Introduction

The Notch cell-to-cell signalling pathway has a central role in animal development, controlling cell diversification and many other processes in a great variety of tissues (Artavanis-Tsakonas et al., 1999; Lewis, 1998). At its core lie the Notch family of receptors and their ligands, the proteins of the Delta/Serrate family. Both the receptors and the ligands are single-pass type I transmembrane glycoproteins, which are thought to interact at sites of cell-cell contact. Signalling is initiated when the extracellular domain of the ligand interacts with that of the receptor, resulting in a proteolytic cleavage that releases an intracellular fragment of Notch, NICD, which translocates to the cell nucleus and regulates transcription of various target genes (Selkoe and Kopan, 2003).

From this perspective, it might seem that the only part of the ligand that really matters is the part that binds to Notch. There are, however, strong grounds for believing that the intracellular (C-terminal) domains of the Deltas also have significant functions. All classes of vertebrates possess a subset of Delta proteins with a conserved ATEV\* motif at their C termini. These ATEV Deltas include Delta1 and Delta4 in mammals and DeltaD and DeltaC in the zebrafish. We show that these Deltas associate with the membrane-associated scaffolding proteins MAGI1, MAGI2 and MAGI3, through a direct interaction between the C termini of the Deltas and a specific PDZ domain (PDZ4) of the MAGIs. In cultured cells and in subsets of cells in the intact zebrafish embryo, DeltaD and MAGII are co-localized at the plasma membrane. The interaction and the co-localization can be abolished by injection of a morpholino that blocks the mRNA splicing reaction that gives DeltaD its terminal valine, on which the interaction depends. Embryos treated in this way appear normal with respect to some known functions of DeltaD as a Notch ligand, including the control of somite segmentation, neurogenesis, and hypochord formation. They do, however, show an anomalous distribution of Rohon-Beard neurons in the dorsal neural tube, suggesting that the Delta-MAGI interaction may play some part in the control of neuron migration.

Key words: DeltaD, DeltaC, MAGI proteins, Notch, PDZ domains, Zebrafish, Morpholino, Rohon-Beard neurons
comparisons among the vertebrates: within this group, it is highly conserved (although it shows no detectable conservation between vertebrates and insects). For example, the Delta1 (DLL1) protein of humans has an intracellular domain that is 67% identical to that of chick Delta1, 61% identical to that of Xenopus X-Delta1 and 56% identical to that of zebrafish DeltaD. In all vertebrates, and in at least one echinoderm (Sweet et al., 2002), we find a subset of Delta proteins that share a conserved motif – ATEV* – at their C terminus. This motif fits the consensus for a PDZ-domain-binding protein (Nourry et al., 2003; Songyang et al., 1997). The ATEV subset of Delta proteins includes Delta1 and Delta4 in mammals and birds, Delta1 and Delta2 in Xenopus, and DeltaC and DeltaD in zebrafish. From their expression patterns, it seems that the ATEV Deltas have some functions in common that set them apart from other vertebrate Deltas that lack this motif. They are expressed in endothelial cells of blood vessels (Beckers et al., 1999; Mailhos et al., 2001; Shutter et al., 2000; Smithers et al., 2000), in the gut epithelium (Schröder and Gossler, 2002) (M. Skipper, G.J.W., L.A.-M. and C. Crosnier, unpublished), and in the presomitic mesoderm, where they play an essential part in the oscillator mechanism that controls somite segmentation (Davis et al., 2001; Holley et al., 2002; Hrabé de Angelis et al., 1997; Jen et al., 1997; Jiang et al., 2000).

On the other hand, the ATEV motif is not required for the core function of Delta proteins in the central nervous system (CNS) as mediators of lateral inhibition during neurogenesis: all members of the vertebrate Delta family seem to share this activity (e.g. Haddon et al., 1998), regardless of whether or not they possess a terminal ATEV. Moreover, removal of the ATEV is not sufficient to confer dominant-negative activity: in the chick retina, a version of Delta1 with a mild C-terminal truncation, removing the ATEV motif along with 96 adjacent amino acids, still showed normal function, activating Notch and delivering lateral inhibition; only with a more severe truncation, eliminating all but 13 amino acids of the intracellular domain, was a dominant-negative effect seen (Henrique et al., 1997).

What then is the function of the ATEV motif? In this paper we show that it mediates binding to MAGI proteins (MAGUK proteins with inverted domain arrangement) – a subset of the MAGUK (membrane-associated guanylate kinase homolog) protein family – through a specific one of the six PDZ domains that these contain. A similar conclusion has recently been reached independently by another group (Pfister et al., 2003), who have shown that the mouse Delta1 protein binds through its C terminus to Acrv1p-1, also known as MAGI2. Our data demonstrate that in fact all three members of the MAGI protein family can bind ATEV Delta proteins in this way. We show that MAGI1 is widely expressed in the developing zebrafish embryo, and that it co-localizes with DeltaD at a subcellular level if and only if the ATEV motif is intact. We find, furthermore, that disruption of the DeltaD-MAGI interaction leaves Delta-Notch signalling practically unaffected; but it appears to alter the migratory behaviour of some neurons in the embryonic neural tube.

Materials and methods

Animals

Zebrafish were maintained at 27.5°C on a 14/10 hour light/dark cycle and embryos collected from spontaneous spawnings. Staging was according to Kimmel at al. (Kimmel et al., 1995).

Isolation of binding partners of Delta proteins

We used a modification of the protocol of Hutchings et al. (Hutchings et al., 2003). A peptide corresponding to the C-terminal 27 residues of human Delta1 was chemically synthesized containing an N-terminal biotin and aliphatic spacer. Peptide-saturated streptavidin-coated paramagnetic beads (Dynabeads® M-280, DYNALE®) were added to an adult mouse brain lysate [0.1 g wet weight per ml of WOP-40 buffer (Wright et al., 2000)]. The brains were crudely chopped in buffer and solubilized using a dounce homogenizer at 4°C. Insoluble material was removed by centrifugation at 400 g and the supernatant filtered. The beads were recovered with a magnet after an overnight rotating incubation at 4°C, and washed; associated proteins were eluted by boiling in SDS loading buffer.

Mass spectrometry

Samples were resolved on 4-12% Bis-Tris NuPAGE™ precast gels and stained using SYPRO Orange dye (BIO-RAD) and scanned on a STORM 860 phosphoimager (Molecular Dynamics). The MAGI-GFP fusion was made in pEGFP-N1 (Clontech) with the introduction of a linker (the protein product contains the sequences VFTP-GGVPVRARDDPVAT-MVS, where the first four and last four amino acids correspond to the C terminus of MAGI1 and N-terminus of EGFP respectively). The coding regions were then subcloned into the pCS2+ vector and mRNA was made using the mMessage mMachine kit (Ambion). For immunohistochemistry, embryos were dechorionated, fixed and stained with zdd2 anti-DeltaD monoclonal antibody and Alexa594-conjugated secondary antibody (Molecular Probes) as previously described (Itoh et al., 2003). MAGI1-EGFP was detected with Alexa488-conjugated rabbit anti-GFP antibody (Molecular Probes). Stained embryos were flat-mounted on slides in Citifluor mounting medium (Citifluor) and examined with a Zeiss LSM510 confocal microscope.

For analysis of phenotypes in the living stage, embryos were anaesthetized, mounted in 3% methyl cellulose, and photographed on a Leitz Diaplan microscope.

PDZ domain binding biochemistry

Sequences (available on request) corresponding to the six individual PDZ domains of zebrafish MAGI1 were amplified and cloned into a frame with a C-terminal 6 histidine tag in pET-23b and expressed in the E. coli strain BL21(DE3)pLysS. Proteins were purified using Nickel-NTA Agarose (Qiagen) using minor modifications to the manufacturer’s protocols and stored at ~70°C.

Biotinylated peptides corresponding to the C-terminal 27 or 26 amino acids of zebrafish DeltaC and DeltaD were synthesized as above, both with, and without, the C-terminal valine residue. 200 µl of streptavidin-coated Sepharose (Amersham Biosciences) was saturated with peptides corresponding to zebrafish DeltaC or DeltaD and divided equally into six aliquots: 12 µg of purified zfMAGI1 PDZ domains were added per tube and incubated with rotation at 4°C for 2 hours,
washed and eluted by adding SDS-PAGE loading buffer and heating to 50°C for 10 minutes. Eluates were resolved by SDS-PAGE on 4-12% NuPAGE gels using MES running buffer. Gels were stained with SYPRO orange as above.

**In situ hybridization**

Wholemount in situ hybridization followed the protocol of Thisse (Westerfield, 2000) or of Ariza-McNaughton and Krumlauf (Ariza-McNaughton and Krumlauf, 2002). DIG-labelled antisense probes for zebrafish MAGI corresponded to nucleotides 3018 to 3530 of the magi1 cDNA sequence. Probes for islet1 (Inoue et al., 1994), col2al (Yan et al., 1995) and myoD (Weinberg et al., 1996) were as previously described.

**PCR and cloning procedures**

Total RNA was extracted from embryos using Trizol (GIBCO-BRL) according to the manufacturer’s instructions and cDNA was made according to Wright et al. (Wright et al., 2000). The primers used to detect the splicing forms of zebrafish deltaD were AGCTGAGGAGCGGGAGCATG (sense) and CTTCAGTTGAGAACCAGCT- deltaD (antisense), usually for 30 PCR cycles. The altered splice forms were characterized by cloning and sequencing.

The full-length zebrafish magi1 cDNA was cloned by both 5’ and 3’ RACE using SMART RACE kit (Clontech) following the manufacturer’s protocols using 24 hour zebrafish cDNA and oligonucleotides based on the zebrafish EST sequence number AW078333. The full-length zebrafish magi1 cDNA has been submitted to GenBank (Accession number AY465352).

**Cell culture, transfection, and immunocytochemistry**

HEK293T cells grown on coverslips were transiently transfected with 1 μg of plasmid DNA per well in a 24-well plate using Superfect Transfection Reagent (Qiagen). Twenty-four hours after transfection, cells were rinsed in PBS and fixed in 4% formaldehyde in PBS for 15 minutes at room temperature. Fixed cells were permeabilized in 0.2% Triton X-100 in PBS for 15 minutes at room temperature, rinsed in PBS, and incubated in blocking solution (1% BSA in PBS) for 30 minutes at room temperature. Cells were then stained with zdd2 antibody, essentially as in Itoh et al. (Itoh et al., 2003). Coverslips were mounted on slides in Citifluor (Citifluor) and examined with a Zeiss LSM510 confocal microscope.

**Results**

**The C terminus of Delta1 binds MAGI proteins**

To identify proteins that might interact with the intracellular domain of Delta in vertebrates, we used a biochemical purification strategy. A synthetic 27-amino-acid peptide corresponding to the C terminus of human Delta1 was used to purify Delta-binding proteins from lysates of either adult mouse brain or the human neuroblastoma NB100 cell line. The altered splice forms were characterized by cloning and sequencing.

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We have chosen to focus on the interaction with the MAGI proteins. This protein family, corresponding (in mammals) to three distinct genes, is widely expressed; MAGI2 is especially plentiful in the adult central nervous system. This may account for its prominence in our purification assay using mouse brain. The domain structure of the MAGI proteins is shown in Fig. 1B: it includes a non-catalytic guanylate kinase homology region, two WW (protein interaction) domains, and six PDZ domains, numbered from 0 to 5, typical of scaffold proteins that hold the multiple components of signalling complexes by their C-terminal tails and keep them together at the plasma membrane. Because of their many interactions, the MAGI proteins have been given many different names (see Data S1 in supplementary material). In particular, the human MAGI1 protein is also known as BAIAP1 (BAI1-associated protein), because it binds BAI1 (brain angiogenesis inhibitor 1). BAI1, like Delta1, is a transmembrane protein with an intracellular C terminus ending in TEV. This strongly suggests that MAGI1, and perhaps the other MAGI proteins, contain a PDZ domain that specifically recognizes proteins with a terminal TEV and is thus responsible for the binding of Delta1.

Regarding the other Delta-binding proteins we identified, MUPP1, like the MAGI proteins, is a membrane-associated scaffold protein, containing no less than 13 PDZ domains; it too may recognize the C-terminal ATEV of Delta1. Meprin A is more puzzling: meprins are transmembrane or secreted proteins that normally act extracellularly as metallopeptidases. In our assay, they may have been detected because they bound to the Delta1 C-terminal peptide as a (non-physiological) substrate, or because they were attached to it indirectly via some scaffold protein such as MAGI1/2/3 or MUPP1. The zinc
finger protein we detected is one for which no function is yet known.

The MAGI protein family is highly conserved between fish and mammals

For further analysis of the interactions of Delta proteins with MAGI proteins, we have used the zebrafish. A BLAST search for counterparts of human MAGI1, -2 and -3 in the Ensembl zebrafish cDNA database reveals one MAGI1 ortholog, two MAGI2 orthologs, and one MAGI3 ortholog. We shall call the corresponding four zebrafish genes magi1, magi2a, magi2b, and magi3. The orthology relationships can be inferred from the percent amino-acid identity when corresponding domains are compared: Table 1 shows values based on PDZ domain 4 (PDZ4). From the sequence comparisons, it seems that the ancestral MAGI1, -2 and -3 genes must have diverged from one another before the divergence of the fish and tetrapod lineages, while divergence of the zebrafish magi2a and magi2b genes may have occurred after this.

Having identified a part of the magi1 coding sequence in the zebrafish EST database, we were able to clone the entire cDNA sequence. The length of the corresponding MAGI1 protein (1247 amino acids) is similar to that of human MAGI1 (1256 amino acids), and the two proteins are 71% identical in sequence overall, with the same domain structure. The degree of conservation of individual domains is remarkably high: for example, the PDZ4 domains of human and zebrafish MAGI1 are identical in 101 out of 102 amino acids. We have used magi1 as representative of the MAGI family for further analysis of Delta-MAGI interactions.

magi1 is widely expressed in the developing zebrafish, becoming most plentiful in the CNS

As a first step towards analysis of the Delta-MAGI interaction in vivo, we examined the expression of magi1, to see whether it overlaps with that of deltaC and deltaD. We used RT-PCR to assess the time course of early expression of magi1 (Fig. 2A). The message is present already in the egg at 0 hours post-fertilization (hpf), has largely disappeared by 3 hpf, but is plentiful again by 6 hpf. This presumably reflects degradation of maternal message followed by a delay of a few hours before zygotic transcripts accumulate. Since the primary transcript is very long (>160 kb), there will presumably be an interval of 2 hours or more (assuming transcription at 1.2 kb/minute) from the initiation of zygotic transcription at 2.5-3 hpf until mature mRNA reaches the cytoplasm. Transcription of deltaC and deltaD begins to be seen by in situ hybridization at 5-6 hpf (Haddon et al., 1998; Smithers et al., 2000). Thus MAGI1 becomes available to interact with the Delta proteins at about the time of their first appearance.

These data are from BLAST alignment of human MAGI sequences with transcribed sequences from the Ensembl zebrafish database. The ENSDARG numbers are Ensembl gene ID numbers.

Table 1. Homology relationships between zebrafish and human MAGI proteins, as reflected in the percentage amino-acid sequence identity for PDZ domain 4

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<th>zf MAGI PDZ4</th>
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<td>Hs MAGI PDZ4 (BAIAP1)</td>
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<td>Hs MAGI2 PDZ4 (AIP1)</td>
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<td>Hs MAGI3 PDZ4</td>
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Hs MAGI1 PDZ4 (BAIAP1) 99 66 67 61
Hs MAGI2 PDZ4 (AIP1) 61 84 86 63
Hs MAGI3 PDZ4 56 67 63 78

Judged by in situ hybridization (Fig. 2B), magi1 seems to be expressed ubiquitously up to 24 hpf. By 48 hpf, expression is seen more in the CNS than elsewhere, and by 72 hpf this heightened expression in the CNS is striking (Fig. 2D,E). Expression in the CNS is, however, evident even at early embryonic stages, while the neural tube is forming and the first neurons are being born – in other words, at the time when delta genes are being expressed and playing their part in regulating neurogenesis. Expression is seen also in other sites where Delta genes are expressed, such as the hair cells of the inner ear (Fig. 2E). Our further experiments imply that interaction between Delta and MAGI proteins indeed occurs and is functionally important.

The C termini of DeltaC and DeltaD bind directly and specifically to PDZ4 of MAGI1

Previous studies (see Data S1 in supplementary material) have shown that the different PDZ (and other) domains in MAGI proteins selectively bind different partners. To understand the place that DeltaC and DeltaD might have in protein complexes

Fig. 2. Expression pattern of magi1 in the zebrafish embryo. (A) Early stages analysed by RT-PCR (data replicated in three separate experiments). Maternal mRNA present in the 0 hpf egg has largely disappeared by 3 hpf, with fresh (zygotically synthesized) transcripts appearing by 6 hpf. EF1α was used as a positive control. (B-E) In situ hybridization patterns. (B) Lateral view at bud stage (10 hpf), showing diffuse expression. (C) Dorsal view at 24 hpf, showing expression still diffuse but strongest in the neural tube. (D) Dorsal view of the head of a whole mount at 72 hpf, showing expression in the retinae and, most strongly, in the diencephalon and telencephalon. (E) Transverse section of embryo stained as a whole mount at 72 hpf, showing expression in the hindbrain and in sensory hair cells in the ear (red arrow).
held together by MAGI1, and to test whether the Delta proteins indeed bind to MAGI1 directly, we examined the binding of artificially synthesized fragments of the proteins in vitro. Peptides corresponding to the C-terminal 26 or 27 amino acids of DeltaC and DeltaD were synthesized chemically. We prepared two variants of each— one with and the other, as a control, without the terminal valine. We also prepared individual His-tagged proteins corresponding to each of the six PDZ domains (~100 amino acids) of zebrafish MAGI1. The Delta peptides, as before, were prepared with an N-terminal biotin to allow coupling to streptavidin-coated beads, and the individual PDZ domains were then assayed for their ability to bind to these. As shown in Fig. 3A, the C termini of DeltaC and DeltaD both bind selectively to PDZ4, with only a faint trace of binding to other PDZ domains. No binding is seen when the Delta peptides lack their terminal valine. We conclude that PDZ4 selectively and directly binds the Delta proteins through a typical PDZ-domain interaction that depends on the terminal valines of these proteins. Since PDZ4 is conserved and distinctive in all members of the MAGI protein family, it is likely that in all of them it is responsible for binding the ATEV Deltas in the same valine-dependent way.

**DeltaD and MAGI1 interact in living cells**

For a further test of the Delta-MAGI1 interaction, we performed cotransfection experiments in HEK293T cells. We constructed expression plasmids containing cDNA coding (1) for MAGI1, tagged at its C terminus with EGFP, (2) for full-length DeltaD, and (3) for DeltaD lacking the C-terminal valine residue (DeltaD-TE*). Both forms of DeltaD are recognized by our zdd2 monoclonal antibody against DeltaD (Itoh et al., 2003). Both full-length DeltaD and DeltaD-TE* when expressed alone were localized to the plasma membrane. In contrast, MAGI1-EGFP when expressed alone appeared cytosolic with scarcely perceptible membrane localization. However, in cells transfected with the full-length DeltaD and MAGI1-EGFP plasmids together, we observed a dramatic increase in the concentration of MAGI1-EGFP at the plasma membrane, where it was co-localized with the full-length DeltaD (Fig. 3B). This localization pattern was not observed when DeltaD-TE* was substituted for full-length DeltaD (Fig. 3C). These data support our in vitro findings as to the specificity of the interaction and imply that Delta proteins can recruit MAGI proteins to the cell surface.

To see whether DeltaD and MAGI1 proteins interact in the...
living embryo, we injected mRNA coding for MAGI1-EGFP into 2- to 4-cell-stage zebrafish embryos and compared the subcellular localization of the tagged MAGI1 to that of endogenous DeltaD at the 10-14 somite stage. DeltaD protein in uninjected embryos has a different intracellular distribution in different cell types: in nascent neurons, cell surface concentrations are below the threshold of detection with our zdd2 antibody, and the protein is seen only in intracellular granules (Itoh et al., 2003); in cells in the anterior parts of somites, DeltaD is detected in a spotty distribution at or near the cell surface (L.A.-M. and François Giudicelli, unpublished). This DeltaD pattern was maintained in embryos injected with the MAGI1-EGFP construct. Although, in these injected embryos, there was no detectable co-localization of MAGI1-EGFP with the intracellular DeltaD granules of the nascent neurons, the MAGI1-EGFP was frequently co-localized with the cell-surface DeltaD of the somite cells (Fig. 3D), suggesting that MAGI1 and DeltaD interact at the cell surface in the intact zebrafish embryo just as they do in cultured HEK293T cells (Fig. 3B). It should be noted that spots of DeltaD without MAGI1 and of MAGI1 without DeltaD were also seen, presumably reflecting the fact that DeltaD is only one of many MAGI1-binding partners (Fig. 1, see Data S1 in supplementary material) and that the levels of MAGI1 protein generated upon mRNA injection may be much higher than endogenous levels.

**A splice-blocking morpholino can be used to deprive DeltaD of its terminal valine in vivo**

We have shown that the terminal valine of DeltaC and DeltaD is required for binding to MAGI1. Removal of this valine should therefore provide a way to test the functional importance of the Delta-MAGI interaction while causing minimal disruption of other processes. By good fortune, the terminal valine of the ATEV Delta proteins is encoded on a separate exon. We were thus able to remove it in vivo by blocking the splicing reaction that links this exon to the rest. For this, we used a morpholino, which we shall call MO[dlD-V], complementary to the exon/intron junction on the 5’ side of the intron preceding the exon that encodes the terminal valine (Fig. 4A). A BLAST search of the zebrafish genome database indicated that this was the only target site for the morpholino. We injected varying doses of MO[dlD-V] into fertilized eggs at the one-cell stage, left them to develop as far as 6, 24, 48 or 72 hpf, and used RT-PCR to discover what splice variants of deltaD mRNA were present. As shown in Fig. 4B, 0.5 ng of MO[dlD-V] was sufficient to give a marked reduction in the amount of the normally spliced mRNA, and with 5 ng or more, this form was undetectable, implying that no DeltaD was being produced with a terminal valine. The block was still fully effective as late as 72 hpf (Fig. 4C). The predominant mRNA instead was a mis-spliced variant with the subterminal intron retained, so as to code for a protein whose terminal valine was replaced by a sequence of 34 amino acids ending in LVLN*.
We examined the effect of MO\[dlD-V\] on the DeltaD-MAGI1 interaction by artificially expressing MAGI1-EGFP alone or together with the morpholino in sibling embryos. In the absence of MO\[dlD-V\], we observed co-localization of DeltaD and MAGI1-EGFP in somite cells, as described above (Fig. 3D). Upon addition of MO\[dlD-V\] to the mRNA injection mixture, however, this co-localization was abolished while the distribution of DeltaD appeared to be unaffected (compare Fig. 3D,E). Although it is difficult to put these observations on a firm quantitative footing, the effect seemed clear. We conclude (1) that treatment with MO\[dlD-V\] is effective in generating a mis-spliced variant of DeltaD which, although expressed at normal levels, is incapable of binding MAGI proteins; and (2) that the co-localization of MAGI1 with DeltaD seen in vivo is indeed dependent on direct interaction between the two proteins.

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Specific and non-specific effects of the DeltaD splice-blocking morpholino can be distinguished by analysis of aei/deltaD mutants

To test the functional significance of the DeltaD-MAGI1 interaction, we examined the phenotype of the embryos that developed from eggs injected with 5 ng of MO\[dlD-V\], so that the only forms of DeltaD produced would be lacking their terminal valine.

At a gross anatomical level, the injected embryos showed clear and reproducible abnormalities in the hindbrain and midbrain, such that at 24 hpf, the width of the roofplate and the lumen of the third and fourth ventricles of the neural tube were markedly reduced (Fig. 5A,B, Table 2). A similar narrowed-ventricle effect was seen when we injected a morpholino (MO\[MAGI1\]) targeted against the translational start of MAGI1 (Fig. 5C), whereas injection of other

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<th>Table 2. Effect of morpholino injections on midbrain and hindbrain structure, scored at 24 hpf</th>
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WT, brain ventricles very similar to uninjected.
Mild, brain ventricles have formed but are misshapen and reduced in size.
Severe, ventricles absent or severely reduced in size and severely misshapen.
morpholinos, including a five base-pair mismatch control for MO[dlD-V], did not produce this phenotype (data not shown, Table 2). These findings strongly suggested that the anatomical abnormality was a specific effect of disrupting the DeltaD-MAGI1 interaction. Similar structural abnormalities have, however, been seen as an effect of morpholino mistargeting (Ekker and Larson, 2001), and they are not easily explainable in terms of known functions of DeltaD or MAGI1. Spurred on by the doubts of a referee, we therefore performed a further, and more decisive, control experiment.

For this, we took advantage of the aei\textsuperscript{AR33} mutant, which has a stop codon in the fifth EGF repeat of the extracellular domain of deltaD, leading to a DeltaD loss-of-function phenotype (Holley et al., 2000). Since the C terminus of DeltaD is already missing in aei\textsuperscript{AR33} homozygotes, we might perhaps expect that they would show the narrowed-ventricle phenotype even without any morpholino injection; and in any case their phenotype should certainly show no further change when MO[dlD-V] is injected, if the only effect of this morpholino is to block the DeltaD-MAGI1 interaction. As it turned out, our expectations were confounded on both scores. The uninjected aei\textsuperscript{AR33} homozygotes did not show a narrowed-ventricle phenotype, but when they were injected with MO[dlD-V] they did show it (Fig. 5D,E, Table 2). We can only conclude that the narrowed-ventricle phenotype resulting from MO[dlD-V] is due to a non-specific toxic effect of the morpholino.

**Disruption of the DeltaD-MAGI interaction does not significantly affect the known functions of DeltaD as a Notch ligand**

The aei\textsuperscript{AR33} mutant is useful not only as a negative but also as a positive control, displaying defects that result from loss of DeltaD: somite segmentation is disrupted (Holley et al., 2002; Jiang et al., 2000; van Eeden et al., 1996); primary neurons are produced in excessive numbers in the embryonic CNS (Holley et al., 2000); and the numbers of hypochord (ventral midline) cells are reduced (Latimer et al., 2002). These abnormalities have been well documented and reflect the functions of DeltaD as a Notch ligand. To see whether the DeltaD-MAGI interaction is important for these functions, we compared wild-type and aei\textsuperscript{AR33} mutant embryos with genetically normal embryos that were injected with MO[dlD-V].

First of all, somite patterning appeared normal in the MO[dlD-V] injected embryos, with no sign of the disruption of somite segmentation that is seen in aei and in other Notch pathway loss-of-function mutants (Fig. 5F-H) (Holley et al., 2002; Jiang et al., 2000; van Eeden et al., 1996).

Our findings with regard to hypochord formation were similar. Defects in hypochord formation are readily observed in aei/deltaD mutants as well as deltaA loss-of-function mutants and morpholina-induced DeltaC knock-down embryos (Appel et al., 1999; Latimer et al., 2002); the phenotype can be seen by in situ hybridization with a probe for alpha-1 collagen type II (col2a1) which labels the floor plate and hypochord (Yan et al., 1995). Using this method, we saw a reduction in hypochord cell number in aei\textsuperscript{AR33} embryos, as expected. However, we detected only a slight increase in hypochord defects in wild-type embryos treated with MO[dlD-V] (Fig. 5I-K). Moreover, we observed no effect of MO[dlD-V] treatment on expression of her4 or ntl (data not shown); these genes are both regulated – her4 positively and ntl negatively – by Delta-Notch signalling during normal hypochord development (Latimer et al., 2002). These findings indicate that the DeltaD-MAGI1 interaction is not important for the function of DeltaD as a Notch ligand in specification of the hypochord.

Lastly, Delta-Notch signalling, mediating lateral inhibition, is well known to regulate the proportion of cells committed to differentiate as neurons (Appel and Eisen, 1998; Chitnis et al., 1995; Haddon et al., 1998), and this is reflected in the phenotype of aei/deltaD mutants, which produce neurons in excess (Holley et al., 2000). If interaction with MAGI1 is important for DeltaD’s function in lateral inhibition, we would expect to see alterations in neurogenesis when the interaction is blocked. We therefore counted neurons, at the 12-16 somite stage, in genetically wild-type embryos injected with MO[dlD-V] and in uninjected controls, using in situ hybridization for islet1 as a neuronal marker (Haddon et al., 1998; Korzh et al., 1993). We found that the MO[dlD-V] injection produced little or no change in the number of islet1-positive cells. In contrast, aei\textsuperscript{AR33} mutants showed a 1.6-fold increase (Fig. 6A-E). Specifically, in the dorsal neural tube, in the region corresponding to the middle five somites, we counted 28.3±0.6 (mean±s.e.m., n=10) islet1-positive neurons per wild-type
embryo, 32.8±1.3 (n=13) per MO[dlD-V]-injected wild-type embryo, 45.3±1.7 (n=10) per aeiAR33 embryo, and 46.2±1.1 (n=13) per aeiAR33 embryo injected with MO[dlD-V]. We conclude that the DeltaD-MAGI interaction has very little influence on the function of DeltaD in the regulation of neuronal commitment.

**Discussion**

All vertebrates, apparently, and some invertebrates such as echinoderms, possess at least one Delta family member with a conserved ATEV motif at its intracellular terminus. We have found by in vitro studies that this terminus of the ATEV Delta binds selectively to members of the MAGI family of PDZ-containing scaffold proteins. We have shown, moreover, that the binding is direct, is to all three MAGIs, and is specifically to their PDZ4 domain. The interaction is demonstrable in cultured cells, where it enables Delta protein to recruit MAGI to their PDZ4 domain. The interaction is direct, is to all three MAGIs, and is specifically conserved A TEV motif at its intracellular terminus. We have shown, moreover, that DeltaD-MAGI interaction is important in some way either in determining the site of production of the neurons, or in governing their migratory behaviour as they move away from their birthplace.

**Morpholino experiments require stringent controls**

To discover what the function of the interaction might be, we exploited a morpholino, MO[dlD-V], which, when injected into the early zebrafish embryo, specifically blocks the interaction by interfering with the splicing of the deltaD message so as to alter the C terminus of the DeltaD protein. In any morpholino experiment, however, there is a possibility that non-specific side-effects may also be produced. In our system, we were indeed initially misled. We saw a marked narrowing of the third and fourth ventricles of the neural tube in embryos injected with MO[dlD-V], performed several controls of the sort that are conventionally done to check specificity of morpholino action, and concluded that the ventricle abnormality was a specific effect of disruption of the DeltaD-MAGI interaction. Fortunately, a deltaD loss-of-function mutant, aei, was available and allowed us to perform a more stringent control experiment. This showed convincingly that the ventricle abnormality was after all a non-specific side-effect of the morpholino. We offer this cautionary tale as a footnote to our other findings: it may serve as a warning of the risks of misinterpreting morpholino experiments, where often there is no mutant available to provide a stringent test for non-specific side-effects.

**Delta-Notch signalling appears to be independent of the Delta-MAGI interaction**

DeltaD has several well-characterized roles in Notch signaling, clearly revealed in the deltaD loss-of-function mutant aei, where disorders are seen in somitogenesis, hypochond formation, and neurogenesis (Holley et al., 2000; Jiang et al., 2000; Latimer et al., 2002; van Eeden et al., 1996). Upon disruption of the DeltaD-MAGI interaction, however, these processes are largely unaffected. The fact that this treatment does not phenocopy aei indicates that the interaction of Delta with MAGI proteins is not important for the function of DeltaD as an activating ligand for Notch, at least in these processes. This finding is perhaps not surprising given that, as explained in the Introduction, several forms of Delta protein lacking the MAGI-binding motif are already known to be effective Notch ligands.

**The Delta-MAGI interaction may be important in the control of neuron migration**

In assaying neurogenesis in embryos injected with MO[dlD-V] to block DeltaD-MAGI interaction, we observed a mislocalization of Rohon-Beard sensory neurons, which frequently strayed into the dorsal midline of the neural tube – a phenomenon rarely seen in wild-type or aei embryos. This effect needs further investigation, but it suggests that the DeltaD-MAGI interaction is important in some way either in determining the site of production of the neurons, or in governing their migratory behaviour as they move away from their birthplace.

We are attracted by the latter possibility, since other studies have reported effects of Delta protein on cell motility: in particular, De Joussineau et al. (De Joussineau et al., 2003) found that Delta in Drosophila sense-organ precursor cells promoted extension of filopodia, while Lowell and Watt (Lowell and Watt, 2001) found that mammalian keratinocytes showed enhanced motility when they expressed a truncated form of Delta1 lacking most of the intracellular domain.
Exert important effects on cell behaviour by influencing the cross-talk between the Delta-Notch pathway and many other Data S1 (see supplementary material), and could mediate is to discover what that function is. The MAGI proteins, with interaction does not appear to be critical for Delta-Notch proteins, not only in vitro but also in the living embryo. The subset of the Delta family – the ATEV Deltas – interact through the vertebrate body. We have established that a conserved development and maintenance of a great variety of tissues in As Notch ligands, the Delta proteins play a central part in the translocation to the nucleus.

MAGI proteins could influence such a reverse signalling that can enter the nucleus and act as a gene-regulatory protein. Delta itself can be cleaved to release an intracellular fragment suggested by recent studies (Ikeuchi and Sisodia, 2003; LaV oie (Franklin et al., 1999); or they could regulate gene expression by binding (or failing to bind) to Notch on neighbouring cells. Delta-MAGI complexes could have direct effects on the possibilities for the detailed molecular mechanism. Delta and des/notch1a reported similar phenomena: they find that mutation of Malicki, 2002). Gray et al. (Gray et al., 2001) have also reported similar phenomena: they find that mutation of des/notch1a alters the migration of neural crest cells and has a non-cell-autonomous effect on axon outgrowth.

Whichever of these interpretations is correct, there are many possibilities for the detailed molecular mechanism. Delta and Delta-MAGI complexes could have direct effects on the adhesive or locomotor properties of the cell surface or actin cortex (Lowell and Watt, 2001); they could influence motility by binding (or failing to bind) to Notch on neighbouring cells (Franklin et al., 1999); or they could regulate gene expression cell-autonomously to exert their effects. This last possibility is suggested by recent studies (Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003) showing that, like Notch, Delta itself can be cleaved to release an intracellular fragment that can enter the nucleus and act as a gene-regulatory protein. MAGI proteins could influence such a reverse signalling activity of ATEV Deltas by regulating their cleavage or their translocation to the nucleus.

**Conclusion**

As Notch ligands, the Delta proteins play a central part in the development and maintenance of a great variety of tissues in the vertebrate body. We have established that a conserved subset of the Delta family – the ATEV Deltas – interact through their intracellular tails with the MAGI family of scaffolding proteins, not only in vitro but also in the living embryo. The interaction does not appear to be critical for Delta-Notch signalling, and yet its evolutionary conservation implies that it must have an important physiological function. The challenge is to discover what that function is. The MAGI proteins, with their multiple PDZ and other protein-recognition domains, have an extraordinary variety of binding partners, as listed in Data S1 (see supplementary material), and could mediate cross-talk between the Delta-Notch pathway and many other pieces of intracellular or extracellular machinery. MAGI may exert important effects on cell behaviour by influencing the location of Delta, or Delta may do so by influencing the location of MAGI. In this paper we have excluded some possible roles of the Delta-MAGI interaction and found preliminary evidence for an effect on cell positioning; further experiments will be needed to explore its functional significance fully.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/131/22/5659/DC1

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