The Neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation

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SUMMARY

Neurofibromatosis-2 is an inherited disorder characterized by the development of benign schwannomas and other Schwann-cell-derived tumors associated with the central nervous system. The Neurofibromatosis-2 tumor suppressor gene encodes Merlin, a member of the Protein 4.1 superfamily most closely related to Ezrin, Radixin and Moesin. This discovery suggested a novel function for Protein 4.1 family members in the regulation of cell proliferation; proteins in this family were previously thought to function primarily to link transmembrane proteins to underlying cortical actin. To understand the basic cellular functions of Merlin, we are investigating a Drosophila Neurofibromatosis-2 homologue, Merlin. Loss of Merlin function in Drosophila results in hyperplasia of the affected tissue without significant disruptions in differentiation. Similar phenotypes have been observed for mutations in another Protein 4.1 superfamily member in Drosophila, expanded. Because of the phenotypic and structural similarities between Merlin and expanded, we asked whether Merlin and Expanded function together to regulate cell proliferation. In this study, we demonstrate that recessive loss of function of either Merlin or expanded can dominantly enhance the phenotypes associated with mutations in the other. Consistent with this genetic interaction, we determined that Merlin and Expanded colocalize in Drosophila tissues and cells, and physically interact through a conserved N-terminal region of Expanded, characteristic of the Protein 4.1 family, and the C-terminal domain of Merlin. Loss of function of both Merlin and expanded in clones revealed that these proteins function to regulate differentiation in addition to proliferation in Drosophila. Further genetic analyses suggest a role for Merlin and Expanded specifically in Decapentaplegic-mediated differentiation events. These results indicate that Merlin and Expanded function together to regulate proliferation and differentiation, and have implications for understanding the functions of other Protein 4.1 superfamily members.

Key words: Protein 4.1, ERM, FERM domain, Apical membrane, Neurofibromatosis-2, Merlin, expanded, Drosophila melanogaster

INTRODUCTION

The regulation of cell proliferation is central to controlling the size and shape of multicellular organisms. Disruption of proliferation control in an organism can have severe consequences; improper activation of cell proliferation can result in the formation of tumors and the development of cancer. Many genes involved directly with the control of the cell cycle, and those involved more indirectly with the control of proliferation, have been identified as oncogenes and tumor suppressors. Among the known tumor suppressor genes, the Neurofibromatosis 2 (NF2) gene is particularly intriguing. NF2 is an inherited disorder characterized by the formation of benign, Schwann-cell-derived tumors on the eighth cranial nerve, and other benign tumors associated with the central nervous system (Martuza and Eldridge, 1988). The NF2 gene encodes a novel member of the Protein 4.1 superfamily, Merlin, that is most highly related to the ERM (Ezrin, Radixin, Moesin) subgroup within the family (Gusella et al., 1999). Members of the Protein 4.1 superfamily all share a conserved region of ~350 amino acids known as the conserved N-terminal region (CNTR) or FERM domain (Chishti et al., 1998; Ward et al., 1998). Some members of the family, including the ERM proteins, interact with transmembrane binding partners through the CNTR and localize them to specific membrane domains via direct interactions between their C-terminal domain and the actin cytoskeleton (Mangeat et al., 1999).

Merlin shares significant sequence identity with ERM proteins, particularly in the CNTR. The C-terminal half of Merlin also contains a predicted coiled-coil domain, but lacks the actin-binding domain found at the C terminus of ERM proteins. Nonetheless, Merlin has been shown to interact with...
the actin cytoskeleton through an N-terminal binding region (Xu and Gutmann, 1998). Consistent with this observation, overexpression of Merlin in vertebrate cultured cells causes transient alterations in the F-actin cytoskeleton (Gutmann et al., 1999). Work from several laboratories indicates that Merlin can form homotypic dimers, and heterotypic dimers with ERM proteins, but the functional significance of these interactions is not known (Gronholm et al., 1999). Other Merlin binding partners include NHE3-RF, the Na+/H+ exchanger regulatory factor that also binds to ERM proteins (Receze et al., 1997; Murthy et al., 1998), and βII-spectrin (Scoles et al., 1998). Although transmembrane partners for Merlin have not been identified yet, the hyaluronin receptor CD44, the adhesion molecule ICAM-2, the cytos fibrosis transmembrane conductance regulator and the B-adrenergic receptor have been shown to bind ERM proteins directly or indirectly (Tsukita et al., 1994; Hall et al., 1998; Heiska et al., 1998). In addition, recent studies indicate that ERM binding is essential for proper recycling of the B-adrenergic receptor, and thereby regulation of its activity (Cao et al., 1999). Thus, it seems that ERM proteins and perhaps Merlin may bind to and regulate the functions of a number of transmembrane proteins. Nonetheless, it is not yet clear why loss of Merlin function results in misregulation of proliferation.

The discovery that a member of the Protein 4.1 superfamily has a tumor suppressor function was initially surprising because its members traditionally have been considered to play a structural role in linking transmembrane proteins to the cytoskeleton. However, another family member, Drosophila expanded (ex), has also been shown to have a tumor suppressor function; loss of ex function can lead to the development of hyperplastic imaginal discs and overgrown adult structures such as the wing (hence the name expanded; Boedigheimer and Laughon, 1993). Expanded has a divergent CNTR and a C-terminal region that contains a putative coiled-coil domain and three potential SH3-binding domains (Boedigheimer et al., 1993). Like NF2, ex seems to function in regulating proliferation, and loss of its function does not appear to significantly disrupt patterning of the affected imaginal discs.

We have identified and characterized an NF2 gene homologue in Drosophila called Merlin (Mer) and shown that it functions similarly to NF2 in regulating cell proliferation (McCartney and Fehon, 1996; LaJeunesse et al., 1998). To understand its cellular roles, we have begun to investigate the molecular framework in which Drosophila Merlin functions by identifying interacting genes and proteins. Given its phenotypic and structural similarities, and previous evidence that other family members can interact heterotypically, we were interested in the possibility that Merlin and Expanded might function together to regulate proliferation during Drosophila development. In this study, we show that Merlin and expanded display dose-sensitive interactions that suggest physical interactions between the proteins that they encode. Cell culture and blot overlay assays indicate that Merlin and Expanded interact directly via the CNTR of Expanded and the C-terminal domain of Merlin. Removal of all Merlin and Expanded function in double mutant clones results in severe differentiation defects that are not observed with either mutation alone. Genetic interactions between Mer, ex and decapentaplegic (dpp) are consistent with the notion that Merlin and Expanded positively regulate DPP function. Taken together, our results indicate that Merlin and Expanded function cooperatively in regulating cell proliferation and differentiation in developing epithelial cells.

METHODS AND MATERIALS

Drosophila stocks, genetic analysis and immunolocalization

All Drosophila cultures were maintained on standard corn meal, yeast, molasses and agar medium. The Mer alleles used in this study were described previously (Fehon et al., 1997; LaJeunesse et al., 1998). ex alleles were a gift from W. Boedigheimer and P. Bryant. K. Wharton provided the dpp-lacZ (B3.0) stock was obtained from the Bloomington Drosophila stock center. Whole-mount preparation and analysis of adult eyes, wings and legs were described previously (LaJeunesse et al., 1998). The distance between wing hairs as a measure of cell size was determined using the measure function in NIH Image. Scanning electron microscopy was preformed as described previously (Rebay et al., 1993). Somatic mosaic clones of Merf were generated at 36 hours after egg laying (AEL) using the FRT/FLP system (Xu and Rubin, 1993). Merf,ex- mutant clones were generated by gamma irradiating (1100 rad) w118 sn3 Merf w118 sn3 Merf; P[w+, UpMER] ex+/exf larvae at 72 hours AEL. Immunolocalization was conducted as described (McCartney and Fehon, 1996) with the following antibodies: anti-Merlin (1:7500 dilution; McCartney and Fehon, 1996), anti-Expanded (1:500, provided by M. Boedigheimer and P. Bryant), anti-β-gal (1:1000, Promega), anti-ELAV (1:500, provided by I. Rebay and G. Rubin)

Expression in Schneider-2 cells

Schneider-2 (S2) cell maintenance, transfection and fluorescent staining were performed as described previously (Fehon et al., 1990). Full-length Expanded (EX1-1429, EX+) and an N-terminal Expanded fragment (EX1-399, both provided by M. Boedigheimer and P. Bryant) were subcloned into the MetallothioneinA promoter vector pRnHa3 (Bunch et al., 1988). Three additional Expanded C-terminal fragments were PCR amplified, Myc-tagged and subcloned into pRnHa3: EX73-1249, EX73-1065 and EX966-1429. Sequencing confirmed that no PCR errors were introduced. Myc-tagged full-length (MER1-635, MER+), N-terminal (MER1-349) and C-terminal (MER345-635) Merlin fragments in pCaspR-9s were described previously (LaJeunesse et al., 1998). Expression of Expanded constructs was induced by addition of 0.7 mM CuSO4 for a total of 12-16 hours. Expression of Merlin constructs was induced during this time by a 20 minute incubation at 38°C followed by 6 hours at 25°C. Cells were stained with anti-Expanded (1:500), anti-Merlin (1:2000) or anti-Myc 9c10 (1:100).

Blot overlay experiments

DNA fragments generated by PCR corresponding to MER1-306, MER345-636, MER350-599, MER223-635, EX1-399 and EX73-1429 were subcloned into the GST fusion protein vector pGEX3 (Pharmacia). The MER350-599 fragment was cloned as an in-frame dimer and therefore contains two tandemly repeated copies of this portion of the protein. The MER1-635 full-length construct was generated by cloning a MunI restriction fragment that contains the entire Mer coding sequence in frame into pGEX. Fusion proteins were expressed in BL21 cells and purified as described (Ferar et al., 1991). Purified proteins (30-100 μg/ml in 50 mM NaHCO3, pH 8) were labeled with the addition of 75 μg/ml NHS-LC biotin (Pierce) for 2 hours on ice, followed by the addition of 50 mM glycine. Proteins were dialyzed at 4°C for 16 hours in 100 mM sodium phosphate pH 7.0/50% glycerol. Target proteins were also expressed as GST fusions. To
merlin and expanded interact genetically. (A-C) Scanning electron micrographs of adult eyes (anterior is to the right). (A) Wild-type eye. (B) Adults hemizygous for Mer3 exhibit a weakly roughened, reduced eye with expansion of ventral and posterior head cuticle, and development of ectopic vibrissae, the bristles found ventral and anterior to the eye (arrow). (C) Recessive loss-of-function mutation ex697a dominantly enhanced the small, roughened eye phenotype exhibited by Mer3. In addition, numerous interommatidial bristle duplications were observed. (D-F) Wings from adult male flies. (G) Wild type. (H) Hemizygotes for Mer3 have broadened wing blades. They may also have disruptions of the posterior cross vein (not shown). (F) Recessive loss-of-function mutation ex697a dominantly enhanced the Mer3/Y broad wing phenotype producing significantly larger wings that show an increased frequency of posterior cross-vein disruptions (arrow). Due to the size of the wing, the blade often folds during mounting (arrowhead) (G-I) Wings from adult female flies. (G) Wild type. (H) Adults homozygous for ex697a display a broad wing phenotype characterized by disruption of the posterior cross vein (arrow) and the anterior cross vein (arrowhead). (G) Recessive loss-of-function mutation Mer1a dominantly enhanced the ex697 broad wing phenotype producing a significantly larger wing. (J) Comparisons of mean wing area and cell size of genotypes shown in D-I. Note that wild-type male wings are smaller than wild-type female wings. By use of the t-test, the increases in wing area observed in the mutants were shown to be significant when compared to wild type (P<0.01). Furthermore, the distance between wing hairs in wing compartment 4 was determined as a measure of cell size. These values indicate that the increase in wing area in the mutants is not due to an increase in cell size.

Binding was detected with SuperSignal chemiluminescent reagents (Pierce).

**RESULTS**

**Merlin and ex display similar phenotypes and interact genetically**

Hypomorphic alleles of either Mer (Mer3) or ex (ex697a) result in very similar adult wing and eye phenotypes. In both cases, mutant adults displayed enlarged wings due to an increase in cell number rather than an increase in cell size (Fig. 1D,E,G,H,J). In fact, the cell size in the mutants appeared to
be slightly decreased (Fig. 1J). Furthermore, this expansion in wing area was often accompanied by the disruption or complete absence of the posterior cross vein (Fig. 1H arrow). In addition, the anterior cross vein was sometimes disrupted in ex<sup>697</sup> adults (Fig. 1H, arrowhead). Mer and ex mutants have smaller, weakly roughened eyes (Fig. 1B; Boedigheimer and Laughon, 1993). Histological sections of Mer<sup>3</sup> eyes revealed only minor perturbations in interommatidial organization and no obvious disruptions in ommatidial polarity (data not shown). Concomitant with the reduction in eye size was an apparent expansion of the ventral peripheral head cuticle and the development of ectopic vibrissae (Fig. 1B arrow). Although stronger alleles of both Mer (Mer<sup>1</sup>, Mer<sup>2</sup>, Mer<sup>4</sup>) and ex (ex<sup>e1</sup>, a null allele) result in lethality, Mer mutant larvae did not develop the hyperplastic discs characteristic of ex mutant larvae (data not shown; Boedigheimer and Laughon, 1993).

Dose-sensitive genetic interactions have been shown to be a reliable indicator of functional interactions between genes (Chang et al., 1994). Reduction of ex function in the Mer<sup>3</sup> hemizygous background with either ex<sup>697</sup> or ex<sup>e1</sup> resulted in an enhancement of the Mer<sup>3</sup> head phenotypes (Fig. 1B,C); the adult eye was reduced in size, more ectopic head cuticle and vibrissae were observed, and the bristles normally found between ommatidia were often duplicated and disorganized (Fig. 1C). Those ommatidia that did form contained the normal complement of photoreceptors, however (data not shown). In addition, Mer<sup>3</sup> wing area and the frequency of posterior cross vein disruptions increased when ex function was reduced (Fig. 1E,F,J arrow). Consistent with these observations, reduction of Mer function in ex<sup>697</sup> mutants caused an increase in ex<sup>697</sup> wing size (Fig. 1H-J).

**Merlin and Expanded colocalize in Drosophila tissues and cultured cells**

If the observed genetic interactions between Mer and ex reflect molecular interactions between the proteins that they encode, then the subcellular distribution of these proteins should be similar in expressing tissues. We have previously reported that Merlin is localized in a punctate distribution at the apical plasma membrane in the region of the adherens junction and in the cytoplasm (McCartney and Fehon, 1996), and Expanded has been reported to localize to the apical membrane domain (Boedigheimer and Laughon, 1993). To confirm that they are similarly distributed, we double labeled the two proteins in imaginal disc cells and found that the staining patterns were almost indistinguishable (Fig. 2A,B).

Given their clear colocalization, we asked whether the localization of Merlin in this specific distribution is dependent on the presence of Expanded. We examined Merlin in ex<sup>e1</sup> wing imaginal discs and did not find any significant alterations in Merlin localization (not shown). In a complementary experiment, we generated Mer<sup>4</sup> mitotic clones at 36 hours AEL using the FRT/FLP system and examined Expanded localization in the Mer mutant tissue. Two notable results were observed. First, we detected no alteration in Expanded localization in the absence of Merlin protein, indicating that Expanded does not depend on Merlin for proper subcellular localization. Second, there was an apparent increase in the level of Expanded autonomously in the Mer<sup>4</sup> tissue as compared with the heterozygous background (Fig. 2C,D arrows indicate a border of the clone). This observation indicates either that loss of Merlin results in increased Expanded expression or that an Expanded epitope is masked in the presence of Merlin protein, perhaps due to direct interactions between these proteins.

Although we did not detect interdependence between Merlin and Expanded for subcellular localization in tissues, it remained possible that these proteins physically interact. For example, each protein could localize to the apical junctional domain of intact tissues via interactions with other proteins in this region of the cell. To examine possible interactions between Merlin and Expanded, we cotransfected constructs expressing these proteins into cultured S2 cells that lack an apical junctional domain to more readily detect possible interactions between them. The localization of Merlin expressed in cultured cells follows a very specific pattern; Merlin is first found at the plasma membrane and is then trafficked into the cytoplasm where it is associated with endocytic compartments (McCartney and Fehon, 1996). Expanded is endogenously expressed at levels virtually undetectable by immunofluorescence in S2 cells (data not shown). When full-length Expanded was expressed in S2 cells,
Merlin and Expanded interact

Merlin and Expanded interact directly

To complement and extend the colocalization experiments, we examined the ability of the Merlin and Expanded proteins to interact directly in vitro. Previous blot overlay experiments with ERM proteins have shown that sequences derived from the ERM CNTR bind to the ERM C-terminal tail region (Bretscher et al., 1995). Recent results using the yeast two-hybrid system have confirmed these interactions, and have shown that heterotypic dimers can form between Merlin and ERM proteins (Gronholm et al., 1999). We therefore employed a blot overlay assay to determine if the CNTR of Expanded interacts directly with the C-terminal tail of Merlin. As shown in Fig. 4B, the CNTR of Expanded (EX1-399) binds strongly to full-length Merlin, and to a C-terminal Merlin fragment (MER345-635). These results are consistent with a head-to-tail heterodimeric interaction between Merlin and Expanded, as suggested by experiments with S2 cultured cells. However, we did not detect interactions between Expanded and the larger C-terminal Merlin fragment (MER345-635), the same fragment that interacted with Expanded in the cell culture assay. This may indicate that in vitro the MER345-635 fragment folds into an inaccessible configuration. In addition, EX1-399 bound to a C-terminal Expanded fragment (EX773-1429, Fig. 4B), suggesting...

Fig. 3. Expanded is redistributed in response to Merlin in S2 cells. Singly or doubly transfected S2 cells were treated as described in Materials and Methods. EX and MER proteins were stained using specific antibodies for each protein, or anti-Myc as necessary. (A) EX+ associates with the C-terminal half of Merlin. EX+ is localized in a punctate distribution in the cytoplasm when ectopically expressed in S2 cells (a). When coexpressed with full-length Merlin (MER+), EX+ colocalizes with MER+ at the membrane and in large cytoplasmic compartments (b-c). The CNTR of Merlin is not necessary or sufficient for this interaction as EX+ does not colocalize with MER1-349 (d-e), but does colocalize with MER345-635 (f-g). (B) Merlin associates with the CNTR of Expanded. The CNTR of Expanded (EX1-399) is sufficient for an interaction with MER+ (a-c), whereas a C-terminal fragment of Expanded (EX773-1429) is unable to colocalize with MER+ (d-f). Coexpression of EX1-399 with MER345-635 (g,h) and indicates that the CNTR of Expanded colocalized with the C-terminal domain of Merlin at the plasma membrane.
that Expanded interacts with itself in a head-to-tail fashion, as has been shown for the ERM proteins and Merlin (Bretscher et al., 1995; Gronholm et al., 1999). We did not observe interactions between EX1-399 and the C-terminal domain of Drosophila Moesin (amino acids 468-578; data not shown), indicating that the observed interaction between Merlin and Expanded is specific to these proteins.

Loss of function of both Merlin and expanded results in differentiation defects

Previous studies indicate that loss of function of either Mer or ex in clones results in a 2- to 3-fold overproliferation of the mutant tissue compared with the wild-type twin spot (LaJeunesse et al., 1998; Boedigheimer et al., 1997). In the wing, loss of either Mer or ex alone in clones has no apparent affect on the differentiation and morphology of the affected tissue (B. M. McC. and R. G. F., unpublished observations; Boedigheimer et al., 1997). Similarly, in the eye, loss of function of Mer results in overproliferation without obvious changes in the underlying morphology (LaJeunesse et al., 1998). In contrast, loss of ex function in the eye results in defects in planar polarization, in addition to proliferation defects (Blaumueller and Mlodzik, 2000). Because the loss of function of either Mer or ex results in overproliferation, we examined the consequences of loss of function of both genes using somatic mosaic analysis.

Double mutant clones were generated by gamma-ray induction of mitotic recombination in the w^{1118} sn^{3} Mer^{4}/w^{1118} sn^{3} Mer^{4}; P[w^{+}, UpMer^{+}] ex^{e4}/ex^{e4} background at 72 hours AEL. The Mer^{+} transgene was localized to the distal portion of the left arm of the second chromosome by in situ hybridization (data not shown). We identified double mutant clones in imaginal tissues by staining with antibodies against Merlin and Expanded, providing evidence that clones were generated in this background (data not shown). Clones were marked by the absence of the w^{+} minigene in the adult eye, and were unmarked in the adult wings. However, we could identify mutant clones in the wing by phenotypes not observed in non-irradiated controls, or in controls that carried a Mer^{+} transgene localized to the right arm of the second chromosome (data not shown).

In the adult wing, we observed that both vein and intervein cells differentiate in mutant tissue (Fig. 5A-C). In contrast, clones that intersected the position of the posterior cross vein disrupted its development (Fig. 5B black arrow), consistent with the variably penetrant disruption of the posterior cross vein observed in hypomorphic alleles of Mer or ex (Fig. 1H). Clones in the position of the anterior cross vein differentiated normally (data not shown). Within the mutant intervein and vein clones, we observed apparent defects in proliferation control. In the proximal region of the wing, clonal vein tissue was observed to form a raised protrusion (Fig. 5A). In other regions of the wing, bulges in the veins were also observed, although more frequently vein clones were merely broadened when compared with the surrounding vein (Fig. 5B, blue arrow). In the intervein regions, the clonal tissue appeared to bulge and crinkle within the confines of the normal tissue, suggesting overproliferation (Fig. 5B,C). Similar cuticular bulges or protrusions have been reported for mutant clones of genes that have tumor suppressor phenotypes, such as warts (Justice et al., 1995; Xu et al., 1995). Thus we interpret this phenotype to indicate that the Mer,ex double mutant clones in the wing proliferate at a greater rate than the single mutant clones, though we were not able to confirm this directly. Based on general morphology, the cells within the clone appeared to differentiate as intervein cells, however, the cuticle deposited at the base of each wing hair within the mutant clone appeared to be thickened (Fig. 5C blue arrow) and was distinct from cuticle produced by either the heterozygous intervein (Fig. 5C red arrow) or vein cells.

Clones that developed within the eye appeared either as small scars with associated clusters of bristles (data not shown), or as elongated scars and associated indentations running from within the eye field toward the anterior margin.
Fig. 5. Merlin-\textit{expanded} mutant clones display defects in proliferation and differentiation. (A-F) Mitotic recombination was induced with gamma rays in \textit{w}^{1118} \textit{sn}^3 \textit{Mer}^4\textit{w}^{1118} \textit{sn}^3 \textit{Mer}^4; \textit{P}[\textit{w}^+, \textit{UpMer}^+] \textit{ex}^+/\textit{ex}^1\textit{l} larvae 72 hours AEL. (A-C) \textit{Mer-}\textit{ex}– mutant clones in the adult wing. (A) Mutant clones within the vein protrude from the surface of the wing. (B) In some cases, vein clones do not bulge from the surface, but broaden within the plane of the wing (blue arrow). Mutant clones in intervein tissue that intersect the position of the posterior crossvein disrupt the differentiation of the vein (black arrow). (C) Overproliferation of mutant intervein cells produced clones that buckled within the confines of the heterozygous tissue. The cuticle at the base of each wing hair in the clone (blue arrow) appears thickened compared with that outside the mutant clone (red arrow). (D-F) \textit{Mer-}\textit{ex}– mutant clones in the adult eye. Wild-type twin-spot clones, marking the site of clone induction, are indicated by white arrows. Mutant clones in the center of the eye appear to disrupt progression of the morphogenetic furrow and result in an elongated scar starting within the eye and extending to the anterior margin (black arrows in D,E). The mutant tissue does not differentiate ommatidia and often appears to differentiate as head cuticle (F, black arrow).

\textit{Merlin and expanded} interact genetically with the \textit{dpp} pathway

Reduction of \textit{dpp} function in the eye imaginal disc (\textit{dpp}^blk) results in reduction of the eye along the dorsoventral axis such that the ventral portion of the eye is replaced by head cuticle (Masucci et al., 1990). A similar, yet less severe, phenotype was observed in \textit{Mer}^3 hemizygotes (Fig. 1B) and \textit{ex}^{697} homozygotes (Boedigheimer and Laughon, 1993). To ask whether \textit{dpp} expression is disrupted in \textit{Mer} mutants, we expressed the \textit{dpp-\textit{lacZ}} transgene (B3.0) in the \textit{Mer}^3 background. In the wild-type eye-antennal complex, \textit{dpp} is expressed at the lateral margins and in the morphogenetic furrow of the eye disc and in a ventral wedge of tissue in the antennal disc (Fig. 6A; anti-ELAV is labeling the developing photoreceptors). In the \textit{Mer}^3 mutants, the ventral portion of the disc is significantly enlarged (Fig. 6B,C). The expression pattern of \textit{dpp} was disrupted such that the cells expressing \textit{dpp} at the margin were displaced to the outer tip of the overproliferated tissue (Fig. 6B arrow). In some cases (<5%), this \textit{dpp} staining was associated with an ectopic furrow and developing photoreceptors (Fig. 6C).

To better understand the functional relationship between \textit{dpp}, \textit{Mer} and \textit{ex}, we next examined genetic interactions between these genes. Reduction of \textit{dpp} dose in \textit{Mer}^3 hemizygotes enhanced the severity of the \textit{Mer}^3 eye phenotype, resulting in a smaller, more roughened eye and expansion of the head cuticle (Fig. 6D-F). Similar reduction of \textit{dpp} function in \textit{ex}^{697} homozygotes resulted in enhanced eye phenotypes (data not shown) and variably penetrant truncated leg phenotypes (Fig. 6G-I arrows indicate the position of the sex combs), reminiscent of those observed in pharate adults null for \textit{ex} function (Boedigheimer and Laughon, 1993).

DISCUSSION

Loss of Merlin function in humans has been linked to the formation of Neurofibromatosis-2, a genetic disorder characterized by slow growing benign tumors associated with the peripheral and central nervous systems. Previous studies have suggested the existence of second-site modifier loci for \textit{NF2} because the severity and age of onset of the disease does not appear to be completely correlated with specific \textit{NF2} lesions (Baser et al., 1996). In \textit{Drosophila}, we have observed that reduction of \textit{Mer} function alone is characterized by an autonomous overproliferation phenotype without significant alterations in the differentiation of affected tissues. Likewise, inactivating mutations in \textit{ex}, another member of the Protein 4.1 superfamily, produce hyperplastic imaginal discs and a broadened wing phenotype, but do not significantly affect cell differentiation (Boedigheimer and Laughon, 1993). In contrast, we have shown that simultaneous loss of both \textit{Mer} and \textit{ex} function results in an inability to differentiate certain cell types, such as photoreceptors of the adult eye. In addition, even a slight reduction in \textit{ex} function (\textit{ex}^{697}+) enhances visible \textit{Mer} head and wing phenotypes, and reduction of \textit{Mer} function enhances the similar \textit{ex} phenotypes (Fig. 1). These results indicate that these two structurally related genes function synergistically in developing tissues.

Dose-sensitive genetic interactions of the sort that we have identified between \textit{Mer} and \textit{ex} often reflect physical interactions between the proteins that they encode (Chang et al., 1994). Several additional lines of evidence support the idea that Merlin and Expanded interact directly. First, these proteins are coexpressed in imaginal epithelial cells and colocalize at the subcellular level. Second, when coexpressed in S2 cells these proteins colocalize at the plasma membrane and in the cytoplasm. Deletion mutagenesis experiments indicate that this colocalization requires the CNTR of Expanded and the C-terminal half of Merlin (Fig. 3). Third, studies of Merlin and the related ERM proteins indicate that they form homotypic and heterotypic dimers via an interaction between the CNTR and a domain of ~100 amino acids at the C terminus of the
protein. It is therefore possible that Merlin can form heterodimers with Expanded via similar interactions. Consistent with this hypothesis, blot overlay and coexpression experiments indicate that Merlin and Expanded interact directly via the CNTR of Expanded and the C-terminal 114 amino acids of Merlin. While technical difficulties prevented us from demonstrating by co-immunoprecipitation that the observed interactions between Merlin and Expanded occur in tissues (Expanded is insoluble in non-denaturing cell lysates – data not shown), the combination of genetic interactions, co-localization and direct in vitro interactions strongly suggest that this interaction is biologically relevant.

What is the functional significance of this interaction? Removal by genetic mutation of either protein does not appear to affect the subcellular localization of the other (Fig. 2 and data not shown), indicating that the interaction is not required for proper subcellular localization in tissues. Studies of ERM proteins have shown that they exist in multiple conformations that appear to regulate their ability to interact with transmembrane and other interacting proteins. (Mangeat et al., 1999). Thus, the formation of homotypic and heterotypic dimers or oligomers via interactions between the CNTR and C-terminal domains may function to maintain ERM proteins in either an active or inactive state. Recent studies of human Merlin indicate that it can form folded monomers, homotypic dimers and heterotypic dimers with ERM proteins (Gronholm et al., 1999). Our results indicate that the CNTR of Expanded interacts directly with the C-terminal domains of Expanded and Merlin, suggesting that Expanded can exist in a similar range of conformations. Based on the results presented here, we propose that Merlin and Expanded form a heterodimeric complex that actively suppresses proliferation (Fig. 7).

According to this model, in the heterodimer, the N-terminal domain of Merlin would be free to interact with other proteins, and therefore would be in an activated state. Consistent with this view, we have shown previously that removal of the 35 amino acid C-terminal region of Merlin results in a constitutively active form of the protein that contains all essential Merlin functions (LaJeunesse et al., 1998). In addition, both Merlin and Expanded must possess growth suppression functions that are independent of each other, because the Mer; ex– double mutant phenotype that we observed was much more severe than either mutation alone (Fig. 5).
Formally, the observed interactions with Expanded could function to positively or negatively regulate Merlin function. Our previous results indicate that overexpression of an activated Merlin allele has no dominant phenotype, indicating that beyond a minimum threshold, tight regulation of the activated form of Drosophila Merlin is not important in vivo. However, Merlin is expressed at low levels in cells, suggesting that specific mechanisms may be required to activate and/or stabilize Merlin function. Thus, Expanded may function by stabilizing the activated conformation, thereby increasing the overall level of Merlin function. Although a complete elucidation of the functions of interactions between Merlin and Expanded will require an in depth understanding of the biochemical properties and interactions of both proteins, this model is consistent with our genetic and biochemical results, and is consistent with known functions of other family members.

Merlin and Expanded in patterning events

Our phenotypic analyses of Mer and ex mutants suggest a relationship between Merlin and Expanded and the DPP signal transduction pathway. We investigated this potential relationship by examining mutant phenotypes and genetic interactions associated with the eye and leg. In developing eye imaginal discs from Mer3 and ex697 hemizygotes, we found that often the ventral portion of the eye disc was overgrown and DPP expression was disrupted so that the morphogenetic furrow did not initiate or failed to progress through the ectopic tissue (Fig. 6A-C). A similar effect has been seen with mutant clones of Mad, a downstream effector of DPP signaling, in the posterior portion of the eye (Wiersdorff et al., 1996). Such clones inhibit furrow initiation and can result in the formation of an ectopic furrow ventral to the clone. In addition, hypomorphic mutant phenotypes of either Mer or ex are enhanced by heterozygosity for the dpp br56 allele (Fig. 6D-I). In the eye, the reduction of dpp function enhances the Mer3 hemizygous phenotype, resulting in an eye that is greatly reduced in size, especially ventrally. Because dpp is required for ommatidial recruitment, this phenotype is consistent with a positive role for Merlin in DPP function. Similarly, in the leg imaginal disc, we observed that reduction of dpp function enhances hypomorphic ex phenotypes so that the distal elements of the leg are reduced significantly. In the developing leg, DPP and the ligand Wingless (WG) are expressed in distinct domains that meet in the center of the disc, the region that corresponds to the most distal segment of the adult leg (Serrano and O’Farrell, 1997). The combined action of DPP and WG activates expression of distal-less, which in turn specifies the formation of the proximal-distal axis. Loss of distal structures in the leg is characteristic of dpp hypomorphs (Spencer et al., 1982). Thus, the truncated leg phenotype produced as the result of reduction of dpp function in ex997 homozygotes indicates that Expanded also has a positive role in the DPP signaling pathway.

This and other genetic studies of Mer and ex mutant phenotypes have revealed at least two functions for these genes. First, these two genes are required for normal cell proliferation in developing imaginal tissues. Second, the data presented here implicate Merlin and Expanded in DPP-mediated patterning events in several Drosophila tissues. Previous studies of dpp and related genes have demonstrated that this pathway has functions in both proliferation and patterning during development of the wing imaginal disc (Burke and Basler, 1996). Although it is appealing to think that the effects of Mer and ex on patterning and proliferation are both mediated through the DPP pathway, this seems unlikely given that loss of either gene seems to negatively affect DPP patterning functions, but simultaneously causes overproliferation of mutant cells. It therefore seems more likely that the proliferation phenotypes of Mer and ex loss-of-function mutations are mediated through effects on one or more other pathways that regulate proliferative events. Recent studies of Merlin and the related ERM proteins indicate that they may have multiple binding partners (Mangeat et al., 1999), and therefore may function to regulate more than one signaling pathway simultaneously in developing tissues.

Conclusion

We have identified Expanded as a novel binding partner for the Drosophila NF-2 tumor suppressor homologue Merlin. Although a vertebrate homologue of ex has not yet been identified, the interaction between Merlin and Expanded and their shared role in the regulation of cell proliferation make ex an intriguing candidate for a second-site-modifier of the NF-2 phenotype. Thus, further study of Expanded and its relationship to Merlin function in developing tissues should provide insights both into the mechanisms by which cell proliferation is regulated, and into the mechanisms of and possible therapeutic approaches toward the NF-2 disease.

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