A family of mammalian Fringe genes implicated in boundary determination and the Notch pathway

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SUMMARY

The formation of boundaries between groups of cells is a universal feature of metazoan development. Drosophila fringe modulates the activation of the Notch signal transduction pathway at the dorsal-ventral boundary of the wing imaginal disc. Three mammalian fringe-related family members have been cloned and characterized: Manic, Radical and Lunatic Fringe. Expression studies in mouse embryos support a conserved role for mammalian Fringe family members in participation in the Notch signaling pathway leading to boundary determination during segmentation. In mammalian cells, Drosophila fringe and the mouse Fringe proteins are subject to post-translational regulation at the levels of differential secretion and proteolytic processing. When misexpressed in the developing Drosophila wing imaginal disc the mouse Fringe genes exhibit conserved and differential effects on boundary determination.

Key words: Fringe, boundaries, Notch, hindbrain, presomitic mesoderm, neurogenesis, mouse, Drosophila

INTRODUCTION

Intercellular signaling is a fundamental feature underlying the establishment of pattern during embryonic development (Jessell and Melton, 1992). Theoretical models predict that an important aspect of embryonic patterning is likely to be the establishment of boundaries (Meinhardt, 1991). These boundaries are expected to demarcate key regions of the embryo, and to pattern those regions by acting as organizers (Lawrence and Struhl, 1996).

Such models have recently received direct experimental support from studies of patterning in the Drosophila wing imaginal disc. This embryonic field is subdivided by cell lineage restrictions into anterior, posterior, dorsal and ventral cell populations termed compartments (Garcia-Bellido, 1975; Blair, 1993). It was recently shown that the dorsal-ventral (D-V) boundary is established through the activities of the Drosophila genes fringe (D-fng), Serrate (Ser), Delta (Dl), and Notch (Irvine and Wieschaus, 1994; Speicher et al., 1994; de Celis et al., 1996; Kim et al., 1995; Diaz-Benzunmea and Cohen, 1995; Doherty et al., 1996). Molecular cloning of D-fng indicated that it encodes a putative secreted signaling protein. Genetic mosaic analysis and ectopic expression studies demonstrated that it is the juxtaposition of D-fng-expressing cells (dorsal) with D-fng non-expressing cells (ventral) that results in fringe-dependent cell signaling. D-fng is thus unique among known signaling molecules in that it is not D-fng expression itself that acts as a signal, but rather its expression boundaries.

The boundary-specific nature of fringe activity suggests that D-fng not only influences signaling to fringe non-expressing neighboring cells, but it also determines a fringe-expressing cell’s inability to respond to fringe-dependent signals.

Although Notch is expressed throughout the entire imaginal disc, at the time of boundary determination it is activated specifically in the dorsal and ventral cells just adjacent to the compartment boundary (de Celis et al., 1996). D-fng activity modulates the ability of Ser and Dl to activate Notch (V. Panin, V. Papayannopoulos, R. W. and K. D. I., unpublished data). This influence of D-fng results in a positive feedback loop between the two Notch ligands at the D-V boundary such that Dl expressed in ventral cells binds and activates Notch in dorsal cells, and Ser expressed in dorsal cells binds and activates Notch in ventral cells. An important consequence of D-fng boundary-mediated signaling for the formation of the wing is that the initially asymmetric boundary between dorsal and ventral cells is translated by reciprocal activation of Notch at the D-V compartment boundary into symmetrical expression of downstream targets and the expression of wingless (wg) as the organizing signaling molecule (de Celis et al., 1996; Doherty et al., 1996; Kim et al., 1995, 1996; Rulifson and Blair, 1995; Diaz-Benzunmea and Cohen, 1995; Zecca et al., 1996).

Given the evolutionary conservation of the Notch pathway (Artavanis-Tsakonas et al., 1995), we searched for and have identified three Fringe genes in mouse and human. Our characterization of their coincident expression with Notch ligands at developmental boundaries, their differential protein secretion...
and processing, and their ability to participate in boundary determination in the Drosophila wing disc provides evidence for both conserved and novel aspects of restricting fringe activities.

MATERIALS AND METHODS

Cloning and sequencing

Ten human cDNAs with homology to Drosophila fringe were initially identified in a screen of The Institute for Genome Research (T.I.G.R.) EST database and additional human and mouse cDNAs were identified by reiteratively screening the Database of Expressed Sequence Tags at the National Center for Biotechnology Information (N.C.B.I.) using the BLAST network service (Altschul et al., 1990). The following human clones were obtained and sequenced: cDNA clone 40887 (GenBank accession number: R56561; IMAGE Consortium) was designated LUNATIC FRINGE, clones HCE0B60 (TIGR) and HSC3MD091 (GenBank accession number: F13368; Genexpress cDNA program) were designated RADICAL FRINGE and clone HNBAA16 (TIGR) was designated MANIC FRINGE. Clones 40887, HCE0B60 and HNBAA16 were used separately to screen a 8.5 d.p.c. mouse embryonic cDNA library (Fahrner et al., 1987). Mouse Lunatic, Radical and Manic cDNAs were identified. Mouse Manic and Radical cDNAs were extended 5' using nested RACE PCR with the Marathon cDNA Amplification Kit (Clontech) on an adult mouse brain linker ligated cDNA library (gift from T. Vasicke). Primer sequences are available on our laboratory homepage (www.molbio.princeton.edu/vogt/vogt.html).

In situ hybridization

Embryos were obtained from pregnant female Swiss mice; 0.5 d.p.c. corresponding to the morning the vaginal plug was detected. RNA in situ hybridizations on serial 8-μm paraffin tissue sections were performed essentially as described by Wilkinson and Nieto (1993). Whole-mount in situ hybridizations using digoxigenin-labeled riboprobes were performed according to the protocol of Riddle et al. (1993). In double-label experiments, the other probe was fluorescein-labeled and detected with anti-fluorescein-AP using FastRed (Sigma) as a substrate. The other probes were NotchI (Amo et al., 1992), Dll1 (Bettencourt et al., 1995), Jagged (Lindell et al., 1995), Sek-I (Nieto et al., 1992) and Mox-I (Candia et al., 1992).

Fringe-AP fusion constructs and expression analysis of Fringe-AP fusion proteins

The ORFs of each cDNA were PCR amplified and cloned into APtag-2 (Cheng et al., 1995). Primer sequences are available on our laboratory homepage (www.molbio.princeton.edu/vogt/vogt.html).

COS 7 and Hep3B cells were grown in 100 mm plates in DMEM with 10% bovine calf serum and were transiently transfected using Lipofectamine (GIBCO BRL). The media and lysed cells were collected and AP activities measured essentially as described by Flanagan and Leder (1990) and Cheng et al. (1995).

[35S]methionine ([35S]Met) labeling of COS cells and immunoprecipitations of labeled media or cell fractions with anti-human alkaline phosphatase (Genzyme Diagnostics) were performed essentially as described by Flanagan and Leder (1990).

A number of controls were performed to diminish the likelihood that the absence of Radical-AP protein in the media fractions was due to analytical techniques including the sequencing of multiple cDNA and genomic clones, analysis of ECM fractions extracted with 1 M NaCl and methods similar to those described by Bradley and Brown (1990) and exchange of the AP-tag for an Fe-tag (Zettlmeissl et al., 1990).

N-terminal sequence analysis of Fringe-AP and Lunatic-AP

N-terminal protein sequencing was performed on an Applied Biosystems 476A Protein Sequencer System. The N-terminal sequence obtained for D-fringe-AP was YPGIQ which corresponds to amino acids 40-45 and the N-terminal sequence obtained for mouse Lunatic-AP was DADPP which corresponds to amino acids 86-90.

Misexpression in the Drosophila wing

cDNAs encoding each respective mouse Fringe were cloned into pUAST (Brand and Perrimon, 1993). Constructs were transformed into Drosophila (Rubin and Spradling, 1982), and several independent lines were established for each transgene. Flies carrying Fringe transgenes were crossed to the ptc-Gal4 driver strain (GALA 559.1; Hinz et al., 1994) and the wings and wing discs of their ptc-Gal4 UAS-Mfrng progeny were examined, mounted and stained for wg protein expression as described previously (Irvine and Wieschaus, 1994). Lunatic expression along the A-P boundary of the wing imaginal disc was confirmed by in situ hybridization and DNA sequencing confirmed that no mutations had been introduced during cloning.

RESULTS

Identification of a mammalian Fringe gene family

We used the D-fng protein sequence to interrogate human expressed sequence tagged (EST) databases and identified three classes of fringe-related sequences. Using the human FRINGE-related ESTs as probes, we identified full length open reading frames for three orthologous mouse genes (Fig. 1). We have named the three mammalian fringe-related family members: Lunatic Fringe, Radical Fringe and Manic Fringe. Examination of the mammalian Fringe genes' sequences suggests conservation of several features consistent with secreted proteins: predicted amino-terminal signal peptides, potential N-linked glycosylation sites, distribution of cysteine residues and conservation of an internal proteolytic processing site in Lunatic (Fig. 1 and Irvine and Wieschaus, 1994).

The presence of the conserved proteolytic processing site among D-fng, mouse Lunatic, and Xenopus Lunatic (Wu et al., 1996), suggests they may be synthesized as inactive pre-pro-protein precursors that would require proteolytic processing for full activity. Interestingly, the mouse Manic and Radical predicted signal sequence cleavage sites align at the same relative position as the pre-processing site in D-fng and Lunatic (Fig. 1A). Therefore, all mature Fringe proteins are predicted to be of similar size and pl (Fig. 1 legend). The existence of three mammalian family members could allow for differential regulation and the deployment of a spectrum of overlapping and specialized activities.

Manic and Lunatic Fringe expression demarcates segment boundaries during mammalian development

Northern analysis shows that each individual Fringe family member is expressed in the embryo and in adult tissues derived from all three germ layers (data not shown). Analysis of their expression in mouse embryos at 8.0-10.5 d.p.c. by whole-mount RNA in situ hybridization reveals that the Fringe genes are dynamically expressed in many different cell types undergoing cell fate decisions (Fig. 2). Strikingly, Manic and Lunatic expression is suggestive of a role for mammalian Fringe genes in the demarcation of segmental boundaries observed during anterior-posterior patterning of the hindbrain and somites (Lumsden and Krumlauf, 1996; Tam and Trainor, 1994).
Fig. 1. Amino acid comparisons of D-fringe to mouse and human Lunatic, Radical and Manic Fringe. (A) PILEUP alignment of the Drosophila, mouse and human Fringe amino acid sequences (accession numbers for mouse Manic Fringe, U93439; Radical Fringe, U94350; Lunatic Fringe, U94351 and human MANIC FRINGE, U94352, RADICAL FRINGE, U94353; and LUNATIC FRINGE, U94354). Human genes are in upper case. The predicted pre regions/signal sequences are underlined (Nielsen et al., 1996). A conserved dibasic processing site between D-fng and Lunatic Fringe is boxed. Conserved cysteines are denoted by arrowheads and predicted N-linked glycosylation sites are double underlined. Amino acids identical among 50% of the sequences are shaded in black and amino acids with a high degree of similarity among 50% of the sequences are shaded in grey. Numbers represent the amino acid number of D-fng. (B) Schematic representation of the Fringe molecules. D-fng and Lunatic are predicted to be pre-pro-protein precursors while Manic and Radical are predicted to be pre-protein precursors. Conserved dibasic processing sites are indicated by arrows. Predicted N-linked glycosylation sites are denoted by branches. Numbers to right indicate amino acid length of the predicted ORFs. The conserved mature domain among Fringe family members, indicated by black boxes, have similar relative molecular masses: D-fng 33.4, Lunatic 32.9, Manic 32.7 and Radical 32.7 (×10³); and pI’s: D-fng 7.5, Lunatic 7.76, Manic 7.88 and Radical 7.89. (C) Pairwise comparisons of percentage amino acid identity in the conserved domain (beginning at Asp 149 of D-fng) among D-fng and the mouse Fringe family members. Values in parentheses represent similarities when allowing for conservative amino acid substitutions. Based on their primary amino acid sequences the two recently identified Xenopus Fringe genes (Wu et al., 1996) are most closely related to the mammalian Radical and Lunatic genes. However, in contrast to mouse Radical, Xenopus Radical predicts a pre-pro protein precursor.
Vertebrate hindbrain development proceeds by a process of internal subdivision resulting in seven metameric units termed rhombomeres (reviewed by Lumsden and Krumlauf, 1996). Both Manic and Lunatic are expressed in anterior-posterior stripes in the hindbrain prior to the formation of morphologically recognizable rhombomeric boundaries. In embryos at the 5 somite stage, Manic is expressed in pre-r3, while Lunatic is expressed in the otic vesicle (asterisks). In the hindbrain region, anterior and slightly posterior to the otic vesicle, different levels of Lunatic expression are observed in the rhombomeres. The neural tube of this embryo has been splayed open and one half of the tube is observed. Three lateral stripes of expression along the D-V axis of the neural tube are apparent (arrows). Lunatic is also expressed in the dermamyotome of the mature somites (arrowheads). Manic is also expressed in the olfactory placode (black arrow), the neural crest migrating into the branchial arches (arrowhead), and in the otic vesicle (white arrow). The expression of Lunatic is dynamic, reflecting the rostral-caudal wave of somitic developmental progression (Fig. 3C,D; Tam and Trainor, 1994). In the posterior presomitic mesoderm, Lunatic is expressed in a broad swath of cells. As somitogenesis proceeds, this band of expression becomes narrower, paralleling the cell condensation observed within the anterior presomitic mesoderm as it organizes into clusters of cells recognizable in scanning electron micrographs as segmental units termed somitomeres (Fig. 3C,D). Stripes of Lunatic expression are observed in the anterior two presumptive somitomeres that are about to form somites. These stripes of expression appear to correspond to what will form the posterior half of the somite (Fig. 3D). Once the somite has formed, transcript levels of Lunatic decline rapidly, such that expression is seldom evident in the newly formed somites. However, Lunatic expression is later observed in the dermamyotome of the mature somites (Fig. 2C; data not shown).

Fringe may participate in a conserved Notch pathway to determine somitic boundaries

In the fly wing D-fng acts upstream of Notch (Kim et al., 1995;
Mouse Notch1 is expressed throughout the presomitic mesoderm and somite segmentation is perturbed in Notch1 mutant embryos (Conlon et al., 1995). The candidate vertebrate Notch ligand, Delta-like 1 (Dll1), is also expressed in a broad domain throughout the presomitic mesoderm (Fig. 3E; Bettenhausen et al., 1995). By performing double label RNA in situ we observe that the initial broad posterior band of Lunatic expression is found in the midst of Dll1-expressing cells. However, as the somitomere matures, Lunatic and Dll1 expressions refine. Lunatic expression is localized to the posterior half of presumptive somitomere 2, while Dll1 expression is localized to the posterior border of this same somitomere (Fig. 3E). Dll1 expression also extends caudally throughout the adjoining presomitic mesoderm. As somitogenesis continues and transcript levels of Lunatic diminish, cells that do not express either gene, the prospective anterior half of the somite, are observed between the Lunatic and Dll1 expression domains (Fig. 3F).

**Fringe expression suggests a role in vertebrate neurogenesis**

Mammalian Fringe genes are expressed in the three contexts in which vertebrate neurogenesis occurs: within the central neuroepithelium, during migration of neural crest cells, and in...
cranial placodes (Figs 2, 4). At 10.5 d.p.c. Fringe family members are expressed in a series of distinct longitudinal stripes within the ventricular region of the hindbrain and the neural tube (Fig. 2C; data not shown). Examination of their expression in cross sections of the neural tube at different rostral-caudal levels reveals Lunatic and Manic Fringe in bilateral symmetrical stripes at different dorsal-ventral levels: a ventral stripe that appears to border the floor plate, a narrow medial stripe and a broad domain that includes most of the alar plate (Fig. 4). As the rostral-caudal development of the spinal cord progresses the dorsal domains of Manic and Lunatic expression refine into two stripes, with the dorsal limit bordering the roof plate. At this developmental stage, Radical does not appear to be expressed in the caudal region and its rostral expression appears overall to be weaker and more diffuse (Figs 2H, 4C,F). At later stages Radical expression is associated with the appearance of ventral motor neurons (data not shown). Therefore, Radical expression is correlated with the rostral-caudal maturation of the spinal cord and the differentiation of neuronal populations.

The vertebrate Notch pathway has been proposed to mediate the maintenance of a population of dividing uncommitted cells in the ventricular zone of the neural epithelium by lateral specification (reviewed in Lewis, 1996; Tanabe and Jessel, 1996; Chitnis et al., 1995). To determine the relationship of Fringe gene expression to Notch ligands within the neural tube, we examined the expression of Manic Fringe relative to Dll1 and to Jagged\Serrate-1. We observe that Manic and Dll1 expression are largely coincident, whereas Manic and Jagged expression appear to be complementary (Fig. 4G,H). Therefore, although within the neural tube there is a juxtaposition of Fringe and Notch ligands, their relative expression is complementary to the expression of D-fng and Ser andDll during wing boundary determination (Kim et al., 1995; Doherty et al., 1996).

Fringe family members are also expressed at other sites and times of neurogenesis. Fringe family members exhibit restricted expression patterns in cranial placodes (e.g. olfactory placode and the otic vesicle) that will give rise to sensory structures (Fig. 2B,C,E,F; data not shown). The development of sensory structures also appears to be associated with the expression of Notch and regionalized expression of its ligands (Lewis, 1996). Lastly, the expression of Fringe family members is evident in the neural crest derived dorsal root ganglia and sensory ganglia (Figs 2, 4).

Fringe family member activities may be regulated at the level of secretion and proteolytic processing

The mammalian Fringe gene family expression profiles provide evidence for the acquisition of different regulatory elements controlling spatial-temporal transcription. The structures of the Fringe precursor proteins suggests an opportunity for posttranslational regulation (Fig. 1). Expression of an epitope-tagged D-fng protein in fly cell lines (S2 and clone-8) and in the wing imaginal disc show that the protein can be secreted (V. Panin, V. Papayannopoulos, R. W. and K. D. I., unpublished data). To determine if Lunatic, Radical and Manic proteins are secreted and processed we generated Fringe-alkaline phosphatase protein fusion constructs (AP-tag) and assayed their expression in COS (monkey kidney) and Hep3B (human liver) cell lines. For comparison, we also examined the secretion and processing of D-fng-AP in COS cells. Protein expression was determined in media and cell lysates by measuring alkaline phosphatase (AP) activity and by immunoprecipitation of [35S]Met labeled Fringe AP-tagged proteins (Flanagan and Leder, 1990).

Significant levels of AP activity in the media were observed for Lunatic-AP, Manic-AP and D-fng-AP constructs, indicative...
of efficient secretion (Fig. 5A; data not shown). In contrast, Radical-AP produced significantly lower AP activity in the media when compared to the other Fringe proteins and to the secreted alkaline phosphatase (Seap) control (Fig. 5A; data not shown). Pulse-chase labeling in COS cells demonstrates that Radical-AP exhibits a similar rate of synthesis and intracellular stability to both Manic-AP and Lunatic-AP; however, over the time period of the chase, Manic-AP and Lunatic-AP appear in the media, whereas Radical-AP does not (data not shown).

In addition, D-fng-AP, Lunatic-AP and Manic-AP are immunoprecipitated from the media of [35S]Met labeled cells while Radical-AP is not (Fig. 5B; Materials and Methods). These observations suggest that although Radical enters the secretory pathway and is processed to the mature form, it is not found as a stable extracellular protein.

We also examined whether the Fringe proteins are processed. Both in [35S]Met labeled COS cell lysates and media, the fastest migrating immunoprecipitated bands correspond to the predicted sizes of the mature proteins for Manic-AP and Radical-AP (Fig. 5B). The slower bands are indicative of glycosylation known to occur on AP. By contrast in COS cell lysates we observe bands corresponding to the predicted sizes of the pro-proteins for Lunatic-AP and D-fng-AP (Fig. 5B). However, in the media we observe two bands for Lunatic-AP that migrate at relative positions consistent with their representing the pro- and mature forms (Fig. 5B). The identity of the major secreted Lunatic-AP band was determined by N-terminal sequencing to represent the fully processed mature form. In contrast to Lunatic, D-fng is observed in the media as a single major band. The identity of the secreted D-fng-AP protein recovered from the media was determined by N-terminal sequencing to represent its pro-form (Fig. 5B). In summary, although individual Fringe family members are evolutionarily conserved within the mature protein region and they all appear to enter the secretory pathway, they differ in their efficiency of secretion and in their requirement for posttranslational proteolytic processing.

**Expression of mammalian Fringe genes in the Drosophila wing affects boundary determination**

The *Drosophila* wing disc offers an established context in which to investigate the potential boundary-determining activities of mouse Fringe family members. We misexpressed Manic, Radical and Lunatic in the wing disc using the UAS-Gal4 system and compared their activity to that of D-fng (Kim et al., 1995). The patched (ptc) promoter driving Gal4 directs UAS-Fringe expression in a broad stripe along the anterior side of the A-P compartment boundary, perpendicular to endogenous, dorsally restricted D-fng expression (schematized in Fig. 6F). Misexpression of D-fng using the ptc-Gal4 driver has two effects on *Drosophila* wing development (Kim et al., 1995). First, wing margin tissue is lost at the D-V compartment border where misexpression has eliminated a fringe expression and non-expression boundary (Fig. 6B). This loss of wing margin tissue can also be visualized in the developing wing imaginal disc as a loss of wg protein expression (Fig. 6G). Second, an ectopic wing margin is induced in ventral cells where misexpression has created a novel fringe expression boundary (Fig. 6B). Induction of ectopic wing margin can be visualized in the developing wing imaginal disc as ectopic wg protein expression (Fig. 6G).

Misexpression of mouse Manic using the ptc-Gal4 driver eliminates wing margin tissue at the D-V compartment border (Fig. 6C) and causes a localized loss of wg protein expression (Fig. 6H). Misexpression of Manic in the ventral compartment is able to induce ectopic wg protein expression but is unable to induce ectopic wing margin formation (Fig. 6C,H). Thus, in comparison to D-fng, Manic’s ability to induce ectopic margin in the ventral compartment, whether assessed by margin bristle formation or wg protein expression, is compromised (compare Figs 6B,G to 6C,H). Similar to Manic, misexpressed Radical also retains an ability to prevent normal margin formation and causes a localized loss of wg protein expression in the wing imaginal disc (Fig. 6D,J). However in contrast to D-fng and Manic, this does not result in ectopic wg protein expression or margin formation (Fig. 6I). Lunatic expression does not affect the margin or wg protein expression (Fig. 6E,J). Lunatic’s inactivity may result from an inability of wing imaginal disc cells to process it to the mature form (see Materials and Methods and Discussion). Overall, misexpression of mouse Fringe genes in the fly wing suggests a conserved ability to participate in aspects of boundary-determination via the Notch pathway, leading to wg expression at the D-V organizing center (Fig. 6K).

**DISCUSSION**

We have identified three mammalian genes (Manic, Radical, and Lunatic Fringe) that constitute a gene family related to *Drosophila* fringe (D-fng). The expansion and divergence of the Fringe family members has allowed for the evolution of differential regulation at the level of sites of their expression, protein secretion and proteolytic processing. Assessment of mammalian Fringe genes in the context of the *Drosophila* wing disc demonstrates that the mouse Manic and Radical genes exhibit a conserved role in the Notch pathway leading to expression of wingless at the D-V compartment boundary. The coordinated expression in mammals of Fringe genes with Notch and its ligands, Delta-like 1 and Jagged/Serrate-1, further suggests that Fringe genes operate in this pathway to determine boundaries during segmentation and in cell fate decisions occurring during neurogenesis.

**Fringe gene family members are subject to posttranslational regulation that restrict activities**

Regulation of secreted signaling molecules at the level of protein processing is an important feature of embryonic development and organismal homeostasis (Barr, 1991; Lee et al., 1994; Thomsen and Melton, 1993; Morisato and Anderson, 1995). The divergence of Fringe family protein precursor structure allows for differential regulation and a restriction of activities amongst individual family members. The differential processing and secretion of D-fng and mouse Fringe family members in mammalian cell lines and their distinct phenotypes when expressed in the *Drosophila* wing lead us to infer these posttranslational controls have biological consequences. The evolutionary conservation of a dibasic processing site amino terminal to the conserved regions of D-fng and vertebrate Lunatic Fringe proteins suggests that proteolytic cleavage plays an important regulatory role in Fringe function (Wu et al., 1996). The processing sites in D-fng (RRSR) and the ver-
tebrate Lunatic proteins (RXRR) both correspond to a recognition sequence for the subtilisin-like pro-protein convertase (SPC/furin family of proteolytic processing enzymes. These processing enzymes have been shown in both mammals and flies to exhibit tissue restricted expression and recognition site specificity (Barr, 1991; Bie et al., 1995; Roebroek et al., 1995). Thus, we suggest that the lack of an effect of Lunatic when misexpressed in the wing imaginal disc is consistent with the absence of a SPC/furin enzyme capable of processing it to its mature form. Recent experiments by Wu et al. (1996) have also provided indirect evidence that the Xenopus Lunatic protein requires regulated proteolytic processing for its extracellular activity in the developing frog embryo.

In contrast to D-fng and Lunatic, the primary sequences of mouse Manic and Radical have evolved such that only signal peptide cleavage is required for production of the mature protein. However, despite their similar structure, the regulation of Manic and Radical extracellular secretion appears to be more complex. In mammalian cell lines Manic is secreted into the medium, whereas Radical is not. Pulse-chase studies further demonstrate that the three mouse Fringe proteins are synthesized and accumulate intracellularly to similar levels; however, only mature Lunatic and Manic appear extracellularly. These observations are unexpected and provocative with respect to Fringe activities.

The boundary-specific nature of D-fng activity in Drosophila has led to the inference that D-fng not only confers fringe-dependent signaling to neighboring cells, but must also prevent fringe-expressing cells from responding to the fringe-dependent signal (Irvine and Wieschaus, 1994). A proposed mechanistic model accounting for the refractory and signaling aspects of fringe-dependent boundary signaling posits that in fringe-expressing cells a fringe protein complex assembles during transit in the secretory pathway that sequesters a component required for reception of a paracrine fringe-dependent signal (Irvine and Wieschaus, 1994). A similar combination of refractory and signaling activities is found in the behavior of the yeast killer toxin K1 protein. Mechanistically, immunity is hypothesized to be achieved by intracellular complexing of the killer toxin protein with its plasma membrane receptor, thus rendering the expressing cell refractory to the secreted killer toxin (Boone et al., 1986; Bussey, 1991).

We observe that the misexpression of mouse Manic and Radical under ptc-Gal4 control recapitulates the ability of D-fng to cause loss of the normal wing margin and extinction of wg expression. We propose that the activity conserved between D-fng and Manic and Radical is the ability to prevent fringe-expressing cells from responding to a fringe-dependent signal. Because Manic and Radical only require signal peptide processing to produce a mature Fringe protein, and because Lunatic lacks this refractory activity, we further suggest that fringe-refractory activity is conferred by the mature form of the protein.

Although both Manic and Radical confer fringe refractory activity, only Manic is able to induce the ectopic expression of wg in the ventral compartment. Radical does not induce ectopic wg or margin. This difference in the ability of Manic and Radical to induce ectopic wg expression correlates with our secretion data. Taken together, we favor a model that suggests that fringe-refractory activity is conferred by the mature form of the protein within the intracellular secretory compartment.
pathway, while fringe-dependent signaling requires extracellular secretion of a mature Fringe protein. Alternatively, the reduced extent of Manic’s (and formally Radical’s) induction of ectopic wg expression and of a morphological margin may reflect either a quantitative difference in the amount of secreted protein or a qualitative difference in mouse Fringe protein interaction with Drosophila proteins.

The expansion and divergence of mammalian Fringe family members has allowed for the acquisition of different regulatory elements controlling the localized expression of the different precursor proteins. This combination of transcriptional and posttranslational control could enable cells expressing different combinations of Fringe family members to fine-tune their overall reception and transmission of Fringe-dependent signaling activities. For example, we envision that it may be important during certain stages of either boundary determination or cellular differentiation to have cells acquire a state where they are refractory to Fringe-mediated signaling, yet are unable to send a Fringe signal. Expression of Radical would achieve this state.

**Fringe expression demarcates segmental boundaries in the mammalian embryo and may act through the Notch pathway**

Our observation that mammalian Fringe family members can affect boundary determination in the fly wing imaginal disc suggests that they operate through the Notch pathway. Furthermore, this result indicates that aspects of boundary organizing functions may be conserved from flies to man. This notion is supported by the observations that Fringe-expressing and non-expressing cells are juxtaposed prior to the formation of morphological segment boundaries during somitogenesis and in the hindbrain.

In mammals the clearest parallel between D-fng and its relationship to Notch in the wing is the probable participation of Lunatic in this pathway during somitogenesis. Absence of Notch1 in the presomitic mesoderm leads to a defect in the transition between presomitic mesoderm and somites (Conlon et al., 1995). Although Notch signaling is essential for somitogenesis, it is still unclear what patterning mechanisms provide the positional information to locate and establish segmental boundaries (Keynes and Stern, 1988). Our expression analysis suggests that the relationship of Lunatic to Dll1 is reminiscent of their expression during Drosophila wing margin boundary formation (Doherty et al., 1996). The expression of Lunatic in presumptive somitomeres suggests it may be a critical factor in determining segmental boundaries by acting to modulate the expression and/or signaling activities of Notch ligands to establish boundary-specific gene expression and possibly differences in cell adhesion and morphogenesis.

Although the role of the Notch pathway in the determination of hindbrain boundaries is unclear, the expression of Manic and Lunatic, as well as Jagged/Serrate-1, in pre-rhombomere 3 and pre-rhombomere 5 (C. R. and T. F. V., unpublished) are consistent with their playing a role in the segmentation of this structure (Lumsden and Krumlauf, 1996).

Fringe expression during neurogenesis suggests a role in developmental decisions mediated by the Notch pathway

D-fng, in addition to its role in boundary determination at the wing margin, is also involved in other cell fate decisions that interface with the Notch pathway (K. D. I., unpublished). Similarly, mammalian Fringe family members are expressed along with Notch and its ligands where obvious boundaries do not exist and this expression may be indicative of a role in interactions that specify cell differentiation. The regionalized expression of Fringe genes, the Notch ligands, Delta and Serrate, and the multiple Notch receptors in the neural tube, cranial placodes and neural crest is highly suggestive of a role for Fringe family members in Notch signaling in the selection of neural fates (Lewis, 1996; Tanabe and Jessell, 1996, Chintis et al., 1995). We suggest that integration of Fringe family activities with the Notch pathway may be required for generation of the complex set of neurons and support cells that originate along the D-V and A-P axis of the neural tube.

The dynamic and localized expression of individual Fringe family members suggest that they may function independently and in concert to influence cell fate decisions that occur during embryonic development and in adult tissue homeostasis. Furthermore, as has been found for deregulated Notch in mammals (Ellisen et al., 1991), we anticipate that alterations in Fringe expression and function may result in developmental defects and disease.

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