of ocellar nerve axon pathfinding in pupae. In *nrt* mutants, misrouting, stalling and defasciculation of the ocellar pioneer nerves are observed at high frequencies (Speicher *et al*., 1998).

One might expect that if AMA and NRT indeed interact *in vivo* as indicated by the co-immunoprecipitation experiment, mutations in these genes would lead to similar phenotypes. Since the ocellar pioneer nerve disruptions are the most consistent phenotype for *nrt* null mutants, we determined whether a loss-of-function *ama* mutant displayed comparable phenotypes. Similarly to *nrt* mutations, a deficiency for *ama* resulted in highly penetrant defasciculation phenotypes of the ocellar pioneer nerves in developing pupae. However, there were significant differences in the expressivity of ocellar nerve defects. Stalling or misrouting of these axons as is found in *nrt* mutants was not observed in *ama* mutant pupae. This result suggests that rather than having an instructive role in pathfinding, AMA maintains proper cohesion between these axons and contributes to the organization of these fascicles.

We have been unable to address critically the role of AMA during embryonic axon guidance due to the lack of specific *ama* mutations. The deficiency that we used in our studies [Df(3R)*ama*] is also deleted for the embryonic dorsal–ventral patterning gene *zerknullt*. We are able to rescue the lethality associated with *zerknullt* mutations with a wild-type *zerknullt* transgene and thus generate an adult viable strain that is deficient for AMA. The difficulty is that partial rescue of *zerknullt* embryonic functions leads to variable defects in germ band extension and retraction (see Seeger *et al*., 1988). These disruptions in morphogenetic movements make it impossible to assay reliably for subtle defects in formation of axon pathways in the embryonic CNS. Therefore, it is possible that loss-of-function mutations in *ama* could result in embryonic axon pathfinding defects that are similar to *nrt* loss-of-function mutations. However, we will have to wait for the isolation of specific *ama* mutations in order to address this issue.

Although the specific roles of NRT and AMA during *Drosophila* development remain quite obscure, we are beginning to fill in different pieces of the puzzle. We have clearly established a link between these two enigmatic proteins. One of the critical missing pieces is what lies downstream of the NRT receptor and how AMA binding to NRT affects these downstream components. Addressing this issue and identifying orthologs in other organisms are probably key steps in our further understanding of this intriguing receptor–ligand interaction.

Materials and methods

Drosophila stocks

All fly stocks were raised on standard media. Oregon R stock was used as a wild-type stock. The viable *ama* deficiency stock was constructed by using two deficiencies within the Antennapedia complex with transgenic copies of *zen* and *bcd*: P[*zen*$^+$]/P[*bcd*$^+$]; Df(3R)*ama, zen*$^-$*, bcd*$^-$*, ama*$^-$/Df(3R)D830 *zen*$^-$*, bcd*$^-$*, ama*$^-$*, Dfd*$^-$*, Scr*$^-$. The ethylmethane sulfonate (EMS)-induced *nrt*^{1} allele was isolated in a search for lethals over deficiency Df (3L) std11 (J.Singer and M.Piovant, unpublished results).

Construction of deleted ΔCYT NRT molecule

The NRT ΔCYT molecule was constructed by using the method of gene splicing by overlap extension (Horton *et al*., 1989). Three rounds of PCR generated this molecule. The first one consisted of the 101 bp ATG region (–44 to 57 bp), amplified by using the primers a (5′-ACGTgcggccgcGAATTCTCGCAGAGTTCTCTAAAC) and b (5′-ACGCAGACGTTGCTGGGCGGCTGTCGTCT). The latter was composed of a large part of the region before the deletion (underlined) and of a small region (nine bases) downstream from the deletion. The second round consisted of the region corresponding to 1448–3513 bp amplified with primers c (5′-GCCCAGCAACGTCTGCGTGGCTACAAGTGC) and d (5′-GGACcgccggcgGTATATTTTGTGATGTTTTATTTGTG). Primer c was composed of a large region downstream from the deletion (underlined) and of nine bases upstream of the deletion. Primers b and c were made complementary to one another by including nucleotides at their 5′ ends that were complementary to the 3′ end of the other primer. Primer d was made specific for the *nrt* distal region by adding the *Not*I cloning site. After their purification, the AB and CD fragments were mixed and submitted to denaturation–renaturation to anneal the overlapping regions of primers b and c. A third round of PCR with excess of the two distal primers a and d led to the NRT ΔCYT molecule in which the presence of the AB region was verified by Southern blot analysis. This molecule was ligated into a pCR™ II vector (Invitrogen). The whole NRT ΔCYT molecule has been sequenced and compared with the cDNA sequence (De la Escalera *et al*., 1990). Three mutations out of 2147 nucleotides were detected, but none altered the NRT amino acid sequence.

After digestion with *Eco*RI, the ΔCYT DNA fragment was ligated either into a pCaSpeR-hs vector (Thummel *et al*., 1988) for transfections or into pBlueScript vector (Stratagene) for sequencing. Genome Express (Grenoble, France) conducted the DNA sequencing.

Cell cultures

Cell transfection experiments were performed as previously described by Darboux *et al*. (1996). AMA transfectants were generated by transfection of S2 cells with the *ama* full-length cDNA (Seeger *et al*., 1988) cloned in blunted pHT4 vector as an *Eco*RI fragment. The membrane-tethered form of AMA was produced by adding the transmembrane and cytoplasmic domains from *Drosophila* NRG (Bieber *et al*., 1989). PCR was utilized to introduce an *Ava*II site immediately before the *ama* stop codon. This altered form of AMA was then spliced into the *Ava*II site in *nrg* that precedes the transmembrane domain by ~20 amino acids. The long cytoplasmic domain isoform of NRG was used for this construct. The AMA-TM construct was then subcloned into the pRmHa3 vector (Bunch *et al*., 1988).

Anti-AMA antibodies

Two polyconal antibodies were raised: the first against an AMA peptide and the second against the entire AMA protein. The peptide was chosen in a region of the Ig domain that was supposed to be exposed to the solvent, from residue 309 to 323 in the AMA putative amino acid sequence. This 15mer oligopeptide was synthesized and bound to diphtheria toxoid protein as a carrier by Chiron Mimotope Peptide Systems (Australia). This protein was mixed with Freund's adjuvant and injected into rats. Full-length *ama* cDNA was cloned into the pHT4 vector and used as a template for PCR. The histidine-tagged AMA molecule was made using PCR: the start codon was removed in order to add a stretch of six histidine residues. This construct was made in the PQE-30 vector from Qiagen (Chestwort, CA) and expressed in *Escherichia coli* M15 according to the manufacturer's instructions. The tagged AMA was recovered in the eluate from a nickel column, separated on SDS–PAGE and transferred to nitrocellulose. The protein bound to nitrocellulose was used as immunogen without Freund's adjuvant (Diano *et al*., 1987) and was injected into rats and rabbits. Anti-AMA antibodies raised against βGal-AMA fusion protein (Seeger *et al*., 1988) were also used for embryo staining.

Cell aggregation assays

These assays were performed as described by Darboux *et al*. (1996). A total of 10^6 NRT transfectant cells were incubated in 500 µl of a soluble fraction obtained either from embryo homogenates or from AMA transfectants, which secreted AMA into the culture medium. After 30 min incubation on a rotatory shaker at 100 r.p.m., the presence of aggregates was monitored by microscopy. NRT cells (aggregates or isolated cells) were collected by centrifugation at 100 *g* for 5 min and washed with Schneider's medium (Gibco-BRL). Pellets were homogenized in sample buffer and proteins were separated by SDS–PAGE and analyzed by

western blotting using anti-NRT monoclonal antibodies (Darboux *et al*., 1996) and rat anti-AMA antibodies.

In order to collect a fraction containing secreted AMA protein, AMA transfectants were grown in suspension in Schneider's medium supplemented with fetal calf serum (FCS) and harvested in the exponential growth phase. They were then washed in Schneider's medium alone to remove FCS proteins, heat shocked for 15 min at 37°C and allowed to recover for 90 min at 25°C. The cells were separated from the culture medium by a gentle centrifugation at 100 *g* for 5 min. For aggregation assays, this medium was filtered through a 0.4 µm membrane filter to remove any cells or debris. For SDS–PAGE analysis, 10% trichloroacetic acid (TCA) was added to the cell extracts and the precipitated proteins were resuspended in sample buffer (Piovant and Léna, 1988), separated on 7.5% polyacrylamide gels and blotted onto nitrocellulose.

Co-immunoprecipitation

Dechorionated embryos were homogenized in lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 1% NP-40, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% bovine serum albumin) in the presence of 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. The lysate was centrifuged at 14 000 *g* for 15 min. The supernatant was collected and pre-cleared on protein A–Sepharose CL-4B (Pharmacia) and divided into two parts. E1C monoclonal anti-NRT antibody was added to the first aliquot at 1:500 dilution. As a control, N2 7A1 monoclonal anti-ARM antibody was added to the second aliquot at 1:200 dilution, and they were both incubated for 1 h at 4°C. A 50 µl aliquot of protein A–Sepharose (diluted 1:1 in lysis buffer) was added and both samples were rocked for 30 min at 4°C. Beads were washed three times with 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP-40, resuspended in loading buffer (Piovant and Léna, 1988), run on a 10% SDS–polyacrylamide gel and blotted onto nitrocellulose. Strips of nitrocellulose were blocked in 50 mM Tris pH 7.4, 150 mM NaCl, 5% powdered non-fat milk, then incubated separately with monoclonal antibodies E1C and N2 7A1 and rat polyclonal anti-AMA antibodies (diluted 1:100) for 1 h at room temperature, and washed with 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% powdered non-fat milk. Staining was carried out with mouse or rat alkaline phosphatase-conjugated secondary antibodies (Promega).

Immunohistochemistry and in situ hybridization

Egg collections were carried out for an 8 h period and aged for an additional 5 h at 29°C. Embryos were dechorionated in 50% chlorox for 2 min, rinsed and fixed for 15 min in 4% formaldehyde in 2 mM EGTA, 1 mM $MgSO_4$, 100 mM PIPES pH 6.5 with heptane (1:1). Fixed embryos were devitellinized in heptane/methanol (1:1), rehydrated successively in 70, 50, 30 and 0% methanol in phosphate-buffered saline (PBS) and equilibrated in PBS buffer containing 0.1% Triton X-100 (PTX) and then in PTX containing 5% powdered non-fat milk. Incubation with primary antibodies was carried out either for 1 h at room temperature or overnight at 4°C. After several washes, embryos were incubated for 1 h with horseradish peroxidase- or alkaline phosphatase-conjugated secondary antibodies and stained with a Vectastain Elite peroxidase kit or with the alkaline phosphatase substrates BCIP and NBT (Boehringer Mannheim), respectively. Pupae were processed as previously described (Garcia-Alonso *et al*., 1996) and stained with monoclonal antibody 22C10 followed by the appropriate biotinylated secondary antibody.

Digoxigenin (DIG)-labeled antisense or sense RNA probes were generated from DNA with T3 or T7 RNA polymerase (Promega) and DIG-UTP (Boehringer). They were used for whole-mount *in situ* hybridization of fixed staged embryos prepared as described in O'Neill and Bier (1994). The DIG-labeled RNA probes were detected with the aid of a pre-absorbed anti-DIG antibody coupled to alkaline phosphatase (Boehringer Mannheim) and NBT/BCIP as substrate. Embryos were mounted in Permount for further observation. For double labeling experiments, the conditions were the same as above. *In situ* hybridizations were performed before antibody staining. After dehydration of the embryos labeled with antisense RNA probes, they were rehydrated in PTX for at least 1 h and saturated in the same buffer containing 5% goat serum before incubation with the primary antibodies.

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