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p38 MAPK is a likely component of the signal transduction pathway triggering rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*

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Summary

Rapid cold hardening (RCH) is an adaptation enabling insects to quickly respond to low temperature, but little is known about the molecular events that trigger this response. In this study of the flesh fly *Sarcophaga crassipalpis*, we explore a possible role for mitogen-activated protein kinases (MAPKs) in the low temperature signaling that elicits RCH. We report that p38 MAPK from *S. crassipalpis*, which shows high cDNA sequence homology to p38 MAPKs from other insects and mammals, is rapidly activated at temperatures around 0°C, temperatures that are most effective for inducing RCH. By contrast, low temperature does not activate either extracellular signal-regulated kinase (ERK) or Jun N-terminal kinase (JNK). An increase in phospho-p38 MAPK was observed within 10 min following exposure to 0°C and reached its maximum level in 2 h. When flies were transferred from 0 to 25°C, the level of phospho-p38 MAPK decreased immediately and reached trace levels by 3 h. Nondiapausing flies were much more responsive to p38 MAPK activation than cold-hardy diapausing pupae. Thus, p38 MAPK activation and RCH both show the same narrow ranges of temperature sensitivity, temporal profiles of activation and decay, and developmental specificity. These correlations suggest that p38 MAPK plays a potential role in regulating the induction of RCH. The p38 MAPK response was not dependent upon the brain, as evidenced by high activation in isolated abdomens exposed to low temperature.

Key words: cold stress, diapause, signal transduction, MAP kinase, phosphorylation.

Introduction

In addition to the cold hardening mechanisms exploited by overwintering insects that are in diapause, most insects have the capacity to quickly enhance their cold tolerance at other times of the year utilizing a mechanism referred to as rapid cold hardening (RCH) (Chen et al., 1987; Lee et al., 1987a; Denlinger and Lee, 1998). For example, nondiapausing pharate adults of the flesh fly *Sarcophaga crassipalpis* that have been reared at 25°C cannot survive direct exposure to –10°C, but if they first receive a brief exposure of a few minutes or hours at 0°C they readily survive –10°C (Chen et al., 1987; Lee et al., 1987a). The RCH response has been documented in diverse insect species and appears to be a mechanism used to track daily changes in temperature (Kelty and Lee, 2001).

Currently, there are several biochemical and molecular events known to be associated with RCH. Glycerol, a classic cryoprotectant, increases during RCH (Lee et al., 1987a), but this increase is rather modest and is unlikely to be the sole agent rendering protection. More recently, changes in composition of cell membranes have been implicated in RCH (Overgaard et al., 2005; Michaud and Denlinger, 2006), and studies based on microarrays (Qin et al., 2005) and metabolomics (Michaud and Denlinger, 2007) suggest that numerous gene networks and metabolic pathways are involved in this response.

One of the major outstanding questions associated with RCH is identifying the signaling system responsible for induction. Somehow, the environmental signal of a decrease in temperature activates the physiological mechanisms associated with RCH. Based on characteristics of the RCH response in *S. crassipalpis*, we know that a candidate signaling agent for this fly would have the following characteristics: maximum responsiveness at temperatures around 0°C (Chen et al., 1991), the capacity to be activated within 10 min and retention of activity for several hours (Chen et al., 1987; Lee et al., 1987a), decay of activation quickly upon return to higher temperature (Chen et al., 1991), developmental loss of activation capacity in developmental stages that are already cold hardy, e.g. diapause (Chen et al., 1987), and a non-dependence on the brain for activation (Yi and Lee, 2004).

In the present study, we propose that phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) meets the criteria of a candidate signaling molecule. The MAPK family is well known for its role in transmitting stress signals from the environment to the cell nucleus. MAPKs are serine/threonine kinases that, when phosphorylated, enter the nucleus and phosphorylate various transcription factors, enzymes and other proteins that modulate cellular activity (Martin-Blanco, 2000; Ono and Han, 2000; Cowan and Storey, 2003). Activation of

the MAPKs involves phosphorylation at both the serine/threonine and tyrosine residues. Previous experiments demonstrating a role for the MAPK family in insect diapause and cold acclimation (Iwata et al., 2005; Fujiwara et al., 2006a; Fujiwara and Shiomi, 2006; Fujiwara et al., 2006b; Kidokoro et al., 2006a; Kidokoro et al., 2006b) suggest that members of this family of signaling molecules may also be involved in RCH.

To establish the validity of using antibodies directed against mammalian members of the MAPK family, we first cloned p38 MAPK from *S. crassipalpis* and determined that flesh fly p38 MAPK has high identity to mammalian p38 MAPK. Antibodies directed against p38 MAPK and the phosphorylated form of p38 MAPK, along with those directed against other members of the MAPK family, were then used to determine the environmental conditions that elicit activation (phosphorylation) and subsequent inactivation. Additional experiments evaluate the responsiveness of different tissues to activation, developmental changes that affect activation, and the potential role of the brain in mediating this response.

Materials and methods

Flies

Flies were from an established laboratory colony of *Sarcophaga crassipalpis* Macquart. Nondiapausing individuals were reared under long-day conditions of 15 h light and 9 h darkness at 20 or 25°C, while diapausing pupae were produced by rearing adults under short-day conditions of 12 h light and 12 h darkness at 25°C, and larvae and pupae at 20°C under the same short-day conditions (Denlinger, 1972). The anterior caps of the puparia were removed to observe development. To initiate diapause termination, 5 μl hexane was applied to the head of diapausing individuals (Denlinger et al., 1980).

For RCH experiments, a single larva, pupa or pharate adult was placed in a thin-walled glass test tube (1.5×10 cm) that was capped with a cotton plug. The tubes were placed in a low-temperature bath filled with 50% glycerol.

For experiments utilizing ligated abdomens, the pupae were first punctured in the head to prevent rupture of the abdomen following ligation. Ligation between the head and thorax was performed using nylon thread (Yoder et al., 2006).

Tissue dissections were performed in phosphate-buffered saline (50 mmol l^{-1} sodium phosphate, pH 7.5 and 150 mmol l^{-1} NaCl) with 10 mmol l^{-1} EDTA under a dissection microscope.

Chemicals

Anti-phospho-p38, anti-phospho-JNK and anti-phospho-ERK MAPK rabbit antibodies and peroxidase-conjugated anti-rabbit IgG goat antibody (used at 1:1000 dilutions) were from Cell Signaling Technology (Beverly, MA, USA). A goat antibody against the N-terminal sequence of *Drosophila* p38 MAPK (anti-total-p38 MAPK antibody; used at 1:400 dilutions) and peroxidase-conjugated anti-goat IgG donkey antibody (used at 1:5000 dilutions) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were described previously (Iwata et al., 2005). A single animal was used for each sample. Whole bodies were homogenized in 10 volumes of SDS-PAGE sample buffer. For experiments using dissected tissues, five brains, head epidermis from four individuals, three midguts or fat body from the head of one pupa were homogenized in 100 μl SDS-PAGE sample buffer. Protein samples were boiled for 5 min, and 5 μl was applied to each lane. Proteins were separated by SDS-PAGE, and gels were then transferred to polyvinylidine difluoride membranes (Immobilon; Millipore, Bedford, MA, USA). Membranes were incubated with primary antibodies and a peroxidase conjugated secondary antibody and were visualized on X-ray film using LumiGLO chemiluminescent reagent (KPL; Gaithersberg, MD, USA). Each treatment was replicated at least three times.

cDNA cloning

Total RNA was isolated from the brain and optic lobes of nondiapausing pupae of *S. crassipalpis* using TRizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). The p38 MAPK cDNA was amplified using degenerate PCR primers p38-1 (GTNAAYGARGAYTGYGA) and p38-2 (TGRTYRTARTGCATCCARTT) (where Y=T or C; R=A or G; N=A, C, G or T) using Platinum Taq DNA Polymerase High Fidelity (Invitrogen). The PCR conditions were described previously (Ijiro et al., 2004; Fujiwara et al., 2006a). The amplified DNA fragments were subcloned into plasmids and sequenced. The 5′ and 3′ regions of the p38 MAPK cDNA were obtained using a SMART RACE cDNA amplification kit (Clontech Laboratory, Mountain View, CA, USA) according to the manufacturer's instructions with primers p38-6 (ATCACGACGTTTCATGGGTGGCAA) and p38-3 (TTGGATTTCGGTTTGGCACGTC) for 5′ and 3′ RACE, respectively. Finally, the whole coding region of the *Sarcophaga* p38 MAPK was amplified using PCR primers p38-7 (AAACGAACGAGTCAGAACGTAGATAAACA) and p38-14 (TGTGCGCTGAATGAACATTATTGTTTCTAA) at the 5′ and 3′ untranslated regions, respectively.

Results

Cloning and amino acid sequence of Sarcophaga p38 MAPK cDNA

To confirm the presence of the p38 MAPK gene in *S. crassipalpis*, and for comparison with that of other species, we cloned a 1.6 kb p38 MAPK cDNA from *S. crassipalpis* (DDBJ, EMBL and GenBank accession number, AB277828). The deduced amino acid sequence revealed that *Sarcophaga* p38 MAPK is a 42 kDa protein with 361 amino acid residues and 91% identity and 94% similarity with *Glossina* p38 MAPK (ABC25081), 86% identity and 94% similarity with *Drosophila* p38 MAPK a (AAC39030) and 82% identity and 91% similarity with *Drosophila* p38 MAPK b (AAC39032). The phosphorylation domains of *Sarcophaga* p38 MAPK were compared and are completely or almost identical with their *Glossina*, *Drosophila* and human counterparts (Fig. 1). The high similarity suggests that p38 MAPK has a conserved function among species and confirms that commercial antibodies against mammalian kinases can likely be used successfully in *S. crassipalpis*.

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                                    + +
Sarcophaga     161 CELRILDFGLARPTENEMTGYVATRWYRAPEIMLNWMHYN
Glossina       161 CELRILDFGLARPTENEMTGYVATRWYRAPEIMLNWMHYN
Drosophila a   166 CELRILDFGLARPTENEMTGYVATRWYRAPEIMLNWMHYN
Drosophila b   223 CELRILDFGLARPAESEMTGYVATRWYRAPEIMLNWMHYN
Human alpha    161 CELKILDFGLARHTDDEMTGYVATRWYRAPEIMLNWMHYN
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Fig. 1. Comparison of predicted amino acid sequences of the phosphorylation domains of *Sarcophaga* p38 MAPK with *Glossina*, *Drosophila* and human kinases. The plus signs (+) indicate conserved amino acid residues that are phosphorylated. Asterisks (*) and periods (.) indicate identical and similar amino acids, respectively. The DDBJ, EMBL and GenBank accession numbers of these sequences are: AB277828 (*Sarcophaga*), ABC25081 (*Glossina*), AAC39030 (*Drosophila* p38a), AAC39032 (*Drosophila* p38b) and Q16539 (human p38a).

p38 MAPK is activated by low temperature

To examine the roles of MAPKs in temperature stress responses in *S. crassipalpis*, we analyzed the phosphorylated state of MAPKs in nondiapausing red-eye pharate adults incubated for 2 h at temperatures ranging from –10 to 40°C (Fig. 2). Both anti-phospho and anti-total p38MAPK antibodies specifically recognized a single 42 kDa band. Increases of phospho-p38 MAPK were observed in the low-temperature range from –5 to 10°C, with the strongest increase observed at 0°C (Fig. 2). No increase of phospho-p38 MAPK was observed by heat shock. Although some fluctuations can be seen, no major changes were observed in levels of phospho-ERK or phospho-JNK by either high or low temperature. The total p38 MAPK protein level was unchanged at all temperatures, indicating that changes in the levels of phosphorylated p38 MAPK were due to phosphorylation rather than protein synthesis. The close congruence between the temperature range inducing RCH (Chen et al., 1991) and the phosphorylation of p38 MAPK noted here suggests a causal link.

Temporal profiles of activation and decay of p38 MAPK

To document the temporal profile of phospho-p38 MAPK activation, red-eye stage nondiapausing pharate adults were held at 0°C for 10 min to 24 h, and phospho-p38 MAPK levels were analyzed (Fig. 3A). An increase of phospho-p38 MAPK was observed within 10 min, the levels continued to increase until reaching a plateau at 2 h, and thereafter the levels remained constant for at least 24 h.

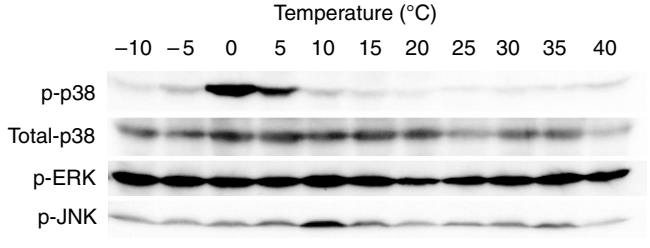

Fig. 2. Temperature-dependent activation of p38 MAPK. Red-eye stage nondiapausing pharate adults were exposed for 2 h at the indicated temperatures. Fly proteins were analyzed using western blotting with anti-phospho-p38, anti-phospho-ERK, anti-phospho-JNK and anti-total p38 MAPK antibodies.

In another experiment, pharate adults were exposed to 0°C for 10 min and then transferred to 25°C. An increased level of phospho-p38 MAPK was observed 10 min after the transfer (Fig. 3B), thus confirming that a 10 min exposure to 0°C was sufficient to increase levels of phospho-p38 MAPK.

Another group of pharate adults was held at 0°C for 2 h and then transferred to 25 or –10°C for 2 h or held for an additional 2 h at 0°C (Fig. 3C). The signal was nearly completely lost at 25 and –10°C but persisted at the same level in individuals held at 0°C. To analyze the temporal profile of decay, pharate adults held for 2 h at 0°C were transferred to 25°C, and protein samples were prepared at various intervals thereafter (Fig. 3D). A decrease in the level of phospho-p38 MAPK was observed 15 min after transfer, and the levels decreased to trace amounts by 3 h (Fig. 3D).

Diapause- and developmental-stage-dependent activation of p38 MAPK

We analyzed phospho-p38 MAPK levels in various developmental stages of diapausing and nondiapausing

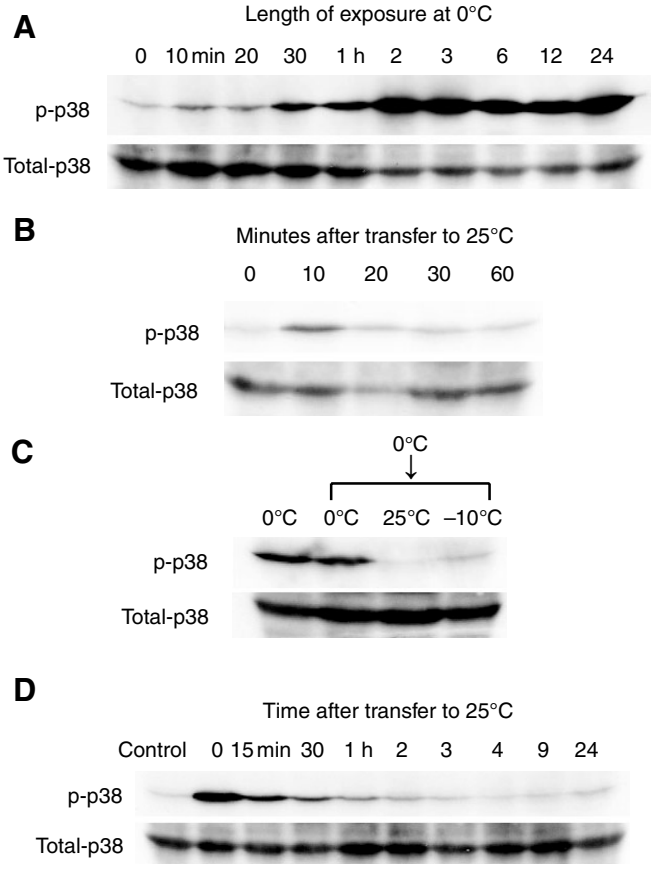

Fig. 3. Temporal profiles of activation and decay of p38 MAPK phosphorylation in red-eye stage nondiapausing pharate adults. (A) Exposure to 0°C for various durations. (B) Exposure to 0°C for 10 min and then transferred to 25°C. (C) Exposure to 0°C for 2 h and then transferred to 25 and –10°C for 2 h, or held for an additional 2 h at 0°C. (D) Exposure to 0°C for 2 h and then transferred to 25°C for various durations. Flies held at 25°C were used as controls. Fly proteins were analyzed using western blotting with anti-phospho-p38 and anti-total p38 MAPK antibodies.

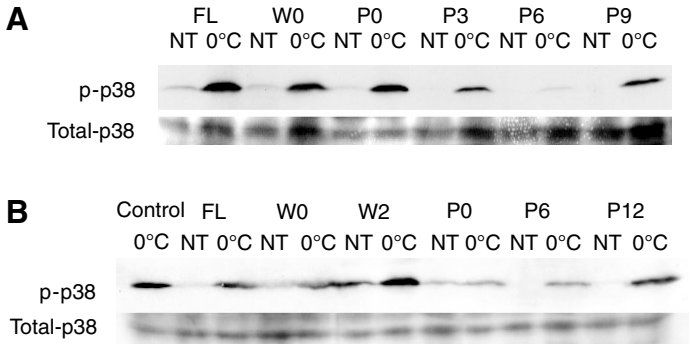

Fig. 4. Development-specific phosphorylation of p38 MAPK in nondiapausing flies at 0°C. Flesh flies were reared at a nondiapausing condition of (A) 25°C or (B) 20°C. Larvae or pupae were held at 0°C for 2 h, and protein samples were prepared. Fly proteins were analyzed using western blotting with anti-phospho-p38 and anti-total p38 MAPK antibodies. FL, 3rd-instar feeding larvae; W0-2, day 0–2 wandering 3rd-instar larvae; P0–12, day 0–12 after pupariation; NT, no treatment; 0°C, held at 0°C for 2 h. Red-eye stage nondiapausing pharate adults reared at 25°C were used as controls.

individuals. In nondiapausing flies reared at 25°C, the phospho-p38 MAPK levels in feeding and wandering third-instar larvae and in day 0 puparia were greatly increased by exposure to 0°C (Fig. 4A). The phospho-p38 MAPK response gradually decreased after pupariation but recovered by day 9 (red-eye pharate adult stage) (Fig. 4A). When nondiapausing flies were reared at 20°C, phospho-p38 MAPK levels in feeding and wandering larvae were greatly increased by exposure to 0°C, but reduced levels of activation at 0°C were observed in day 0 and 6 puparia. The response was again evident by day 12, when the flies reached the red-eye pharate adult stage (Fig. 4B).

In diapause-inducing conditions, phospho-p38 MAPK levels were activated by 0°C during the prediapause larval period, but the responsiveness suddenly dropped to trace levels at pupariation (Fig. 5A) and remained low throughout the cold-hardy pupal diapause stage (Fig. 5B). Responsiveness returned following termination of diapause (Fig. 5C), by which time the flies had lost much of their diapause-associated cold hardiness.

Tissue-specific and brain-independent activation of p38 MAPK

To determine whether specific tissues respond differently to p38 MAPK phosphorylation, red-eye pharate adults were held at 0°C for 2 h, and phospho-p38 MAPK levels were examined in different tissues (Fig. 6A). Pronounced increases were observed in the fat body and midgut. No change was observed in the brain, and only a slight increase was observed in the optic lobes. p38 MAPK protein was not detected in the epidermis.

To evaluate the role of the brain in activation of p38 MAPK, we analyzed the p38 MAPK response in isolated abdomens. Phospho-p38 MAPK levels increased in isolated abdomens that were exposed to 0°C for 2 h (Fig. 6B).

Discussion

This study shows a close correlation between phosphorylation of p38 MAPK and RCH. Phosphorylation of p38 MAPK and RCH show the same temperature sensitivity and temporal patterns of activation and inactivation in nondiapausing pharate adults of *S. crassipalpis*. A temperature of 0°C is the most effective temperature for both p38 MAPK activation (Fig. 2) and RCH induction (Chen et al., 1987), and 0°C is required not only to activate p38 MAPK but also to maintain the activated phospho-p38 MAPK levels (Fig. 3C).

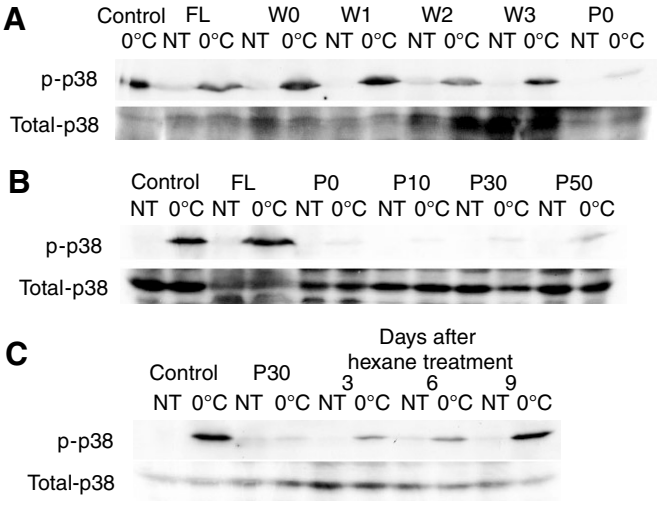

Fig. 5. Phosphorylation of p38 MAPK in diapause-programmed flies reared at 20°C and exposed to 0°C. (A) Feeding 3rd instar to day of pupariation, (B) diapausing pupae 0 to 50 days after pupariation and (C) day 20 diapause pupae to which 5 μl hexane was applied to elicit diapause termination. Larvae or pupae were held at 0°C for 2 h, and protein samples were prepared. Whole body proteins were analyzed using western blotting with anti-phospho-p38 and anti-total p38 MAPK antibodies. FL, 3rd-instar feeding larvae; W0–3, day 0–3 wandering 3rd-instar larvae; P0–50, day 0–50 after pupariation; NT, no treatment; 0°C, held at 0°C for 2 h. Red-eye stage nondiapausing pharate adults reared at 25°C were used as controls.

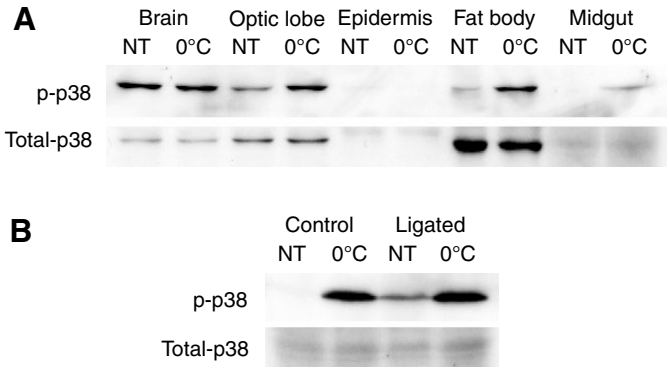

Fig. 6. Tissue-specific and head-independent phosphorylation of p38 MAPK at 0°C. (A) Red-eye stage nondiapausing pharate adults were exposed at 0°C for 2 h, and specific tissues were dissected. Tissue proteins were analyzed using western blotting with anti-phospho-p38 and anti-total p38 MAPK antibodies. (B) Red-eye stage nondiapausing pharate adults were ligated between the head and thorax with a nylon thread and exposed at 0°C. Protein samples from the posterior part were analyzed using western blotting with anti-phospho-p38 and anti-total p38 MAPK antibodies. NT, no treatment; 0°C, held at 0°C for 2 h.

The most striking feature of RCH is the rapidity of its induction. A 10 min chilling at 0°C is sufficient for both p38 MAPK activation (Fig. 3B) and RCH induction (Chen et al., 1987; Lee et al., 1987a). The temporal profiles for the induction and the decay responses of RCH (Chen et al., 1987) clearly mirror the changes in levels of phospho-p38 MAPK that we noted in the current study (Fig. 3A,D).

Developmentally, p38 MAPK activation is most robust in life stages where the RCH response is most pronounced, i.e. in life stages that are inherently less cold tolerant. For example, nondiapausing flies reared at 25°C are less cold tolerant than those reared at 20°C (Adedokun and Denlinger, 1984), and in this study we see higher phospho-p38 MAPK activation in flies reared at 25 than at 20°C (Fig. 4). Also, mid-pupal stages of nondiapausing flies reared at 25°C have higher cold tolerance than other stages (Chen et al., 1987; Lee et al., 1987b), and during this period p38 MAPK is activated less strongly by 0°C (Fig. 4). Diapausing pupae, which have the highest level of cold tolerance (Chen et al., 1987; Lee et al., 1987b), showed almost no p38 MAPK activation (Fig. 5), but when diapause is terminated, cold hardiness gradually decreases (Lee and Denlinger, 1985; Lee et al., 1987b), and the flies concurrently show increased p38 MAPK responsiveness (Fig. 5). Thus, there is a consistent relationship showing high p38 MAPK responsiveness in stages with low inherent cold tolerance. It is these stages that can most benefit from RCH. The other extreme is diapause, a stage that is already developmentally programmed for cold hardiness and thus has no further need for RCH. We thus suggest that p38 MAPK plays a key role in regulating RCH and is likely to be a component of the signal transduction pathway that switches on the RCH response.

The speed of p38 MAPK activation suggests that the low-temperature signal directly activates p38 MAPK rather than being activated by cellular damage caused by cold stress. p38 MAPK activation is specific to low temperature (Fig. 2), thus heat shock elicitation of RCH (Chen et al., 1987) would necessitate the use of a different switch.

Regulation of glycerol production is a candidate target for p38 MAPK. The ontogeny of cold tolerance in flesh flies correlates with the pattern of glycerol synthesis (Lee et al., 1987b). Glycerol concentrations are increased by exposure to 0°C (Lee et al., 1987a), and injection of glycerol increases cold tolerance (Yoder et al., 2006). Possibly, p38 MAPK regulates glycerol synthesis in the fat body of *S. crassipalpis* during RCH. Glycogen phosphorylase, which is activated rapidly at 0°C, is a key enzyme for glycerol production (Ziegler and Wyatt, 1975; Chen and Denlinger, 1990). Activation profiles of glycogen phosphorylase correspond to some extent with those of p38 MAPK. Glycogen phosphorylase can be activated within 30 min at 0°C. Both glycogen phosphorylase and p38 MAPK show higher levels of activation in nondiapausing than in diapausing flies. However, there are some discrepancies as well: glycogen phosphorylase also can be activated by heat stress, and no activation of glycogen phosphorylase was observed in larvae and young pupae (Chen and Denlinger, 1990), despite the fact that p38 MAPK activations at 0°C can be observed in these stages (Figs 4 and 5).

Synthesis of heat shock proteins (Hsps) is activated by both cold as well as heat stress, but they are unlikely targets of p38 MAPK because Hsp expression in this fly requires –10°C, not 0°C, and the Hsps are synthesized during the recovery phase rather than during the actual cold stress (Joplin et al., 1990; Yocum et al., 1998; Rinehart and Denlinger, 2000; Rinehart et al., 2000). By contrast, p38 MAPK is activated by 0°C, not –10°C, and it is not activated during the recovery period from cold stress (Figs 2 and 3).

Recent studies indicate that proteins besides Hsps and compounds in addition to glycerol may increase in response to RCH. RCH elevates the expression of many genes, including stress proteins and membrane-associated protein genes, in *Drosophila melanogaster* (Qin et al., 2005). Chilling increases oleic acid, which in turn can stabilize the structure of the cell membrane in *S. crassipalpis* (Michaud and Denlinger, 2006), and a recent metabolomics study has also revealed RCH-induced changes in the levels of carbohydrates, polyols and amino acids (Michaud and Denlinger, 2007). Numerous changes in gene expression and metabolic shifts are thus elicited during RCH, and we are not yet able to link p38 MAPK to one specific pathway.

There are two seemingly contrasting opinions concerning the role of the brain in regulating the RCH reaction. Yi and Lee reported that isolated cells and tissue can undergo RCH (Yi and Lee, 2004), while Yoder et al. reported that the presence of the brain enhances the accumulation of glycerol during RCH (Yoder et al., 2006). Both are likely to be correct. While the tissues can display an RCH response independent of the brain (Yi and Lee, 2004), the response is more robust when the brain is present (Yoder et al., 2006). In the present study, we show that p38 MAPK is activated by 0°C in a limited number of tissues, most notably the fat body and midgut (Fig. 6A), and it can be activated in an isolated abdomen that lacks a brain (Fig. 6B). These results suggest that p38 MAPK is activated autonomously in specific tissues rather than regulated remotely by nervous or endocrine systems.

We thus conclude that the phosphorylation of p38 MAPK has all of the attributes of a switch that could initiate the RCH response. This hypothesis is further supported by the widespread use of MAPKs as environmental signaling molecules in other eukaryotes (Kyriakis and Avruch, 1996; Cowan and Storey, 2003), including temperature responses in other insects (Stronach and Perrimon, 1999; Iwata et al., 2005; Fujiwara et al., 2006a; Fujiwara and Shiomi, 2006; Kidokoro et al., 2006a; Kidokoro et al., 2006b).

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