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The PP1 phosphatase Flapwing regulates the activity of Merlin and Moesin in *Drosophila*

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ABSTRACT

The signalling activities of Merlin and Moesin, two closely related members of the protein 4.1 Ezrin/Radixin/Moesin family, are regulated by conformational changes. These changes are regulated in turn by phosphorylation. The same sterile 20 kinase-Slik co-regulates Merlin or Moesin activity whereby phosphorylation inactivates Merlin, but activates Moesin. Thus, the corresponding coordinate activation of Merlin and inactivation of Moesin would require coordinated phosphatase activity. We find that *Drosophila melanogaster* protein phosphatase type 1 β (flapwing) fulfils this role, co-regulating dephosphorylation and altered activity of both Merlin and Moesin. Merlin or Moesin are detected in a complex with Flapwing both in-vitro and in-vivo. Directed changes in *flapwing* expression result in altered phosphorylation of both Merlin and Moesin. These changes in the levels of Merlin and Moesin phosphorylation following reduction of *flapwing* expression are associated with concomitant defects in epithelial integrity and increase in apoptosis in developing tissues such as wing imaginal discs. Functionally, the defects can be partially recapitulated by over expression of proteins that mimic constitutively phosphorylated or unphosphorylated Merlin or Moesin. Our results suggest that changes in the phosphorylation levels of Merlin and Moesin lead to changes in epithelial organization.

Introduction

Epithelial tissues are composed of polarized cells with specific apical and basal domains, defined by intercellular junctions. Proliferation of cells within an epithelial layer requires remodelling of these intercellular interaction domains. One group of proteins with known roles in both proliferation and epithelial integrity includes Merlin (Mer) and Ezrin, Radixin, Moesin (ERM) (Bretscher et al., 2002; McClatchey and Fehon, 2009). Mer is a critical regulator of proliferation in mammalian and *Drosophila* tissues, and is defined as a tumour suppressor protein (Fehon et al., 1997b; McCartney and Fehon, 1996; Rouleau et al., 1993; Trofatter et al., 1993). Mer has also been shown to be required for establishment of stable adherens junctions (Gladden et al., 2010; Lallemand et al., 2003). There is clear functional conservation for Mer activities, as human Mer can rescue loss of *Drosophila* Mermutant flies (LaJeunesse et al., 1998). ERM proteins are thought to primarily regulate and maintain epithelial integrity through organization of the apical cytoskeleton (Bretscher et al., 2002; McClatchey and Fehon, 2009). In addition, both Mer and ERM proteins function as membrane-cytoskeletal linkers and as potential regulators of multiple signalling pathways (Bretscher et al., 2002; Curto et al., 2007; Hamaratoglu et al., 2006; Maitra et al., 2006; McClatchey and Fehon, 2009; Shaw et al., 2001; Speck et al., 2003).

Mer and the ERM proteins have >45% sequence identity (Bretscher et al., 2002). All are predicted to have intramolecular interaction between the N-terminal 4.1 ERM (FERM) head domain and the C-terminal tail domain (Berryman et al., 1995; Gonzalez-Agosti et al., 1999; Gronholm et al., 1999; Meng et al., 2000; Nguyen et al., 2001; Sherman et al., 1997). Change in protein conformation can alter function by affecting interaction(s) with protein partners via selective masking or unmasking of specific amino acid sequences (Gary and Bretscher, 1995; Henry et al., 1995; Martin et al., 1995; Reczek and Bretscher, 1998). For example, phosphorylation of a conserved threonine in the C-terminal tail of mammalian ERM proteins relieves the intramolecular head to tail interaction and is required for activation (Fukata et al., 1998; Hayashi et al., 1999; Nakamura et al., 1995; Tran Quang et al., 2000). In general, closed hypophosphorylated ERM proteins are thought to not interact with transmembrane proteins or the cytoskeleton (Matsui et al., 1998; Nakamura et al., 1999). However, in mammalian cells, the closed, hypophosphorylated form of Mer is thought to be active as it has been shown to inhibit

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proliferation which is thought to occur via dephosphorylation of a conserved serine at residue 518 upon serum withdrawal or cell–cell or cell–matrix contact (Morrison et al., 2001; Shaw et al., 2001).

The single *Drosophila* ERM orthologue, Moesin (Moe), negatively regulates Rho signalling, thus maintaining epithelial integrity (Speck et al., 2003). Multiple kinases have been shown to regulate Mer and ERM protein activity. This includes Rho (Oshiro et al., 1998) and Protein kinase C (Pietromonaco et al., 1998) which also phosphorylate Moe. Both p21 activated kinase (PAK) (Kissil et al., 2002; Xiao et al., 2002) and PKA (Laulajainen et al., 2008) have also been implicated in Mer phosphorylation. In *Drosophila*, the kinase Slik has been shown to phosphorylate both Mer and Moe thereby inactivating Mer and activating Moe which results in a coordinate regulation of proliferation and epithelial integrity (Hughes and Fehon, 2006).

During development, epithelial cells alternate between strongly adherent and proliferative states. This would require repeated alteration between Mer and Moe phosphorylation states. Therefore, we hypothesized that one or more corresponding phosphatases co-regulate these proteins. The conserved Serine 518 of mammalian Mer is a known target of phosphatase PP1δ, in a complex with MYPT-1 (myosin phosphatase), leading to Mer activation (Jin et al., 2006). This was demonstrated by using the cellular inhibitor of MYPT-1-PP1, CPI-17, which results in a loss of Mer function concomitant with changes in Mer phosphorylation, activation of Ras and cellular transformation (Jin et al., 2006). Previous studies have provided some hints as to the identity of the phosphatases that might regulate Moe. Moe binds to a regulatory subunit of myosin/moesin phosphatase (MMP), containing a protein type 1 phosphatase catalytic subunit (Eto et al., 2000). Supporting a role for this phosphatase in the regulation of Moe, this enzyme is important for the dynamic remodelling of the cytoskeleton in fibroblast cells as determined using a specific MMP inhibitor (CPI-17) (Eto et al., 2000). Similarly, changes in Sds22, a PP1 phosphatase regulatory subunit that binds to all four *Drosophila* PP1 proteins, affect epithelial cell shape and polarity in *Drosophila* cells (Grusche et al., 2009). Loss of Sds22 leads to increased phosphorylation of Moe in *Drosophila* follicle cells and the disruption of epithelial polarity. Sds22 function has been shown to be conserved in human cells (Grusche et al., 2009). However, up to now, no specific phosphatase has been shown to directly regulate Moe activity, and none are known to co-regulate both Mer and Moe, like Slik (Hughes and Fehon, 2006).

Protein phosphatase type 1 (PP1) defines a large group of serine/threonine phosphatases (Shi, 2009). These are found in all types of eukaryotic cells and are important regulators of a vast array of cellular functions including cell signalling, protein synthesis, RNA splicing, cell cycle, and muscle contraction (Lin et al., 1999; Shi, 2009). There are multiple catalytic sub-units of the PP1s, which interact with different regulatory subunits. The regulatory subunit of the PP1 enzyme complex, in turn, regulates substrate specificity and sub-cellular localization (Ceulemans and Bollen, 2004; Cohen, 2002; Lin et al., 1999). Based upon specific sequence similarity, PP1s have been classified into three isoforms; PP1α, PP1β (also called PP1δ) and PP1γ (Lin et al., 1999). Mammals have three PP1 genes, whereas *Drosophila* have four, encoding highly related (>85% identity) PP1catalytic proteins (Dombradi et al., 1990a, 1993). In *Drosophila*, there are three PP1α type enzymes (PP1-13C, PP187B and PP196A) while *flapwing* (*flw*) or *PP1β9C* encodes a PP1β type protein. *Drosophila* PP1α is homologous to mammalian PP1α and PP1γ whereas *Flw*/PP1β is homologous to mammalian PP1β/δ. It is particularly notable that PP1α (PP187B) contributes 80% of the total phosphatase activity within *Drosophila* larvae, while the *flw* locus contributes only 10% of the total PP1c activity (Axton et al., 1990; Dombradi and Cohen, 1992; Dombradi et al., 1990b). However, the *flw* gene is the only *Drosophila* PP1 gene that is essential for viability. Loss of function *flw* clones in follicle cells show increased myosin levels, and disorganization of the actin cytoskeleton (Vereshchagina et al., 2004).

We set out to test if Flw co-regulates Mer and Moe activities during cell proliferation and adhesion. We found that Flw is able to dephosphorylate both Mer and Moe. This coordinate modification positively regulates Mer and represses Moe activity, which subsequently leads to epithelial disorganization during the development of *Drosophila* tissues such as wing imaginal discs.

Materials and methods

Drosophila stocks

y cho flw^{1}, UAS *Flw* HA (stock # 23703) (Kirchner et al., 2007) was obtained from the Bloomington *Drosophila* Stock Center. The *flw* RNAi line (stock #104677KK) was obtained from the Vienna *Drosophila* RNA Center (Dietzl et al., 2007). UAS transgenes and RNAi (inverted repeats; IR) lines were expressed by crossing to *apterous*-Gal4 (expressed in dorsal surface), *patched*-Gal4 (expressed along the anterior/posterior boundary) or MS1096-Gal4 lines (expressed in dorsal surface) (Brand and Perrimon, 1993; Capdevila and Guerrero, 1994). Flies were raised on the standard media currently used by the Bloomington Stock Centre.

Transfection of Schneider 2 (S2) cells

2 μg each of *Ubiquitin*-GAL4, UAS HA Mer and UAS HA *Flw* plasmid DNA was incubated with 120 μl of 250 μg/ml DDAB (dimethyl dioctadecyl ammonium bromide; Sigma Aldrich) transfection reagent and 60 μl of Hyclone Serum free SFX insect cell culture medium (Thermo Scientific) at room temperature for 20 min (Han, 1996). The transfection mix was then added dropwise into 3 ml of S2 cells (10^{6} cells per ml) in a six well plate and were then incubated overnight at 25 °C.

Antibody preparation

A N-terminal GST-Merlin fusion protein was expressed and purified as described previously (Rebay and Fehon, 2009) except that the GST protein was purified by column chromatography and following elution by glutathioine, electroeluted and then dialyzed into 1× PBS. Polyclonal sera were raised in guinea pigs against the Merlin fusion protein (Pocono Rabbit Laboratory and Farms).

Co-immunoprecipitation and immunoblotting

Cells were collected by centrifugation at 1000×*g* for 3 min. Cell pellet was lysed and cross-linked into 1 ml of mild lysis buffer (50 mM HEPES pH 7.0, 50 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM DTT, 1% Triton X-100, 1× Roche complete EDTA free inhibitor cocktail, 1× Roche Phos STOP) + 0.1% formaldehyde for 10 min at 4 °C. Cellular debris was cleared by centrifugation at 16,000×*g* for 10 min at 4 °C. The supernatant was divided into two 1.5 ml tubes and incubated overnight at 4 °C with mouse anti-HA (Sigma) dimethyl pimelimidate dihydrochloride crossed linked protein G beads (IP for *Flw*), or protein G beads alone (Control), or alternately were incubated overnight at 4 °C with guinea pig anti-Merlin dimethyl pimelimidate dihydrochloride crossed linked protein A beads (for Mer IP), or protein A beads alone (Control). Anti HA (1:500) and anti-Merlin (1:1000) was used in the crosslinking to sepharose beads. Beads were pelleted at 1000×g for 30 s and washed four times in mild lysis buffer. Beads were eluted two times using 75 μl of Gentle Ag/Ab elution buffer (Thermo Scientific). The protein was precipitated (chloroform and methanol), resuspended in 50 μl of 1× SDS sample buffer. Protein samples were heated to 95 °C for 5 min, loaded and were separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose (Biorad).