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Glycogen Synthase Kinase 3β Negatively Regulates Both DNA-Binding and Transcriptional Activities of Heat Shock Factor 1.

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RUNNING TITLE: GSK3β negatively regulates HSF1 in vivo.
SUMMARY

Stress-activation of Heat Shock Factor (HSF1) involves conversion of repressed monomers to DNA-binding homotrimeric complexes with increased transcriptional capacity, and results in transcriptional upregulation of the heat shock protein gene family. Cells tightly control the activity of HSF1 through interactions with Hsp90 chaperone complexes and integration into a number of different signaling cascades. A number of studies have shown that HSF1 transcriptional activity is negatively regulated by constitutive phosphorylation in the regulatory domain by glycogen synthase kinase isoforms GSK3α/β. However, previous studies have not examined the ability of GSK3 to regulate the DNA-binding activity of native HSF1 in vivo under heat shock conditions. Here we show that GSK3β inhibits both DNA-binding and transcriptional activities of HSF1 in heat shocked cells. Specific inhibition of GSK3 increased the levels of DNA-binding and transcription after heat shock and delayed attenuation of HSF1 during recovery. In contrast, overexpression of GSK3β resulted in significant reduction in heat-induced HSF1 activities. These results confirm the role of GSK3β as a negative regulator of HSF1 transcription in cells during heat shock, and demonstrate for the first time that GSK3β functions to repress DNA-binding.

INTRODUCTION

The stress response is a highly conserved cellular reaction leading to rapid expression of cytoprotective heat shock proteins (hsp). Hsps are molecular chaperones that aid in folding, transport, regulation, and degradation of cellular proteins under normal conditions, and their expression during stress is essential for cell survival (1). In higher organisms the response is controlled at the transcriptional level by transient activation of heat shock transcription factor (HSF1) (2). Under normal conditions HSF1 is maintained as latent non-DNA-binding monomers that upon stress assemble into active homotrimers capable of interacting with heat shock elements upstream of hsp genes. Full activation is a
multi-step process involving additional modifications to the transcriptional activation domain, i.e., transcriptional competence is regulated independently of oligomerization/DNA-binding (3). Upon removal of stress, or after expression of a critical level of hsps, trimers are disassembled back to inactive monomers in a process called attenuation.

The functional domains responsible for oligomeric switching, DNA-binding and transcriptional activation of HSF1 have been determined, and it is known that intrinsic properties of the molecule control its own activity (2). However, the cellular mechanisms governing HSF1 regulation appear to be quite complex, and are not fully understood. Recent studies have shown that HSF1 is regulated by association with a dynamic series of Hsp90-chaperone complexes (4-7). In addition, it is a highly phosphorylated molecule that appears to be integrated into a number of stress-activated signaling cascades. Signaling pathways/kinases activated in stressed cells include: PKB (8), PKC (9,10), PKR (for review see (11)), and mitogen activated protein kinase (MAPK) family members: ERK1/2 (extracellular regulated kinase), SAPK/JNK (stress-activated protein kinase/c-jun N-terminal kinase), and p38/Hog-1 (for review see (12)). Glycogen synthase kinase GSK3α/β has also been implicated in the stress response (13-15).

HSF1 is constitutively phosphorylated on several serine residues, and the molecule is hyperphosphorylated after heat shock (13,16-18). Upon stress, some residues remain constitutively phosphorylated, some become dephosphorylated, and several others are phosphorylated. The magnitude and complex dynamics of HSF1 phosphorylation throughout the activation-deactivation process has made it difficult to ascertain the functional role of these modifications, and the links to various kinases still remain unclear. There is a great deal of contradictory evidence in the literature regarding the role of hyperphosphorylation. For example, hyperphosphorylation has been shown in some studies to accompany transcriptional activation, suggesting it is important for this process (14,16,19,20). In support of this, HSF1 is not hyperphosphorylated by salicylate which
activates DNA-binding but not transcription (21), and hsp expression is either reduced by serine/threonine kinase inhibitors (H7, staurosporine, 2-aminopurine, calphostin) or increased by serine/threonine phosphatase inhibitors (calyculin A, okadaic acid, NaF) (17,22-26). In contrast, other studies suggest hyperphosphorylation functions to deactivate transcription after heat shock (27), or even that it plays no role in regulating HSF1 activity (28,29).

A number of studies using general kinase inhibitors to examine the potential regulatory role of phosphorylation on the DNA-binding activity of HSF1 have also yielded contradictory information. For example the serine/threonine kinase inhibitor H7 was reported either to inhibit DNA-binding (26) or to have no effect (17,28). Other general kinase inhibitors (GF-X, staurosporine, K252b, KT5720) have no effect on DNA-binding (17,23,28). General phosphatase inhibitors have been shown to have inconsistent effects, either to increase activation (25), delay activation (20), or delay attenuation of DNA-binding (17,28).

Previous studies have clearly demonstrated that amino acids 303, 307, and 363 in the regulatory domain of human HSF1 are constitutively phosphorylated respectively by GSK3, ERK1/2, and PKC, and in vivo analyses of specific mutants have demonstrated that these phosphorylations function to repress the transcriptional activity of HSF1 (13,14,16,30,31). Repression can be overcome by heat shock, suggesting that phosphorylations in the regulatory domain, including those by GSK3, act in concert with other negative regulatory mechanisms to tightly control HSF1 activity in unshocked cells. However, the potential role for regulatory domain phosphorylations in controlling trimerization and DNA-binding has never been directly examined. This is because past work has involved phosphorylation mutants of either GAL4- or LexA-HSF1 chimeras that did not contain the DNA-binding and oligomerization domains of HSF1 (16,30,31), or overexpressed but constitutively active (under non-shock conditions) full-length HSF1 mutants (13,14).
Here we directly examined the in vivo role of GSK3β on the regulation of endogenous HSF1 under physiological stress conditions. In microinjection experiments with Xenopus oocytes, overexpression of GSK3β repressed both the DNA-binding and transcriptional activity of endogenous HSF1. In contrast, inhibition of GSK3 with LiCl or by overexpression of GBP (GSK3 binding protein) increased DNA-binding and transcription and significantly delayed attenuation of DNA-binding during recovery. These results confirm that GSK3β acts as a negative regulator of HSF1 transcription in vivo during heat shock, and demonstrate that GSK3β represses the DNA-binding activity of HSF1.

EXPERIMENTAL PROCEDURES

Oocyte manipulations.

*Xenopus laevis* frogs were purchased from *Xenopus* I (Ann Arbour MI). Ovaries were surgically removed from adult female frogs and follicular cells were removed from oocytes by treatment in calcium free OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM NaH₂PO₄, 5 mM HEPES-pH 7.8, 10 mg/L streptomycin sulfate, 10 mg/L benzyl penicillin) containing 2 mg/ml collagenase (type II, Sigma) for 3 h at 18°C. Oocytes were washed extensively and allowed to recover for 4 h in OR2 (as above + 1mM CaCl₂) at 18°C, and maintained in OR2 during experimental treatments. Only stage VI oocytes were selected for experiments. Nuclei and cytoplasm were obtained by scoring animal hemispheres with a needle and gently squeezing the equatorial region with watchmaker’s forceps.

Oocyte microinjection and stress treatments.

Capped mRNAs encoding active or inactive (kinase dead) forms of GSK3β and GSK3 binding protein (GBP) were synthesized *in vitro* from pXG73/CS2, pXG114/CS2, and pBP20 (32) respectively using SP6 RNA-polymerase (Pharmacia Biotech).
pXG73/CS2 encoding active GSK3β was generated by PCR cloning from XG73 (kindly provided by D. Kimelman) using PCR primers 5'-cgcggatccatgtcgggaaggccgagaac-3' and 5'-cgcggatcctcaggaggagttggaggcag-3' (33). The PCR product was ligated into the PCR TA3.1 cloning vector (Invitrogen) and the BamH1 insert was further isolated and cloned into pCS2 expression vector. pXG114/CS2 encoding the kinase dead mutant containing a lysine to arginine substitution at position 114 was constructed as above by PCR-cloning from XG114 (provided by D. Kimelman). For expression in oocytes, 20 nl of respective mRNA solutions (2 mg/ml) were injected directly into the cytoplasm. For immediate elevation of nuclear levels of GSK3β, purified recombinant rabbit skeletal muscle GSK3β (New England Biolabs) was injected directly into oocyte nuclei. Cells injected with mRNA were incubated for 12 h at 18°C to allow for translation of expressed protein prior to heat shock. Cells injected with purified enzyme were incubated for 1 h at 18°C prior to heat shock. For stress treatments with LiCl, oocytes were incubated in OR2 containing 10, 25 and 50 mM LiCl for 1 h prior to heat shock in LiCl-free OR2. Heat shock was at 33°C for 1 h (unless otherwise indicated). For recovery, oocytes were transferred to OR2 at 18°C for the indicated times. In all experiments, a minimum of 25 oocytes were used for each sample.

Protein extracts and gel mobility shift assays.

Protein extracts were prepared by homogenizing oocytes in Buffer C (50 mM Tris-Cl pH 7.9, 20% glycerol, 50 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10 μg/ml aprotinin and 10 μg/ml leupeptin) in a volume of 10 μl buffer per oocyte. Homogenates were transferred to eppendorf tubes and centrifuged for 5 min at 15,000 g (4°C). The resultant supernatants were immediately frozen in liquid nitrogen and stored at -80°C.

DNA mobility shift assays were performed using radiolabeled oligonucleotide probes as previously described (34,35). DNA-binding reactions contained 10 μl extract (one oocyte equivalent by volume, or 20 μg soluble protein). Binding reactions were
performed with 1 μg poly (dl-dC), 10 mM Tris (pH 7.8), 50 mM NaCl, 1mM EDTA, 0.5 mM DTT, and 5% glycerol, in a final volume of 20 μl. Reactions were incubated on ice for 20 min, and immediately loaded onto 5% non-denaturing polyacrylamide gels containing 6.7 mM Tris-Cl (pH 7.5), 1 mM EDTA, 3.3 mM sodium acetate. Gels were electrophoresed for 2.5 h at 150 V, dried and exposed to X-ray film. Quantitation of DNA-binding activity was performed using NIH Image (Version 1.6.1) and expressed as arbitrary densitometry units.

**Immunoblotting**

Protein extracts were fractionated by SDS-PAGE (10% acrylamide) then electroblotted onto PVDF membranes. Blots were blocked for 2 h in TBST (20 mM Tris-Cl, pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween 20) containing 5% milk powder. Blots were incubated in primary antibody (1:5,000) in TBST with 2.5% milk and for 2 h, washed in TBST, and incubated with secondary antibody (HRP-conjugated goat anti-rabbit or anti-mouse IgG, BioRad) diluted 1:10,000 in TBST and 2.5% milk, for 2 h. Blots were washed and proteins were visualized by chemiluminescence (Renaissance system; Dupont NEN). Antibodies against GSK3β, IκB, FLAG tag and PCNA were obtained from Santa Cruz Biotechnology.

**Transcription and kinase assays**

CAT assays were performed using 1 oocyte equivalent of whole cell extract as previously described (35). CAT expression vectors (36) and CAT assay protocols were as previously used in (5). Heat shock treatments were performed 4 h after CAT plasmid injections, then oocytes were incubated 18˚C for 12 hours to allow for CAT expression. GSK3 activity was assayed as previously described (37). Activity was measured by scintillation counting of label transfer from (γ-32P)-ATP to the CREB phosphopeptide.
substrate (New England BioLabs) and expressed as a percentage of activity in un.injected unshocked cells.

RESULTS

_GSK 3β represses HSF1-mediated DNA-binding_.

In order to examine whether GSK3β modulates HSF1 in vivo under relevant stress conditions we overexpressed GSK3β and observed the effects on HSF1 activity in _Xenopus_ oocytes. Overexpression was achieved either by microinjection of mRNA into the cytoplasm or by direct injection of purified GSK3β into the nucleus. Increased levels and activity of GSK3β in cells was confirmed by immunoblotting and by specific kinase assays (Fig. 1A). We typically attained a 4 to 5-fold increase in GSK3β protein and activity levels over uninjected control cells (Fig. 1A). By comparison, heat shock for 1 h resulted in a 2-fold increase in endogenous GSK3 activity. In repeated experiments, overexpression of GSK3β consistently resulted in a 5-fold decrease in the amount of HSF1-mediated DNA-binding activity after 1 hr of heat shock (Fig 1B). Microinjection of purified GSK3β enzyme also resulted in decreased DNA-binding after heat shock (Fig 1B). Heat-inducible DNA-binding activity was almost completely inhibited after injection of 0.5 U of GSK3β. In order to control for potential non-specific effects on HSF1 due to the microinjection procedure, and to confirm that the effects observed were attributable specifically to alterations in GSK3β activity, we performed similar experiments with an inactive GSK3β mutant lacking kinase activity (kinase dead GSK3β) (38). Expression of similar levels of kinase-dead enzyme did not change total GSK3β kinase activity (Fig. 1A) and had no measurable effect on the amount of DNA-binding by HSF1 after heat shock (Fig. 1B). Comparison between HSF1 and GSK3 activation during time course of heat shock showed rapid induction of HSE-binding (at 5 min) well before the increase in GSK3 activity which was initially observed after 30 min (Fig. 1C). Control experiments showed that endogenous HSF1 was not activated by the injection procedure itself, and
Manipulation of GSK3β had no apparent effect on other DNA-binding activities. (Fig. 1D). Therefore, the results of these experiments appear to be specific effects of GSK3β on the DNA-binding activity of HSF1.

In converse experiments, we inhibited endogenous GSK3 by pre-treating cells with LiCl (39) or by overexpression of GBP (GSK3 binding protein), a *Xenopus* protein which specifically binds to and inactivates GSK3 (32). LiCl and GBP inactivate both GSK3α and β subtypes. Endogenous oocyte GSK3 activity was reduced to less than 10% of controls after treatment with LiCl (25 mM) or after overexpression of GBP (Fig 2A). Under non-shock conditions, the DNA binding activity of HSF1 was unchanged by GBP expression, but was significantly increased at different time points during heat shock induction (Fig. 2B). Inhibition by LiCl also resulted in a dose-dependent increase in DNA-binding. DNA-binding was apparently activated in the absence of heat shock at 50 mM LiCl, suggesting that GSK3 may function to repress HSF1 under non-shock conditions.

GSK3β has been hypothesized to facilitate inactivation of HSF1 transcription and dispersal of stress-granules following heat stress (15). Therefore we next determined the potential role of GSK3 on the DNA-binding activity of HSF1 during recovery. Inhibition of GSK3 by LiCl or GBP delayed attenuation of DNA-binding activity relative to untreated cells (Fig. 3A). Overexpression of GSK3β appeared to accelerate recovery (Fig. 3A), an opposite effect to that seen with GSK inhibition. Kinase assays confirmed the magnitude of changes in the levels of GSK3 activity in these experiments (Fig. 3B). The prolonged retention of HSF1 activity in LiCl and GBP-inhibition experiments, and accelerated recovery after overexpression, suggests that GSK3 normally functions in efficient disassembly of trimers during attenuation. These results, combined with previously reported evidence of elevated GSK3 activity in cells recovering from heat shock (15), suggests that one of the physiological roles of HSF1 phosphorylation by GSK3 might be to inactivate DNA-binding and transcription at the later phases of the activation
process. Overall, the results of experiments shown in Figs. 1-3 clearly demonstrate that GSK3β represses the DNA binding activity of HSF1 during and after heat shock.

**GSK 3β represses HSF1-mediated transcription during heat shock in vivo.**

The role of GSK3β in regulating HSF1-dependent transcription was determined in oocytes by measuring CAT expression from a microinjected reporter construct (hsp70-CAT). This construct is under control of the hsp70 promoter which is known to be switched on by heat shock and various other stresses in oocytes (36,40,41). The heat shock-induced transcriptional activity of HSF1 was significantly reduced in cells with elevated levels of GSK3β activity achieved either by microinjection of mRNA or active kinase (Fig. 4). Kinase-dead GSK3β had no apparent effect on HSF1-mediated transcription. Conversely, inhibition of GSK3 activity by LiCl or microinjection of GBP elevated the level of HSF1-mediated transcription in heat shocked cells. The possibility that GSK3β manipulation caused a general inhibition of transcription was ruled out by controls in which equal expression from the cytomegalovirus (CMV) promoter was observed after each treatment (Fig. 4). It is possible that the decreased transcription observed in oocytes with elevated GSK3β activity was attributable to decreased DNA-binding, however in the experiment in which GSK3β was expressed from microinjected mRNA, DNA-binding was not completely inhibited (Fig. 1B) whereas transcription was nearly abolished in similarly treated oocytes (Fig. 4). This suggests that GSK3β has dual effects on DNA-binding and transcription. Therefore, these results confirm that GSK3β serves to repress HSF1 mediated transcription under stress conditions in vivo.

**Intracellular localization of endogenous GSK3β during heat shock**

We have previously shown that HSF1 is a nuclear protein in oocytes before and after heat shock (42). Therefore, direct phosphorylation of HSF1 by GSK3β would require co-localization of these proteins during activation or deactivation. To assess the
intracellular distribution of GSK3β before and after heat shock, nuclear and cytoplasmic extracts from manually enucleated cells were analyzed by immunoblotting and kinase assays. GSK3β protein and GSK3 activity were mostly cytoplasmic under non-shock and heat shock conditions and were barely detectable in the nuclei of unshocked cells (Fig. 5). In intact cells, GSK3 activity was elevated by heat shock. The nuclear levels of GSK3β protein were elevated after heat shock, and nuclear kinase activity rose accordingly (Fig 5.). No significant change in GSK3 activity was seen after inhibition of de novo protein synthesis with cycloheximide in both non-shock and heat shock conditions, suggesting that heat shock upregulates endogenous kinase activity. However, the increase in nuclear GSK3 activity could have been caused by a combination of nuclear translocation and activation of the enzyme. In order to demonstrate that the nuclear preparations used in these assays were free of contaminating cytoplasm, we assayed for the presence of IκB and PCNA which are strictly cytoplasmic and nuclear proteins respectively. The cytoplasmic marker IκB was not detected in nuclear extracts and PCNA was not detected in cytoplasmic extracts (Fig. 5).

**DISCUSSION**

GSK3 is a proline directed serine/threonine kinase that was originally discovered as the major kinase phosphorylating glycogen synthase (43). Various other GSK3 substrates have since been identified, including protein phosphatases, kinases, adhesion molecules, myelin basic protein, and several transcription factors including c-Jun/AP-1, JunD, c-Myb, c-Myc, L-Myc (44). The results shown here clearly demonstrate for the first time that GSK3β negatively regulates the DNA-binding activity of HSF1 in vivo, and confirms that it functions to repress transcription in cells exposed to heat shock. It is interesting that GSK3 phosphorylates c-Jun in the DNA-binding domain and down-regulates its DNA-binding activity (45), and that it negatively regulates Myc family
members and Myb (46). Therefore, it appears that the inhibitory action of GSK3 may be common to a variety of transcription factors including HSF1.

The role of GSK3β as a negative modulator of HSF1 was supported here by observations that heat-induced DNA-binding was inhibited in cells overexpressing GSK3β, and conversely that DNA-binding was stimulated after inhibition of GSK3. In addition to effects on DNA-binding, our results confirm the role of GSK3 in modulating transcription. HSF1-mediated transcription was either decreased after increasing GSK3β or increased after inhibition of GSK3. In each experiment in which GSK3 was either increased or decreased, we observed consistent and reciprocal effects on HSF1 activities. The degree to which GSK3β is important for HSF1 regulation in vivo is illustrated by the observation that nuclear enzyme injection almost completely inhibited DNA-binding in response to heat shock. Transcriptional effects on HSF1 by manipulating GSK3 were likely not due to changes in the ability of HSF1 to form trimers and bind DNA because we observed complete transcriptional repression but incomplete repression of DNA-binding with 4-fold increases in GSK3β activity. This leads us to suggest that GSK3 modulates both DNA-binding and transcriptional activities, and this could be through targeting at single or multiple sites on the molecule. The conclusion that GSK3β regulates both activities separately is consistent with uncoupling of DNA-binding and transcription as seen when HSF1 is activated by anti-inflammatory agents such as salicylate and indomethacin (21).

When during the activation-deactivation process does GSK3 exert its influence on HSF1? Previous work suggests that GSK3 might act to constitutively repress HSF1 (13,14,16,30). Consistent with this, we found HSF1 was inhibited in GSK3β-overexpressing cells during early phases of the activation process. We also observed activation of HSF1 DNA-binding under non-shock conditions after inhibiting GSK3 with LiCl, although it is possible that this effect was caused by toxic effects of LiCl. Therefore, it appears that GSK3β represses the DNA-binding and transcriptional activity of HSF1
under normal conditions, and so is important for maintenance of the transcriptionally inactive monomeric conformation. However, several observations lead us to suggest that its primary role is in the later stages of heat shock and attenuation. Our experiments showed that the total level of GSK3 activity increased in response to heat shock well after HSF1 was activated, nuclear levels of GSK increased after 1 hr of heat shock, and inhibition of GSK3 delayed attenuation during recovery. This is consistent with the findings of He et al (15) showing localization of GSK3β to stress granules in vivo and facilitates the disappearance of transcriptionally active HSF1 granules. The current data suggests that GSK3β is required for efficient recovery of HSF1 following resumption of normal conditions.

What is the mechanism by which GSK3β-mediated phosphorylation represses HSF1? GSK3β has been shown to phosphorylate human HSF1 at serine 303 (13,14), and so it is likely that it acts directly on HSF1 in oocytes. As with several other GSK3 substrates (47,48), phosphorylation at this site on HSF1 is dependant on hierarchical phosphorylation at an upstream site, (serine 307) by ERK1/2 (13,14). Sequence alignment of human and *Xenopus* HSF1 (Fig. 6) reveal that the (T286) of frog aligns with serine 303 and matches the consensus phosphorylation site for GSK3β (S/T*XXXS(P)) (47,48). The effects of GSK3β manipulation observed in these experiments could have been through phosphorylation at this site or other site(s) in the *Xenopus* HSF1 molecule. It will be interesting to address whether the dual regulation of HSF1 by GSK3 involves targeting of single or multiple sites, and to determine the dynamics of phosphorylations throughout the activation profile.

We hypothesize that GSK3β acts as the terminal kinase of several stress signal transduction pathways that regulate HSF1 activity. GSK3 is known to be the terminal effector kinase of a number of signal transduction pathways. The upstream activators of GSK3 have not been identified, but it has been shown that the PI(3)K-PKB pathway, p70S6K, p90rsk, and certain isotypes of PKC phosphorylate and downregulate GSK3β.
Of these, PKC and PKB are known to be activated by stress, but only PKC has been implicated as a negative regulator of HSF1. Treatment of human erythroleukemia K562 cells with TPA (12-O-tetraecanoylphorbol 13-acetate), a specific activator of PKC, has been shown to enhance heat induced activity, and accelerate attenuation (52) and this effect was abolished by concurrent treatment of cells with a specific PKC inhibitor (53). In addition, PKC has been shown to directly phosphorylate human HSF1 near the transcriptional activation domain and inhibit transcription (14). It is difficult with the current evidence to determine the relationship between HSF1, PKC and GSK3 at different phases of the stress response. It is interesting that PKC and GSK3 are both activated by stress and are negative regulators of HSF1. Therefore, initial events in the activation of HSF1 probably involves integration of multiple signals and a complex series of Hsp90 chaperone interactions (5-7).

Complete understanding of the role of HSF1 phosphorylation will require incorporation of the effects of other kinases known to phosphorylate HSF1 such as ERK1/2 and PKC. What remains to be answered is whether the constitutively phosphorylated sites in the regulatory domain are actually modified during stress, or if GSK3β acts through other sites on the HSF1 molecule. This is part of the larger question concerning the role of multiple signaling pathways and phosphorylation events in activation and attenuation.

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FIGURE LEGENDS

FIG 1. Overexpression of GSK3β results in decreased DNA binding. (A) Expression and activity of kinases from in vitro synthesized mRNA. Immunoblot of whole cell extracts of control and heat shocked (33°C, 1h) oocytes injected with mRNA coding for active and inactive (kinase dead) GSK3β, and purified enzyme (0.5U). The activity of overexpressed GSK3β was confirmed by specific kinase assay. Kinase activity is expressed as a percentage of uninjected control (100%) and are means of values obtained from five experiments. (B) Gel mobility shift assay of non-shocked (NS) and heat shocked (HS; 33°C) stage VI oocytes. Oocytes were microinjected (cytoplasmic) with in vitro synthesized mRNA coding for active or inactive GSK3β, or were microinjected (nuclear) with different concentrations of purified GSK3β 30 min prior to heat shock for 60 min. Results shown are representative of at least five separate experiments performed with different batches of oocytes. Quantitation of DNA-binding is shown below each panel. (C) A comparison between HSF1 activation and GSK3 activity during time course of heat shock induction. Top panel: gel mobility shift assay; bottom panel: GSK3 activity. (D) Gel mobility shift assays showing a comparison of HSE-binding in uninjected (control) and H2O-injected oocytes (upper panel), and effect of GSK manipulation on CCAAT (middle) and Sp1 (lower panel) binding activities.
FIG 2. Inhibition of GSK3β elevates heat shock induced DNA binding of HSF1.

(A) Expression of GBP from in vitro synthesized mRNA. Immunoblot of whole cell extracts from oocytes injected with mRNA coding for FLAG-GBP. The inhibition of GSK3β by LiCl and GBP was confirmed using kinase assays as described in Fig 1. (B) Gel mobility shift assay of heat shocked oocytes pretreated with various concentrations of LiCl in OR2 buffer for 1 h prior to heat shock (upper panel). The pretreated oocytes were heat shocked at 33°C for 1 h. Separate sets of oocytes were microinjected with mRNA coding for GBP (lower panel). Quantitation of DNA-binding is shown below each panel.

FIG 3. Inhibition of GSK3β delays attenuation of HSF1 during recovery from heat shock. (A) Oocytes pretreated with LiCl (25 mM, 1h), overexpressing GBP, or overexpressing active GSK3β were heat shocked at 33°C for 1 h and then allowed to recover at 18°C for the times indicated, and the DNA binding activity of HSF1 was analyzed by gel mobility shift assay. Quantitation of DNA-binding is shown below each panel. (B) Relative GSK3 activity in control and treatment groups (as indicated) during recovery (time of recovery below panel). Kinase activity is expressed as a percentage of uninjected control (100%) and are means of values obtained from five experiments.

FIG 4. GSK3β modulates HSF1-mediated transcription in heat shocked cells.

CAT assays of oocytes microinjected with hsp70-CAT or CMV-CAT. Cells were injected with purified GSK3β (0.5U), with mRNA coding for active and inactive GSK3β, mRNA encoding GBP, or oocytes were pretreated with 25 mM LiCl for 1 h before heat shock (HS). In each experiment, the same batch of oocytes was used to minimize variation. Each experiment was repeated at least five times with different batches of oocytes, and representative assays are shown. The lower panel expresses relative heat shock-inducible CAT activity as a ratio between control (untreated) and treated groups.
FIG 5. **Intracellular localization of GSK3β.** Western immunoblot of whole cell, cytoplasmic and nuclear fractions and detection with anti-GSK3β antibody. Control blots with nuclear (PCNA) and cytoplasmic (IκB) marker proteins are also shown. One oocyte equivalent of protein extract was used for whole cell and cytoplasmic samples. One nuclear equivalent was loaded for detection of GSK3β and PCNA, but 10 nuclear equivalents protein was used for detection of IκB. Cycloheximide treatment was at 150 μg/ml in OR2 for 1 hr. GSK3β activities are as described in Fig 1.

FIG 6. **Sequence alignment of human and Xenopus HSF1.** Human serine 303 aligns with threonine 286 as indicated by a closed triangle.
FIG 1 Xavier et al.
FIG 1 Xavier et al.

B

Control
HS (min)
NS 5 15 30 60

Active GSK3β (mRNA)
HS (min)
NS 5 15 30 60

Inactive GSK3β (mRNA)
HS (min)
NS 5 15 30 60

Relative HSF1 binding

GSK3β enzyme (U/oocyte)
Control
0.005 0.05 0.5
NS HS NS HS NS HS NS HS

Relative HSF1 binding
FIG. 2 Xavier et al.

A

Uninjected

NS | HS | NS | HS

Control

+LiCl

+GBP

B

LiCl (mM)

0 | 50 | 25 | 10

NS | HS | NS | HS | NS | HS | NS | HS

GBP

Control

Relative GSK3 activity

Relative HSF1 binding

Relative HSF1 binding
FIG 3 Xavier et al.

A

| Recovery from heat shock (min) | Control | LiCl (25 mM) |
|-------------------------------|---------|--------------|
| 2    5   10   20   30  60  120|         |              |

| GBP mRNA |
|----------|
| 2    5   10   30  60|

| Relative HSF1 binding |
|-----------------------|
| 2    5   10   20    2    5   10   30  60 |

B

| Relative GSK3 activity |
|------------------------|
| NS HS 2  5  10  30  60|

| Control | LiCl | GBP mRNA | Active GSK mRNA |
|---------|-----|----------|-----------------|
| NS HS 2 5  10  30  60 | NS HS 2 5  10  30  60 | NS HS 2 5  10  30  60 | NS HS 2 5  10  30  60 |
FIG 4 Xavier et al.

|                | hsp70-CAT          | CMV-CAT          |
|----------------|--------------------|------------------|
|                | Control | Injected/treated | Control | Injected/treated |
| Purified GSK3β | NS       | HS                | NS       | HS                |
| Active GSK3β mRNA |        |                   |          |
| Inactive GSK3β mRNA |        |                   |          |
| LiCl (25 mm)  |         |                   |          |
| GBP mRNA      |         |                   |          |

Relative CAT activity (fold increase/control)
FIG 5 Xavier et al.
FIG 6 Xavier et al.

240 SAPSPAYSSSSLYAPDAVASSGP1SDITELAPAMSPMASPGGSIDERPFLSPLVLRKVEE 300 human
240 SYPVSGFTDS--------SAGPIISDVTLEPESSPSPSPCPSLEASPSPVILIKTE 300 xenopus

305 PFSPPQSPRVEEASPGRFSSVDTLLSPTALIDSILRESEPAVPASVTALTDGHTDEGR 360 human
305 PLTPSQSP---EQSPAPPKLDDTP1SPF1STFIDSILLETETSVCPGGKNDE----MESH 360 xenopus

365 PFSPPPTSTPEKCLSVACLKNELSDHUDAMDSNLNLQTMGLSSHGFSDTSLALLDFSP 420 human
365 PPEP--------CLSVACLDNISLRSQMEVSR--------LFPTSCSVGSPARPPGLDM 420 xenopus

420 SVTVPDMLPDLSLASSIQLSPQEPDRPPEAENSSPDGQLVLHYTAQPLFLDHPGS 480 human
420 AVALNDHVDNLDHNLQNLNGQS------------------FSVDTALMDLFSP-- 480 xenopus
Glycogen Synthase Kinase 3b Negatively Regulates Both DNA-Binding and Transcriptional Activities of Heat Shock Factor 1
Ilungo J. Xavier, Phillipe A. Mercier, Christine L. McLoughlin, Adnan Ali, J. R. Woodgett and N. Ovsenek

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