The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in *Drosophila* by promoting nuclear localization of HIF-α/Sima

Andrés Dekanty¹, Sofía Lavista-Llanos¹, Maximiliano Irisarri¹, Sean Oldham² and Pablo Wappner¹,*

¹Instituto Leloir and IIB, FCEyN-Universidad de Buenos Aires, CONICET, Patricias Argentinas 435, Buenos Aires (1405), Argentina
²The Burnham Institute, La Jolla, CA 92037, USA

*Author for correspondence (e-mail: pwappner@leloir.org.ar)

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Summary

The hypoxia-inducible factor (HIF) is a heterodimeric transcription factor composed of a constitutively expressed HIF-β subunit and an oxygen-regulated HIF-α subunit. We have previously defined a hypoxia-inducible transcriptional response in *Drosophila melanogaster* that is homologous to the mammalian HIF-dependent response. In *Drosophila*, the bHLH-PAS proteins Similar (Sima) and Tango (Tgo) are the functional homologues of the mammalian HIF-α and HIF-β subunits, respectively. HIF-α/Sima is regulated by oxygen at several different levels that include protein stability and subcellular localization. We show here for the first time that insulin can activate HIF-dependent transcription, both in *Drosophila* S2 cells and in living *Drosophila* embryos. Using a pharmacological approach as well as RNA interference, we determined that the effect of insulin on HIF-dependent transcriptional induction is mediated by PI3K-AKT and TOR pathways. We demonstrate that stimulation of the transcriptional response involves upregulation of Sima protein but not *sima* mRNA. Finally, we have analyzed in vivo the effect of the activation of the PI3K-AKT pathway on the subcellular localization of Sima protein. Overexpression of dAKT and dPDK1 in normoxic embryos provoked a major increase in Sima nuclear localization, mimicking the effect of a hypoxic treatment. A similar increase in Sima nuclear localization was observed in *dPTEN* homozygous mutant embryos, confirming that activation of the PI3K-AKT pathway promotes nuclear accumulation of Sima protein. We conclude that regulation of HIF-α/Sima by the PI3K-AKT-TOR pathway is a major conserved mode of regulation of the HIF-dependent transcriptional response in *Drosophila*.

Introduction

The mammalian hypoxia-inducible factor-1 (HIF-1) is an α/β heterodimeric transcription factor composed of two basic helix-loop-helix-PAS (bHLH-PAS) subunits (Wang et al., 1995). HIF-1 binds to HIF responsive elements (HREs) in the regulatory region of a wide array of hypoxia-regulated target genes, thereby inducing hypoxia-dependent transcription (Maxwell et al., 1993; Wang and Semenza, 1993). Over the last decade, substantial progress in understanding the mechanisms that underlie HIF-1 oxygen-dependent regulation has been reported (Bruick, 2003; Schofield and Ratcliffe, 2004). It is now clear that, whereas the β subunit is constitutive, the α subunit is regulated by oxygen tension at several different levels that include control of protein half-life, transcriptional co-activator recruitment and regulation of subcellular localization. Regulation of HIF-α degradation depends on oxygen-dependent hydroxylation of two key prolyl residues (P402 and P564), that is catalyzed by specific prolyl-4-hydroxylases (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan, 2001; Jaakkola, 2001). This post-translational modification enables interaction with the ubiquitination machinery, thus promoting proteosomal degradation (Maxwell et al., 1999).

Transcriptional coactivator recruitment is regulated by a second oxygen-sensing system in which an asparaginyl hydroxylase, a different oxoglutarate and iron-dependent dioxygenase, promotes hydroxylation of a specific asparaginyl residue located within the HIF-α C-terminal domain. Hydroxylation of this asparagine prevents interaction with the transcriptional co-activator p300, resulting in the inhibition of transactivation (Dann et al., 2002; Elkins et al., 2002; Hewitson et al., 2002; Lando et al., 2002). A third regulatory mechanism, the control of subcellular localization determines that, in normoxia, HIF-α is mostly cytoplasmic, and in hypoxia it accumulates in the nuclear compartment (Kallio et al., 1998). Structure-function studies have led to the identification of a functional nuclear localization signal and of additional domains that are required for regulated nuclear localization (Kallio et al., 1998; Luo and Shibuya, 2001). Nevertheless, the cellular and molecular mechanisms controlling HIF-α oxygen-dependent subcellular localization are poorly understood.

In addition, it has been shown that several non-hypoxic...
accumulated in the cytoplasm in normoxia and became nuclear overexpression of Sima in transgenic fly lines, Sima subcellular localization also depends on oxygen tension. When the activity of a conserved prolyl-4-hydroxylase that we have stabilized in hypoxia. Normoxic Sima degradation depends on life of Sima protein, which is rapidly degraded in normoxia and largely conserved because oxygen tension determines the half-life of Sima protein, which is rapidly degraded in normoxia and stabilized in hypoxia. Normoxic Sima degradation depends on the activity of a conserved prolyl-4-hydroxylase that we have named Fatiga. In fatiga loss-of-function mutants Sima is stabilized, and high expression of the HRE reporter occurs constitutively in normoxia. Conservation of the cellular machinery that regulates the hypoxic response extends even further, because Sima subcellular localization also depends on oxygen tension. When rapid degradation was overwhelmed in vivo through the overexpression of Sima in transgenic fly lines, Sima accumulated in the cytoplasm in normoxia and became nuclear in hypoxia (Lavista-Llanos et al., 2002). The mechanism controlling HIF-α/Sima oxygen-dependent subcellular localization is so far unclear.

Involvement of the insulin pathway in the induction of the HRE response in Drosophila has not yet been reported. Here, we show both in cell culture and in vivo that the PI3K-AKT signaling pathway can activate an HRE response in Drosophila that is mediated by the bHLH-PAS proteins Sima and Tango. HRE induction depends on TOR and its upstream regulator Rheb and involves upregulation of Sima protein levels as well as an increase of its nuclear localization.

Materials and Methods

Cell culture

Schneider’s line (S2) cells were maintained at 25°C in Schneider Drosophila medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), containing 50 units/ml penicillin and 50 μg/ml streptomycin in 25 or 75 cm² T-flasks (Greiner). Cells were plated in 12-, 24- or 96-well cell culture dishes (Greiner), cultured for 3 days and then treated with 1-50 μg/ml insulin (Gibco), deferoxamine (Sigma) or were exposed to hypoxia in a Forma Scientific 3131 incubator.

Plasmids, transfections and luciferase assays

To generate the HRE-luciferase reporter plasmid (HRE-Luc), a dimerized 51-bp sequence derived from the murine lactate dehydrogenase-A enhancer (Lavista-Llanos et al., 2002) was inserted into KpnI and Xhol sites of the polylinker of a pGL3 vector (Promega), containing a Drosophila Hsp70 basal promoter (Darlington et al., 1998). Drosophila Sima and human AKT coding sequences were inserted into EcoRI-Xbal and EcoRI-HindIII sites, respectively, of a pAC/V5His plasmid (Invitrogen); human AKT cDNA was a gift from Marcelo Katzanietz (Department of Pharmacology, University of Philadelphia, PA).

For transfections, 10⁶ cells were plated in a 12-well plate and cultured overnight. Transfections were performed with a calcium phosphate transfection kit (Invitrogen) with 5 μg of total DNA using pcDNA3 plasmid as filler DNA when necessary. To generate a stable cell line bearing the HRE-Luc reporter, cells were co-transfected with the pGL3 plasmid and with a second plasmid encoding a blasticidin resistance gene (pBLAST) in a 19:1 ratio. Forty-eight hours after transfection, 10⁻⁶ g/ml of blasticidin (Invitrogen) were added to the cells and selection was carried out for 3 weeks. For transcription assays in the stable cell line treated with various stimuli, luciferase activity was measured, normalized to protein concentrations and expressed as fold-induction with respect to non-stimulated cells. For transient transfection experiments, expression vectors were co-transfected together with a Renilla luciferase plasmid (pAC/Renilla) to normalize transfection efficiency. Forty-eight hours after transfections cells were harvested, lysed and firefly and Renilla luciferase activities were determined. Normalized luciferase activity (i.e. Renilla: luciferase activity ratio) was expressed as fold-induction with respect to the transfection control (empty vector). Luciferase activity was measured using Steady-Glo or Dual-Glo luciferase reagents (Promega) by following instructions of the manufacturer. Protein quantification was performed by the Bradford method (BIO-RAD).

RNA isolation, northern blots and RT-PCR

Total RNA from cells exposed to different treatments was isolated using the Trizol reagent (Invitrogen). Thirty micrograms of total RNA were separated on denaturing 6% formaldehyde 1% agarose gels and transferred in 10× SSC buffer to Zeta-Probe GT Genomic
membranes (BIO-RAD) and hybridized with $^{[32P]}$CTP-labeled sima, LDH or 18S RNA probes; a storm 840 phosphomimetager (Molecular Dynamics) was used to detect $^{32}$P emission. Probes were synthesized using 25 ng of the corresponding PCR products and labeled with $^{[32P]}$CTP using a Random Primer Labeling Kit (Invitrogen). PCR products used to prepare probes were: sima (nucleotides 612-1312), 18S rRNA (complete sequence), LDH (nucleotides 157-1118).

For RT-PCR experiments, reverse transcription was carried out with a superscript reverse transcriptase enzyme (Invitrogen), according to the instructions of the manufacturer, using oligo-dT as a primer. Semi-quantitative RT-PCR experiments were performed at different cycles of amplification using a hot-start DNA polymerase enzyme (Taq platinum, Invitrogen) and the analysis was carried out in each case at the exponential phase of amplification. For normalization, actin42A was amplified along with the particular transcript of interest.

RNA interference

Fragments from the sima transcript (nucleotides 4099-5058), tango (nucleotides 901-1413), dPTEN (nucleotides 205-912), dAKT (nucleotides 784-1510), dPKDK1 (nucleotides 1749-2261), dTOR (nucleotides 3694-4208), dRhb (nucleotides 455-645) and ds6K (nucleotides 376-1165) were amplified by reverse transcriptase (RT)-PCR using specific primers containing part of the T7 RNA-polymerase-binding site at the 5′ end of each primer, and a second PCR using the complete sequence of the T7 RNA-polymerase-binding site (5′-GAATTAATAGCTCATATGGAG-3′) as primer. PCR products were purified in Microspin G25 columns (Amersham) and $1\mu$g of these DNA products was used as a template to synthesize double-stranded (ds)RNA with the T7 Megascript kit (Ambion) following manufacturer’s instructions. The synthesized RNA was precipitated with LiCl, resuspended in water and annealed by heating at 65°C for 30 minutes followed by slow cooling until reaching room temperature. RNA interference (RNAi) experiments in S2 cells were performed as previously described (Clemens et al., 2000). Briefly, S2 cells were plated at a density of $10^6$ cells/ml in 96- or 24-well plates in serum-free medium, and 1 to 5 g of dsRNA were added. After 1 hour incubation, serum-containing medium was added and cells were cultured for 3 days at 25°C. Then, cells were stimulated with insulin, or exposed to hypoxia or the iron chelator deferoxamine (DFO) for luciferase reporter assays. To confirm silencing after the RNAi treatment, cells were harvested and endogenous mRNA levels were analyzed by semi-quantitative RT-PCR.

Immunofluorescence and β-galactosidase assays

Immunofluorescence was performed as previously described (Lavista-Llanos et al., 2002). Briefly, embryos from different developmental stages were bleach-dechorionated and fixed with 3.7% formaldehyde for 20 minutes; fixed embryos were blocked for 2 hours in PBS containing 1% BSA and 0.1% Triton X-100 (PT) and then incubated overnight with the primary antibody. After washing, embryos were incubated with the secondary antibody for 2 hours in PT containing 10% normal goat serum. Analysis of subcellular localization was performed in an Olympus BX60 microscope or in a Carl Zeiss LSM5 Pascal confocal microscope. For X-Gal stainings, embryos were bleach-dechorionated and fixed with 0.5% glutaraldehyde for 20 minutes; fixed embryos were washed three times in PT and incubated with the β-galactosidase synthetic substrate X-gal.

To quantify the subcellular localization of Sima, at least two independent experiments were carried out and 30 or more embryos from each analyzed developmental stage were recorded. The subcellular localization was scored in three categories (as described in the Results section) and data are represented as histograms showing the percentage of embryos in each category. The $\chi^2$-test was applied to compare different samples.

SDS-PAGE and immunoblotting

Twenty-five micrograms of nuclear protein extracts were prepared as described in Gorr et al. (Gorr et al., 2004), separated in 6% SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto PVDF membranes (BIO-RAD). Thereafter, membranes were blocked for 1 hour at room temperature with 5% nonfat milk in TBS with 0.1% Tween 20 (TBS-T) and then incubated overnight with rat anti-Sima antiserum (1:1000) or mouse anti-Hsp70 antibody (1:1000) in 5% nonfat milk in TBS-T (Bacon et al., 1998). Membranes were washed in TBS-T and incubated for 1 hour at room temperature with a peroxidase-conjugated anti-rat or anti-mouse secondary antibody (Jackson) diluted 1:5000 in 5% nonfat milk in TBS-T. Immunoblots were developed with the ECL detection reagent (Cell Signalling).

Fly stocks and genetics

The following fly strains were used: PTEN2L117 (Oldham et al., 2000), UAS-dAKT, PDK1EP10557 (Rintelen et al., 2001), UAS-dPTEN (Huang et al., 1999) UAS-Sima and LDH-LacZ (Lavista-Llanos et al., 2002), engrailed-Gal4 (en-GAL4, Bloomington Stock Center).

Embryos collected in overnight egg-laying agar plates were kept in normoxia or exposed to hypoxia for 4 hours; then embryos were recovered, fixed and immunostained. Hypoxia was applied by regulating the proportions of oxygen and nitrogen in a Forma Scientific 3131 incubator at 25°C.

In Sima subcellular localization experiments, ectopic expression of Sima was performed by crossing en-GAL4 and UAS-Sima fly strains. To study the effects of the mutation dPTEN2L117 on Sima subcellular localization, dPTEN2L117 UAS-Sima and dPTEN2L117 en-GAL4 recombinant chromosomes were generated by meiotic recombination. Briefly, heterozygous yw; dPTEN2L117/Cyo flies were crossed with yw; UAS-Sima/Cyo or yw; en-GAL4 flies and non-Cyo females from the F1 were crossed with yw; Xa/Cyo males. Males bearing UAS-Sima or en-GAL4 elements, identified by red eyes, were tested for lethality by crossing them with yw; dPTEN2L117/Cyo females. Once established, the recombinant strains yw; dPTEN2L117 UAS-Sima/Cyo males were crossed with yw; dPTEN2L117 en-GAL4/Cyo females and embryos from the offspring were immunostained. To restore PTEN expression in the PTEN mutant background, a strain with the following genotype was generated yw; dPTEN2L117 UAS-Sima/Cyo; UAS-dPTEN and males from this strain were crossed to yw; dPTEN2L117 en-Gal4/Cyo females and analyzed as above. To study whether overexpression of dAKT and dPKDK1 affect Sima subcellular localization, recombinant chromosomes bearing UAS-Sima and UAS-dAKT were generated by meiotic recombination as above. Males bearing UAS-Sima and UAS-dAKT elements were identified by strong red eyes and used to establish individual strains; the presence of both UAS constructs was confirmed by RNA in-situ hybridization. Subcellular localization of Sima was studied in crosses with en-GAL4 flies and processed for anti-Sima immunostaining as above.

Results

Hypoxic induction of an HRE response in Drosophila S2 cells

In a previous report, we have shown that a dimerized 51-bp sequence derived from the murine lactate dehydrogenase-A (LDH-A) enhancer can drive hypoxia-dependent transcription of a Gal4 reporter element in transgenic fly lines (Lavista-Llanos et al., 2002). To generate a cell culture system that enables pharmacological intervention, we generated an S2 cell line bearing an HRE reporter. The dimerized regulatory sequence of the LDH gene was cloned upstream of a firefly luciferase reporter gene bearing a fly Hsp70 minimal promoter (HRE-Luc) in a pGL3 plasmid (Fig. 1A). With this construct, stable
Drosophila S2 cell lines were generated and challenged with diverse hypoxic treatments. As depicted in Fig. 1B, exposure of the cells to 1% O₂ for 20 hours elicited a 23-fold increase of luciferase activity, 2% O₂ caused a 9-fold increase and 3% O₂ led to 1.5-fold increase. These results show that the LDH regulatory element can drive hypoxia-dose-dependent transcription in Drosophila S2 cells, as it does in transgenic embryos (Lavista-Llanos et al., 2002). In mammalian cell culture systems, iron chelation was reported to mimic the effect of hypoxia, because prolyl hydroxylases involved in oxygen sensing have an absolute requirement of iron. In agreement with this notion, addition to the cells of the iron chelator DFO provoked a consistent 6-fold increase in luciferase activity (Fig. 1B), confirming that the HRE-Luc reporter drives a bona fide transcriptional response to hypoxia in the transfected S2 cell line. Exposure of the cells to 2% oxygen for 16 hours caused an increase in the expression of endogenous dLDH mRNA, which was detected by northern blot (Fig. 1C) and semi-quantitative RT-PCR (not shown). Induction of this hypoxia-responsive gene parallels induction of the HRE-Luc reporter, demonstrating that this element can report an endogenous physiological response to hypoxia. To confirm that HRE-Luc hypoxic induction is driven by the Drosophila HIF-1 homologue, we treated the cells with sima or tango dsRNA and, as expected, silencing of sima or tango expression caused complete abrogation of luciferase induction by hypoxia or DFO (Fig. 1D). Consistent with these results, transfection of an expression vector encoding Sima was sufficient to elicit a strong induction of the HRE-Luc reporter in a dose-dependent manner (Fig. 1E). We conclude from these experiments that, induction of the HRE-Luc reporter in the stable cell line depends on the Drosophila HIF-1 functional homologue, which is composed by the bHLH-PAS subunits Sima and Tgo.

Insulin can induce an HRE response in S2 cells through the bHLH-PAS proteins Sima and Tango

Having characterized the behavior of the HRE-Luc reporter in the stably transfected Drosophila S2 cell line, we sought to investigate whether insulin can trigger the HRE response in this setting. Upon treating the cell culture with 50 µg/ml insulin, expression of the HRE-Luc reporter was increased about 20-fold, attaining levels similar to those observed upon hypoxia and higher levels to those recorded upon DFO treatment (Fig. 2A). Moreover, insulin induction of the reporter was dose-dependent (Fig. 2B), suggesting that graded activation of an insulin-dependent pathway can render proportional induction of the HRE response. Northern blot analysis revealed that dLDH mRNA levels were also upregulated upon insulin treatment (Fig. 2C), suggesting that...
Insulin induces an HRE-response in S2 cells. (A) Effect of insulin on HRE-Luc reporter induction. S2 cells stably transfected with HRE-Luc were cultured for 3 days and then exposed or not to hypoxia (1% O2), DFO (100 μM) or insulin (50 μg/ml) for 20 hours. Luciferase activity was determined and normalized to protein concentration. HRE-Luc was induced by insulin, and attained levels similar to those upon exposure to hypoxia and higher than those observed upon DFO treatment. (B) Treatment with different doses of insulin. Cells were stimulated with 1-50 μg/ml insulin for 20 hours, lysed and luciferase activity was determined and normalized to protein concentration. HRE-Luc was induced as a function of insulin concentration. (C) Endogenous dLDH mRNA levels upon insulin treatment. To assess the effect of insulin on the expression of the endogenous dLDH gene, cells were treated for 4, 8, or 16 hours with insulin (10 μg/ml) and then total RNA was prepared and analyzed by northern blot using a specific dLDH probe (upper panel) or 18S rRNA as a control (lower panel); expression of dLDH mRNA increased as a function of the time of exposure to insulin. (D) Role of Sima and Tango in insulin-dependent induction of HRE-Luc. Cells were incubated in the absence (control) or presence of dsRNA directed against GFP, sima or tango and 3 days later, they were treated or not with 10 μg/ml insulin for 20 hours. Cells were then lysed and luciferase activity determined. Induction of the HRE reporter by insulin was totally abrogated by sima or tango dsRNA.

Activation of the insulin-elicited HRE response depends on the PI3K-AKT pathway

It has been reported that activation of the Drosophila InR can trigger the induction of MAPK and PI3K signaling pathways (Oldham et al., 2002; Olivier et al., 1993). To assess which pathway transduces insulin-dependent induction of the HRE response in Drosophila, we treated the cells with LY294002, a specific inhibitor of PI3K. Insulin-triggered induction was strongly suppressed in a dose-dependent manner (Fig. 3A), suggesting that the PI3K signaling pathway is crucially involved in the transduction of the signal that results in HRE induction. Consistent with this, LY294002 treatment significantly reduced dLDH mRNA induction by insulin, confirming involvement of the PI3K-AKT pathway (Fig. 3B). By contrast, treatment with U0126, a specific inhibitor of MAPK kinase (MEK), did not have an effect on insulin-dependent HRE-Luc induction (data not shown), suggesting that the MAPK pathway does not contribute significantly to HRE induction by insulin. To gather additional evidence for a role of the PI3K-AKT pathway in the HRE response, we silenced the expression of dAKT or dPDK1 by RNAi treatment. Complete silencing of dAKT and dPDK1 was attained upon addition of dsRNAs (data not shown) and, concomitantly, strong suppression of insulin-dependent induction of the HRE reporter was observed (Fig. 3C). These results are consistent with the notion that dAKT and dPDK1 are both required for this transcriptional response. Further confirming that activation of the PI3K-AKT pathway can induce an HRE response, transfection of an expression vector encoding AKT elicited a fivefold induction of the HRE-Luc reporter (Fig. 3D). To obtain further evidence of an involvement of the PI3K-AKT pathway in the HRE response, we altered dPTEN levels. PTEN is a negative regulator of the PI3K-AKT pathway and Drosophila PTEN (dPTEN) mutants were shown to upregulate PtdIns(3,4,5)P3 levels, leading to constitutive activation of the PI3K-AKT pathway (Oldham et al., 2002; Stocker et al., 2002). Consistent with this, dPTEN RNAi treatment caused a 5 fold induction of the HRE response in the absence of insulin stimulation (Fig. 3E), further demonstrating that activation of the PI3K-AKT pathway can elicit an HRE transcriptional response in S2 cells. We next wanted to test whether augmented activity of the PI3K signaling pathway can also induce an HRE response in vivo. To this end, we analyzed the expression of the hypoxia-responsive reporter LDH-LacZ in transgenic embryos that were mutant for dPTEN (PTEN2L117). As previously described (Lavista-Llanos et al., 2002), the LDH-LacZ reporter element was silent in wild-type normoxic embryos (Fig. 4A) and induced upon hypoxic exposure in a characteristic expression pattern that corresponds to some of the developing tracheal branches (Fig. 4B). Interestingly, in PTEN2L117 homozygous mutant embryos the LDH-LacZ
reporter was clearly activated in normoxia (Fig. 4C), mimicking the expression pattern of wild-type embryos exposed to hypoxia (Fig. 4B,C). These results suggest that activation of the PI3K-AKT pathway can induce the HRE response not only in cell culture but also in vivo.

Activation of the PI3K-AKT pathway provokes upregulation of Sima protein but not of its mRNA

To gain insights into the molecular mechanism that mediate induction of insulin-dependent HRE response, we analyzed whether Sima protein levels were modified upon insulin stimulation. As depicted in Fig. 5A, western blot analysis revealed that Sima protein accumulated in insulin-treated cells, attaining levels comparable to those of cells exposed to hypoxia (2% O₂ for 16 hours). By contrast, northern blot analysis showed that sima mRNA levels remained constant after exposure to insulin (Fig. 5B), indicating that Sima protein levels are increased through a post-transcriptional mechanism. To analyze whether in Drosophila S2 cells insulin-dependent accumulation of Sima depends on TOR, we studied the effect of rapamycin on HRE-Luc induction. Indeed, rapamycin treatment provoked a strong inhibition of the HRE response (Fig. 5C) and, consistent with this, treatment of the cells with dsRNA for TOR, for its upstream regulator Rheb or for S6 kinase (S6K), which is known to be activated by TOR (Oldham and Hafen, 2003; Li et al., 2004; Saucedo et al., 2003; Stocker et al., 2003; Zhang et al., 2003) led to complete abrogation of insulin-dependent induction of HRE-Luc (Fig. 5D). These results suggest that the insulin-PI3K pathway can modulate Sima protein levels through the activation of TOR.

Sima nucleo-cytoplasmic localization is developmentally modulated and depends on oxygen levels in a dose-dependent manner

We have shown above that insulin-PI3K stimulation leads to upregulation of Sima protein levels. Therefore, we sought to assess whether this pathway can regulate the subcellular localization of Sima. For comparison, it was important to first study the effect of oxygen levels on Sima localization in detail. In a previous report, we showed that it was possible to override rapid Sima degradation by overexpressing the protein in Drosophila transgenic embryos through an engrailed-Gal4 driver, and determined that Sima is mostly cytoplasmic in normoxia and accumulates preferentially in the nucleus in hypoxia (Lavista-Llanos et al., 2002). To better characterize this regulation, we used the same overexpression strategy (Brand and Perrimon, 1993) and for quantitative purposes, because Sima subcellular localization is not an all-or-none phenomenon, thirty or more embryos of each developmental stage were analyzed. Each observation was classified into one of the following three arbitrary categories of Sima subcellular localization: (a) cytoplasmic; (b) ubiquitous and (c) nuclear (Fig. 6A) (see supplementary material Tables 1-5, for statistical analysis). With this criterion, we first studied whether subcellular localization is influenced by developmental

![Fig. 3. HRE-Luc induction in S2 cells is mediated by the activation of the PI3K-AKT pathway.](#)
parameters in normoxia. As depicted in Fig. 6B, in normoxia (21% O₂) Sima was completely cytoplasmic at embryonic stages 11-14 but later in embryogenesis, at stage 16, a proportion of the embryos showed ubiquitous (61.8%) or nuclear (14.7%) localization, indicating that the subcellular localization of Sima is developmentally modulated. Next, we addressed whether localization depends on graded hypoxia and performed the analysis at different oxygen levels throughout development. We observed that, indeed at all stages, Sima became gradually more nuclear when oxygen levels decreased (Fig. 6B) and, confirming developmental modulation, at embryonic stage 11 the protein tended to be more cytoplasmic and became gradually more nuclear as embryogenesis progressed (Fig. 6B). We therefore conclude that the subcellular localization of Sima depends on oxygen levels in a dose-dependent manner and that this regulation is under developmental control.

Activation of the PI3K pathway promotes Sima nuclear localization
To test whether Sima subcellular localization also depends on the PI3K-AKT pathway, we initially analyzed the effect of ectopic expression of dAKT. In transgenic embryos that overexpress dAKT under the control of the engrailed-Gal4 driver in normoxia, Sima localized in the nucleus in a larger proportion than in wild-type embryos all throughout development (Fig. 6C). When dAKT was overexpressed together with dPDK1, an additional enhancement of Sima nuclear localization was observed (Fig. 6C), further suggesting that activation of the pathway can induce the HRE response at this level. Moreover, in PTEN<sub>2L17</sub> homozygous mutant embryos, Sima nuclear localization was also increased, and overexpression of dPTEN in a mutant PTEN<sub>2L17</sub> background completely reverted Sima localization to wild-type levels (Fig. 6C). Altogether, these results strongly suggest that activation of the PI3K-AKT pathway promotes nuclear localization of HIFα/Sima, thereby contributing to the induction of an HRE-dependent transcriptional response in Drosophila cells.
Discussion

Regulation of Sima through the PI3K-AKT pathway

We have used a combination of biochemical and genetic strategies to show both in cell culture and in vivo that activation of the PI3K-AKT pathway promotes HIF-dependent transcription in Drosophila cells. We demonstrated that insulin stimulation or exposure to hypoxia can induce common target genes, and that such transcriptional response depends on the Drosophila HIFα and HIFβ homologues Sima and Tango. We provided evidence that insulin-stimulated HRE response is transduced by the PI3K-AKT pathway and, furthermore, that the effect depends on TOR and involves an increase in Sima protein levels, whereas mRNA levels are not affected. Our results are in good agreement with the reported effect of the PI3K-AKT/TOR pathway on mammalian HIF, because in several cell lines activation of this pathway led to an increase in HIF protein levels (Zundel et al., 2000) caused by augmented translation (Fukuda et al., 2002; Laughner et al., 2001; Treins et al., 2002) or stabilization of the protein (Hudson et al., 2002).

In addition, we have shown that the subcellular localization of Sima depends on oxygen tension in a dose-dependent manner and that activation of the PI3K-AKT pathway also causes a major increase in Sima nuclear localization. This regulatory mechanism might represent another conserved aspect of HIF regulation, because one recent report suggests that HIF-α accumulates in the nuclei of retinal epithelial cells upon IGF-1 treatment (Treins et al., 2005). The molecular bases of HIFα nuclear accumulation upon hypoxia (Kallio et al., 1998) or PI3K activation are so far unclear. Nucleocytoplastic localization of many transcription factors results from a steady-state equilibrium between nuclear import and nuclear export, and accumulation in one or the other compartment depends on the relative rate of import versus export (Fabbro and Henderson, 2003; Swameye et al., 2003). Whether HIF nuclear accumulation upon hypoxia or growth factor stimulation depends on regulated nuclear import or regulated nuclear export remains to be determined.

Conservation of HIF regulation in evolution

We have previously shown that all major cellular features of oxygen-dependent regulation of HIF proteins are conserved in Drosophila (Lavista-Llanos et al., 2002) and, thus, activation of the HRE response in Drosophila by the PI3K-AKT and TOR pathways extends even further the notion of a conserved HIF system in evolution. The functional significance of the regulation exerted by PI3K-AKT and TOR pathways over the HRE response was previously discussed in the context of cancer biology (Brugarolas and Kaelin, 2004; Hudson et al.,...
Role of the PI3K-AKT and TOR pathways in growth control and metabolism

A cardinal function of the PI3K-AKT and TOR pathways throughout evolution is to regulate growth, and to determine the final size of developing organs and whole organisms (Garofalo, 2002; Oldham and Hafen, 2003). Genetic studies in Drosophila have shown that a reduction of the activity of the PI3K-AKT pathway results in flies with a reduced body size, bearing smaller cells (Bohni et al., 1999; Goberdhan et al., 1999; Huang et al., 1999; Rintelen et al., 2001). Likewise, a reduction in TOR signaling provokes growth decrease (Oldham et al., 2000; Zhang et al., 2000) and, conversely, over-activation of TOR signaling due to loss-of-function of its negative regulators TSC1 and/or TSC2, leads to an increase in cell and body size (Gao and Pan, 2001; Potter et al., 2001). The effect of TOR on cell growth was reported to be mediated at least in part by S6K, a kinase that phosphorylates the ribosomal protein S6, leading to translational activation (Gingras et al., 2004; Miron et al., 2003).

Besides its role in growth control, the insulin-PI3K-AKT pathway has been traditionally implicated in the regulation of circulating glucose levels and anabolic metabolism (Saltiel and Kahn, 2001). It was recently demonstrated that the cellular bases of glucose sensing and regulation of serum glucose are conserved between mammals and Drosophila (Kim and Rulifson, 2004; Rulifson et al., 2002), and it has been proposed that PI3K-AKT signaling in conjunction with the TOR pathway coordinates growth according to environmental conditions and the nutritional status of the organism (Britton et al., 2002). The mechanism involved in this coordination is still unclear (Jacinto and Hall, 2003; Oldham and Hafen, 2003). Oxygen tension is one environmental factor that has been shown to modulate growth in Drosophila, because hypoxic flies have a reduced body size (Frazier et al., 2001). We recently provided a mechanistic explanation to this phenomenon, by showing that overexpression of Sima protein causes a reduction in cell size in an autonomous manner (Centanin et al., 2005). Consistent with this, it has previously been shown that hypoxia provokes a reduction in Drosophila TOR pathway activity and that such reduction results from hyperactivation of the TSC1-TSC2 tumor suppressor complex (Reiling and Hafen, 2004). In a simultaneous publication, Brugarolas et al. reported similar results in mammalian cells, implying that TOR regulation by hypoxia (Arsham et al., 2003) is an evolutionary conserved feature (Brugarolas et al., 2004). Furthermore, both studies (Brugarolas et al., 2004; Reiling and Hafen, 2004) demonstrated that hypoxia-dependent TSC1-TSC2 stimulation and growth inhibition are mediated by the product of a HIF/Sima-inducible gene called scylla in Drosophila and RTP801/REDD1 in mammals.

Sima/HIF as a modulator of animal growth

The results presented here establish a direct link between pathways largely implicated in growth regulation (PI3K-AKT and TOR) and the hypoxia-responsive machinery (HIF/Sima). A mechanistic explanation for hypoxia-dependent inhibition of growth is depicted in Fig. 7. In hypoxia, HIF prolyl hydroxylase (Hph/Fatiga) activity is reduced, resulting in HIF/Sima stabilization and induction of an HRE response. One of the genes induced by hypoxia is scylla/RTP801/REDD1, that in turn activates TSC1-TSC2. Then, stimulation of the TSC complex provokes reduction of TOR activity and decreased S6K phosphorylation, resulting in growth inhibition (Brugarolas et al., 2004; Reiling and Hafen, 2004). According to this model, PI3K-TOR activation of HIF-α/Sima might generate a negative feedback loop to limit or downregulate growth; in this scenario, low oxygen levels are expected to enhance Sima-dependent inhibition of growth.

Frei et al. have recently reported that mitochondrial dysfunction inhibits Hph/Fatiga activity, thereby triggering the transcriptional response to hypoxia, and also that it concomitantly provokes growth defects (Frei et al., 2005). They propose that Hph/Fatiga operates as an integration node between oxygen levels and growth regulation (Frei and Edgar, 2004; Frei et al., 2005). Our results have shown that the effect of Hph/Fatiga on growth regulation is conveyed at least in part

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Fig. 7. Model of insulin signaling through HIF/Sima. The activated InR can signal through AKT to prevent TSC1-TSC2 from inhibiting TOR, thereby activating S6K leading to growth stimulation. In addition, TOR can activate Sima/HIF-1 that induces transcription of several target genes, including scylla/RTP801 that in turn stimulates TSC1-TSC2, resulting in a negative feedback loop that reduces TOR activity. Activated AKT can stimulate nuclear localization of Sima/HIF-1 through an unknown mechanism (dashed line), contributing to the negative regulation of TOR. Low oxygen levels can inhibit growth by reducing the activity of Hph/Fatiga, which provokes stabilization of HIF-α/Sima and, in consequence, induction of scylla/RTP801, thereby reducing TOR activity.
by Sima (Centanin et al., 2005). Further studies will reveal whether Hph/Fatiga also plays Sima-independent roles in cell and organ size determination.

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