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Phosphorylation of Ind by MAP kinase enhances Ind-dependent transcriptional repression

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ABSTRACT

The *Drosophila* neuroectoderm is initially subdivided into three longitudinal domains that give rise to columns of neuroblasts. This subdivision is coordinately accomplished by the action of the signaling pathways, Dorsal and Epidermal Growth Factor Receptor (EGFR), in conjunction with the homeodomain proteins, Ventral nervous system defective, Intermediate neuroblasts defective (Ind) and Muscle Segment Homeobox. We previously demonstrated that Ind expression is activated in response to the EGFR pathway. Here we show that EGF signaling subsequently mediates the direct phosphorylation of Ind by MAP kinase, which enhances the capacity of Ind to repress target genes, such as *achaete*. Specifically, we show that reduced EGF signaling results in diminished repression of *achaete* in the intermediate column, despite the presence of high levels of Ind protein. We also demonstrate that ectopic activation of MAP kinase results in the lateral expansion of the Ind expression domain with a corresponding reduction in *achaete* expression. This regulation is also dependent on the co-repressor, Dichaete. Our data indicate that EGF signaling, acting through MAP kinase, impinges on multiple aspects of Ind regulatory activity. While it has been often demonstrated that MAP kinase phosphorylation of transcriptional repressors attenuates their repressor activity, here we provide an example of phosphorylation enhancing repressor activity.

Introduction

In *Drosophila* the Map kinase (MAPK), Rolled, plays a key role in several different developmental processes. Initially, it is activated in the termini of the embryo by Torso signaling (Furriols and Casanova, 2003). Later, during the early stages of central nervous system (CNS) development, Rolled is activated in the developing neuroectoderm by the Epidermal Growth Factor Receptor (EGFR) pathway (Gabay et al., 1997; Skeath, 1998), and in the mesoderm by the Fibroblast Growth Factor Receptor (FGFR) pathway (Shishido et al., 1997; Vincent et al., 1998). Ultimately, activation of Rolled by its immediate upstream effector, MAP Kinase Kinase (MEK), results in its translocation to the nucleus where it phosphorylates a number of nuclear targets. ETS-class DNA binding proteins are among the primary group of transcription factors modified by MAPK. In the *Drosophila* neuroectoderm, the *pointed* (*pnt*) protein, Pnt P2, is activated by MAPK-mediated phosphorylation (Brunner et al., 1994). Other known targets include the negative regulators, Yan and Capicua, both of which are exported to the cytoplasm and degraded upon phosphorylation (Astigarraga et al., 2007; O'Neill et al., 1994; Rebay and Rubin, 1995). Another non-DNA binding target of MAPK is Groucho, a transcriptional co-repressor that interacts with numerous DNA binding proteins (Cinnamon et al., 2008; Hasson et al., 2005). In this paper we uncover a novel transcription factor substrate for MAPK.

The expression domains of the three homeodomain proteins, Ventral nervous system defective (Vnd), Intermediate neuroblasts defective (Ind), and Muscle Segment Homeobox (Msh) define the ventral, intermediate and lateral columnar domains across the dorsoventral (DV) axis of the *Drosophila* embryo (Chu et al., 1998; Isshiki et al., 1997; Mc Donald et al., 1998; Weiss et al., 1998). Additionally, the high mobility group (HMG) proteins, Dichaete and Sox Neuro, participate in the columnar sub-division of the neuroectoderm (Buescher et al., 2002; Overton et al., 2002; Zhao et al., 2007). Notably, MAPK signaling downstream of EGFR is crucial to the dorsal–ventral patterning of the *Drosophila* neuroectoderm. The activated form of MAPK is present in the ventral and intermediate neuroectoderm, but not in the lateral domain (Skeath, 1998). Previously, we demonstrated that the EGFR pathway is essential for expression of *ind* in the intermediate column (Helman et al., 2011; Von Ohlen and Doe, 2000), which ultimately leads to the formation of the intermediate column of neuroblasts (Weiss et al., 1998). In the ventral column, EGF signaling plays a key role in the specification of neuroblasts as well as in the maintenance, but not initiation, of Vnd expression (Gabay et al., 1996; Skeath, 1998; Udolph et al., 1998; Von Ohlen and Doe, 2000). EGF signaling does not affect patterning or specification of the Msh-dependent lateral column (Skeath, 1998; Udolph et al., 1998).

Maximal activation of the EGFR pathway, and ultimately MAPK, in the neuroectoderm is accomplished by the activity of two EGF-

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like ligands: Vein, a neuregulin-like molecule that is expressed in the early embryonic neuroectoderm; and Spitz, a TGFα family member, which requires the activity of the transmembrane protein, Rhomboid, for proper secretion (Noll et al., 1994; Schweitzer et al., 1995). There are two waves of EGF pathway activation in the neuroectoderm, initially at stages 5 and 6 and again at stage 9. The first wave requires both Vein and Spitz, while the second wave apparently only requires Spitz (Skeath, 1998). The CNS phenotype of embryos mutant for both *rhomboid* and *vein* (*rho vn*) mimics that of EGFR mutant embryos with respect to CNS mis-patterning (Skeath, 1998), suggesting that these are the only EGFR ligands relevant to this developmental process.

Here we demonstrate that the homeodomain protein, Ind, is directly phosphorylated by MAPK in vitro. We genetically demonstrate that phosphorylation of Ind by MAPK is a regulatory mechanism essential for accurate repression of target genes, particularly *achaete*, and to a lesser degree *msh*. Furthermore, we show that this repression requires the co-regulator, Dichaete. Taken together our results strongly suggest that phosphorylation of Ind by MAPK is a key modulator of Ind regulatory activity, which possibly involves physical association of Ind with Dichaete. Thus, we provide molecular and genetic insights into how the EGFR signaling pathway modulates Ind repressor activity and how gene expression is regulated in the neuroectoderm. Our previous work demonstrated that Vnd is also phosphorylated by MAPK (Zhang et al., 2008). To date, Vnd and Ind are the only two examples of homeodomain proteins involved in D–V patterning that are phosphorylated by MAPK. Furthermore, in each previously described example of MAPK phosphorylation of transcriptional repressors, phosphorylation results in attenuation of repressor activity (Ajuria et al., 2011; Astigarraga et al., 2007; Cinnamon et al., 2008; Hasson et al., 2005; Helman et al., 2011; O'Neill et al., 1994; Rebay and Rubin, 1995). Our work provides the first example, to our knowledge, where repressor activity is enhanced by MAPK phosphorylation.

Methods

Fly lines

ind^{RR108}*/TM3ftz-LacZ* (Weiss et al., 1998), *UAS ind* is a full length wild type *ind* cDNA inserted into the pUAST vector described in (Von Ohlen et al., 2007b, 2009). The lines used include *rho*^{7M43}*/TM3ftzLacZ*, *pnt*$^{\Delta 88}$*/TM3ftzLacZ*, *pnt*$^{\Delta 18}$*/TM3ftzLacZ*, *vn*^{C221}*/TM3ftzLacZ*, *vn*$^{\gamma 4}$*/TM3ftzLacZ*, *vn*^{L6}*/TM3ftzLacZ*, *msh*^{rH96}*/msh*^{rH96} (Isshiki et al., 1997), *UAS Rhomboid* (de Celis et al., 1997), *EGF*^{F2}*/CyOftz LacZ. UAS Rho* and *dichaete*^{87}*/TM3 ftz LacZ* (Mukherjee et al., 2000). All EGF pattering mutants were obtained from either the Bloomington Stock center, Amanda Simcox (Ohio State University), Jocelyn Mc Donald (Cleveland Clinic), or from Dr. James Skeath (Washington University, St. Louis).

In situ hybridization and antibody stains

A rabbit anti-Ind antibody was used at a dilution of 1:2000 as described in Von Ohlen and Moses (2009), mouse Anti-β-Gal antibody (Promega) was used at 1:500 dilution. Mouse anti-diphosphorylated ERK 1 and 2 antibody (dpERK; Sigma) was used at 1:2000 dilution. Digoxigenin (dig) labeled antisense *dichaete*, *achaete* and *msh* probes were made from cDNAs according to standard procedures (Tautz and Pfeiffle, 1989). For confocal images rabbit anti-Ind (1:1000) and rat anti-Msh (1:500) antibodies were used. Secondary antibodies were Rhodamine- (1:2000) or Cy5-conjugated (1:800) (Jackson Laboratories). Embryos were mounted using DakoCytomation medium. Images were captured with a Leica DM500 light microscope and digital camera. All images were processed with photoshop software.

Phosphorylation assays

Ind and mutant derivatives were synthesized and labeled with the Quick TNT-coupled rabbit reticulocyte lysate system (Promega) in the presence of [35S]-methionine. Labeled proteins were then incubated with (or without) 0.2 mg of active Erk2, in a total volume of 50 μl of kinase reaction buffer (20 mM HEPES, 0.1 mM benzamidine, 25 mM beta-glycerophosphate, 0.1 mM DTT, 1 mM Na3VO4, 10 mM MgCl2, and 0.1 mM ATP) for 30 min at 30 °C. Reactions were stopped by addition of 3× SDS sample buffer (0.25 M Tris pH 6.8, 6% SDS, 30% glycerol, B-mercaptoethanol, and a few grains of bromophenol blue). The phosphorylation state of the proteins was analyzed by SDS-PAGE and autoradiography. To activate Erk2, a His-tagged Erk2 fusion protein was expressed in *Escherichia coli*, purified on nickel beads (QIAGEN), and activated with active MEK1 (Upstate).

Site directed mutagenesis and constructs

Mutagenesis of *ind* cDNA was done in pBluescript KS using the Quick Change Mutagenesis multi Kit (Stratagene) according to package directions.

Primers used are as follows:

Ind S199A: cacgcgtcctcctgcgctccgaatagctcacc
Ind S308A: caaagcatctccagtggctccccaagtcaaagtc
Ind S135A: atccatcgccaccgctcgcggccaatcccaactccc
Ind S305, 308A: tggagtccccaagcagctccagtggctccccaag
Ind S135D: atccatcgccaccgctcgacgccaatcccaactccc
Ind S308D: caagcatctccagtggacccccaagtcaaagtccac

The Ind S135, 308A (IndAA) and IndS135, 308D (IndDD) constructs were amplified by PCR, digested with SalI and XhoI, and subsequently cloned into the pUAST vector XhoI site with the following primers:

Ind Upper: ccgctcgagaaacccaagatgtcgcgttcatttttgatgg
Ind lower: acggcgtcgacggtgattgattctacgcc

These primers and conditions were also used to clone the same mutant and wild type versions of *ind* cDNA into the Ract-Hadh vector at the SalI site for expression in S2 cells. UAS-IndAA and UAS-Ind-DD transgenes were sent to the Non-Mammalian Model Systems unit at Duke University for injection. At least three independent transformants for each transgene were tested for both expression of Ind protein and ability to repress *achaete* and *msh*. Anti-Ind stains were done on all lines in parallel and those that appeared most similar to the WT UAS Ind transgenics in expression levels were selected for further experiments.

Results

Repression of achaete by Ind is more effective in the ventral neuroblast column than in the lateral column

achaete is expressed in a subset of proneural clusters and neuroblasts in the ventral and lateral columns, but is specifically excluded from the intermediate column by the repressor activity of Ind (Fig. 1A–B) (Von Ohlen et al., 2007a; Weiss et al., 1998; Zhao et al., 2007). In *ind* mutant embryos *achaete* expression is expanded into the intermediate column (Fig. 1C). Ectopic expression of Ind across the DV axis using the *Kruppel* (*Kr*) *Gal4* driver reveals a differential ability of Ind to repress *achaete* in the ventral column when compared to the lateral column, with repression being stronger in ventral column cells (Fig. 1D). Initially the repression of *achaete* by Ind in the lateral column is stronger (compare to Fig. 6A). However, by the end of stage eight approximately 90% of embryos observed (16 of 18) show a pattern similar to that shown in Fig. 1D. This timing coincides precisely with MAPK activity narrowing to the ventral column (Helman et al., 2011; Skeath, 1998). Since the activity of MAPK is high in ventral column cells but absent in lateral column cells,